Prostate Cancer And The Role Of Cell Adhesion Molecules

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by

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For Mum and Dad

Being here now is simply because of the ears, shoulders, and hearts of what seems like an endless number of people. You all know who you are. I am incredibly fortunate. However, a few people need to be mentioned for their unconditional friendship, love and support. Special thanks to Maria, who has now completed the crash course in "Prostate Cancer And Cell Adhesion Molecules": thanks also to Sam, Angela, Lins, Gillian, Angela Gillian, Emma, Gabsi, and lastly, but by NO means least, Clarkie. I can't think how to say thanks enough to you, but I once heard these four lads...........

"I get by with a little help from my friends,

...,

With a little help from my friends."

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Prostate Cancer And The Role Of Cell Adhesion Molecules

Julie Marie Hastings

Prostate cancer is the most prevalent cancer in man. The development of metastatic cancer involves a complex cascade of events that include release of neoplastic cells from the primary tumour, movement of the tumour cells into the vasculature and arrest at distant sites via interactions with vascular endothelial cells. These steps involve changes in the adherent characteristics of tumour cells. Cell adhesion molecules mediate this adhesion. This study proposes a role for cell adhesion molecules in the metastatic spread of prostate cancer.

Frozen sections of benign and malignant prostate tissue were immunohistologically analysed for ICAM-1, VCAM-1, alpha-4, alpha-5, alpha-L, beta-1, CD44, and E-selectin. The effect of HUVECs on the expression of cell adhesion molecules by PC3 and Du145 cells investigated. The effect of PC3 and Du145 cells on the expression of cell adhesion molecules by HUVECs was investigated.

PC3 and Du145 conditioned medium and endothelial cell-conditioned medium did not induce changes in cell adhesion molecule expression by endothelial, and PC3 / Du145 cells, respectively.

The prevalence and level of expression of ICAM-1 in prostate tumours appear to be significantly greater than in their benign counterparts. Co-culture of HUVECs with Du145 cells induced an upregulation of ICAM by the Du145 and a down-regulation in CD44 by HUVECs. Co-culture of PC3 cells with HUVECs induced a down-regulation in CD44 and upregulation of alpha-5 by the PC3 cells.

The expression of ICAM-1 by Du145 metastatic prostate cancer cells may be involved in the stabilised attachment of Du145 cells to HUVECs. The expression of CD44 by HUVECs may play a role in the initial attachment of Du145 cells to HUVECs. The expression of CD44 by PC3 prostate cancer cells may be important in the initial attachment of PC3 cells to HUVECs, while α5 may play a role in the stabilised binding and / or transendothelial migration of PC3 cells.

Conclusion: ICAM-1 confers an invasive phenotype to prostatic epithelial cells.

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Abbreviations

AB/PBS	AB serum In Phosphate Buffered Saline
APPAP	Alkaline Phosphatase and Anti-Alkaline Phosphatase
Az	Sodium Azide
bFGF	basic Fibroblast Growth Factor
Ca^{2+}	Calcium ion
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
CD44H	Haematopoietic CD44
CD44s	Standard CD44
CD44v	CD44 isoforms
cDNA	cloned Deoxyribonucleic acid
CLA	Cutaneous Lymphocyte Antigen
CNS	Central Nervous System
CO_2	Carbon Dioxide Gas
Da	Dalton
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EC	Endothelial Cell
ECCM	Endothelial Cell Culture Medium
ECM	Extracellular Matrix
ECLM	Established Cell Line Medium
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal Growth Factor
ERM	Ezrin, Radixin, Moesin
ESL	E-Selectin Ligand
FACScan	Fluorescence Activated Cell Scan
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FGF-R	Fibroblast Growth Factor-Receptor
FITC	Fluorescein Isothiocyanate
g	gram
GF	Growth Factor
HA	Hyaluronate / Hyaluronic Acid
HBSS	Hanks Balanced Salt Solution
HCl	Hydrochloric acid
HEPES	n-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)

HEV	Hinge Endothelial Venule
HLA	Human Leukocyte Antigen
H_2O	Water
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Cell Adhesion Molecule
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
IU	International Unit
k	kilo
LASER	Light Amplification by Stimulated Emission of Radiation
L	Litre
LFA	Lymphocyte Function-associated Antigen
LPS	Lipopolysaccharide
McAb	Monoclonal Antibody
MESF	Molecular Equivalent of Soluble Fluorochrome
Mg^{2+}	Magnesium ion
MHC	Major Histocompatability Complex
mg	milligram
ml	millilitre
MMP	Matrix Metalloproteinase
mRNA	messenger Ribonucleic acid
MTT	3(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
N_2	Nitrogen
NA	Not Applicable
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCAM	Neural Cell Adhesion Molecule
ND	Not Done
NGF	Nerve Growth Factor
NGS	Normal Goat Serum
NMS	Normal Mouse Serum
O_2	Oxygen Gas
OCT	Tissue Tek OCT Compound
OD	Optical Density
OGF	Osteoblast Growth Factor
PAP	Prostatic Acid Phosphatase
PBS	Phosphate Buffered Saline
PBS/Az	Phosphate Buffered Saline / Sodium Azide

PBS/Az/NGS	Phosphate Buffered Saline / Sodium Azide / Normal Goat Serum
PBS/Az/NGS/NMS	Phosphate Buffered Saline / Sodium Azide / Normal Goat Serum / Normal Mouse Serum
PE	Phycoerythrin
PECAM	Platelet Endothelial Cell Adhesion Molecule
PEGM	Primary Epithelial Growth Medium
PLN	Peripheral Lymph Node
PSGL	P-Selectin Glycoprotein Ligand
RGD	Arginine-Glycine-Aspartic Acid
RPMI	Roswell Park Memorial Institute
SCR	Short Consensus Repeat
sLe	Sialyl Lewis Antigen
TCGF	Tissue Culture Grade Flask
TcR	T cell Receptor
TEM	Transendothelial Migration
TGF	Transforming Growth Factor
TM	Transport Medium
TNF	Tumour Necrosis Factor
TRIS/HCl	Trishydroxymethylaminomethane hydrochride
TURP	Transurethral Resection of the Prostate
µl	microlitre
uPA	urinary Plasminogen Activator
USM	Urogenital Sinus Mesenchyme
VAP	Vascular Adhesion Protein
VCAM	Vascular Cell Adhesion Molecule
VLA	Very Late Antigen

Chapter 1

Introduction

Contents

1.1 Cell Adhesion Molecules

The organisation of animal cells in differentiated organs depends upon cell-surface interactions with molecules on the surface of other cells and with extracellular matrix (ECM) components (Springer, 1990). Cell adhesion molecules (CAMs) regulate these interactions and their functional importance is richly illustrated within the immune system. In order to patrol the body effectively for foreign antigen, the cells of the immune system must both circulate as non-adherent cells in the blood and lymph and migrate as adherent cells through tissues. In the presence of non-self they must be capable of congregating in lymphoid organs, crossing endothelial and basement membrane barriers to aggregate at sites of infection, and adhere to the cells bearing foreign antigen. Rapid transition between non-adherent and adherent states (controlled by the expression of CAMs) is of key importance to the dual functions of immune surveillance and responsiveness (Springer, 1990)

Adhesion is only one of the functions that CAMs perform: it is through their adherence that they are thought to act as signalling receptors, influencing patterns of gene expression, differentiation and proliferation (Fawcett, 1992). As such, CAMs are operational throughout the biology of multicellular organisms and are fundamentally important in embryonic development and haematopoiesis. Disruption of CAM expression results in the aberrant development of biological entities (McCarthy, 1991). For example, knockout experiments in mice that restricted the expression of Neural Cell Adhesion Molecule (NCAM) caused distortion of their central nervous system. Moreover, deletion of the Neural-cadherin (N-cadherin) gene in mice is lethal, and animals die in mid-embryogenesis with heart malformations (Albelda, 1993).

CAMs have been grouped into several distinct classes according to structural and / or functional similarities. To date, five families of CAMs have been identified. These are the cadherin family, the cartilage link protein family, the integrins, the immunoglobulin superfamily, and the selectins.

1.1.1 The Cadherin Family

1.1.1.1 Introduction

Cadherins are single chain transmembrane proteins that mediate homotypic and homophilic adhesion in a Ca^{2+}- dependent manner (Fawcett, 1992). Cadherins are rapidly degraded by protease action in the absence of Ca^{2+} (Pignatelli and Vessy, 1994). More than ten subclasses of cadherins have been identified (Angres *et al*, 1991, Donalies *et al*, 1991, Ginsberg *et al*, 1991, Napolitano *et al*, 1991, Rauscht *et al*, 1991, Suzuki *et al*, 1991). Three "classical cadherins" have been identified; E-cadherin, N-cadherin, and P-cadherin, which are expressed primarily on epithelial cells, muscle cells, and placental cells, respectively (Albelda, 1993). More recently, novel cadherins have been described including cadherin-10. Cadherin-10

is largely expressed in the brain, but has been demonstrated in glandular epithelial cells of the prostate (Kools *et al*, 1999).

1.1.1.2 Structure Of Cadherins

"Classical cadherins" share a common basic structure consisting of 723 to 748 amino acids (Umbas *et al*, 1992, Albelda, 1993). Cadherins are composed of five extracellular domains, a transmembrane domain and a long intracellular domain (Diagram 1.1). Within the extracellular domain are three homologous putative Ca^{2+} -binding repeats and a 113-residue conserved NH_2 terminal region (Rimm 1995). The tri-peptide His-Ala-Val, located proximal to the amino terminal, is the cell adhesion recognition sequence of cadherins (Jothy *et al*, 1995).
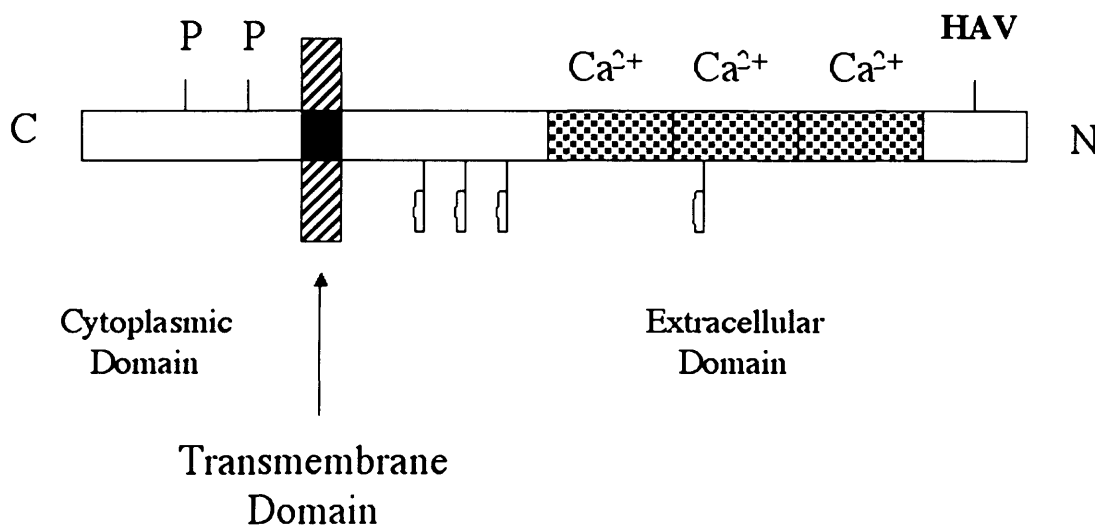
Diagram 1.1 Schematic Representation Of E-cadherin. Cadherins have five extracellular domains, a transmembrane domain and a cytoplasmic domain. The cadherin cell adhesion motif, His – Ala – Val (HAV), is located in the N-terminal (N) extracellular domain. The extracellular domain also contains three putative Ca^{2+} -binding domains and a number of glycosylation sites (represented by flags). The C-terminal (C) domain contains an unknown number of phosphorylation sites (P).

1.1.1.3 Function Of Cadherins

Cadherins participate in the establishment and maintenance of intercellular connections, and as such are considered to be one of the most important groups of CAMs participating in the formation of cell-cell associations (Albelda, 1993). The sizeable cytoplasmic domain is non-covalently associated with the cytoplasmic proteins α-, β-, and γ- catenin and plakoglobin which are indirectly linked to the actin-based microfilament network (Takeichi, 1993, MacCalman *et al*, 1994) (Diagram 1.2). β-catenin binds directly to the carboxy-terminal of cadherins and is thought to bind α-catenin, which binds α-actinin (Jothy *et al*, 1995). Deletion

5

experiments with the catenin binding domain of cadherins clearly show that the domain is essential for cadherin binding to the cytoskeleton and for subsequent connection to adjacent cells (Pignatelli and Vessy, 1994).
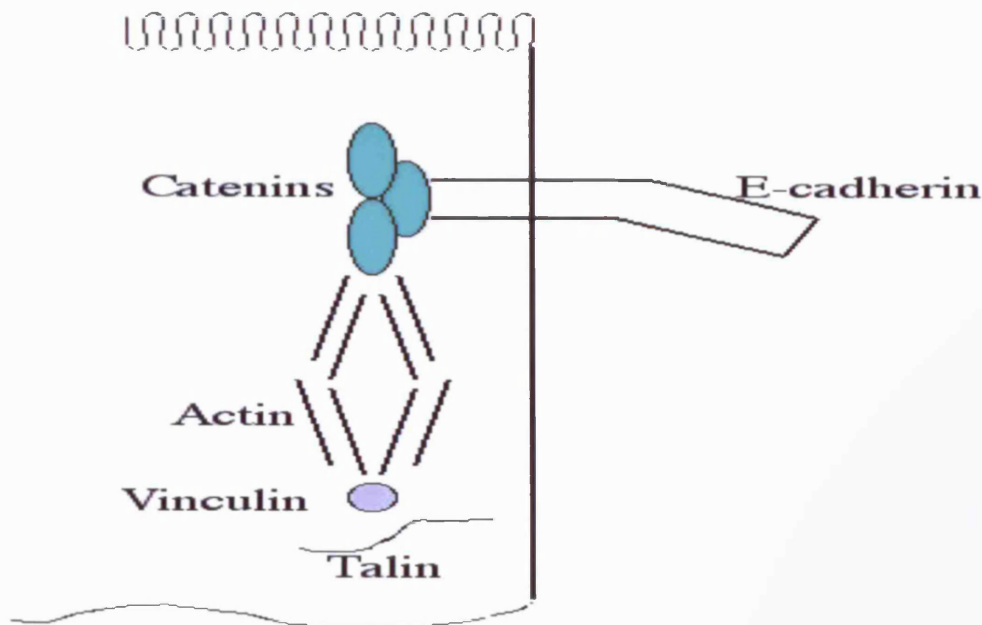
Diagram 1.2 Schematic Representation Of The Relationship Of E-cadherin With The Actin Cytoskeleton. Cytoplasmic E-cadherin associates directly with α, β and γ -catenins and indirectly with the actin-based microfilament network, including vinculin and talin.

1.1.2 The Cartilage Link Protein Family

1.1.2.1 Introduction

Cartilage link protein or standard CD44 (CD44s) is a type I transmembrane glycoprotein previously known as the Hermes antigen, Homing CAM (HCAM), phagocytic glycoprotein-1 (pgp-1), and ECM-RIII (Lazaar and Pure, 1995). Isolation of the cDNA revealed the identity of these molecules to be based on a 37kDa core protein. The extracellular region of CD44s spans 250 amino acids and is highly glycosylated by O and N linked oligosaccharides and chondroitin sulphate side chains to yield an 85kDa mature protein (Staudert and Gunthert, 1995). Human genomic CD44 is localised on chromosome 11p13, consisting of 20 exons over a length of approximately 60 kbases (Goodfellow *et al* 1982, Screaton *et al* 1992, 1993).

1.1.2.2 Structure Of CD44

The 85kDa CD44s or haematopoeiticCD44 (CD44H) spans a region of 7 extracellular exons (designated 1s to 7s, inclusively), transmembrane exon (8s), and a cytoplasmic exon

Variant Exons 1v-10v

← Amino Acid 223

S — S
S — S
S — S

Serine 291
Serine 316
Serine 323 — P
Serine 325 — P

■ Chondroitin Sulphate

● N-linked glycosylation

◉ O-linked glycosylation
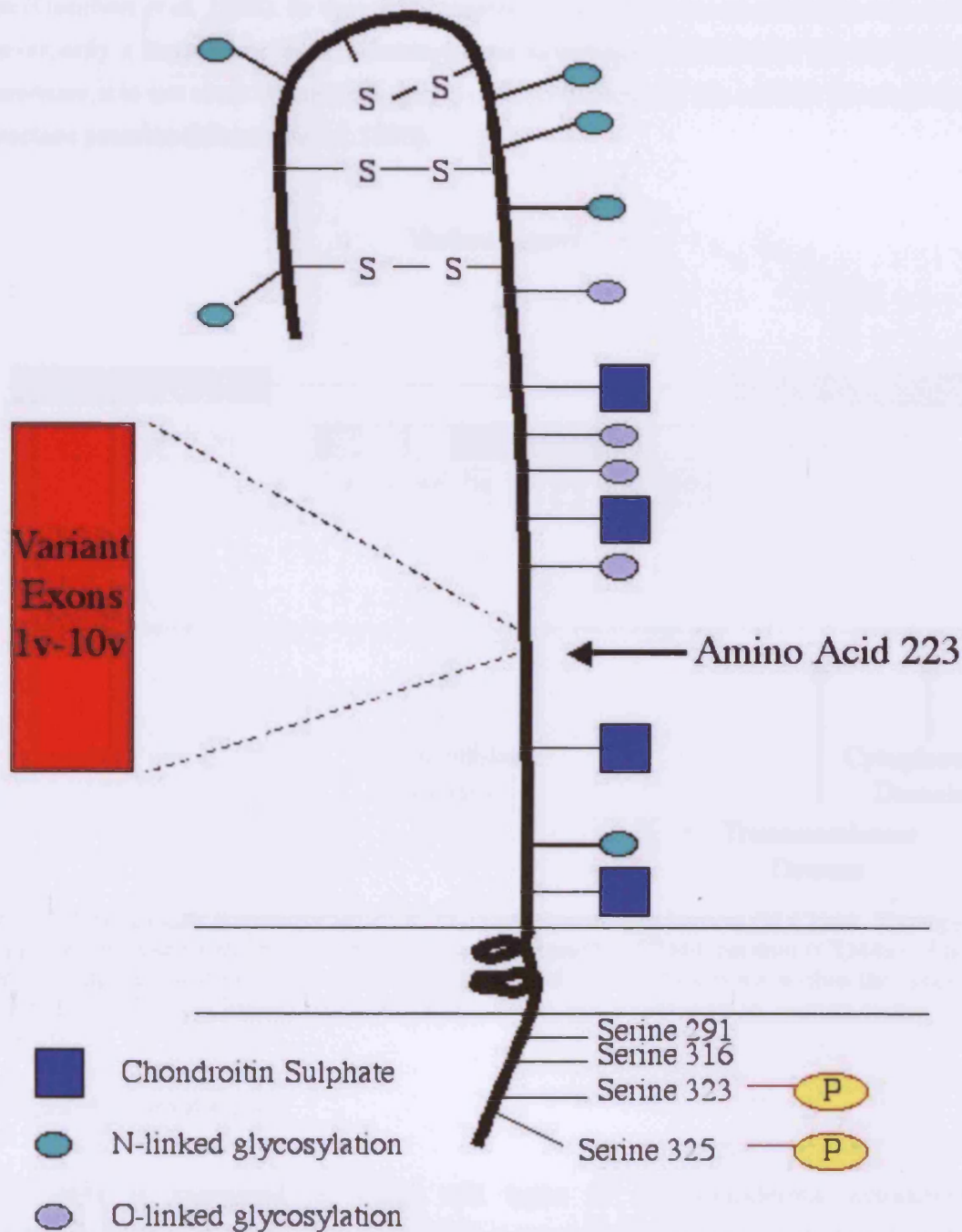
Diagram 1.3 Schematic Representation Of The Standard CD44 Protein. The extracellular domain, which contains a number of glycosylation sites and chondroitin sulphate side-chains, spans 250 amino acids and contains a splicing site at amino acid 223. CD44 is constitutively phosphorylates at serine 325 and / or 323 within the cytoplasmic domain.

which can be either short (exon 9s generating 3 amino acids) or long (10s generating 70 amino acids) (Diagram 1.3). The remaining ten exons are not expressed in CD44s: only by alternative

splicing of the pre-mRNA can they be inserted between exons 5s and 6s in different combinations to generate CD44 variant isoforms (CD44v's), which contain up to 420 additional amino acids (Diagram 1.4). These variant isoforms, which exhibit molecular masses of up to 300kDa, endow the molecules with further glycosylation sites and chondroitin sulphate side chains (Gunthert *et al*, 1995). In theory, the number of variant exon combinations exceeds 1000. However, only a limited number of combinations have been identified for the CD44 isoforms. Furthermore, it is not clear whether all splice variants detected at the mRNA level are translated into surface proteins (Sleeman *et al*, 1995).
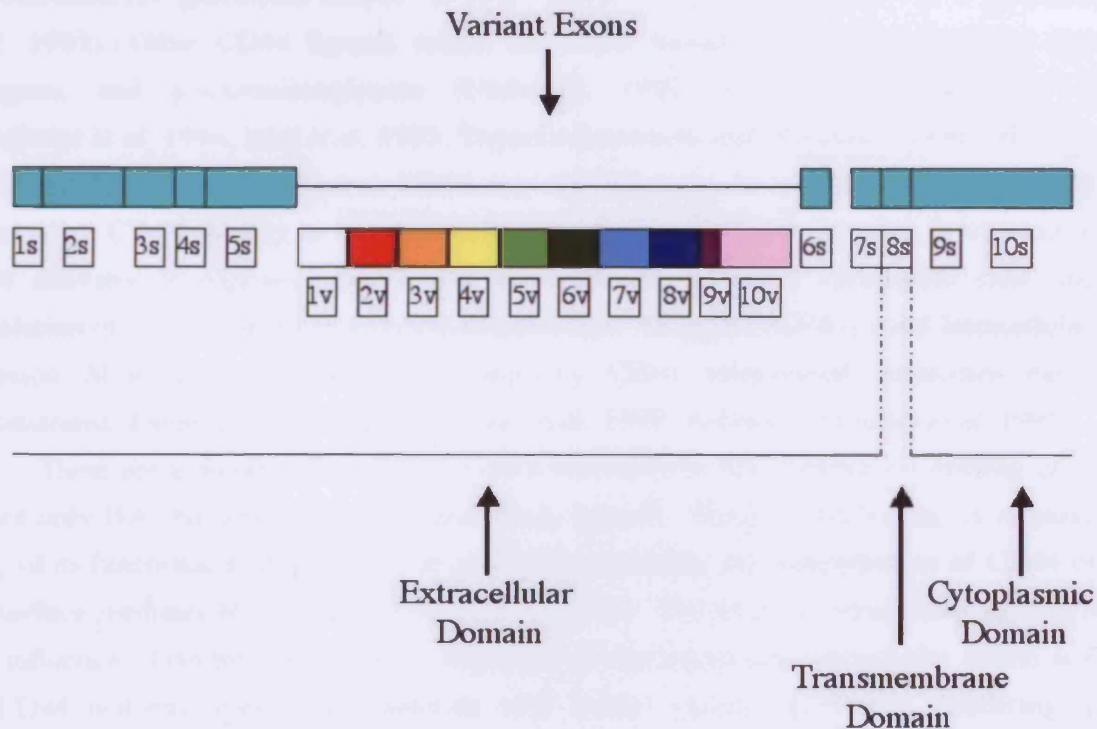
Diagram 1.4 Schematic Representation Of The Genomic Organisation Of CD44. The upper line of rectangles represents the exons found within the standard CD44 protein (CD44s). The lower line of rectangles represents the variant exons found in combinations within the alternatively spliced variant CD44 (CD44v) isoforms. Exon 1v is not expressed in human tissue due to a stop codon.

1.1.2.3 Distribution Of CD44

CD44s is expressed on many cell types of neuroectodermal, ectodermal and mesenchymal origin, as well as on cells of haematopoietic lineages, including lymphocytes, macrophages, fibroblasts, glial cells, epithelial cells, endothelial cells, and smooth muscle cells (Lazaar and Pure, 1995). In contrast, the expression of CD44v's is relatively restricted. Although most epithelia and haematopoietic organs are CD44v$^+$ during ontogeny, CD44v expression in the adult is mainly restricted to the skin and epithelia of the gut and a variety of glands (Wirth *et al*, 199, Fox *et al*, 1994).

1.1.2.4 Function Of CD44

CD44 was first described as a lymphocyte homing receptor, mediating the attachment of circulating lymphocytes to high endothelial venules allowing entry to the lymphatic tissue (Jalkenen *et al*, 1987). Subsequent studies have implicated CD44 in a number of cellular functions including, lymphocyte activation, differentiation and extravasation, haematopoiesis, inflammation, tissue regeneration, and pattern formation in embryogenesis (Stauder and Gunthert, 1995). The cellular ligands utilised by CD44 in many of these processes remain to be identified. Within the ECM, the assembly of which involves CD44, the major ligand for CD44 is hyaluronate (D -glucuronic acid (1 - β- 3) *N* - acetyl - D- glucosamine (1 - β- 4)$_n$) (Knudson *et al*, 1993). Other CD44 ligands within the ECM include fibronectin, laminin, type IV collagens, and glycosaminoglycans (Underhill, 1992, Jalkenen and Jalkenen, 1992, Lokeshwar *et al*, 1994, Ishii *et al*, 1993, Toyama-Sorimachi and Miyasaka, 1994). However, it is believed that on many occasions CD44 may act indirectly. In lymphocyte extravasation, for instance, no CD44 binding to the endothelial cell surface has been detected. It is possible that CD44 activates or exposes other CAMs required for binding to endothelial cells: indeed, modulation of CD2, Lymphocyte Function-associated Antigen-1 (LFA-1) and Intercellular Cell Adhesion Molecule-1 (ICAM-1) expression by CD44 monoclonal antibodies has been demonstrated (Denning *et al*, 1989, Koopman *et al*, 1990, Vermot-Desroches *et al*, 1995).

There are a number of possible control mechanisms that regulate the binding of CD44 to, not only HA, but also other, yet undefined, ligands. Firstly cross-linking is required for many of its functional effects (Lesley *et al*, 1993): secondly, the redistribution of CD44 on the cell surface mediates HA binding (Lesley *et al*, 1992). The level of extracellular glycosylation may influence CD44 binding. Gunthert hypothesises that additional extracellular amino acids of the CD44 isoforms endow the molecule with further glycosylation sites, rendering CD44 hydrophobic and that this could mediate supplementary binding properties (Gunthert *et al*, 1995). CD44 is constitutively phosphorylated on serine 325 and/or serine 323 and 327. T cells expressing mutations at any of these three residues were not phosphorylated and did not bind HA (Pure *et al*, 1995). Lastly, deletions of the cytoplasmic CD44 domain abrogated binding of HA, and subsequent replacement of the transmembrane region of CD44 with the CD3ζ chain, which mediates homodimerisation via disulphide bonds, restores HA binding (Perschl *et al*, 1995). Therefore, the cytoplasmic associations may control the cell surface conformation and distribution of CD44, highlighting the complexity in the regulation of CD44 functional expression.

1.1.3 The Immunoglobulin Superfamily

1.1.3.1 Introduction

Monoclonal antibodies against the integrin molecule, Lymphocyte Function-associated Antigen-1 (LFA-1), or αLβ2, first defined its ligand, Intercellular Cell Adhesion Molecule-1, ICAM-1 (Rothlein *et al*, 1986). Since this first find, ICAM-2 and ICAM-3 have been identified (Staunton *et al*, 1989, de Fougerolles and Springer, 1992). This family of CAMs also embraces the antigen-specific receptors of T and B lymphocytes; for example, the MHC molecules, CD4, CD8, the T cell Receptor (TcR), the VCAMs (Vascular Cell Adhesion Molecules) and the NCAMs (Neural Cell Adhesion Molecules) (Albelda, 1993). The immunoglobulin CAMs most heavily studied are the ICAMs and VCAM-1.
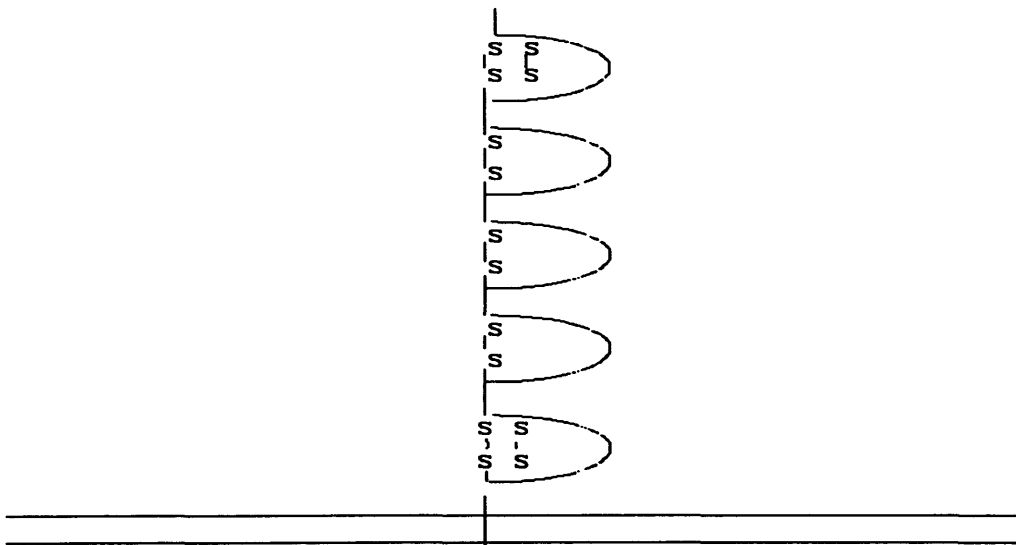
Diagram 1.5 Schematic Representation Of Intercellular Cell Adhesion Molecule (ICAM)-1. ICAM-1 contains five immunoglobulin unit repeats, each held together by di-sulphide bonds. Other ICAM molecules contain a different number of immunoglobulin repeat units.

1.1.3.2 Structure Of Immunoglobulins

Members of the immunoglobulin superfamily of CAMs share the same basic architecture of the immunoglobulin unit. This structure consists of 70-100 amino acids organised into 7 to 9 β-pleated sheets. Each unit is stabilised by a constant disulphide bridge formed between two of the strands (Hunkapiller and Hood, 1989). ICAM-1 consists of five immunoglobulin domains (Diagram 1.5). ICAM-2 has two domains and ICAM-3 has five domains. All the ICAMs contain immunoglobulin domains consisting of 7 β strands (Simmons, 1995). The most closely related domains of the ICAMs are the N-terminal domains and the cytoplasmic tails are the most divergent regions of the molecules. This led to the suggestion that

ICAMs may be involved in intracellular signaling. ICAMs are heavily glycosylated with up to half of the mass being accounted for by oligosaccharides, most of which are N-linked.

1.1.3.3 Function Of Immunoglobulins

Most members of the immunoglobulin CAM family are involved in cell-cell recognition. Most of these molecules are immune regulators. Springer has elucidated most of what is currently known about immunoglobulin adhesion receptors and this has been beautifully reviewed (Springer, 1990). The interaction of ICAM-1 with LFA-1 has a pivotal role in a wide range of leucocyte interactions, including those between antigen presenting cells and T and B lymphocytes, helper and cytotoxic T cells and their targets, natural killer cells and their targets, and antibody dependent cell-mediated cytotoxicity. ICAM-1 is utilised as a major adhesion CAM in the multi-step cascade of leucocyte interaction with the vascular endothelium. However, it must be remembered that the ICAM-1 / LFA-1 interaction is only one of many co-stimulatory signals required for activation of effective cellular function. Other immunoglobulin CAMs involved in these processes include VCAM-1 and CD2 which bind to Very late antigen-4 (VLA-4) and LFA-3, respectively (Springer, 1990).

The dominant contact points for ICAMs with the LFA-1 ligand seem to reside in immunoglobulin domain 1. Furthermore, there appears to be a common, short, linear motif that is an essential component of the immunoglobulin CAM – integrin interactions (Vonderheide *et al*, 1994). This is thought to form the basis of immunoglobulin interaction with integrins, but other regions must provide the specificity, so that ICAM-1 binds LFA-1 and VCAM-1 binds VLA-4.

ICAMs have a role as signal transducers in mediators of cell adhesion. Cross-linking of ICAM-1 can deliver a signal to neutrophils, for example, to induce oxidative burst and to T cells to activate expression of surface proteins.

1.1.3.4 Distribution Of Immunoglobulins

Both ICAM-1 and ICAM-2 are present at low levels on resting leucocytes, whereas ICAM-3 is constitutively expressed. ICAM-1 is expressed at virtually undetectable levels on vascular endothelial cells. However, ICAM-1 is rapidly up-regulated by cytokines, such as interferon γ, interleukin-1β and tumour necrosis factor-α. ICAM-1 expression can be induced on a wide range of cell types, including leucocytes, endothelium, keratinocytes, epithelial cells and fibroblasts. Therefore, ICAM-1 can be viewed as a rapid response-ICAM, present at low levels in quiescent states, but capable of induction in appropriate circumstances. ICAM-3 is expressed by "professional" antigen presenting cells (Simmons, 1995). Wang *et al* (1999) demonstrated zonal up-regulation of ICAM-1 and VCAM-1 by liver endothelial cells after *in*

vivo treatment with Lipopolysaccharide (LPS), suggesting that different agents may induce up-regulation of CAMs at specific sites on the cell membrane.

1.1.4 The Integrin Superfamily

1.1.4.1 Introduction

The integrin family of receptors was discovered in the mid 1980's upon the realisation that several distinct groups of adhesion proteins, both human and non-human, possessed related structures and activities: the name 'integrin' was coined to signify their role of 'integrating' the intracellular cytoskeleton with the ECM (Ruoslahti, 1991). Integrins are a family of transmembrane heterodimeric glycoproteins composed of non-covalently associated α and β polypeptide chains (Fawcett, 1992).

1.1.4.2 Structure Of Integrins

There are currently 16 known α and 9 β subunits (Danen *et al*, 1995). Both α and β subunits have a large extracellular domain, a transmembrane region and a short cytoplasmic tail of 50 amino acids or less (Hynes, 1992). The α subunits, which vary in size between 120 and 180kDa, display extensive intrachain disulphide bonding (Calvete *et al*, 1991). The N terminal of all α chains contains a 7-fold repeat of a homologous sequence that has been partially sequenced as Asp-x-Asp-x-Asp-x-x-Asp (Hynes, 1992). This repeat sequence is believed to contain a divalent cation-binding domain (Danen *et al*, 1995). Some α subunits are subject to post-translational modification of the extracellular domain. This revision can lead to what appears as a double-chain extracellular region (Hynes, 1992).

The β subunit, which is smaller than the α subunit, varies in size between 90 and 110kDa and is rich in internal disulphide bonds, which is partly due to a 4-fold repeat sequence rich in cysteine. The β chain N terminal configuration is tightly folded with more intrachain disulphide bonds (Calvete *et al*, 1991).

The N terminal regions of both the α and β chains combine to form the ligand binding domain of each integrin (Diagram 1.6). This ligand binding domain lies in close proximity to the cation binding domain of the α subunit which is critical for integrin function (D'Souza *et al*, 1988, Gailit and Ruoslahti 1988, Kirchhofer *et al*, 1991).

Upon synthesis, new $\alpha\beta$ heterodimers are transported from the cytoplasm to the membrane. The association of the subunits is promoted by divalent cation binding at the α chain N terminal and may be chaperoned by calnexin. Indeed, the N terminal domains of the α and β subunits are crucial for dimerisation: truncated chains lacking both their transmembrane and cytoplasmic domains cannot produce functional $\alpha\beta$ dimers (Lenter and Vestweber, 1994).

Diversity within the integrin family is generated by the large number of α subunits that can combine with the 9 different β subunits. The practical combinations appear to be much more restricted than the number of theoretical combinations, although a high degree of diversity is maintained with the existence of alternative splicing of the cytoplasmic tail of the $\alpha\beta$ heterodimers (Sastry and Horwitz, 1993). To date it appears that β subunits can dimerise with more than one α subunit and (with the exception of αv) α subunits are capable of binding only 1 β subunit (Table 1.1) (Hynes, 1992)
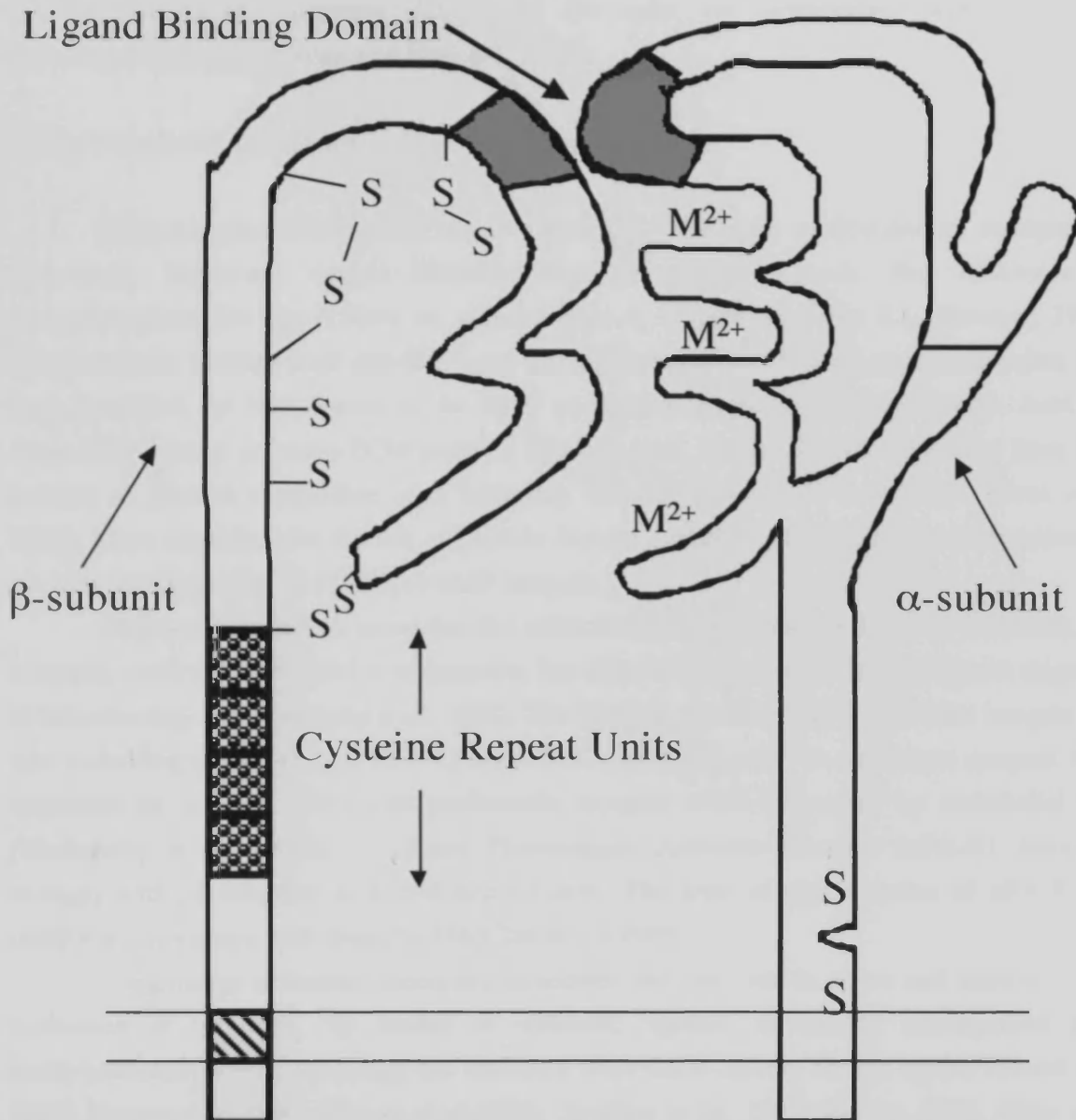
Diagram 1.6 Schematic Representation Of The Alpha And Beta Subunits Of Integrin Molecules. The beta unit contains four cysteine repeat units and internal di-sulphide bonds within the head region. The head region of the alpha unit contains several divalent cation binding domains (M^{2+}). The N-terminal regions of the alpha and beta subunits combine to form the ligand binding domain (represented by the shaded boxes).

1.1.4.3 Distribution Of Integrins

The cellular expression of integrins is variable. For example, the β1 integrins, known collectively as the Very Late Antigen (VLA) proteins, are widely distributed on connective tissue
cells and mononuclear cells (Chapman *et al*, 1995). This VLA subfamily of CAMs aid in mediating leucocyte adhesion to ECM components. In contrast, the expression of the β2 integrins is restricted to leucocytes upon which they serve to aid in, amongst other processes, the transendothelial migration (TEM) of the cells via interactions with endothelial immunoglobulins (Chapman and Haskard, 1995).

1.1.4.4 Function Of Integrins

Most integrins bind ligands found within the ECM such as fibronectin, collagen and vitronectin. However, certain integrins bind to soluble ligands like fibrinogen or counterreceptors like the ICAMs on adjacent cells as detailed in Table 1.1 (Fawcett, 1992). Some integrins mediate both cell-ECM and cell-cell interactions. Several recognition sites have been identified; the best known is the RGD tri-peptide (Arginine-Glycine-Aspartic Acid, see Appendix 8) found in many ECM proteins (Dedhar *et al*, 1987). Several sequences have been isolated as putative recognition sites including KELLPGNNNRKV in ICAM-1 (Ross *et al*, 1992). More recently with the use of peptide libraries, Kraft *et al* (1999) have demonstrated a novel recognition site, DLXXL, for αvβ3 integrin.

Different ligands that recognise the same integrin may mediate different functions. For example, αvβ3 and αvβ5 bind to vitronectin, but only αvβ5 promotes the subsequent migration of the adhering cells (Leavesly *et al*, 1992). The binding specificity of a particular integrin may vary according to the cell type that expresses it. For example, α2β1 is a collagen receptor when expressed by platelets and a collagen/laminin receptor when expressed by endothelial cells (Kirchhofer *et al*, 1990). Urokinase Plasminogen Activator-Receptor (uPA-R) associates strongly with β1 integrins of normal thyroid cells. The level of glycosylation of uPA-R may control its interaction with integrins (McClatchey, 1999).

Integrins are activation-dependent molecules and can exist in active and inactive forms. Activation of integrins, by natural or synthetic ligands, is usually accompanied by a conformational change rendering the molecule with more affinity for its ligand (Shattil *et al* 1985, Gulino *et al* 1990, Kouns *et al* 1990, Andrieu *et al*, 1991, Parise, 1987, Sims *et al*, 1991, Frelinger *et al*, 1991). The effective activation stimuli for integrins again vary depending on the integrin and upon the cell type on which it is expressed. Integrin expression can be temperature-regulated: for example, temperatures of 40°C can *in vitro* increase the avidity of leucocyte α4β7 for MAdCAM-1 on endothelial cells (Evans *et al*, 2000). *In vitro* Activation can also be accomplished by phorbol esters and more physiologically by various inflammatory mediators such as Tumour Necrosis Factor (TNF) and the complement protein C5a or by cross-

linking of the ligand itself (Hynes, 1992, Chammas, 1991). Integrins are not only active in adhesive events but also act as signal transducers allowing the extra- and intra-cellular environments to communicate with each other. This intracellular signalling utilises several second messenger pathways, including activation of protein kinases and G-proteins, cytoplasmic alkalisation and tyrosine phosphorylation (Schwartz, 1993, Hynes, 1992).

β-subunit	α-Subunit	Ligand
β1	α1	Collagen, laminin
β1	α2	Collagen, laminin, α3β1
β1	α3	Collagen, entactin, epiligrin, fibronectin, laminin, α2β1, α3β1
β1	α4	Fibronectin, VCAM-1
β1	α5	Fibronectin
β1	α6	Laminin
β1	α7	Laminin
β1	α8	?
β1	αv	Fibronectin, Vitronectin
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αm	iC3B, fibrinogen, Factor X, ICAM-1
	αx	iC3B, fibrinogen
β3	α11b	Fibrinogen, fibronectin, thrombospondin, vWF, vitronectin
	αv	Collagen, fibrinogen, fibronectin, osteopontin, thrombospondin, vitronectin, vWF
β4	α6	Laminin
β5	αv	Vitronectin
β6	αv	Fibronectin
β7	α4	Fibronectin, MAdCAM, VCAM-1
	αIEL	?
β8	αv	?
β9	α?	?

Table 1.1 Dimers Of Alpha And Beta Integrin Subunits And Their Ligands
iC3B=inactivated complement component C3, ICAM=intercellular cell adhesion molecule, MAdCAM=mucosal addressin cell adhesion molecule, VCAM=vascular cell adhesion molecule, vWF = von Willebrand factor.

Acting as true signalling receptors, ligand binding of certain integrins affects gene expression and differentiation of specific cell types; these include induction of specific protease genes in synovial fibroblasts via $\alpha5\beta1$, inhibition of terminal keratinocyte differentiation by fibronectin acting via $\alpha5\beta1$, and modulation of myogenesis and apoptosis in leukaemia (Werb *et al*, 1989, Menko and Boettiger, 1987, Sugahara, 1994). The occupation of integrin receptors leads to focal adhesion kinase (FAK) activation (Ning Wen,, 1999). Therefore, many of the integrin-mediated signaling events may be downstream of FAK activation.

1.1.5 The Selectin Superfamliy

1.1.5.1 Introduction

Selectins are a CAM family of three highly homologous transmembrane glycoproteins; namely, L-selectin (CD62L) expressed by leucocytes, E-selectin (CD62E) expressed by the endothelium and P-selectin (CD62P) expressed by platelets and endothelial cells (Laffon and Gonzalez-Amaro, 1995).

1.1.5.2 Structure Of Selectins

All three selectins have a cytoplasmic domain, a transmembrane region and a unique and characteristic extracellular domain (Tedder *et al*, 1995). The extracellular domain comprises an amino terminal Ca^{2+}-dependent lectin region, an EGF-like motif, and a variable number of repeated units homologous to the short consensus repeats (SCR) of the complement binding proteins such as CR1, CR2 and decay accelerating factor (Diagram 1.7) (Chapman *et al*, 1995).

1.1.5.3 Function Of Selectins

Selectin function is uniquely restricted to the vascular system. Selectins mediate heterotypic interactions between blood cells and high endothelial venules (HEV) of peripheral lymph nodes (PLN) during lymphocyte homing, as well as the initial attachment of leucocytes to endothelial cells in inflammation (Pignatelli and Vessy, 1994).

The expression of E-selectin, with six SCRs, is induced and tightly regulated at the transcriptional level by inflammatory mediators such as, Interleukin-1β (IL-1β), Tumour Necrosis Factor-α (TNFα), Interferon-γ (IFNγ), substance P and lipopolysaccharide (LPS) (Bevilacqua *et al*, 1989, Bevilacqua and Nelson, 1993). E-selectin expression reaches maximum levels four to six hours after activation of the endothelial cell and declines to basal levels by twenty four to forty eight hours post-stimulation (Ley *et al*, 1993).

L-selectin, which is the smallest selectin with only two SCRs, is constitutively expressed by almost all circulating leucocytes and is involved in leucocyte trafficking,

extravasation and homing. Optimal L-selectin function involves a change in receptor affinity after cellular activation (Tedder *et al*, 1995). Subsequent reversible loss of L-selectin after cellular stimulation results from endoproteolytic release of the receptor from the cell surfaces (Chen *et al*, 1995).
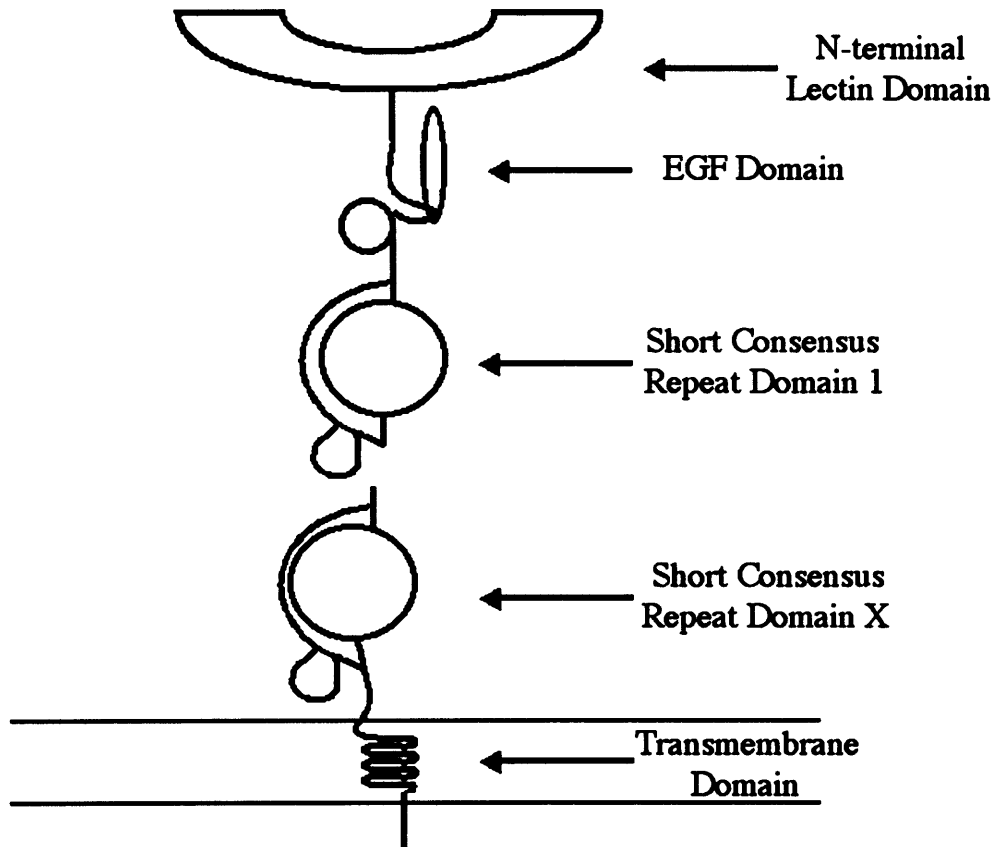
Diagram 1.7 Schematic Representation Of The Selectins. All selectins contain an N-terminal lectin domain, an Epidermal Growth Factor (EGF)-like domain, a variable number of short consensus repeat domains, and a transmembrane domain.

L-, P- and E-selectin are most closely related in amino acid sequence in the lectin and EGF domains. Although these three molecules are distinct molecules with little apparent associations, they are directly involved in cell adhesion and may determine the specificity of ligand binding (Graves *et al*, 1994, Kansas *et al*, 1994). It has been postulated that the SCR domains contribute indirectly to adhesion by serving as structural elements necessary for proper presentation of the lectin-EGF domain: these SCR domains may function to stabilise receptor structure, mediate receptor oligomerisation, or extend the lectin-EGF domains the appropriate distance from the membrane for optimal ligand binding activity (Tedder *et al*, 1995).

Selectins primarily bind to carbohydrate determinants that are sialylated and fucosylated. The prototype ligand for E- and P-selectin is the tetrasaccharide sialyl Lewis^x antigen (sLe^x) or CD155: others include sLe^a, cutaneuos lymphocyte antigen (CLA) and CD34 on endothelial cells (Feizi, 1994, Rosen and Bertozzi, 1994). The precise proteins and lipids that express these carbohydrates are under investigation.

Although E- and P-selectin bind to similar, if not identical, carbohydrate moieties, a host of glycoprotein ligands unique to E- or P-selectin have been identified, including E-selectin ligand-1 (ESL-1), a variant of the Fibroblast Growth Factor-Receptor (FGF-R). There are some glycoprotein ligands, however, that both E- and P-selectin recognise, including P-selectin glycoprotein ligand-1 (PSGL-1). There is the suggestion that E- and P-selectin recognises two categories of glycoprotein ligands: one class being monospecific and the second being common for both endothelial cell selectins (Tedder *et al*, 1995).

1.2 The Prostate Gland

1.2.1 Anatomy of the Prostate Gland

The prostate, the largest male accessory gland, is located deep in the pelvis surrounding the urethra at the neck of the urinary bladder (Kumar and Majumder, 1995).

a)

b)

c)
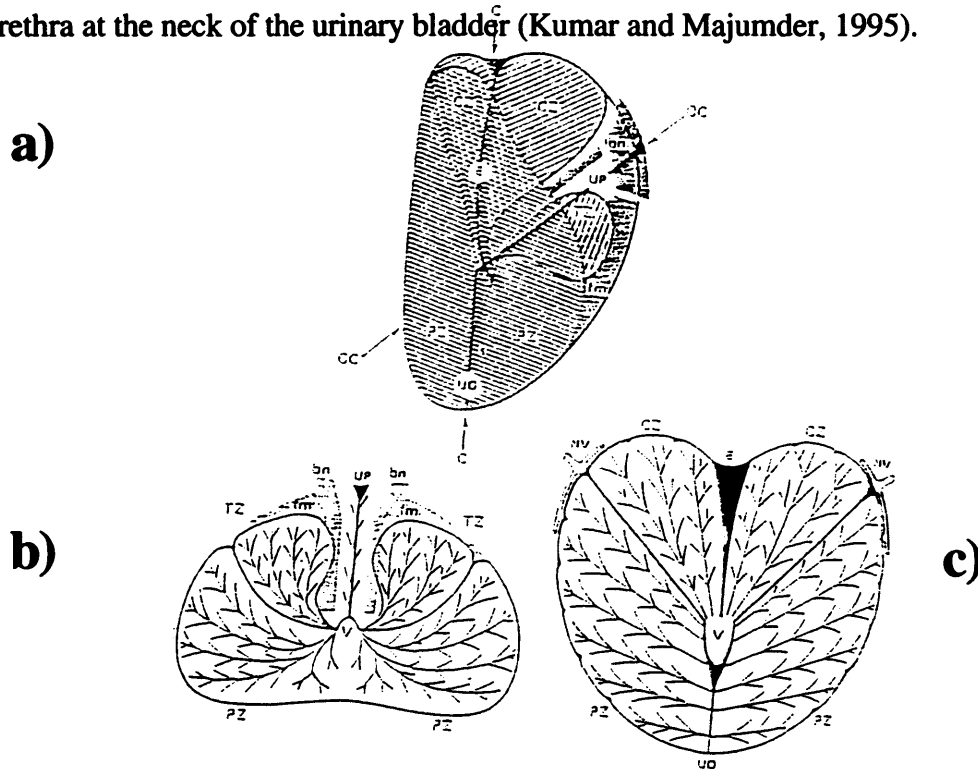
Diagram 1.8 Schematic Representation Of The Prostate. a) A sagittal diagram of distal prostatic urethral segment (UD), proximal urethral segment (UP) and ejaculatory ducts (E) and their relationship with the non-glandular tissue, of the bladder neck (BN), the fibromuscular stroma (fm), and the preprostatic sphincter (s). b) Oblique coronal (OC) section of the prostate showing location of the transition zone (TZ) and peripheral zone (PZ) and their relationship to the verumontanum (V), the preprostatic sphincter (s) and the bladder neck (bn). c) Coronal (C) section of the Prostate showing the central zone (CZ) and peripheral zone (PZ) in relation to the ejaculatory ducts (E). The neurovascular bundles (NV) are located at the junction between the central and peripheral zone.

The human prostate is a composite organ consisting of several glandular and non-glandular components that are tightly fused together within a common capsule (McNeal, 1988). Each of the distinct prostatic glandular regions drains in to a different segment of the urethra. The prostatic urethra shows a sharp $35°$ angulation of its posterior wall at its midpoint between the prostate apex and the prostate base at the bladder neck. This point of angulation, where the verumontanum is found, divides the prostatic urethra into proximal and distal segments of equal length but markedly different anatomical features (Figure 1.5a) (McNeal, 1972).

The distal urethral segment receives the ejaculatory ducts and the ducts of about 95% of the glandular prostate, known as the peripheral and central zones (Figure 1.5c). The peripheral zone comprises about 70% of the glandular prostate mass: its ducts arise from the urethral wall

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as a double row extending from the base of the verumontanum to the prostate apex (McNeal, 1968). The central zone comprises 25% of the total glandular volume of the prostate: its ducts arise in a small focus on the convexity of the verumontanum and immediately surrounding the ejaculatory ducts openings. The central zone ducts branch directly towards the base of the prostate along the entire course of the ejaculatory ducts (McNeal, 1968). The most lateral central zone ducts run parallel to the most proximal peripheral zone ducts, separated only by a narrow band of stroma (McNeal, 1988).

The proximal urethral segment is associated with approximately 5% of the prostatic glandular tissue, and the transition zone (McNeal, 1978) represents almost all of this. Two small, independent lobes, whose ducts leave the urethra at a single point just proximal to the point of angulation, represent the transition zone (Figure 1.5b). The transition zone ducts branch out towards the bladder neck at the prostate base (McNeal, 1988). The glandular tissue of the transition zone is histologically identical to that of the peripheral zone, as described above. The periurethral gland region is the smallest region of the glandular prostate, being only a fraction of the size of the transition zone. The ducts of this region are scattered along the length of the proximal urethral segment and branch into the surrounding periurethral-urethral smooth muscle stroma (McNeal, 1988).

The non-glandular tissues of the prostate are the preprostatic sphincter, the striated sphincter, the anterior fibromuscular stroma, and the prostatic capsule. The preprostatic sphincter is a cylinder of smooth muscle fibres surrounding the proximal urethral segment (Figure 1.5)(McNeal, 1972).

A thin fibroelastic tissue layer (Kumar and Majumder, 1995) encapsulates the prostate. This prostatic capsule consists of an inner layer of smooth muscle fibres and an outer collagenous membrane. There is no capsule at the bladder neck and where the ejaculatory ducts enter the prostate. The terminal acini of the central and peripheral zone, but not those of the transition zone and periurethral glands, abut on the capsule (McNeal, 1988).

The prostate is innervated by the autonomic nervous system. Branches arise from the pelvic plexus, which is formed by parasympathetic visceral efferent preganglionic fibres that arise from the sacral centre, and sympathetic fibres that arise from the thoracolumbar centre (Lepor et al, 1985). Visceral branches arising from the pelvic plexus spread to several ganglia on the prostate capsule. Small nerve trunks originating in these ganglia form smaller branches that penetrate the capsule, extending distally towards the prostate apex innervating the corpora cavernosa at the base of the penis (Eggleston and Walsh, 1985).

There are three vascular zones within the prostate. The capsular zone consists of vessels that branch from the connective tissue surrounding the gland. These branches then radiate centripetally and downwardly through the peripheral and central glandular zones, and these are known as the intermediate zone of vessels (Clegg, 1956). A major arterial branch enters the prostate at each side of the bladder neck and runs towards the verumontanum parallel to the course of the proximal urethral segment. These branches supply the periurethral gland region and the medial transition zone (McNeal, 1988). The third vascular zone, the urethral plexus,

lies accompanying the ejaculatory ducts, surrounding and supplying the urethra itself (Clegg, 1956).

1.2.2 Physiology of the Prostate Gland

The prostate gland participates in the control of urine output from the bladder and in the transmission of seminal fluid during ejaculation. These expulsions are induced by adrenergic stimulation of the smooth muscle cells in the prostate and bladder neck (Blandy, 1989).

The prostate contributes to the seminal fluid, constituting approximately 15% of the normal human ejaculate. The ingredients of the prostatic secretions include various enzymes, lipids, metal ions and amines, as seen in Table 1.2 (Kumar and Majumder, 1995). These secretions are thought to facilitate male fertility. Fibrinolysin and coagulase, amongst other prostatic enzymes, participate in the liquefaction of the seminal coagulates. Prostatic fluid reduces the acidity of the urethra safeguarding sperm viability. The hydrolysis of phosphorycholine to choline by prostatic acid phosphatase (PAP) provides nutrition for spermatozoa. Prostatic-secreted albumin enhances the motility of epididymal washed spermatozoa (Walsh *et al*, 1992). The high level of zinc in human seminal plasma appears to originate primarily from the prostate and acts as an antibacterial agent (Fair and Wehner, 1976). The prostatic production of 5-α-reductase induces rapid metabolism of local testosterone to more potent dihydrotestosterone (DHT), thereby influencing processes under hypothalamic and hypophyseal control (Williams and Chisholm, 1976).

Prostatic epithelium contains a small population of isolated, randomly scattered endocrine-paracrine cells, which contain a variety of peptide hormones (Di Sant'Agnese and De-Mesy-Jensen, 1984). These cells rest on the basal cell layer, which is a mantle of cells separating the secretory cells from the basement membrane and stroma (McNeal, 1988). Their specific role in prostate biology is unknown.

1.2.3 Regulation of Normal Development in the Prostate Gland

The proliferative pool of prostatic epithelium is localised in the basal cell layer. The secretory epithelium represents the differentiated compartment of the prostate and is of limited proliferative potential. The differentiating pathway from basal cell to luminal secretory cells is an androgen-dependent procedure (Bonkhoff and Remberger, 1995). Indeed, the regulation of normal prostatic differentiation and growth requires a hormonal balance between circulating androgens and oestrogens and locally derived growth factors (Aumuller, 1991).

Testosterone is the most important androgen in the male. Testosterone is transported from the testicles and adrenal cortex to the prostate via vascular circulation. It is translocated to the nucleus and reduced to DHT by 5-α-reductase, an integral protein of the outer nuclear membrane (Sinowatz *et al*, 1995). The interaction of DHT with defined sequences of certain

genes regulates prostatic behaviour (Davies and Eaton, 1991). Withdrawal of this hormonal support results in drastic metabolic changes and involution of the prostate. This regression is reversible upon re-instatement of hormonal support (Wright *et al*, 1996).

While mesenchymal effects on epithelial development form the basis of organogenesis during foetal and neonatal periods, analogous stromal-epithelial interactions continue throughout life and presumably have a homeostatic role (Hayward *et al*, 1996). Stroma is an imprecise term that denotes the 'non-epithelial' compartment of an organ. For most internal urogenital organs, the principal cells in stroma are fibroblasts and smooth muscle cells. The immediate microenvironment of adult prostatic epithelium comprises primarily smooth muscle cells as well as ECM surrounding the epithelial ducts. One of the novel characteristics of prostatic smooth muscle is its dependence on androgenic stimulation for differentiation and maintenance of its phenotype: the smooth muscle cells of the prostate express androgen receptors (Prins *et al*, 1991). As a working hypothesis, Hayward *et al*, (1996) propose that prostatic cell growth and differentiation are regulated by reciprocal smooth muscle- epithelial cell interactions mediated by the local production and action of GF's and other paracrine-acting mediators, as described below.

Acid Phosphatase	Fibrinolytic Enzymes
Albumin	Inositol
α-amylase	Magnesium, Zinc, Sodium
β-glucoronidase	Peptide Hormones
Cephalin	Plasminogen Activator
Cholesterol	Phospholipids
Choline	Seminin
Citric Acid	Proteolytic Enzymes
Dermatan	Spermine
Diastase	Spermidine

Table 1.2. Compounds Secreted By the Normal Adult Prostate Gland

A number of polypeptides, which either stimulate or inhibit growth, have been identified in the prostate. These additional growth factors (GF) include members of the fibroblast growth factor (FGF) family, transforming growth factor-β (TGFβ), epidermal growth factor (EGF), nerve growth factor (NGF), and the less well-characterised osteoblast growth factor (OGF) (Gregory *et al*, 1986, Traish and Wotiz, 1987, Jacobs *et al*, 1988, Kyprianou and Isaacs, 1988, Miller-Davies *et al*, 1988, Peehl *et al*, 1989, Wilding *et al*, 1989, Moses *et al*, 1990, Fiorelli *et al*, 1991, Graham *et al*, 1992, Sinowatz *et al*, 1995).

1.3 Carcinogenesis

1.3.1 Introduction

To quote the Chambers Science And Technology Dictionary, cancer is "a disorderly growth of epithelial cells which invade adjacent tissue and spread by the lymphatics and blood vessels to other parts of the body".

In order to understand the development and differentiation in tissues that have adopted abnormal growth patterns, as in cancer, one must first understand the nature and regulation of the normal cell cycle.

Most cells capable of reproduction begin processes of cell division once they reach a critical size and/or receive an appropriate signal. The principle objective of a dividing cell is to achieve the production of a pair of identical daughter cells, both of which contain exact and complete copies of the DNA present in the parent cell. Additionally, the cell mass and sub-cellular organelles must be doubled. The result is two daughter cells with an identical functional potential as the parent cell (Leake, 1996).

1.3.2 The Cell Cycle

The cycle is divided into different phases, each representing a period with a particular function. Mitosis (the M phase) is the division of the nucleus that results in equal distribution of duplicated genetic material to the two daughter cells. Mitosis is a continuous process that is divided into four stages; namely, prophase, metaphase, anaphase, and telophase.

In prophase the genetic material condenses into coiled chromosomes, which are visible under light microscopy. The nuclear membrane begins to break down during prophase and this process is completed by the late stages of prophase, allowing the chromosomes to migrate towards the centre of the cell. The microtubules of the cell begin to form the spindle apparatus, necessary for chromosome movement (Leake, 1996). During metaphase the duplicated chromosomes line up across the middle of the cell along the spindle microtubules (Leake, 1996). The beginning of anaphase is marked by the segregation of the two subunits (or chromatids) of each chromosome. Each chromatid then becomes an independent chromosome. The mitotic spindle elongates at this point, aiding the chromosomal separation (Leake, 1996). During telophase, the two daughter nuclei begin to separate from each other to each pole of the spindle apparatus. The spindle apparatus then disintegrates and the condensed chromosomes begin to uncoil. This allows nuclear re-organisation, including the assembly of the nuclear membranes and the reappearance of the nucleoli (Johnson, 1987). Non-nuclear cellular material, including the plasma membrane, separates during the process of cytokinesis and this marks the end of the M phase (Johnson, 1987).

Following completion of mitosis dividing cells can then enter one of three phases. Cells that are programmed to continually divide proceed into G1 phase (where G is for growth). During G1, gene transcription and protein synthesis occurs, providing the necessary enzymes for DNA replication. Following G1 cells enter a period of DNA synthesis known as the S phase. Upon completion of the S phase G2 arises, during which it is thought that the enzymes and proteins required for mitosis are synthesised. From G2 cells progress into the M phase and the cycle begins again (Leake, 1996). However, cells with evidence of damaged DNA can enter G1, but not the S phase: instead, these cells are sent into programmed cell death or apoptosis. Terminally differentiated cells enter a sustained resting period known as the G0 phase.
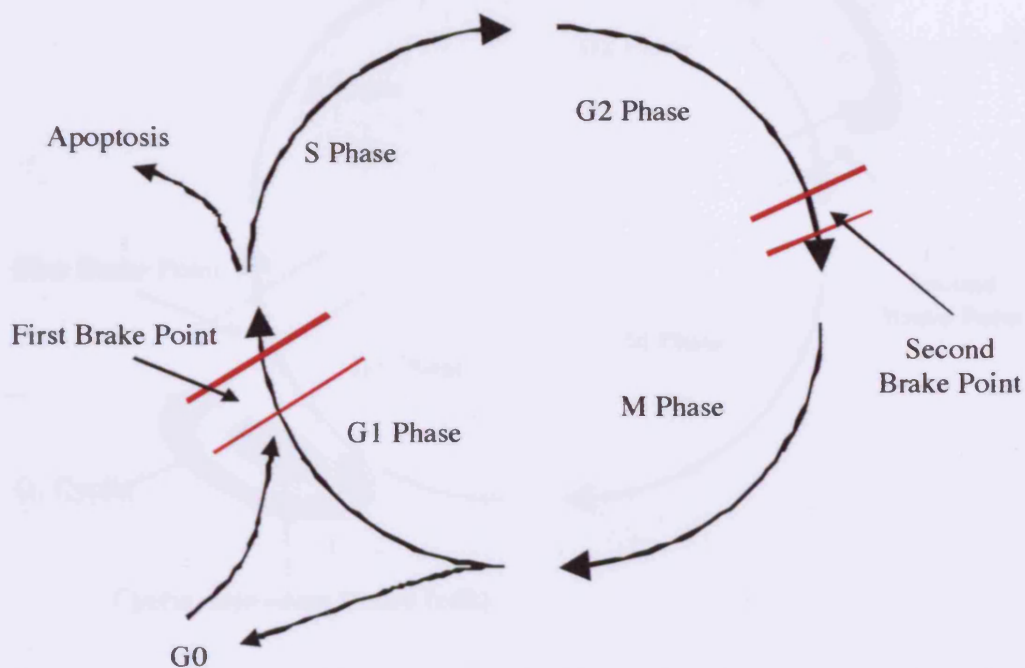
Diagram 1.9 Schematic Representation Of A Typical Cell Cycle. Following mitosis cells can either enter a resting phase (G0) or the first growth phase (G1), a period of DNA synthesis (the S phase) and the second growth phase (G2) before another round of mitosis, or enter the first growth phase by progress into programmed cell death (apoptosis). Two brake points must be overcome to enter the S phase and the M phase.

1.3.3 Regulation of the Cell Cycle

There are two principal control points, or brake-points, at which progress through the cell cycle can either be stopped or promoted. The first is in the late stages of the G1 phase and the second is at the end of the G2 phase immediately before entry into the M phase. It is the action of both exogenous factors, such as hormones and GF's, and endogenous factors, such as cell size, protein content, Ca^{2+} concentration, DNA condition, levels of metabolic stress, and the cyclins and cyclin-dependent protein kinase (Cdk) family, at these two brake points that push a dividing cell through the cell cycle (Diagram 1.10). It is believed that exogenous factors

are most likely to act only during the G1 phase, whereas the endogenous factors are capable of acting at both brake-points. For example, two classes of cyclins have been designed by nature to act specifically at either the G1 brake point (the G1 cyclins) or at the G2 brake-point (the mitotic cyclins) (Leake, 1996). It has been hypothesised that the exogenous factors, such as hormones and growth factors, act to stimulate the transcription of 'early response genes' such as *myc*, *jun*, and *fos*. The products of these genes, in turn, induce the transcription of the Cdk's and cyclins (Schuchard *et al*, 1993, Alvarez *et al*, 1991). Cdk-activating kinases then stimulate the activity of the Cdk's by inducing their phosphorylation (Leake, 1996).
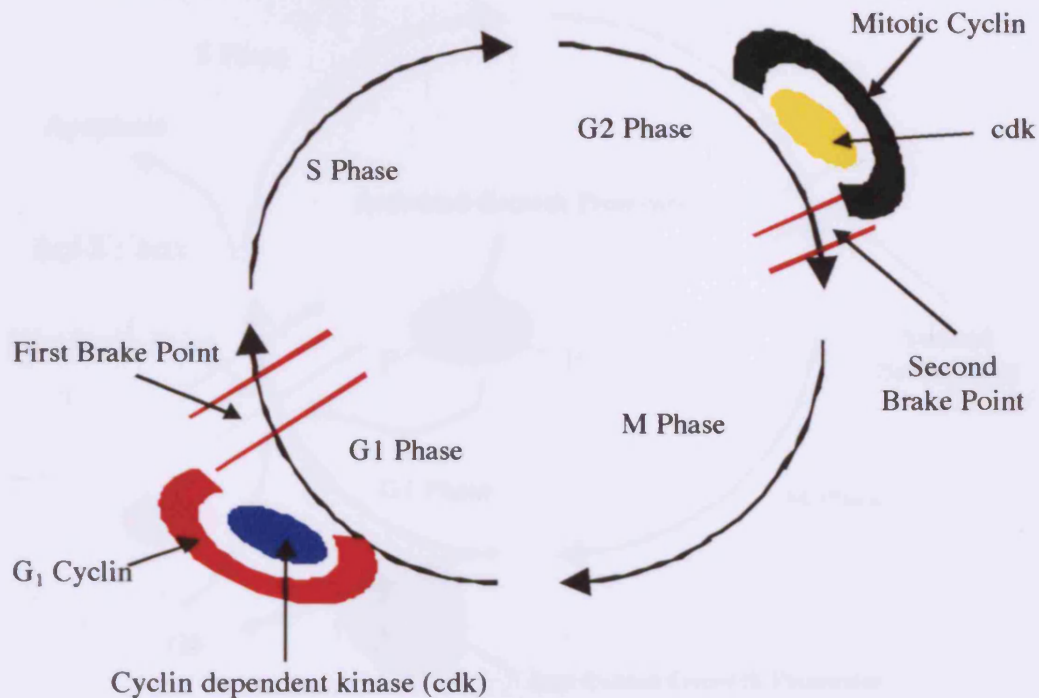
Diagram 1.10 Schematic Representation Of The Cyclin Control Mechanisms Of The Cell Cycle. G1 and G2 cyclins combine with cyclin dependent kinase (cdk) in order to push the cell through the first and second brake points of the cell cycle.

The product of the retinoblastoma (Rb) gene and the p53 protein are two proteins that inhibit the entry of cells into the S phase, unlike the cyclin family of proteins that promote entry of the cells into this phase. Dephosphorylated Rb protein binds to and inactivates transcription factors of the genes for *myc* and *fos*, thereby inhibiting progression of the cell into S phase and promoting entry of the cells into G0. However, once the Rb protein is phosphorylated the transcription factors are released and *myc* and *fos* are then able to induce an increase in the cyclins, which, in turn, pushes the cell through the G1 brake point (Leake, 1996) The p53 protein monitors the quality of the DNA in the cell before replication can take place. If the p53 protein detects any defects in the DNA the S phase is blocked (Diagram 1.11). The p53 protein promotes transcription of growth inhibiting-proteins and blocks the transcription of growth-promoting proteins (Diagram 1.11). This allows time for DNA repair enzymes to restore the damaged DNA to its original state. Indeed, the p53 protein is only detectable in cells that contain damaged DNA. If the amount of DNA damage is too great the cell is pushed into

programmed cell death (apoptosis). Similarly, cells that are likely to be damaged can be pushed into apoptosis: however, these cells are protected from apoptosis by the *bcl-2* protein (Diagram 1.11). The *bcl-2* protein and its close family member *bax* function as dimers. The *bcl-2 / bcl-2* dimer promotes cell survival, the *bax / bax* dimer promotes apoptosis and the *bcl-2 / bax* dimer has intermediate effects (Oltvai *et al*, 1993). Interactions between these proteins provide further control mechanisms of the cell cycle. p53 is thought to influence the relative amounts of *bcl-2* and *bax*, thereby influencing the nature of the active dimer and thus pushing for either cell division or apoptosis (Selvakumaran *et al*, 1994).
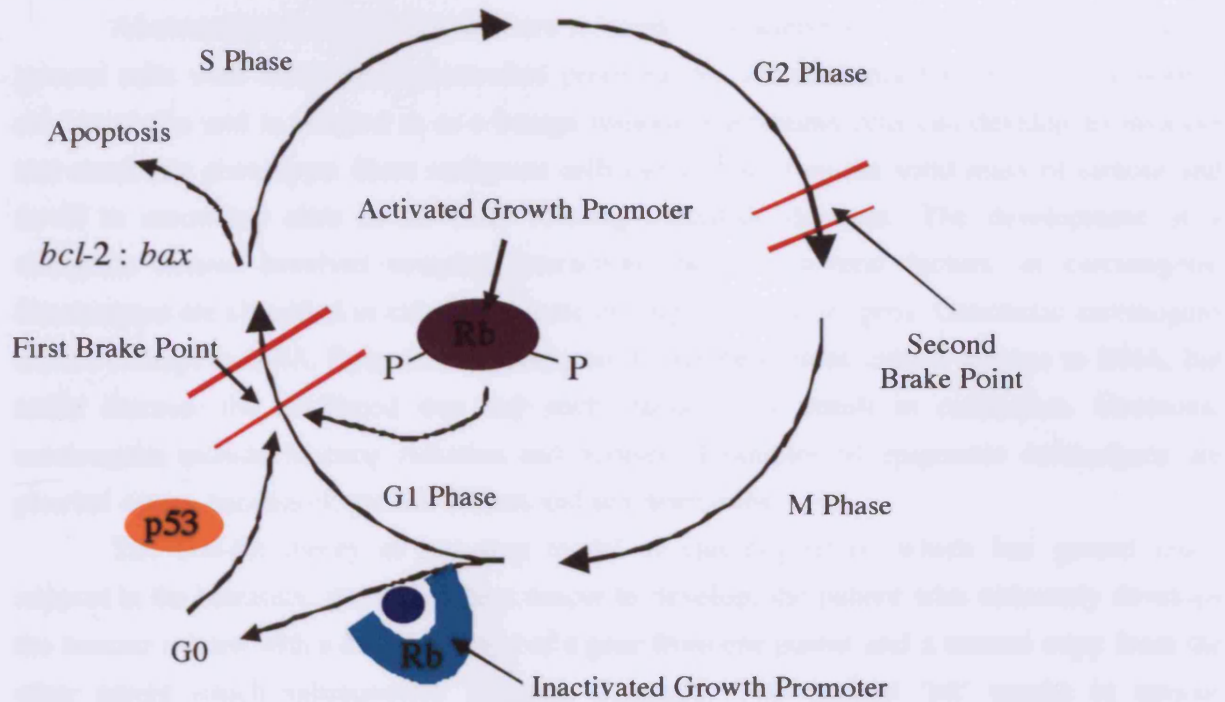
Diagram 1.11 Schematic Representation Of The Critical Control Mechanisms Of The Cell Cycle.

1.3.4 Abnormalities of the Cell Cycle

Successful regulation of the cell cycle ensures that most tissues remain healthy throughout life, providing a balance between cell division and programmed cell death (apoptosis). However, clinical complications in replicating tissues reflect a breakdown in this policing mechanism.

The Rb gene was first discovered in patients who suffer from very rapid and excessive proliferation of the cells of the immature retina. These patients had lost both copies of the Rb gene (Cobrinik *et al*, 1992). Two proteins, E6 and E8, coded by Human Papilloma Virus bind and activate the p53 and Rb proteins (Leake, 1996). This action is thought to be the molecular basis of HPV-induced cervical cancer.

PTEN is a phosphatidylinositol phosphatase that antagonises activation of the PIP3 kinase pathway involved in cell growth by directly dephosphorylating two tyrosine

phosphorylated proteins: it is mutated in many ovarian cancers (Tamura *et al*, 1999). The catalytic subunit of PIP3-kinase is frequently activated in ovarian cancer. Transfection of PTEN into ovarian cancer cell lines significantly inhibited their growth. Therefore, PTEN has been suggested as an oncogene. Mechanisms involved appear to be arrest of the cells in G1 and increased expression of the αv integrin (Minaguchi *et al*, 1999).

1.3.5 General Aspects of Carcinogenesis

Abnormalities of epithelial cells are referred to as adenomas and / or carcinomas. Both present cells with localised, uncontrolled proliferation. An adenoma has no other abnormal characteristics and is referred to as a benign tumour. Carcinoma cells can develop an invasive and metastatic phenotype: these malignant cells can escape from the solid mass of tumour and travel to secondary sites in the body forming metastatic deposits. The development of a malignant tumour involves complex interactions between several factors, or carcinogens. Carcinogens are classified as either genotoxic or epigenetic carcinogens. Genotoxic carcinogens induce damage to DNA. Epigenetic carcinogens do not themselves induce damage to DNA, but rather increase the likelihood that any such damage will result in carcinoma. Genotoxic carcinogens include ionising radiation and viruses. Examples of epigenetic carcinogens are phorbal esters, saccharin, growth factors and sex hormones.

The two-hit theory or two-step model of carcinogenesis, which has gained much support in the literature, states that for a cancer to develop, the patient who ultimately develops the tumour is born with a damaged copy of a gene from one parent and a normal copy from the other parent which subsequently becomes damaged. This second 'hit' results in tumour development (Vogelstein and Kinzler, 1993). It is unlikely, however, that a single 'second hit' results in a full malignant phenotype: this requirement that a 'normal' cell must pass through a series of sequential changes on its way to displaying this malignant phenotype, ensures that the end point of this process is only rarely reached.

1.3.6 Development of Metastatic Carcinoma

In 1829 Recaimer first coined the term metastasis to describe the process of tumour cell dissemination (Morgan-Parkes, 1995). In 1878 Billroth reported the presence of neoplastic cells within vascular thrombi and hypothesised that tumour metastasis occurs when fragments of such thrombi break off and embolise in the circulation. In 1889 Paget postulated the 'seed and soil' theory: this theory stated that a metastasis arose from a proliferation of tumour cells (the 'seeds') in the favourable milieus provided by certain organs (the 'soil'). Forty years later Ewing postulated the 'mechanical entrapment theory': he hypothesised that the first organ encountered by the tumour cells would be the site of greatest tumour arrest and the largest

number of metastatic colonies. The process of tumour growth and metastasis is clearly a complex process: it is probable that all four hypotheses are correct and not mutually exclusive.

Cancer is defined clinically as a breakdown of tissue organisation and the acquisition of invasiveness and is a complex cascade of events: a) tumour growth, invasion, and release of neoplastic cells from the primary tumour: b) movement of tumour cells into the lymphatics and vasculature: c) survival of the tumour cells in the circulation and interactions of the cells with platelets and with the clotting system: d) arrest of the tumour cells in distant sites via interactions with the vascular or lymphatic endothelium and/or the subendothelial basement membrane: e) migration of the tumour cells into the tissue parenchyma; and f) growth of the tumour at the metastatic site (Albelda, 1993). A very small fraction of the tumour cells found at the primary loci are thought to possess any metastatic ability and, of the small percentage that do, successful migration may only occur in the event of one cell: however, that one cell is sufficient to initialise the growth of distant metastases (Kerbel, 1990). Many of the above steps involve either increases or decreases in the ability of the tumour cells to adhere to each other and the surrounding extracellular matrix (ECM), thereby disturbing the integrity of their local environment (Albelda, 1993). CAMs mediates this adhesion: these molecules are functional in many processes including leucocyte recirculation and extravasation (Fawcett, 1992). The process of leucocyte transendothelial migration has been compared to that of tumour cells at distant metastatic sites (as in steps d) and e) above).
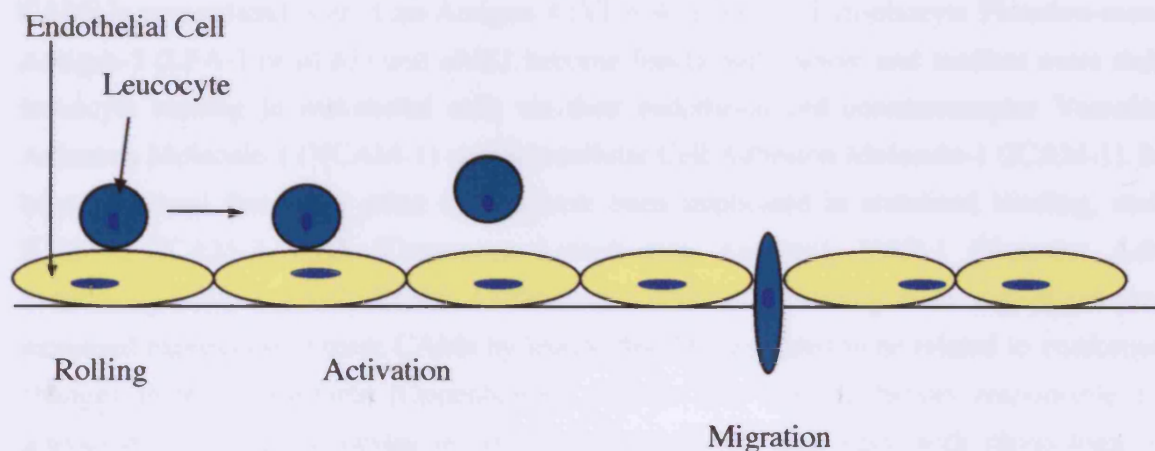
Diagram 1.12 Schematic Representation Of Transendothelial Migration (TEM) of Leucocytes. TEM is a multi-step process including leucocyte rolling, leucocyte and endothelial cell activation, followed by migration of the leucocyte through the endothelial cell layer.

Leucocyte extravasation is exquisitely regulated *in vivo* by mechanisms that display extraordinary specificity. A number of leucocyte and endothelial CAMs are thought to participate in the interaction between these two cells, including members of the adhesion receptor families above (Picker, 1992). Leucocyte-endothelial cell (EC) interactions are regarded as active processes requiring at least three sequential events, namely; a) reversible rolling, b) leucocyte activation and stabilised binding, and c) trans-endothelium migration (Butcher, 1991) (Diagram 1.12)

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Step 1 Reversible Rolling Mediated By The Selectins

Firstly, free flowing leucocytes interact loosely with the endothelial cells, "rolling" along affected segments of the venular wall. This primary adhesion, initiated by binding constitutively active leucocyte CAMs to endothelial cell counterparts, is transient under physiologic shear force and reversible unless secondary adhesion mechanisms are stimulated. This rolling temporarily slows the transit of leucocytes through inflamed venules, allowing them to investigate the endothelial cell surface for activating or chemoattractant signals (Butcher 1991). *In vitro* studies suggest that L-selectin presents leucocyte carbohydrate ligands such as sialyl Lewis x (sLe^x) Antigen to the endothelial E- and P-selectin mediating this temporary rolling along the venule wall (Picker, 1992). Other constitutively expressed CAMs, including CD44 and CD31 (Platelet Endothelial Cell Adhesion Molecule-1, PECAM-1) have been implicated in this primary adhesion (Butcher, 1991). Indeed, relatively recently it was shown that this rolling of T and B cells could be blocked with monoclonal antibodies against leucocyte CD44 and endothelial cell HA (Degrendele *et al*, 1996). HA is expressed on vascular endothelial cells. IL-15 can augment this HA expression and aid in transendothelial migration (Estess *et al*, 1999).

Step 2 Leucocyte Activation

Activation of leucocytes stimulates rapid and dramatic changes in the cells' activity (Butcher, 1991). L-selectin is shed and the functional expression of several integrin CAMs is upregulated. Very Late Antigen-4 (VLA-4 or α4β1), Lymphocyte Function-associated Antigen-1 (LFA-1 or αLβ2) and αMβ2 become functionally active and mediate more stabilised leucocyte binding to endothelial cells via their endothelial cell counterreceptor Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intracellular Cell Adhesion Molecule-1 (ICAM-1). It must be emphasised that many other CAMs have been implicated in stabilised binding, including ICAM-2, ICAM-3, CLA (Cutaneous Lymphocyte Antigen), VAP-1 (Vascular Adhesion Protein-1), LFA-2, CD2, CD48, CD58 and CD59: this binding does not result from an increased expression of these CAMs by leucocytes, but appeared to be related to conformational changes in their structures (Oppenheimer, 1994). The specific factors responsible for the activation of rolling leucocytes *in vitro* is unknown and may vary with physiologic setting (Butcher, 1991).

Step 3 Leucocyte Transendothelial Migration

In contrast to leucocyte-endothelial cell binding, a limited number of CAMs have been identified that mediate the transendothelial migration (TEM) of leucocytes. Receptor blocking experiments with monoclonal antibodies have demonstrated that ICAM-1 and LFA-1 independent of the activation state of endothelial cells mediate TEM of T cells. VCAM-1, VLA-4 or E-selectin play no role in this migratory process (Oppenheimer-Marks, 1991). The signalling events that lead to TEM have not been delineated, but they appear to involve protein kinase C, as treatment of T cells with phorbol esters stimulates motility and TEM (Oppenheimer-Marks *et al*, 1990). Binding of adhesion receptors may induce signals that regulate TEM. For example, enhanced activation of T cells occurs when cells are pre-treated

with monoclonal antibodies that recognise two different surface antigens, including the combinations of CD3 and HLA Class I, CD3 and CD4/8, HLA Class I and CD4/8 or LFA-1 and CD3, followed by cross-linking (Wacholtz *et al*, 1989). Therefore, the LFA-1 molecule itself can transmit a stimulatory signal to T cells that results in enhanced activation. This ligation of receptors involved in leucocyte – endothelial cell binding may not only be important in mediating cell-cell contact, but also in transmitting signals that alter the functional capacity of cells (Diagram 1.13).
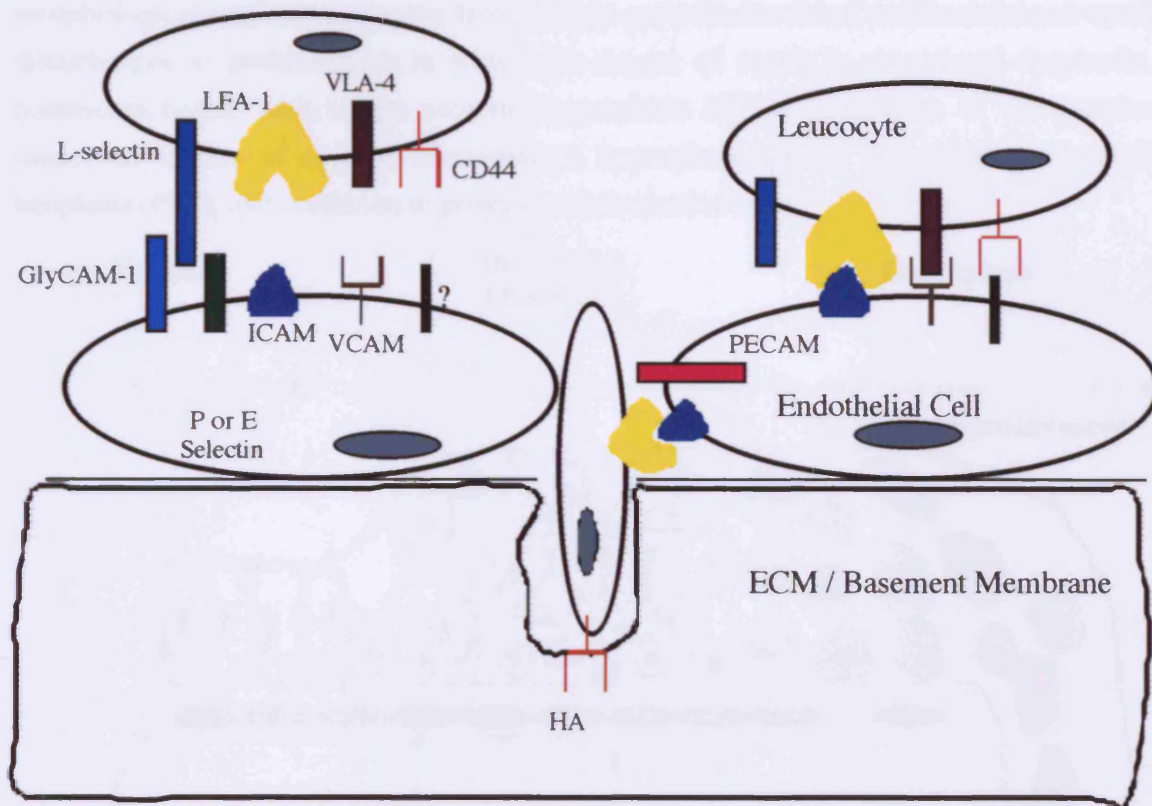
Diagram 1.13 Schematic Representation Of Leucocyte Extravasation. This is the current multi-step model of leucocyte interaction with endothelial cells. Initial rolling is mediated through E-selectin on the endothelial cell. Stabilised binding is negotiated via Lymphocyte Function-associated Antigen-1 (LFA-1) and Very Late Antigen-4 (VLA-4) on the leucocyte and Intercellular Cell Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) on the endothelial cell. Transendothelial migration is thought to involve endothelial Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) and ICAM-1 and leucocyte CD44 and LFA-1.

This model implies that leucocyte-endothelial cell recognition and extravasation can be controlled at any one of these three steps, therefore providing a combinatorial mechanism for generating both specificity and diversity. There may be additional steps beyond those discussed above permitting even more diversity in leucocyte-endothelial cell recognition (Butcher, 1991).

The rationale that tumour cell metastasis may arise in a similar manner to leucocyte extravasation has prompted investigations into CAM expression in different cancers.

1.4 Carcinoma Of The Prostate Gland

1.4.1 Introduction

Disorders of the prostate gland range from bacterial induced inflammation (prostatitis) to prostatic hypertrophy or hyperplasia. Irregular proliferations within prostatic ducts form a morphological continuum, ranging from benign growths devoid of architectural and cytological disturbances to proliferations in which the degree of atypia is considered dysplastic. This continuum begins with benign prostatic hyperplasia (BPH), progresses to the putative, pre-cancerous lesions of atypical adenomatous hyperplasia (ATH) and prostatic intraepithelial neoplasia (PIN), and continues to prostatic adenocarcinoma.
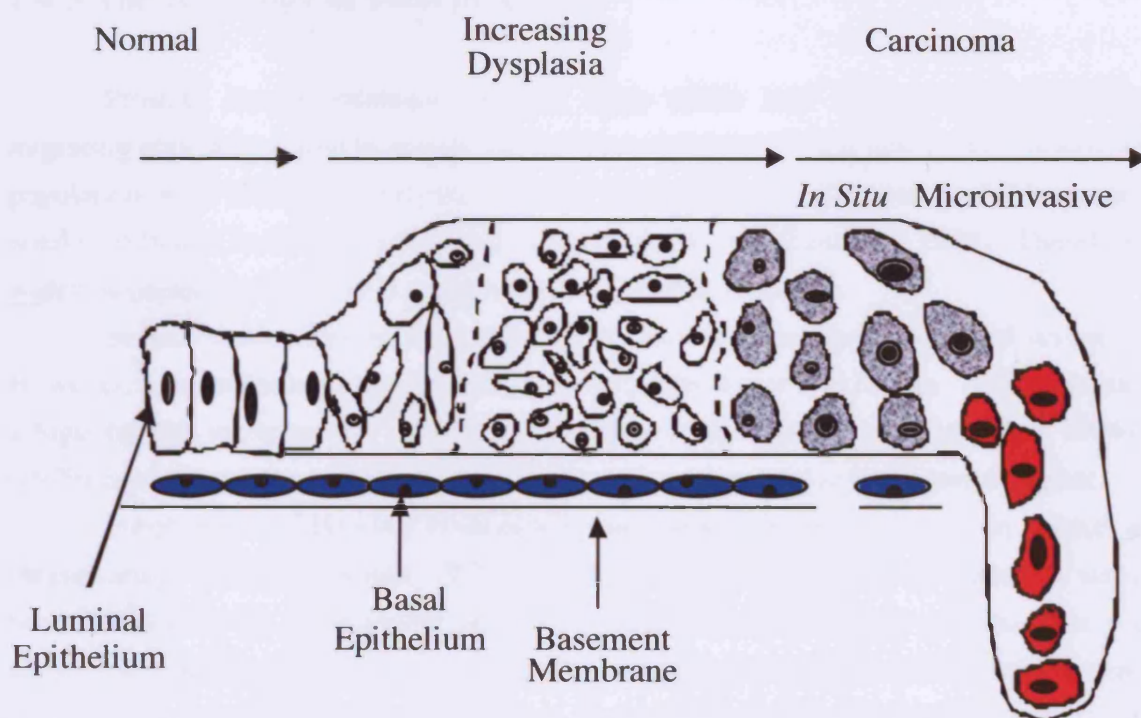
Figure 1.14 Schematic Representation Of The Dysplasia That Occurs During The Development of Carcinoma Of The Prostate. According to the continuum, disease initiation corresponds to very mild dysplasia with epithelial cell crowding and irregular spacing. As dysplasia and disease progress, epithelial cell crowding and spacing increase. Following the onset of Prostatic Intraepithelial Neoplasia (PIN), a state that displays severe dysplasia, luminal epithelial cells have large nuclei. In advanced PIN (grey cells), also considered by some to be the equivalent, histologically, of early stage carcinoma, the basement membrane is disrupted and luminal epithelial cells have large nuclei with increased levels of chromatin and large, visible nucleoli. The endstage dysplasia is represented by invasive carcinoma (red cells). (Adapted from Bostwick, 1989).

1.4.2 Incidence of Prostate Cancer

The incidence of prostate cancers has risen in recent years throughout most of the western world: in 1995, 12000 new cases were diagnosed in England and Wales and over 8500

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deaths were reported (Savage and Waxman, 1996). Prostate cancer is now the most prevalent cancer in men. However, prostate cancer is a slow growing cancer with an *in vivo* doubling time estimated to be between four months and two years: this growth has led many clinicians to report that men will die with, rather than because of, their (prostatic) cancer. Indeed, 244000 cases of prostate cancer were reported in the United States in 1995, but only 35000 deaths were reported (George, 1996). Clinicians also report difficulty in predicting the mortality risk of newly diagnosed prostate cancers. This has resulted in great controversy, with regard to the development of prostate cancer screening programmes and subsequent treatment regimes. However, prostate cancer remains the most commonly diagnosed cancer in men: despite this, relatively little is known about the aetiology of the disease.

1.4.3 The Aetiology of Prostate Cancer

Prostate cancer incidence exhibits large ethnic and international differences and migrating populations tend to acquire the incidence patterns of their new home. Groups within a population with different lifestyles, such as Seventh Day Adventists and Mormons, have notably different incidences of prostate cancer (Boyle and Zardidze, 1994). Therefore, it is widely accepted that lifestyle is a relative risk of prostate cancer.

Several studies have investigated the relative risk of diet, body mass and sexual activity. However, few studies have clearly established prostate cancer risk factors. It is established that a high fat diet increases the incidence of prostate cancer (Boyle and Zardidze, 1994). Lew (1979) confirmed links with overweight males and an increased risk of prostate cancer.

Imbalances in hormonal homeostasis can induce prostate tumours in animal models (Mainwaring, 1979, and Noble, 1977). Sexual activity, an indicator of hormonal status, has been linked with the development of prostate cancer. Prostate cancer patients have a greater sexual drive, but are less sexual active than their normal counterparts: cancer patients experience puberty and first intercourse at a later age. Sexually transmitted infections, such as Herpes Virus 2, simian virus 40 (SV40) and cytomegalovirus have been implicated, inconclusively, in prostate cancer development (Boyle and Zardidze, 1994).

A genetic aetiology had been proposed for prostate cancer, but studies have presented inconsistent results (for review see Sandberg, 1992). However, the "two-hit" or "two-step" theory of cancer genesis has gained much support in recent years, as described in Chapter 1.3.5. It is most probable that a series of "second-hits" induces a malignant phenotype and that each change on it's own is highly unlikely to promote malignancy. In general, although a great amount of research has been completed, very little has conclusively been described as a risk factor for prostate cancer.

1.4.4 Histological Characterisation of Prostate Cancer

Single epithelial cells or groups of epithelial cells that have a specialised secretory function are collectively known as glands. Prostatic epithelial cells form what is known as an exocrine gland where the secretory products are discharged into a ductal system, as described in Chapter 1.2. *In vivo*, epithelial cells form continuous sheets of tightly adhering cells. *In vitro*, epithelial cells grow in cobblestone-like aggregates with little motility. Tight junctions, adherens junctions and desmosomes maintain the tight intercellular contacts. A consequence of this structure is the polarised characteristic of epithelial cells where distinct proteins are expressed on the basolateral (i.e. adjoining the basement membrane) or apical surface (Rodriguez-Boulan and Nelson, 1989). The integrity of the adherens junctions, maintained by E-cadherin and associated cytoplasmic proteins, is critical for the maintenance of the functional characteristics of epithelial cells. Hemidesmosomes are located on the basal surface of the epithelial cells connecting them to the basement membrane (Sonnerberg *et al*, 1991A, Garrod, 1993). The basement membrane separates epithelial cells from the underlying mesenchymal cells. Characteristic constituents of a basement membrane include laminin, collagen type IV, entactin, and basement membrane proteoglycans. Interactions between epithelial cells and the basement membrane include $\alpha6\beta1$ and $\alpha6\beta4$ binding laminin and $\alpha1\beta1$ binding collagen (Sonnerberg *et al*, 1991B).

Prostatic carcinomas are defined clinically and histologically using the TNM classification and Gleason Scoring systems. The TNM classification system describes the extent of malignant disease in an individual patient, thereby facilitating the categorisation of a patient and, more importantly, the possibility to compare groups of patients in multi-centre clinical trials and studies. The TNM classification system considers the primary tumour (T), the regional lymph node involvement (N), and development of distant metastases (M) (Chisholm *et al*, 1994). The Gleason scoring system examines prostatic carcinomas microscopically to determine the level of epithelial disruption and interaction with the surrounding stroma. The Gleason system defines prostatic carcinomas as having a score of 1 to 5 (Table 1.3). A Grade 1 tumour is described as a sharply defined rounded tumour with compact, distinct glandular structures of uniform size and shape, with little or no infiltration of the surrounding stroma. Conversely, a Grade 5 tumour is described as an anaplastic carcinoma with poorly defined margins that severely infiltrate surrounding stroma (Gleason, 1977).

The cytological changes occurring throughout carcinogenesis of a normal prostate can also be documented histologically as the stage of differentiation of a tumour. A very well differentiated prostatic tumour bears no real histological alteration from the normal prostate and contains columnar epithelial cells with clear cytoplasms. Tumours can progressively become well differentiated, moderately differentiated, poorly differentiated and finally very poorly differentiated. Cells within a poorly differentiated tumour are polygonal, pleomorphic with a non-polarised nucleus, which has prominent nucleoli (Table 1.4).

Thus, one of the prominent morphological changes in malignant adenocarcinomas is a loosening of intercellular adhesion. This is a consequence of a functional disturbance of the cell-

	Glandular-Architectural Differentiation	Tumour-Stromal Relation	
	Distinctive Gland Formation	**Boundary of Tumour Mass**	**Stromal Infiltration**
Grade 1	Distinct glands; uniform size and shape; closely packed	Sharply defined, rounded	Negligible
Grade 2	Distinct glands, irregular size and shape; variable interglandular spacing	Defined, but less sharp than Grade 1	Smooth along major stromal planes
Grade 3A	Distinct glands; irregular size and shape; increased interglandular spacing	Ill defined, ragged	Buts upon major and smaller fibre planes
Grade 3B	Abortive, minute and cell clusters **Uncohesive Growth**	Ill defined, ragged	Buts upon major and smaller fibre planes
Grade 3C	Rounded masses, cribiform or papillary	Sharply defined, rounded	Capable of expansion
Grade 4A	Apparently fused glandular tumour	Ill defined, ragged	Severe, across smaller fibre planes
Grade 4B	Gatherings of pale cells with hypernephroid (kidney shaped) appearance	Ill defined, ragged	Severe, across smaller fibre planes
Grade 5A	Solid tumour masses	Sharply defined	Capable of expansion
Grade 5B	Diffusely infiltrating anaplastic carcinoma	Poorly defined, ragged	Severe, across stromal fibres

Table 1.3 The Gleason System Of Grading Prostatic Carcinoma.

cell contacts described above. This loss of intercellular adhesion is a crucial step in carcinogenesis progression and was first proposed nearly 60 years ago (Coman, 1944). Once the epithelial cells loose their homotypic adhesiveness, they become more capable of escaping from the primary tumour: they can invade through the basement membrane and mesenchyme, gaining access to the lymphatic and vascular circulatory systems. With the knowledge that CAMs are crucially responsible for maintaining intercellular adhesion, along with their role in leucocyte extravasation, their role in the progression of metastatic prostatic carcinoma becomes an obvious line of investigation

Some cancers, or a genetic predisposition for some cancers, can be identified by the presence of tumour specific antigens, tumour oncogenes or tumour proto-oncogenes. For example, colorectal cancers can be identified by their absence of the DCC (Deleted in Colorectal Carcinoma) gene product. The presence of BRCA1 (Breast Cancer Antigen1) and BRCA2 in

Tumour Grade	Cytoplasmic	Nuclear	Nucleolar
Very Well Differentiated	Columnar cells; clear cytoplasm	Basal location; condensed, uniform chromatin	Very rarely present
Well Differentiated	Columnar cells; clear cytoplasm	Basal location; condensed, uniform chromatin	Very rarely present
Moderately Differentiated	Cuboidal cells; granular, non-clear cytoplasm	Central location; "open" chromatin network	Frequent, prominent, basophilic
Poorly Differentiated	Cuboidal, polygonal, pleomorphic cells	No polarisation; pleomorphic, hyperchromatic or vesicular	Frequent, prominent, often acidophilic
Very Poorly Differentiated	Cuboidal, polygonal, pleomorphic cells	No polarisation, pleomorphic, hyperchromatic or vesicular	Frequent, prominent, often basophilic

Table 1.4 Cytological Changes In The Various Tumour Differentiation Grades.

the germline is highly correlated with a genetic predisposition for breast carcinoma. However, while it is relatively easy to characterise a prostatic carcinoma using the TNM and Gleason systems, it is more difficult to isolate tumour specific antigens or genetic markers. Some early studies raised antibodies that appear to show specificity for prostatic tumours (Bazinet *et al*, 1988, Beckett *et al*, 19991, Berthon *et al*, 1995, Brawer *et al*, 1988, Kim *et al*, 1988, Murakami *et al*, 1995). However, these studies contained only a few isolated cases of prostatic carcinoma and the antigens could not be identified on all prostatic tumours. More than one tumour can exist within the prostate gland and these tumours may have different histological characteristics. Therefore, the immense heterogeneity of and within prostatic tumours is the most likely explanation for the lack of a prostatic carcinoma specific antigen.

1.4.5 Metastatic Progression of Carcinoma of the Prostate

The mode and pathway of metastasis of carcinoma is under debate. There are two basic mechanisms of metastatic development: a) a one step process, in which primary cancer cells directly disseminate to the site of metastasis, and b) a multi-step or cascade process, in which primary cancer cells are seeded in key metastatic sites and the key sites are then responsible for producing numerous metastases.

Experimental models of tumour metastasis to bone marrow in rodents have helped in the elucidation of metastatic spread. Intracardiac injection of human melanoma cells into a nude rat resulted in bone marrow deposits in almost all animals. However, intravenous injection of the human tumour cells resulted in lung metastases only (Kjonniksen *et al*, 1990). Likewise, intravenous injection of large numbers of B16 melanoma tumour cells into the mouse resulted in lung metastases only, while those injected intracardiacally resulted in widespread organ involvement, including the skeleton (Arguello *et al*, 1988). Notably, intracardiac injection of fewer cells resulted in only skeletal and ovarian metastases. These data support the theory that tumour cells are seeded in key metastatic sites and then disseminate to tertiary sites.

Approximately 50% of men with cancer of the prostate have clinically advanced disease at the time of presentation (Rinker-Schaeffer *et al*, 1994). With the exception of the local pelvic lymph node involvement, bone and more specifically bone marrow of axial bones are the almost exclusive locality of such metastatic disease (Cumming *et al*, 1990, Scalliet, 1996). However, prostatic carcinoma cells also frequently metastasise to the lungs, liver, and bladder and, less frequently, to other organs of the body, including the ureter and seminal vesicles (Saitoh *et al*, 1984). Bone marrow metastases generally consists of clumps or sheets of large pleomorphic cells with prominent nuclei, but cells can also be associated with myofibroblasts (Papac, 1994). Bone metastases, originally thought to be osteoblastic in nature, are increasingly becoming associated with bone erosion. These erosive changes are also seen at sites that are remote from the actual deposits (Clarke *et al*, 1991). In some men, these metastases develop rapidly, while others survive for many years with localised disease (Johannson *et al*, 1989). However, with a poor prognosis for men with metastatic cancer of the prostate, it is crucial that it is understood how and why the cancer spreads

In prostatic carcinoma, which is highly heterogeneous, the 'seed and soil' theory of metastatic spread is suitable. As discussed in Chapter 1.3.6, this theory supports the idea that a particular tumour cell has a favourable milieu or optimal requirement and therefore, that each kind of tumour has its own preferential site of metastasis. However, in the process of dissemination to that soil, the tumour cells could still employ one of the two mechanisms above. The main argument concerning the metastatic spread of prostatic carcinoma specifically is whether the prostatic cells employ the vertebral venous system or the systemic circulation. Vesalius first identified the vertebral venous system, but the identification of its importance is attributed to Batson (1940). He injected the cadaveric dorsal vein of the penis with radio-opaque material and showed the connection with the prostatic plexus and subsequently the pelvic veins, pelvic bones and the sacral canal. Therefore, the vertebral venous system, which directly connects the prostatic plexus of blood vessels to the sacral canal of blood vessels, provides direct access of prostatic carcinoma cells to the favourable milieu of the bone. Batson argued that he had replicated the spread of prostatic metastases, providing evidence to support the vertebral venous system being the channel through which the malignant cells pass to their implantation sites in the bones. However, this has not received uniform acceptance and others have argued that the systemic circulation is the major pathway of prostatic carcinoma

dissemination. Willis, for example, was of the opinion that prostate cancer spread to the pulmonary circulation initially and from here tertiaries metastasised to the bone, amongst other organs: i.e. that the carcinoma cells seeded in the lung, which served as a key metastatic site, and then disseminated to other organs, including the bone. Franks felt that the early spread of prostate cancer was probably through the vertebral venous system but that later, as the tumour mass increased, the systemic circulation was involved (Cumming *et al*, 1990). The systemic circulation provides an indirect route for the prostatic carcinoma cells to the bone, via many other organs, and therefore possible metastatic sites.

One needs to remember that, while prostatic carcinomas metastasise to the bone and bone marrow primarily, metastatic deposits are also seen in many other organs, including the lungs. Therefore, both the vertebral venous system and systemic circulation are likely to be employed by metastatic prostatic cells.

It is not altogether surprising that the bone marrow provides a favourable milieu for metastasising carcinoma cells. The bone marrow is the main site of haematopoietic growth factor (HGF) production and action, including Stem Cell Factor (SCF), members of the Fibroblast Growth Factor (FGF) family, the Transforming Growth Factor (TGF) family, the Interleukin (IL) family, the colony stimulating factor family (CSF), and the bone morphogenetic protein (BMP) family (Nicola, 1989). IL-1, IL-6, and GM-CSF stimulate, while IL-4 inhibits myeloma tumour cell growth (Kawano *et al*, 1989, Zhang *et al*, 1990, and Herrmann *et al*, 1991). These and other HGFs could be responsible for the migration and / or secondary growth of metastatic prostatic adenocarcinoma cells. Many of these growth factors (GFs) are known to promote or inhibit the growth and development of normal prostatic cells, as described in detail in Chapter 1.2.3. Many tumour cells of non-haematopoietic origin have been shown to express both SCF and its receptor, *c-kit* (Turner *et al*, 1992). Prostate cancer cells themselves are known to secrete and express receptors for TGFβ and bFGF (Mansonn *et al*, 1989). Neoplastic human and rat prostatic tissue contain mRNA transcripts for members of the BMP family (Harris *et al*, 1994). Two prostatic carcinoma cell lines, PC3 and Du145 proliferate in response to conditioned medium from unstimulated human, rat and bovine bone marrow. Non-prostatic tumour cells lines show little or no response to the same medium (Chackal-Roy *et al*, 1989). The proliferative activity found in the bone marrow can not be duplicated by biological concentrations of a variety of growth factors alone or in combination, including EGF, aFGF, bFGF, Platelet Derived Growth Factor (PDGF) or thrombospondin, TGF, Granulocyte (G)-, Monocyte (M)- or GM-Colony Stimulating Factor (CSF). However, conditioned medium from bone marrow stromal cells specifically increases growth of PC3 and Du145 cells to equivalent levels observed with the bone marrow conditioned medium (Chackal-Roy *et al*, 1989). Primary tumour cells from mammary carcinomas, a carcinoma that metastasises to the bone marrow, demonstrate increased growth on monolayers of both irradiated and non-irradiated bone marrow stromal cells (Strobel *et al*, 1989). These data suggest that the bone marrow stromal cells, and perhaps specifically radioresistant stromal cells, may provide the favourable milieu to which prostatic carcinoma cells metastasise.

1.4.6 Materials Used in the Study of Cancer of the Prostate

The rat has been used heavily to investigate diseases of the prostate. Unfortunately, the human prostate differs considerably from the rodent prostate in embryological development, adult anatomy and aetiology of disease. Therefore, the rat is an inadequate model for investigating human malignancy of the prostate.

From the late 1970s attempts were made to cultivate and serially passage human prostate cells, both benign and malignant, in cell cultures. Three immortal cell lines have been established from metastatic deposits of prostate cancer found in the brain, supraclavical lymph node and bone marrow, Du145, LNCaP and PC3, respectively (Stone *et al*, 1978, Horozewicz *et al*, 1983, Kaighn *et al*, 1979). The reliability of these cell lines has been questioned, most importantly with respect to their prostatic origin. PC3 and Du145 cells do not express or secrete the widely accepted biochemical markers of prostatic cells, PAP or PSA. PC3 cells do not express the androgen receptor and are androgen-insensitive (Kozlowski *et al*, 1992). Controversy exists over the androgen status of Du145 cells: clonal variation has arisen so that some Du145 cells express while other cells do not express the androgen receptors (Brolin *et al*, 1992). LNCaP cells express both PAP and PSA and are androgen-sensitive, but not androgen-dependent. LNCaP cells do express the androgen receptor, but it appears to be mutated (Kozlowski *et al*, 1992). More recently, new cell lines have been established. However, these cell lines have been derived by genetic manipulation of the already existing Du145, PC3 and LNCaP cell lines (Terouanne *et al*, 2000).

These limitations highlighted the need for a primary cell line of prostatic carcinoma. This need has been meet with limited success. Some success has been reported with serum free growth media, which are highly supplemented with various growth factors including EGF and bovine pituitary extract in the culture of prostatic epithelial cells (Peehl *et al*, 1992, Kozlowski *et al*, 1992). Loop *et al* (1993) established the primary prostatic tumour cell line, ALVA-31, from a biopsy taken at the time of radical prostatectomy. This cell line grows in standard serum-supplemented RPMI1640 medium. PSA, which was expressed by the cells at the initial stages of culture, was lost throughout continued passage of the cell line. Brothman *et al* (1989) established the PPC-1 cell line from a poorly differentiated prostatic adenocarcinoma; however, this cell line is maintained in a medium that is heavily supplemented with serum.

Xenotransplantation, or heterotransplantation, of human prostate tumours into immunodeficient mice, such as the Severe Combined ImmunoDeficient (SCID) mouse, which has a mutation on the T cell maturation pathways, or the Nude mouse, which has no thymus, has been proposed as a model for studying prostate cancer. Unfortunately, the success rate of this has been very poor and only two models have been established: PC-82 and HONDA that are derived from an androgen dependent primary and secondary of prostate cancer, respectively (Hoehn, *et al*, 1980).

1.5 The Role of Cell Adhesion Molecules in Metastatic Cancer

1.5.1 The Role of Cadherins in Cancer

It is generally accepted that cell surface expression of E-cadherin is down regulated in solid tumours. This has been demonstrated in prostate, colorectal, gastric, stomach, oesophageal, breast, lung and squamous cell cancers (Umbas *et al*, 1992,1994, Dorudi *et al*, 1993, Pignateli and Vessey, 1993, Bongiorno *et al*, 1995, De Bruin *et al*, 1999). Bongiorno *et al* (1995) also demonstrated that the E-cadherin expression seen in lung cancers is disorganised. Many cancer cell lines also show reduced levels of E-cadherin expression, including breast cancer MCF-7 cells (Bracke *et al*, 1994). An inverse relationship has been demonstrated, in both rats and humans, between E-cadherin expression and that of the integrin β2 subunit (Murant *et al*, 1997 and MacCalman *et al*, 1994).

More recently, a clinical correlation has been demonstrated between the expression of P-cadherin and the progression of carcinoma. For example, the expression of P-cadherin by breast cancers is indicative of poor clinical prognosis (Soler *et al*, 1999). Smythe *et al* (1999) show reduced levels of P-cadherin expression in non-small cell lung cancers.

Cytoplasmic E-cadherin is connected to the cytoskeleton via many cytoplasmic proteins, including p120 and the catenins. Decreased levels of p120 are seen in bladder cancers compared to their normal counterparts. Moreover, this downregulation correlates with histological grade and clinical progression of the tumour (Syrigos *et al*, 1998). Davies *et al* (1999) further show that the loss of both E-cadherin and its cytoplasmic protein, β-catenin, by bladder cancer cell lines increases their metastatic capacity *in vivo*.

The importance of these cytoplasmic proteins in the activity of E-cadherin has mostly been demonstrated in cancer studies. For example, a lung cancer cell line (PC9), that does not demonstrate good cell to cell aggregation, shows normal E-cadherin expression but no α-catenin (Shimoyama *et al*, 1992). Likewise, PC3 cells show decreased α-catenin expression, but normal E-cadherin expression (Morton *et al*, 1993). This prompted investigators to analyse the expression of E-cadherin in association with its cytoplasmic proteins. The expression of the E-cadherin – catenin complex is decreased in nasopharyngeal cancers (Lou *et al*, 1999). Huang *et al* (1999) found normal expression of E-cadherin and α-catenin, but decreased levels of β- and γ-catenin in neoplastic thyroid when compared to normal thyroid. Re-distribution of β-catenin to the nucleus is observed in colorectal cancers: this re-distribution is associated with the stage of differentiation of the cancer (Hugh *et al*, 1999). Desmoplakin is a protein associated with desmosomes and associates here with the desmosomal cadherins to form a cell adhesion complex. In breast cancers, these complexes redistribute below the plasma membrane. Moreover, an inverse correlation occurs between the level of cell surface desmoplakin expression and the clinical progression of breast cancers (Davies *et al*, 1999). A decrease in

plakoglobin, another desmosomal-associated protein, is associated with decreased E-cadherin expression in breast cancer cell lines. These E-cadherin⁻ cells lines are N- and P-cadherin⁺ (Giroldi *et al*, 1999). Squamous cell carcinomas show decreased expression of E-, N- and P-cadherin, but increased levels of cadherin-associated proteins, desmoglein-1 and -2 and desmocollin (De Bruin *et al*, 1999). This suggests that the tumour cell may attempt to overcome the loss of E-cadherin expression. Indeed, some advanced solid adenocarcinomas that show loss of E-cadherin but retain weak Ca^{2+}-dependent adhesion: this has been shown to be due to cadherin-11 expression, which also associates with α and β catenins. Two variant forms of cadherin-11 appear to be expressed in invasive, but not in non-invasive, adenocarcinoma cell lines of the breast (Pishvaian *et al*, 1999). There is the possibility that cadherin-11 variants interact with ECM components and actively promote invasion and migration.

Most investigators demonstrate a poor prognostic correlation between the loss of E-cadherin and / or its cytoplasmic proteins (Bongiorno *et al*, 1995, Syrigos *et al*, 1998, Nanashima *et al*, 1999). Madin Darby Canine Kidney (MDCK) epithelial cells transformed with a Harvey-sarcoma express lower levels of E-cadherin and adopt a fibroblastic phenotype. When both the transfected and the normal cells are incubated in the presence of antibody against E-cadherin their invasion into both collagen gels and embryonic chick hearts decreases, indicating that the lack of E-cadherin increases the invasiveness of the cell line (Behrens *et al*, 1989). The lung carcinoma cell line PC9 described above possesses poor cell to cell aggregation (Shimoyama *et al*, 1992). These data suggests that E-cadherin must be functionally present to maintain tight epithelial structures: i.e. that E-cadherin must form homodimeric complexes that associate with a series of intracellular proteins to maintain epithelial integrity. Christofori and Semb (1999) suggest that the loss of E-cadherin by tumour cells may actively convey signals that induce tumour cell invasion and metastasis.

Mutations and point mutations were found in colon cancers in the genomic sequence, 16q, which encodes E-cadherin, (Efstathiou *et al*, 1999). Deletion and mutation of this genetic region correlates positively with metastatic deposits and aggressive tumours of prostatic origin (Li *et al*, 1999). AXIN2, which plays an important role in the regulation of β-catenin stability maps to 17q24, a region that shows frequent loss of heterozygousity in breast cancers (Mai *et al*, 1999). This could be the cause of the reduced E-cadherin and associated protein expression seen in these cancers.

To summarise, the clinical progression of solid cancers, both locally and metastatically, is highly correlated with abnormalities in the complexes formed between E-cadherin and the cytoplasmic catenin and desmosomal proteins.

1.5.2 The Role of CD44 in Cancer

The role of CD44 and its variant isoforms in cancer is unclear. The evidence to date mostly supports the hypothesis that the expression of CD44 and / or its isoforms promotes the progression of carcinoma: there is additional evidence that contradicts this theory.

For example, Denno *et al* (1998) demonstrated that the expression of CD44s by gastric cancers is strongly associated with the development of metastases. However, Sato *et al* (1999) later showed that gastric cancers expressed lower levels of CD44s than their benign counterparts and, furthermore, that the reduction was a result of hypermethylation in the CD44 promotor region.

The gastric cancer cell line, SCM1 expresses high levels of CD44v4-v7. The additional glycosylation sites present in this isoform appear to contain binding domains for Hyaluronan (HA) (Hsieh *et al*, 1999). This suggests that the gastric cancer cells expressing higher levels of CD44 than usual could use stromal HA as an anchor to migrate through the extracellular matrix. Epithelial CD44 (CD44E) is expressed more highly in malignant gastric tissue than its benign counterpart: this increased expression correlates with lymphatic and vascular invasion, but not with the state of differentiation of these tumours (Miwa *et al*, 1996). Likewise, some colorectal cancers have been found to express increased levels of CD44E over their normal counterparts (Imazeki *et al*, 1996). Hara *et al* (1999) observed increased levels of CD44v8-v10-containing isoforms in gastric cancer. The expression of CD44v8-v10 was higher in both the primary and metastatic deposits of liver-metastasising colorectal cancers than in non-metastatic cancers (Takeuchi *et al*, 1995).

Increased levels of CD44s expression was found in prostatic carcinomas than in benign prostatic hyperplastic tissue (Zhang *et al*, 1996). Conversely, no differences were found in the expression of CD44s, CD44v4 and CD44v7-v8 by benign prostatic hyperplastic and prostatic carcinoma tissue by Jethwa *et al* (1997). The prostatic carcinoma cell lines PC3 and Du145 are both found to express CD44s, while LNCaP does not. This lack of expression is thought to be due to hypermethylation in the CD44 promotor as seen by Sato in gastric cancer (Verkaik *et al*, 1999, Sato *et al*, 1999). However, LNCaP does express CD44v6, while PC3 and Du145 cells do not (Stevens *et al*, 1996). This suggests that the differential expression of CD44 isoforms may regulate the method of metastatic spread: i.e. that CD44v6 may be involved in the lymphatic spread of LNCaP cells, and that CD44s may be involved in the haematogenous spread of PC3 and Du145 cells.

Focal loss of CD44v3 and CD44v6 is associated with recurrence-free periods in superficial bladder cancer (Toma *et al*, 1999). Squamous cell lung cancers have been found to express high levels of CD44v6 isoforms that their normal counterparts do not normally express (Fasano *et al*, 1999). Nanashima *et al* (1999) demonstrated the expression of CD44v6 in primary colorectal carcinomas: they demonstrated lower levels of expression on tumours that metastasised than on those that did not. The hepatic metastatic deposits have lower CD44v6

4 1

levels than the primary tumours. There did not appear to be any correlation with histological grade and expression of CD44v6. Increased levels of CD44v6 is seen in both the primary and metastatic deposits of cervical carcinoma (Dong *et al*, 1999). The expression of CD44v6 by primary breast carcinomas appears to correlate with a good clinical prognosis (Foekens *et al*, 1999).

Primary brain tumours rarely metastasise: they do not express CD44 isoforms, but do express CD44s. Conversely, brain metastatic deposits of lung, testicular, cervical, colorectal, tonsil, skin, and kidney cancers are found to express CD44v6-v7 (Li *et al*, 1995). While brain tumours do not metastasise, a cell line derived from a glioma has the capacity to form lesions when injected into the rat. Pre-treatment of these rats with antibodies against CD44s inhibits the development of these lesions, suggesting that CD44 plays a pivotal role in the metastatic spread of these cells (Gunia *et al*, 1999).

CD44v2 is associated with the recurrence and poor clinical prognosis of Duke's B colorectal cancer (Haruyama *et al*, 1999). Some gastric cancers have been found to express higher levels of CD44v5 than their non-malignant counterparts (Stachura *et al*, 1999). Gansauge *et al* (1995) found a correlation between the expression of CD44v5-v6-including isoforms and the progression of pancreatic cancer. The distribution of CD44s, CD44v3, CD44v5, and CD44v6 is increased in basal cell carcinoma cells, especially at the tumour cell – stromal cell level of interaction (Dingemans *et al*, 1999). This suggests that a stromal factor may control the expression of CD44 on the tumour cell.

CD44 is not normally expressed by hepatocytes. Hepatocellular carcinoma cells express CD44s, CD44v5, CD44v6, and CD44v7-v8. Simultaneous expression of all isoforms correlates with poor clinical prognosis: the expression of CD44v6 in particular correlates highly with poorly differentiated hepatocellular carcinomas (Endo and Terada, 2000). El-Wahad and Asaad (1998) also showed that CD44 expression by hepatocellular carcinoma cells correlates with the occurrence of microvascular invasion and increased tumour size. Conversely, Yokoyama *et al*, (1999) found that hepatic cancers with decreased levels of CD44s and CD44v6 had poor clinical prognosis, with increased lymph node metastatic deposits. They further correlated expression of CD44s and CD44v6 with a good clinical prognosis.

Mulder *et al* (1995) examined the expression of CD44 in bowel cancers. Depending on the position of the tumour within the bowel, tumour cells can be either non-invasive or invasive, whether found proximally or distally, respectively. Those non-invasive, proximal tumours are usually more proliferative and are found to express increased levels of CD44v5 and CD44v6 isoforms: these tumours are found to be less well differentiated. These data suggest that CD44, and in particular CD44 isoforms, may play a role in supporting local growth of tumour cells. Supporting this theory, Grimme *et al* (1999) provide evidence that CD44v3$^+$, and not CD44v3$^-$, melanoma cells proliferate in response to basic Fibroblast Growth Factor (bFGF). Further evidence was provided by Schroder *et al* (1999), who found early stage ovarian cancers expressing increased levels of CD44v5 and CD44v6 than their benign

counterparts. This suggested the involvement of CD44 isoforms at the early stages of carcinogenesis, perhaps in a growth regulatory capacity.

Contact of melanoma cells with HA up-regulates their expression of CD44s and CD44v3: increased levels of adhesion to HA is seen with these cells (Yoshinari *et al*, 1999). This suggests that tumour cell-CD44 may employ stromal HA to migrate through the extracellular matrix. High grade eosophageal carcinomas appear to show a decreased level of HA expression. However, the stroma surrounding the malignant lesions expressed more HA than that surrounding non-malignant tissue (Wang *et al*, 1996). These data supports the theory that extracellular HA may act as an anchor aiding in the invasion by tumour cells towards sites of extravasation. Stroma surrounding ovarian cancer lesions is also high in HA (Anttila *et al*, 2000). Although this group did not find a corresponding increase in CD44 isoform expression by the ovarian tumour cells, they did not examine the expression of CD44s. Therefore, this data is still suggestive of tumour cell-CD44 interaction with stromal-HA as a means of metastasising. Indeed, the adhesion of NIH OVCAR6 ovarian cancer cells to peritoneal mesothelial cells can be inhibited with antibodies against CD44 (Lessen *et al*, 1999).

Price *et al* (1996) demonstrate that melanoma cells use their surface HA to adhere to CD44 expressed by endothelial cells. This is highly suggestive that CD44 may play an important role in the haematogenous spread of melanoma. More interestingly, it shows that endothelial CD44 may be important and that the HA expressed by the tumour cell may be important. This is different to the proposed role of stromal HA and tumour cell CD44. Indeed, melanoma cells transfected with HA demonstrate increased migration *in vitro*: this migration could be inhibited with antibodies against CD44 (Ichikawa *et al*, 1999).

Fifteen of 16 leukaemia cell lines injected into Severe Combined ImmunoDeficient (SCID) mice produced tumour cell deposits at the site of injection. These 15 cell lines express CD44v6. Pre-treatment of these cells with antibodies against CD44 reduced the local growth of cells as well as the incidence of organ and lymph node metastatic deposits (Kawasaki *et al*, 1996). Likewise, antibodies against CD44s can inhibit the metastatic deposits seen when glioma cells, 9L, are injected into rats (Gunia *et al*, 1999). The adhesion to collagen, matrigel and Boyden Chamber migration of endometrial carcinoma cells, of the SNG-11 line, is mediated, in part, via CD44: conophylline, a vinca alkaloid, reduces their expression of CD44s and adhesion to reconstituted basement membrane and collagen specifically (Irie *et al*, 1999). This suggests that tumour cell-CD44 may employ extracellular matrix components other than HA to migrate through the stroma. B Cell Chronic Leukaemia (BCCL) cells expressing high levels of CD44 isoforms adhere to HA better than low level expressing cells: these high CD44 expressing cells were highly aggressive and of poor clinical outcome (Zarcone *et al*, 1998). Cross-linking of CD44 on acute myeloid leukaemia cell lines, with either antibodies against CD44 or HA, induces their terminal differentiation, stopping the differentiation blockade that causes acute myeloid leukaemia (Charrad *et al*, 1999). Transfection of PC3 cells with various CD44 isoforms increased their adhesion of HA. However, it decreases their *in vitro* and *in vivo* growth and metastasis (Miyake *et al*, 1998).

Cross-linking of CD44 on colon carcinoma cells enhances their adhesion to endothelial cells *in vitro*, mediated in part through an up-regulation in their β2 integrins. This integrin up-regulation is thought to be mediated, in part, by an increased *c-met* expression and its subsequent interaction with its ligand, HGF (hepatocyte growth factor) (Fujisaki *et al*, 1999).

To summarise, CD44 and CD44 isoforms may be involved in the metastatic progression of many cancers. CD44 expressed by the tumour cell may interact with stromal HA to promote the invasion of the ECM by the tumour cell. Alternatively, HA expressed by the tumour cell may interact with endothelial CD44 thereby promoting the extravasation of the tumour cell and its subsequent haematogenous spread. However, there is also evidence to support an inhibitory role of CD44 in the metastatic spread of some cancers. CD44 may also be involved in the promotion of tumour cell growth, both within the primary and metastatic deposit. In conclusion, the role of CD44 and its isoforms in the progression of metastatic cancer is unclear and requires further investigation.

1.5.3 The Role of Immunoglobulins in Cancer

Circulating VCAM-1 concentrations are increased in many cancer patient groups, including breast, ovarian, gastrointestinal and melanoma (Banks *et al*, 1993). ICAM-1 and VCAM-1 has been demonstrated on the cells of lymph node metastases of malignant lymphoma: high levels of circulating ICAM-1 has been associated with the presence of liver metastases of many cancers (Totsuka *et al*, 1993). ICAM-1 is also expressed on the bone marrow metastatic deposits of breast, colon and prostatic cancers (Putz *et al*, 1999). Increased ICAM-1 expression is seen on adenocarcinoma cells over their non-malignant counterparts (Jiang *et al*, 1998).

CD146, which is also known as Mel-CAM, has only recently been identified as a cell adhesion molecule and its physiological role has still to be determined. It is expressed by melanoma cells and its presence correlates with poor clinical prognosis. Conversely, breast tumour cells that express Mel-CAM have a good clinical prognosis (Shih *et al*, 1999).

T cell lymphoma cells have been shown to release factors that induce the up-regulation of ICAM-1 by endothelial cells (Totsuka *et al*, 1993). Incubation of multiple myeloma cell lines with bone marrow stromal cells induces the secretion of IL-6 by the stromal cells. While this effect does not appear to be contact-dependent it is contact-sensitive and can be inhibited by antibodies against ICAM-1 (Thomas *et al*, 1998). Therefore, it could be postulated that the interaction of myeloma cells with bone marrow stromal cells might involve ICAM-1. Antibodies against VCAM-1 can inhibit adhesion of melanoma cells to cultured endothelial cells (Rice and Bevilacqua, 1989).

C-CAM1 functions as a tumour suppressor agent in carcinoma of the prostate and is diminished in both Prostatic Intraepithelial Neoplasia (PIN) and prostatic adenocarcinoma. This indicates a role for C-CAM1 in the early stages of prostatic carcinogenesis. Mutation of

prostatic carcinoma C-CAM1 increases the incidence of tumour deposits and their volume when cells are transferred into the athymic mouse (Hsieh *et al*, 1999). Recombinant C-CAM1 adenovirus therapy reduces the metastatic development of prostatic carcinoma cells, PC3 cells, when injected into the nude mouse. However, this treatment needs to be maintained, as sustained C-CAM1 expression is required for optimal suppression (Lin *et al*, 1999).

Interleukin-1 (IL-1) increases the expression of ICAM-1 by vascular endothelial cells and aids in the transendothelial migration of neutrophils (Sano *et al*, 1995). Injection of a metastatic melanoma cell line, A375M, into the nude mouse results in metastatic deposits in the lung. These deposits are increased in number by the administration of IL-1, which increased the expression of VCAM-1 by the vascular endothelial cells of the mouse (Garafalo *et al*, 1995). Bone marrow stromal cells express ICAM-1 and VCAM-1. IL-1 increased the expression of VCAM-1 and ICAM-1 by these cells and increases their attachment to CD43 progenitor cells (Teixido *et al*, 1992). These data suggest that tumour cells metastasising to the bone and bone marrow may utilise ICAM-1 and VCAM-1 expressed by these stromal cells.

TGFβ is a known growth factor for hepatic stellate cells. TGFβ was shown to decrease ICAM-1 and VCAM-1 expression, while increasing NCAM-1 expression (Kuehn *et al*, 1999). Nitric oxide induces up-regulation of ICAM-1 by the squamous carcinoma cell line, NA (Toyoshima *et al*, 1999). Anti-tumour agents cisplatin and 5-fluorouracil were found to increase ICAM-1 expression by these NA cells: there was a synergistic effect of the two agents (Takizawa *et al*, 1999).

In conclusion, CAMs of the immunoglobulin superfamily appear to be highly involved in the metastatic progression of many cancers. ICAM-1 and VCAM-1, in particular, appear to be involved in the development of metastatic deposits within the bone and bone marrow.

1.5.4 The Role of Integrins in Cancer

Early studies in metastatic cancer demonstrated a role for integrins in the metastatic process. Humphries *et al* (1986) demonstrate that migration of tumour cells through tissue can be inhibited by RGD peptides. *In vivo* experiments show that dissemination of tumour cells in mice can be inhibited by simultaneous intra-venous injection of RGD peptides together with tumour cells (Rouslahti and Giancotti, 1989).

Normal prostatic cells have been shown to express α2β1 (a collagen receptor), α3β1 (a epilligrin receptor), α4β1 (a fibronectin receptor), α6β1 (a laminin receptor), αvβ1 (a vitronectin receptor), and α6β4 (a hemidesmosomally associated laminin receptor). These integrins are expressed by basal epithelial cells at the basal lamina interphase (Bonkhoff *et al*, 1993). These molecules are expressed in PIN, but the majority are not observed on prostatic carcinoma cell surfaces (Bostwick *et al*, 1989, Cress *et al*, 1995). However, the expression of α3 and α6 can be demonstrated in some prostatic tumours: the distribution of these subunits is no longer polarised at the basal surfaces and appears diffuse upon the whole cell membrane (Cress *et al*,

1995). These data suggest that focal loss of the α6 at the hemidesmosome results in the breakdown of epithelial integrity and a subsequent increase in cell migration through the basement membrane via ligand interaction with laminin. This theory is supported by the data of Pyke *et al* (1994) that demonstrates the presence of laminin at the invasion front of many tumours. Increased expression of α6β4 by Lewis lung carcinoma cells increases the invasiveness of these cells (Perrotti *et al*, 1990). The squamous carcinoma cell line, A431, which expresses α6β4, migrates in response to Epidermal Growth Factor (EGF). EGF induces a rapid redistribution of α6β4 from the hemidesmosome to newly formed cell protrusions (Rabinovitz *et al*, 1999). Hakkinen *et al* (1999) also show depolarisation of the α9 integrin subunit in squamous carcinoma cells. This further suggests that a metastatic phenotype may be conferred by a simple redistribution of integrins and may not necessarily require changes in the level of their transcription and / or expression. α6β4 has also been shown to induce apoptosis when transfected into carcinoma cells that do not normally express it: expression and subsequent clustering of α6β4 with monoclonal antibody against β4 promoted p53-dependent apoptosis (Bachelder *et al*, 1999).

Prostatic tumours and PC3 cells express αvβ3, while normal prostatic epithelial cells and LNCaP cells do not. Transfection of LNCaP cells with αvβ3 confers adhesion to vitronectin (Zheng *et al*, 1999). This suggests different pathways for the lymphatic and venous spread of prostate cancer cells. Ovarian cancer tumour cells express αvβ3 co-localised with the focal contact proteins and focal adhesion kinase (FAK) at the focal contact points (Cruet *et al*, 1999). The migration of ovarian adenocarcinoma cells, IGROV1, in a transfilter migration assay is mediated by their αvβ3 and vitronectin, which is co-localised with the actin stress fibres. The interaction leads to activation of PKC, and other intracellular signaling molecules, PIP-3 and PTK (Carreiras *et al*, 1999). αvβ3 may play a role in the metastatic spread of malignant melanoma. Antagonists of αvβ3 induce apoptosis of melanoma cells, M21: adhesion of these cells to collagen via αvβ3 induces a 5-fold increase in the *Bcl-2:Bax* ratio promoting cell growth (Petitclerc *et al*, 1999). *In Situ* renal carcinomas that have invaded through the basement membrane express elevated levels of αvβ3 compared to those cells which are still confined within the glandular structures of the kidney (Wechsel *et al*, 1999). These data support the role of αvβ3 in the metastatic spread of carcinoma cells and provide further evidence of cell signaling via the integrins: they also emphasise the importance of integrins in the regulation of cell growth as well as in adhesion.

The expression of β1 integrins is increased in poorly differentiated prostatic carcinomas. Conversely, decreased levels of β2 integrins are present in these tumours: however, there does not appear to be any clinical or histological correlation with this decrease (Murant *et al*, 1997). In contradiction to these finding, Lang *et al* (1997) hypothesised a role for α1 and β2 integrins in the adhesion of prostatic epithelial cells and fibroblasts to bone marrow stromal cells. This data and the expression of ICAM-1 (a ligand for α1β2) by bone marrow stromal cells

demonstrated by Teixido *et al* (1992) suggests a role for α1 and β2 in the process of metastatic prostatic carcinoma.

Monoclonal antibodies against α2β3 have been found to inhibit the formation of lung metastases that develop after intra-venous injection of Du145 prostatic carcinoma cells into the SCID mouse (Trikha *et al*, 1998). This suggests that metastatic prostate carcinoma cells may employ the α2β3 integrin at the site of extravasation and α1 and β2 integrins during interaction with the bone marrow cells within the metastatic site itself. Indeed, Festuccia *et al* (1999) show that TGFβ-enhanced invasion of a reconstituted basement membrane by PC3 cells, in the presence of osteoblast-conditioned medium, was accompanied by attachment and spreading of the cells via α2β1 and α3β1.

Normal breast epithelial cells express α2β1 and α5β1. However, in breast carcinoma, expression is reduced and their loss correlates with differentiation of the tumour (Zutter *et al*, 1993). Surprisingly, MCF-7 breast cancer cells do not express the α5 subunit. Whilst transfection of α5 into MCF-7 cells increases their adhesion to fibronectin, the proliferation of these attached cells is decreased. Interestingly, TGFβ, which is described above to increase PC3 basement membrane attachment via α2β1 and α3β1, was secreted in higher quantities by these α5-transfected MCF-7 cells. TGFβ secretion was higher still when these cells were grown on fibronectin (Wang *et al*, 1999). These data suggest that TGFβ may be involved in a) a negative-feedback loop controlling the growth of MCF-7 cells, and b) the regulation of α5β1 expression and may itself be under the control of α5β1 expression and interaction with its ligand. Boku *et al* (1995) demonstrated secreted levels of TGFβ and increased expression of α3 by invasive gastric cancer cells, providing further evidence to support control mechanisms between TGFβ and integrin expression. Supporting α5β1 as a metastasis-promoting integrin, sub-cutaneous injection of B cell tumour cells expressing α5β1 into SCID mice resulted in vascular dissemination. Whilst tumour cells lacking α5β1 did not disseminate after injection, they did show local growth at the site of injection (Blasé *et al*, 1995).

Activation of Protein Kinase C (PKC) in breast carcinoma cells with phorbal ester (TPA) induces their adhesion to laminin and type I collagen. Whilst no increase in α2 or β1 integrin expression can be demonstrated, this increased adhesion can be inhibited with blocking antibodies against them (Rosfjord *et al*, 1999). Antibodies against these two subunits and α1 and α6 reduce invasion by mouse mammary glandular epithelial cells of reconstituted basement membrane (Lochter *et al*, 1999). From these data, one could suggest that breast cancer cells have the potential to metastasise, but that they require a signal to activate the process, via activation of constitutively expressed integrins. In other words, breast cancer metastasis fits the "second hit" theory of carcinogenesis.

Induced expression of α4β1 on B16 melanoma cells suppresses the development of pulmonary metastases when cells are injected into mice sub-cutaneously, but not when injected intra-venously (Qian *et al*, 1994). This further supports the hypothesis that different cell adhesion molecules are involved in the invasion of the basement membrane and extracellular matrix by the tumour cell and their subsequent extravasation. This data specifically suggests

that $\alpha4\beta1$ may act as a metastasis-suppressor integrin maintaining the primary lesion. This correlates with the data above documenting reduced $\alpha4\beta1$ expression in advanced prostatic tumours (Cress *et al*, 1995).

MV3 melanoma cells migrate through a 3D collagen lattice via their collagen receptor, $\alpha2\beta1$ (Maaser *et al*, 1999). This parallels the invasion of reconstituted basement membranes by prostatic carcinoma cells, PC3 (Festuccia *et al*, 1999).

The adhesion of rat bladder cancer cells to a collagen matrix via $\alpha2\beta1$ also activates PKC (Petit *et al*, 1999). Monoclonal antibodies against $\beta1$ inhibit the adhesion of ovarian carcinoma cells, SKOV3, to peritoneal mesothelial cells and of liver carcinoma cells to reconstituted basement membrane, implying a role for $\beta1$ integrins in the metastatic spread of these cancers (Lessen *et al*, 1999, Torimura *et al*, 1999). Indeed, invasive hepatocellular carcinoma cells express higher levels of $\beta1$ integrins than their non-invasive counterparts (Masumoto *et al*, 1999). The induction of $\beta1$ expression on epithelial cells that do not normally express it induces redistribution of the α-catenin network of cytoskeletal proteins and transformation from an epithelial to a spindle-shape fibroblastic phenotype: tight junctions are no longer intact and a loss of polarity is also seen (Gimond *et al*, 1999).

The fibronectin receptor, $\alpha5\beta1$, is decreased on some malignant cells when compared to their normal counterparts (Schwartz, 1993). Some carcinoma cells express novel receptors for extracellular matrix proteins. For example, $\alpha5\beta x$ binds vitronectin, but does not bind von Willebrand Factor or fibrinogen, as $\alpha5\beta3$ does: these cells are incapable of invading out through the extracellular matrix (Cheresh *et al*, 1989). Therefore, the expression of novel integrin heterodimers may act to inhibit and not to promote the progression of a carcinoma.

To summarise, the increased expression or induction of expression of integrin heterodimers by tumour cells appears to act mostly to promote the metastatic spread of the cells. However, a simple redistribution of integrin expression or integrin activation may deliver the same result. As with CD44 expression by tumour cells, the expression and activation of integrins by a tumour cell may also promote its growth at both the primary and metastatic site.

1.5.5 The Role of Selectins in Cancer

As detailed in Chapter 1.1.5, the naturally occurring vascular ligands for the selectin family of cell adhesion molecules are mucin-type glycoproteins, including sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a) antigens. Increased expression and altered glycosylation of mucins are prominent features of carcinoma progression. Colorectal cells bind to E-, L- and P-selectin. The development of a tumour lesion after injection of colorectal carcinoma cells into P-selectin deficient mice is decreased when compared to those seen in normal mice (Kim *et al*, 1999). Metastatic colorectal carcinoma cells express sLe^x antigen and the adhesion of these cells to cytokine-activated endothelial cell can by inhibited by antibodies against E-selectin (Pigott and Power, 1993). Intravenous injection of E-selectin antibodies with an intra-splenic injection of

H-59 lung carcinoma cells inhibits the development of liver metastases (Brodt *et al*, 1997). Nineteen of 20 bladder cancer cell lines examined by Skorstengaard *et al* (1999) were found to adhere to E-selectin coated plates. Moreover, the expression of sLe^a by the cancer cells is strongly correlated with their ability to adhere to the E-selectin. These data are strong support for the involvement of selectins in the development of metastatic carcinoma.

L-selectin expression is described on some malignant leucocytes (Pigott and Power, 1993). Metastatic deposits of breast cancer contain cells that express E-selectin (Krause and Turner, 1999). Increased sLe^x antigen is seen in aggressive and hormone-resistant prostatic tumours (Satoh *et al*, 1998). Epithelial cells of head and neck tumours express lower levels of sLe^x than their non-malignant counterparts (Renkonen *et al* 1999)

The endothelial cells of blood vessels surrounding head and neck tumours, of both lymphoid and epithelial origin, express decreased levels of sLe^x, sLe^a, E-selectin, and P-selectin (Renkonen *et al*, 1999). Circulating levels of E-selectin are increased in patients with ovarian, breast and gastrointestinal cancer (Banks *et al*, 1993). SLe^a, sLe^x, sLe^b an sLe^y antigens all show decreased expression on apoptotic cell, irrespective of the manner of apoptosis induction (Rapoport and Le Pendu, 1999).

To summarise, the selectins and their receptors show higher levels of expression on some cancers. One could suggest that, as with integrins, the selectin group of cell adhesion molecules are important not only in cell adhesion, but also in the control of proliferation of the cells.

1.5.6 The Role of the Basement Membrane in Cancer

During metastatic cascades, malignant cells must attach to adhesion proteins in the extracellular matrix, or basement membrane. This basement membrane, described in Chapter 1.4.4 above, represents a major barrier to the invasive cells; the migration through this matrix is one of the rate limiting steps in the metastatic cascade. Each basement membrane is unique, but in general is composed of collagens, proteoglycans, (including hyaluranon and heparin sulphate) and glycoproteins (including elastin, fibronectin, laminin). Receptors for these matrix components include the integrins, selectins and cartilage link proteins. Proteases of several classes including aspartic acid proteases (Cathepsin D), cysteine proteases (Cathepsin B), matrix metalloproteinases (MMPs) and serine proteases can degrade these adhesion proteins (Monsky and Chen, 1993). This proteolysis allows cells to detach from the basement membrane.

The basement membrane surrounding melanoma lesions is rich in Type IV collagen and laminin: the melanoma cells are the source of these two proteins, as well as MMP-2 (Schaumburg-Lever *et al*, 2000). Melanoma cells adhere to collagen *in vitro*. Therefore, the production of collagen and laminin by the tumour cell itself may promote its invasion through the extracellular matrix. The production of MMP-2 suggests that again the tumour cell itself provides the components required for the degradation of the basement membrane. However, destruction of the basement membrane is not required for the invasion of these melanoma cells

(Schaumburg-Lever *et al*, 2000). Therefore, the basement membrane may act here to protect the tumour cells from an attacking immune system, preventing cells getting into the tumour lesion instead of preventing their escape.

Boyd and Balkwill (1999) have demonstrated that co-culture of ovarian cancer cells with tumour-associated fibroblasts increases the levels of MMP-2 and one of the naturally occurring inhibitor of MMPs, Tissue Inhibitor of Metalloproteinase-1 (TIMP-1). This effect was contact-dependent. The fibroblasts are the source of the MMP-2: the MMP-2 is activated through matrix collagen and a membrane bound metalloproteinase.

It has been suggested that the contact between luminal epithelial cells of the mammary gland and the basement membrane maintains the polarity of these cells (Slade *et al*, 1999).

Bone sialoprotein and osteopontin are secreted glycoproteins found in the extracellular matrix of bone. They contain an RGD sequence that is thought to mediate attachment of osteoclasts and osteosarcoma cells. Both these proteins are detectable in metastatic and *in situ* breast cancers, but not on the eleven breast cancer cell lines tested (Sharp *et al*, 1999). These data suggest close communication of tumour cells with their surrounding stromal compartments, in that upon removal of the stromal cells the induction of protein expression is lost.

Cell adhesion molecules also controls the expression and activation of matrix proteins. For example, activation of $\alpha1$ and $\alpha2$ expressed on mouse mammary carcinoma cells inhibits the transcription and expression of stromelysin, and conveys invasive behaviour to the cells (Lochter *et al*, 1999). This suggests that the tumour cell itself can control the composition of the extracellular matrix via its cell adhesion molecule expression and activation. Binding of urokinase-type Plasminogen Activator-Receptor (uPA-R) by uPA may potentiate signals conveyed by integrins and promote degradation of the basement membrane and cell migration (Yebra *et al*, 1999). Activation of this uPA-R has also been shown to stimulate the migration of MCF-7 breast cancer cells and HT1080 fibrosarcoma cells on vitronectin coated plates via $\alpha v\beta1$ and $\alpha v\beta5$. Ligation of uPA-R activated *Ras*, Mitogen Activated Protein Kinase (MAPK), Myosin Light Chain Kinase (MLCK) and Extracellular signal Regulated Kinase (ERK). Activation of these intracellular proteins is required for the uPA-mediated adhesion (Nguye, *et al*, 1999).

Two colon carcinoma cell lines express similar levels of $\alpha2$, $\alpha3$, $\alpha5$, αv, $\beta1$, $\beta4$, and $\beta5$ integrin subunits. However, the two cell lines adhere to different proteins of the basement membrane and have different metastatic capabilities. HT-29P cells are weakly metastatic and adhere strongly to collagen I and IV: this adhesion can be inhibited by antibodies against $\beta1$ and $\alpha2$ integrins. The highly metastatic cells, HT-29LMM, adhere strongly to laminin and fibronectin: this adhesion can be inhibited by antibodies against $\beta1$, $\beta6$ and αv (Haier *et al*, 1999). These data suggest that the metastatic potential of a cell may not only be conferred by the expression of a particular cell adhesion molecule, but may also be under the control of the interaction that that molecule may have with proteins within the basement membrane. All the

above data provide strong evidence for a role of cell adhesion molecules in carcinogenesis, not only in the metastatic cascade, but also in the control of growth of the primary tumour.

5 1

1.6 Experimental Aims

Cancer is defined clinically as the breakdown of tissue organisation and the acquisition of invasiveness and as such is a complex cascade of events. One of the prominent morphological changes in malignant adenocarcinomas is a loosening of intercellular adhesion. In particular, the metastatic progression of carcinomas involves the escape of the tumour cells from the primary deposit, invasion through the basement membrane and extracellular matrix, gaining access to the lymphatics and / or vasculature, extravasation at distant sites, and invasion through the basement membrane of the site of metastatic deposit. With the knowledge that CAMs are crucially responsible for maintaining intercellular adhesion, along with their role in leucocyte extravasation, it is the hypothesis of this study that invasive prostate cancer cells employ cell adhesion molecules to facilitate their progression.

Therefore, prostatic tissue will be obtained from patients undergoing radical prostatectomy or transurethral resection of the prostate for clinical disease of the prostate. Tissue will be snap frozen in liquid nitrogen and the expression of E-selectin, ICAM-1, VCAM-1, CD44, $\alpha4$, $\alpha5$, αL, and $\beta1$ will be examined on frozen sections by immunohistochemistry, using the alkaline phosphatase and anti-alkaline phosphatase (APAAP) method of detection. Haematoxylin and Eosin (H & E) sections of all tissues will be analysed histologically by a trained, consultant histopathologist, to determine the state of differentiation of the tissue microscopically. Patient notes will be consulted to determine the clinical background of all patients, including the TNM classification of all carcinoma patients. The expression of CAMs will be compared between samples from prostatic carcinomas and benign prostatic hyperplasia (BPH).

With the exception of the local pelvic lymph node involvement, bone and more specifically bone marrow of axial bones are the almost exclusive locality of prostatic metastatic disease (Cumming *et al*, 1990, Scalliet, 1996). The bone marrow is the main site of haematopoietic growth factor production and action, including the colony stimulating factor family (CSF) (Nicola, 1989). In order for prostatic carcinoma cells to migrate to the bone marrow, they encounter vascular endothelial cells, both at the site of intravasation (invasion into the blood vessel) and extravasation (invasion out of the blood vessel). During leucocyte extravasation, the endothelial cell surface is rich in leucocyte-attracting signals. This study proposes that vascular endothelial cells may supply similar signals for invasive, prostatic carcinoma cells. Conversely, invasive, prostatic carcinoma cells may convey signals that activate the vascular endothelial cells and, thereby prime the vasculature for intra- or extravasation. I propose that bone marrow cytokines may also act as chemo-attracting signals for prostatic carcinoma cells into the bone marrow.

Therefore, the effect of Granulocyte Macrophage (GM)-CSF on the expression of CAMs by prostatic carcinoma cells will be examined. Conditioned medium will be prepared from prostatic carcinoma cell lines, PC3 and Du145. The effect of this medium on the activation of

vascular endothelial cells, HUVECs (Human Umbilical Vein Endothelial Cells) will be examined. Conditioned medium will be prepared from HUVECs and the effect this has on the expression of CAMs by prostatic carcinoma cells will be determined.

Leucocyte extravasation and the changes in CAM expression seen on both cells in this process are dependent upon cell – cell contact between the endothelial cell and the leucocyte. Therefore, the molecular interactions that occur between vascular endothelial cells (HUVECs) and the prostatic carcinoma cells, PC3 and Du145, will be examined in this study. In order to examine these interactions, a co-culture system will be devised. In this co-culture, endothelial and epithelial cells will be cultured in direct contact with each other for both a short one hour co-culture and a long 24 hour co-culture. The two cell populations will then be analysed individually for the expression of CAM expression. This will be performed by investigating the use of various fluorophores to label one, but not the other cell population.

The overall aim of this thesis is to determine the role of cell adhesion molecules in the progression of prostate cancer.

Chapter 2

Methods

Contents

2.1. Culture of Established Prostatic Cell Lines

2.1.1 Basic Cell Culture

Dr. F K Habib, of the Edinburgh University Department Of Surgery, supplied PC3 and Du145 cell lines. These cell lines were grown in Tissue Culture Grade Flasks (TCGFs) in RPMI 1640 medium supplemented with FCS, L-glutamine, penicillin, streptomycin, and amphotericin B (Established Cell Line Medium, ECLM, Appendix 4.4), at 37^0C and in a Sanyo MCO175 incubator providing an atmosphere of 5% CO_2 / 95% air.

2.1.2 Subculture of Established Cell Lines

PC3 and Du145 cells were subcultured at 70-90% confluence. Spent medium was pipetted out of the flask and the cells were rinsed with Ca^{2+} / Mg^{2+} free HBSS (Appendix 4.1). Trypsin / Ethylene Diamine Tetra-acetic Acid (EDTA, Appendix 4.5) was pipetted into the flask. An equal volume of RPMI 1640 (Appendix 4.2) with 10% FCS was added to the flask when detachment was observed under an Olympus CK2 inverted microscope. The cells were pipetted into a 25ml universal and collected by centrifugation at 167xg for 5 minutes. The cells were washed twice in Ca^{2+} / Mg^{2+} free HBSS and seeded at 10-20% confluence with fresh medium in TCGFs.

2.1.3 Freezing Of Established Cell Lines

Stocks of immortalised cell lines were kept in liquid N_2. Cultures were trypsinised (Chapter 2.1.2). Following the first centrifugation the cell pellet was re-suspended in a small volume of 50% Ca^{2+} / Mg^{2+} free HBSS: 50% FCS (usually 1-2ml). An equal volume of 80% Ca^{2+} / Mg^{2+} free HBSS: 20% Dimethyl sulphoxide (DMSO) was added to the cell suspension. 1ml aliquots of cells were pipetted into appropriately labelled cryovials. These cryovials were stored at -70^0C overnight: the vials were then transferred to a liquid N_2 fridge for long-term storage.

2.1.4 Preparation Of Established Cell Line-Conditioned Medium

The culture medium of confluent PC3 and Du145 cell cultures was transferred to a 25ml universal. This solution was centrifuged at 167xg for 10 minutes to pellet any remaining cells. The supernatant was carefully transferred to a fresh universal taking care to avoid any cellular deposit. This suspension was filtered through a 0.2 micron sterile filter and stored at -20°C until required.

2.2. *In Vitro* Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were co-cultured with established prostate cancer cell lines in Chapter 5.4. The generation and maintenance of HUVECs is described below. All procedures were conducted in a Class II Laminar Flow Cabinet unless otherwise stated. All equipment was sterile. All resulting cultures were incubated at 37^0C in a Leec GA150 CO_2 incubator providing an atmosphere of 5% CO_2 / 95% air.

2.2.1 Collection of Tissue

Human umbilical cords available from the Maternity Department of the Leicester General Hospital were collected in 150ml sputum tubs. Once in the laboratory, cords were stored at 4^0C until use. Cords were used within 24 hours of availability.

2.2.2 Extraction of Endothelial Cells

The exterior of the cord was swabbed with tissue saturated in 70% propan-2-ol (Appendix 4.6): visible blood clots were gently massaged. One or 2cm were sliced from each end of the cord removing any tissue damaged by clamping during labour. A cannular was inserted into the lumen of the vein at one end of the cord and secured by a clamp and a piece of thick string. 30ml of Ca^{2+} / Mg^{2+} free HBSS was slowly perfused through the vein via a syringe and the cannular. Air was diffused into the vein expelling residual HBSS. A second cannular and syringe were secured into the second end of the vein. Preheated (to 37^0C) Sigma collagenase solution (Appendix 4.7) was introduced into the vein by syringe through the cannular. The cord was wiped with 70% propan-2-ol, as above, and carefully placed into an opened 50ml universal containing a sufficient level of pre-warmed HBSS to cover the cord: the universal and cord were left in a heated waterbath for 15-20 minutes (Diagram 2.1). The cord was wiped with 70% propan-2-ol and transferred back to the Laminar Flow Cabinet. 10ml HBSS containing 10% FCS was flushed through the vein via a syringe and the first cannular. The outflowing solution was collected in the syringe at the second end and expelled into a 25ml universal. Air was diffused through the vein expelling residual media. This cord effluent was centrifuged at 167x*g* for 5 minutes and the pellet washed in Endothelial Cell Culture Medium (ECCM, Appendix 4.8) at 167x*g* for 5 minutes. This pellet was resuspended in 10-12ml ECM and transferred to an 80cm² TCGF.

Diagram 2.1 The Extraction Of Human Umbilical Vein Endothelial Cells. The umbilical cord is incubated, with collagenase perfused into the vein, in a heated waterbath for 15-20 minutes.

2.2.3 Culture of Endothelial Cells

Endothelial cells were incubated in 95% air / 5% CO_2, at 37^0C. Approximately 24 hours following seeding the TCGF was washed in pre-warmed Ca^{2+}/Mg^{2+} free HBSS, removing any unwanted debris, such as red blood cells: spent medium was replaced with fresh medium.

2.2.4 Subculture of Endothelial Cells

Endothelial cells were subcultured by trypsinisation, as described in Chapter 2.1.2

2.2.5 Preparation Of HUVEC-Conditioned Medium

The culture medium of confluent HUVEC cultures was transferred to a 25ml universal. This solution was centrifuged at 167xg for 10 minutes to pellet any remaining cells. The supernatant was carefully transferred to a fresh universal taking care to avoid any cellular

deposit. This suspension was filtered through a 0.2 micron sterile filter and stored at $-20^{\circ}C$ until required.

2.3. *In Vitro* Culture of Miscellaneous Cell Lines

2.3.1 A549

A549 was an adherent epithelial cell line derived from a human lung carcinoma. The cell line was obtained from Dr. B Shenton, Department of Surgery, University of Newcastle. The culture conditions of A549 were identical to that of PC3 and Du145 (Chapter 2.1.1). A549 cells were passaged by trypsinisation (Chapter 2.1.2).

2.3.2 LLC PK1

LLC PK1 was an adherent tubular epithelial cell line derived from a porcine kidney. The cell line was a gift from Dr. A Bevington, Department of Renal Laboratories, Leicester General Hospital. Culture conditions were the same as those of A549. However, cells were grown in DMEM / HAMs F12 medium supplemented with FCS, glutamine, penicillin, streptomycin, and amphotericin (Appendix 4.9). LLC PK1 cells were subcultured by trypsinisation (Chapter 2.1.2).

2.4. Immunohistochemical Studies

2.4.1 Analysis by Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) Staining

In Chapter 4 cell surface expression of cell adhesion molecules was examined on frozen sections of prostatic tissue. Preparation and analysis of tissues are described below. Monoclonal antibody details are listed below.

Monoclonal Antibody	Specificity	Distribution	Supplier	Cat. No.	IgG Isotype
HLA-ABC	MHC Class I	All nucleated cells	Serotec	MCA 673	IgG1
CD3	T cell- CD3ε chain	T lymphocytes	Dako	M756	IgG1
CK-pan	All Cytokeratin	Epithelial cells, from simple glandular to stratified squamous epithelia	Dako	M 717	IgG1
CD62E	E-selectin	Activated endothelial cells and some T lymphocytes	R&D Systems	BBA16	IgG1
CD31	PECAM-1	Endothelial cells, platelets, T lymphocytes, monocytes, and granulocytes	R&D Systems	BBA 7	IgG1
CD106	VCAM-1	Activated endothelial cells	R&D Systems	BBA 5	IgG1
CD54	ICAM-1	Activated and non-activated endothelial cells	R&D Systems	BBA3	IgG1
CK-8	52.5kDa protein, Cytokeratin-8	Epithelium of liver, intestine, pancreas, urinary bladder, salivary gland, thyroid, prostate, and placenta	Sigma	C5301	IgG1
PAP	Prostatic Acid Phosphatase	Normal and neoplastic prostatic epithelium	Sigma	P9808	IgG2a
PSA	Prostate Specific Antigen	Prostatic epithelium and prostatic carcinoma cells	Euro-Diagnostica (Euro-Path)	2222 MPA	IgG1

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Monoclonal Antibody	Specificity	Distribution	Supplier	Cat. No.	IgG Isotype
CD44	All CD44 isoforms	Peripheral blood leucocytes, liver Kupffer cells, fibroblasts, epidermal keratinocytes, some pancreatic acinar cells, and brain cells	Sigma	C7923	IgG1
CD49d	Alpha chain of VLA-4	Monocytes, T and B lymphocytes, thymocytes, and Langherhans cells.	Serotec	MCA 697	IgG1
CD49e	Alpha chain of VLA-5	T lymphocytes, granulocytes, platelets, some melanoma cells	Serotec	MCA 698	IgG1
CD11a	Alpha chain of LFA-1		R&D Systems	BCA 1	IgG2a
CD29	Beta 1				
CD31-PE	PECAM-1	Endothelial cells, platelets, T lymphocytes, monocytes, and granulocytes	Becton Dickinson	340297	IgG1

Table 2.1 The Specificity, Distribution And Supplier Details Of Monoclonal Antibodies Employed In This Study.

Monoclonal Antibody	Immunohistochemistry Dilution Factor	Flow Cytometry Dilution Factor
HLA-ABC	100	100
CD3	100	10
CK-pan	100	Not Applicable
CD62E	500	100
CD31	1000	1000
CD106	1000	100
CD54	500	100
CK-8	250	Not Applicable
PAP	400	Not Applicable
PSA	50	Not Applicable
CD44	900	50
CD49d	500	50
CD49e	1000	10
CD11a	500	50
CD29	1000	100
CD31-PE	Not Applicable	Neat

Table 2.2 Working Concentrations Of Monoclonal Antibodies Employed In This Study

2.4.1.1 Cryofixation of Solid Tissue

All solid tissue was of prostatic origin. Within a Class II Laminar Flow Cabinet freshly collected tissue was placed on the thin half of a petri-dish. A sample was cut to a maximum size of 3mm in depth and 10mm in length. Care was taken to ensure all outer margins of tissue were free of theatre excision markings. The tissue was removed from the Class II Laminar Flow Cabinet. Under non-sterile conditions a drop of Tissue Tek OCT Compound (OCT) was poured onto a cork sliver of dimensions large enough to house the tissue, but small enough to fit inside a cryovial. The tissue was positioned in the OCT so that one section would contain the entire width and length of the tissue. Using disposable forceps the cork was plunged into, and held under, liquid nitrogen (N_2) contained in a dewar flask. When the bubbling ceased the sample was placed in a labelled cryovial and replaced in the liquid N_2. Once temperature equilibrium was reached the cryovial was transferred to a liquid N_2 fridge. Residual liquid N_2 in the dewar was left to evaporate in a fume cabinet.

2.4.1.2 Cryosectioning of Solid Tissue

$5\mu m$ sections were cut using the Frigocut cryostat, which was set at a temperature of -20^0C and a knife block clearance of 7.5^0. Samples being cut were transferred from the liquid N_2 fridge to the cryostat cabinet and were left to warm to -20^0C. The cork was mounted with OCT on a mounting block and once solid was secured on the cutting block. Sections were collected on the knife edge using the anti-roll plate of the cryostat onto labelled gelatinised microscope slides (Appendix 2). The sections were air dried for 30-60 minutes and fixed in acetone (Appendix 3). Slides were stored in sealed bags with silica gel at -20^0C until use.

2.4.1.3 Preparations of Cytospins

Single cell suspensions were analysed by transfer to ungelatinised microscope slides using a Shandon cytospin 2. Cytospin buckets were assembled. Cell suspensions were prepared in Ca^{2+} / Mg^{2+} free HBSS to a concentration not more than 2×10^5 cells/ml: $100\mu l$ of each solution was dispensed by pipette into each bucket. The cytospin was run for 7 minutes at 149g at low acceleration. The buckets were carefully dismantled and the slides left to air dry for at least 12 hours, when they were stored at -20^0C in sealed specimen bags with silica gel.

2.4.1.4 APAAP Staining

All incubations were conducted at room temperature in humid conditions, unless otherwise stated.

Microscope slides with tissue sections and cytospin preparations were stored at -20°C. These specimen bags were left on the bench top, sealed, for 30-60 minutes to allow the specimens to defrost. Microscope slides were separated from each other. Tissue sections or areas of cells were circled with a wax pen to minimise the amount of reagents required. Cytospin preparations were fixed in acetone (Appendix 3). All samples, whether sections or cytospin preparations, were re-hydrated for 15 minutes with phosphate buffered saline (PBS, Appendix 4.11) using microscope slide racks. 20% AB serum (prepared from blood type AB) was prepared in PBS (AB/PBS). 100μl of AB/PBS was pipetted onto the samples and left for 30 minutes. The AB/PBS was drained from the slide and excess fluid was carefully blotted from the slide using tissue paper. Following this, 100 μl of mouse anti-human monoclonal antibody (McAb), diluted appropriately in AB/PBS, was pipetted onto the samples and left to incubate for 1 hour. Slides were washed 3 times, using slide racks, in PBS for 5 minutes each time. Excess fluid was again blotted off the slide. A 1:50 dilution of rabbit anti-mouse immunoglobulin (Ig) was prepared (in AB/PBS): 100μl of this preparation was pipetted onto the sample and left to react with the bound McAb for 30 minutes. After a further 3 washes with PBS as above, 100μl of a 1:50 dilution of soluble complexes of alkaline phosphatase and anti-alkaline phosphatase (APAAP) in AB/PBS was pipetted onto the sample. This was incubated for 30 minutes. The slides were washed 3 times in PBS; the 3rd wash contained 1-2% levamisole. Levamisole inhibits the reaction of endogenous alkaline phosphatase with the enzyme substrate. The Vector Red Enzyme Substrate, prepared in 100mM Trishydroxymethylaminomethane hydrochloride (TrisHCl) pH8.2 (Appendix 4.12) as per the manufacturer's instructions, also contained levamisole (100mM): 2 drops of this solution was incubated on the sample for 30 minutes. The slides were rinsed in Elga-purified water. The slides were placed on a plastic rack over a sink. The samples were counterstained with a few drops of Mayer's Haemalum for 10 minutes. The slides were rinsed again and washed in Elga-purified water for 2-3 minutes. The samples were protected with coverslips using pre-warmed glycerol gelatin. The slides were left to harden overnight before storage.

2.4.1.5 Immunohistochemical Scoring

A Leitz Dialux 22 invert microscope was used to score all sections of tissue and cytospins. The epithelial and stromal compartments were scored independently of each other. Each slide was examined twice and given a score of 0-6, depending upon the extent of red stain produced, as described in Table 2.3. Each section was scored twice and the mean value was calculated.

Immunohistochemical Score	Percentage Of Epithelial / Stromal Compartment Stained
0	0
1	<5
2	5-20
3	20-50
4	50-80
5	>80, but <100
6	100

Table 2.3 The Immunohistochemical Scoring System Adopted For Analysis of Frozen sections Of Solid Prostate And Cytospin Preparations of Cell Suspensions.

2.4.2 Analysis by Flow Cytometry

In Chapter 5 suspensions of established prostate cancer cell lines were analysed for surface expression of cell adhesion molecules. A flow cytometer was used for these experiments, and procedures are detailed below. All investigations with a flow cytometer were conducted on a Becton Dickinson FACScan (Fluorescence Activated Cell Scan).

2.4.2.1 Preparation of Cells

Single cell suspensions of the cultured cells were generated by trypsinisation (Chapter 2.1.2). Trypsinised cells were transferred to 5ml FACS tubes and washed once in PBS/Azide (Appendix 4.13) at 663xg for 7 minutes.

2.4.2.2 Staining With a Single Fluorophore

Following centrifugation of Chapter 2.4.2.1, the supernatant was decanted. 50μl of McAb, diluted appropriately in 0.1% Normal Goat Serum (NGS) in PBS/Azide (PBS/Az/NGS, Appendix 4.14), was pipetted into the tube, which was then vortexed to resuspend the pellet with the McAb. Cells were incubated at 4°C for 30 minutes, when they were washed twice in PBS/Azide at 663xg for 7 minutes. A 1:50 dilution of goat anti-mouse Ig conjugated to the fluorophore Fluorescein Isothiocyanate (FITC) was prepared in PBS/Az/NGS. 50μl of the Ig-FITC solution was pipetted into the tube after the supernatant was discarded. The cells were vortexed as before, incubated for 30 minutes at 4°C and washed once in PBS/Azide at 663xg for 7 minutes. The supernatant was discarded and the cells were resuspended in 150μl FACSFlow if assayed by FACScan immediately, or in 150μl 1% paraformaldehyde (Appendix 4.15) if assayed more than 1 hour later: both were added using a Scocorex multi-stepper.

In each FACScan experiment cells were incubated in the absence of any antibody. Auto-fluorescence of these cells was detected on the FACScan, serving as one of three controls. A

second group of control cells were incubated in the absence of the primary antibody but in the presence of the goat anti-mouse Ig conjugated to FITC. These cells served to control for any cellular binding by the FITC-conjugated antibody. Thirdly, cells were incubated with an irrelevant primary antibody and FITC-conjugated secondary antibody, before analysed on the FACScan. Two irrelevant antibodies were used in this study; anti-human MHC Class I and anti-human CD3 (the human T cell Receptor).

2.4.2.3 Double Staining With 2 Fluorophores

2.4.2.3.1 Double Staining With Phycoerythrin-conjugated Antibodies And FITC

Anti-CD31 (PECAM-1) McAb conjugated to the fluorophore phycoerythrin (PE) was used in the co-culture assays of PC3 and Du145 with HUVECs. Cells were prepared as described in Chapter 2.4.2.1. They were stained with the primary McAb and FITC goat anti-mouse Ig conjugate (Chapter 2.4.2.2). Following the final wash of Chapter 2.4.2.2 100µl of PBS/Az/NGS/normal mouse serum (NMS, Appendix 4.16) was pipetted into the tube. Cells were vortexed and left to incubate at room temperature for 5 minutes. The addition of the mouse serum inhibited background reaction of the PE conjugated McAb that was raised in mouse. 20µl of the stock PE-CD31 was pipetted directly into the tube. The tube was vortexed and cells were incubated at $4^{0}C$ for 20 minutes. The cells were washed in PBS/Az for 7 minutes at 663xg. After the supernatant was discarded the cells were resuspended in either FACSFlow or paraformaldehyde, as in Chapter 2.4.2.2.

2.4.2.3.2 Double Staining With PHK26 And FITC

In comparison to the use of PE, fluorescent cell linking with PKH26 is performed before investigations were conducted. PKH26 was used, as was PE-CD31, in co-culture assays of PC3 and Du145 cells with HUVECs. PC3 and Du145 cells were pre-labelled with the dye.

Du145 and PC3 cells were trypsinised (Chapter 2.1.2). Following the 1st wash in Ca^{2+} / Mg^{2+} free HBSS the total cell number was adjusted to approximately 10^{6}. This cell suspension was washed at 167xg for 5 minutes in Ca^{2+} / Mg^{2+} free HBSS. The resulting pellet was resuspended in Ca^{2+} / Mg^{2+} free HBSS and centrifuged at 400xg for 5 minutes at $25^{0}C$. During this centrifugation 5µl of stock PKH26 dye (10^{-3} M) was pipetted into 995µl of dye diluent in a 25ml universal. Before re-suspending the pellet of cells, excess supernatant was carefully removed by pipette. After re-suspending the dry pellet 1ml of the dye diluent was pipetted into

the cells. The cell suspension was pipetted into the dye. This mixture was gently inverted and left to incubate for 4 minutes at room temperature: the universal was inverted 2 or 3 times during this period. 2ml of foetal calf serum (FCS) was pipetted into the cells and dye and gently mixed. This suspension was left to incubate for 1 minute at room temperature to stop the dying process. 4 ml of established cell line medium (ECLM) was then pipetted into the universal. This suspension was centrifuged at 400xg and 25^{0}C for 10 minutes to remove the cells from the staining solution. The supernatant was decanted and the pellet of cells was resuspended and transferred to a fresh 25ml universal. The cells were washed 3 times in Ca^{2+} / Mg^{2+} free HBSS and once in culture medium at 167xg for 5 minutes. The cells were then resuspended in culture medium to the appropriate concentration: for co-culture assays this concentration was 9 x 10^{4} cells/ml. 500µl of this suspension was pipetted into a 24-well tissue culture grade plate that contained confluent HUVECs.

Following any culture period the cells were subjected to further trypsinisation and staining (Chapters 2.4.2.1, 2.4.2.2 and 2.4.2.3.1).

2.4.2.4 Standardisation Of FACScan Analysis

The Becton Dickinson FACScan was calibrated weekly with DAKO fluorospheres. This controlled for variation in the performance of the LASER and for the sensitivity of the detectors of the machine. Fluorospheres contained a mixture of 5 bead populations: one of these did not contain fluorochrome, while the remaining four bead populations were stained to different intensities. A sample of the beads was analysed on the FACScan. Four positive levels of fluorescence were demonstrated, with one negative peak. These values were plotted into a line graph, with the equation:

$$y=mx+c,$$

where the m value represented the slope of the graph and c represented the intersection of the y axis. These m and c values were transferred to the equation;

$$MESF= EXP ((Median\ level\ of\ fluorescence + c) / m),$$

where MESF is the molecular equivalent to soluble fluorochrome. FACScan analysis measurements throughout this study are represented as MESF values when FITC was used.

Chapter 3

Optimisation Of The Co-culture Assays

Contents

3.1 Introduction

During this study co-culture experiments will be performed: these investigations will examine the expression of cell surface molecules by vascular endothelial and prostatic adenocarcinoma epithelial cells when they were grown in direct contact with each other. The level of expression will be analysed by flow cytometry. The simplest way to distinguish two cell populations using flow cytometry is by size and granularity utilising the forward and side scatter detectors of the Fluorescence Activated Cell Scan (FACScan) machine. However, endothelial and epithelial cells are indistinguishable in both size and granularity by the FACScan machine used in this study. Therefore, cells need to be distinguished by the level and type of fluorescence exhibited.

The Becton Dickinson FACScan employed in this study measures fluorescent emission induced by an ionic (argon ion) LASER (Light Amplification by Stimulated Emission of Radiation). The light radiation has a wavelength of 488nm. The argon ion laser excites fluorophores with absorption maxima of 488nm or longer. However, the three fluorescence detectors of the flow cytometer only measures the emitted radiation of fluorophores with emission maxima around 500nm, 585nm, or 650nm, as described in Figure 3.1. Fluorescein, the fluorophore within Fluorescein Isothiocyanate (FITC), has an absorption maximum of 495nm and an emission maximum of 520nm (Table 3.1). Other frequently used fluorophores are phycoerythrin, and phycoerythrin-Texas red. The fluorophore propidium iodide (PI) was readily available in this laboratory. PI preferentially binds double-stranded DNA, but is also capable of binding double-stranded RNA. However, PI only binds DNA in dead cells: PI is incapable of crossing the intact cellular membrane of living cells, much like trypan blue in Appendix 1. Therefore, PI is of no use in distinguishing between 2 populations of viable cells.

Fluorophore	Excitation Maximum (nm)	Emission Maximum (nm)	Fluorescence Detector
Fluorescein	495	520	FL1
R-phycoerythrin	564, 495	576	FL2
Phycoerythrin-Texas Red Conjugate	495	620	FL3
PKH26	551	567	FL2
Propidium Iodide	495, 342	639	FL3
Acridine Orange	503	530 (DNA), 640 (RNA)	

Table 3.1 The Excitation And Emission Maxima Of Frequently Used Fluorophores. All fluorophores have excitation maxima close to the 488nm wavelength of the argon-ion laser used in the Becton Dickinson FACScan. All fluorophores emit light of wavelengths that can be measured by the FL1, FL2, and FL3 detectors of the FACScan.

FL1 Detector

Holder

530 / 30nm
Band Pass
Filter

Brewster Window

Side Scatter
Detector

FL2 Detector

585 / 42nm
Band Pass Filter

560nm Short
Pass Dichroic
Mirror

FL3 Detector

640nm
Long Pass
Dichroic Mirror

650nm
Long Pass Filter

Collection Lens

Forward
Scatter
Detector

Collection
Lens

Bar

Flow
Chamber
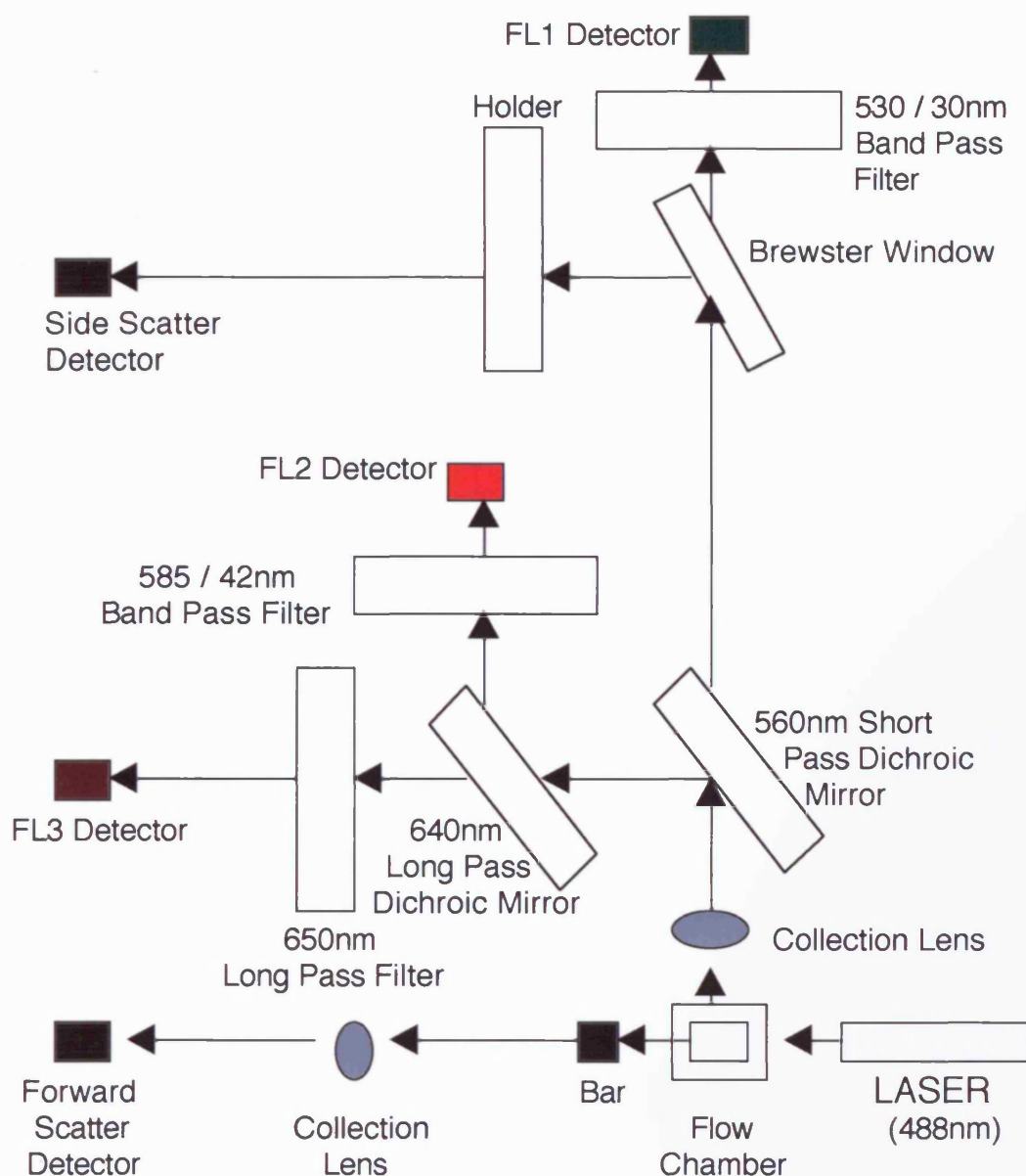
LASER
(488nm)

Diagram 3.1 Optical Layout Of A Becton Dickinson FACScan. When a fluorophore absorbs light (and hence energy) emitted from the LASER, electrons are raised from a ground state to an excited state. The electrons return to the ground state and emit their energy, often in the form of a quantum of light. The interference optical filters, known as bandpass and edge filters, serve to separate the mixture of scattered and fluorescent light produced by this shift of energy. Bandpass filters filter light of a given wavelength over a narrow band, e.g. 530nm +/- 30nm for the FL1 detector. Edge filters, or dichroic filters, are either short wavelength pass or long wavelength pass filters: these filters transmit light below a certain wavelength and reflect light of a longer wavelength, or transmit light of a longer wavelength and reflect light of a shorter wavelength, respectively. Light of three particular wavelengths fall onto the three fluorescent detectors where the intensity is measured.

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3.2. Double Labelling with FITC and Phycoerythrin

3.2.1 Differentiation of Epithelial and Endothelial Cells

The usefulness of fluorescein (FITC) and phycoerythrin (PE) in the epithelial / endothelial co-culture system was examined. As detailed in Table 3.1, the FL1 and FL2 detectors measure the energy emitted by FITC and PE, respectively. The PE fluorophore was directly conjugated to anti-human Platelet Endothelium Cell Adhesion Molecule-1 (PECAM-1), and thereby binds to only the endothelial cells in the endothelial - epithelial suspensions. This experiment was designed to determine two factors. Firstly, the usefulness of FITC and PE in distinguishing between the epithelial and endothelial cells in a cell suspension and secondly, the concentration of epithelial cells that produces optimal binding to endothelial cells.

Firstly, PC3 cells and HUVECs were individually subjected to FACScan analysis (Chapter 2.4.2). Briefly, cells were incubated with either a) goat anti-mouse immunoglobulin conjugated to FITC, b) mouse anti-human CD31 conjugated to PE (CD31-PE), or c) mouse anti-human CD44, FITC-conjugated immunoglobulin, and mouse anti-human CD31-PE. Neither cell population emits fluorescent light in the wavelengths visible by either the FL1 or FL2 detectors of the FACScan when incubated with anti-mouse FITC-conjugated immunoglobulin (Figure 3.2.1.1). HUVECs, but not PC3 cells, emit fluorescent light measured by the FL2 detector of the FACScan when incubated with PE-anti-CD31 monoclonal antibody. Therefore, the PC3 cells and HUVECs can be distinguished (Figure 3.2.1.2). To ensure that a) CD44 monoclonal antibody binding does not physically or electronically interfere with CD31-PE monoclonal antibody binding, cells were incubated with both antibodies. Figure 3.2.1.3 demonstrates that a) PC3 cells bind CD44, but not CD31-PE monoclonal antibodies, b) HUVECs bind both CD44 and CD31-PE monoclonal antibodies, and c) these two cell populations can be differentiated. When all these different cell populations are combined and analysed by the FACScan all four cell types are distinguishable, i.e. CD44⁻ CD31⁻ PC3 cells, CD44⁺ CD31⁻ Du145 cells, CD44⁻CD31⁺ HUVECs, and CD44⁺ CD31⁺ HUVECs (Figure 3.1.2.4).

Having established that epithelial and endothelial cells could be differentiated fluorescently by using an antibody against CD31 expressed by the endothelial cells only, Du145 cells and HUVECs were now cultured in direct contact with each other for 1 hour and the expression of CD44 was examined by FACScan. HUVECs were cultured on 24-well TCGPs until confluent (Chapter 2.2.3). Confluent Du145 cells were trypsinised from their culture flask (Chapter 2.1.2). Serial dilutions of Du145 cells were prepared in Endothelial Cell Medium (ECM) and 500µl of each preparation was added to the confluent monolayers of HUVECs in the 24-well TCGP, in triplicate. These cells were left to incubate under standard tissue culture conditions for 1 hour. Unattached cells were then carefully aspirated off the HUVECs by pipette and transferred to 5ml polystyrene tubes. A small volume of Ca^{2+} / Mg^{2+} -

a) PC3 Cells
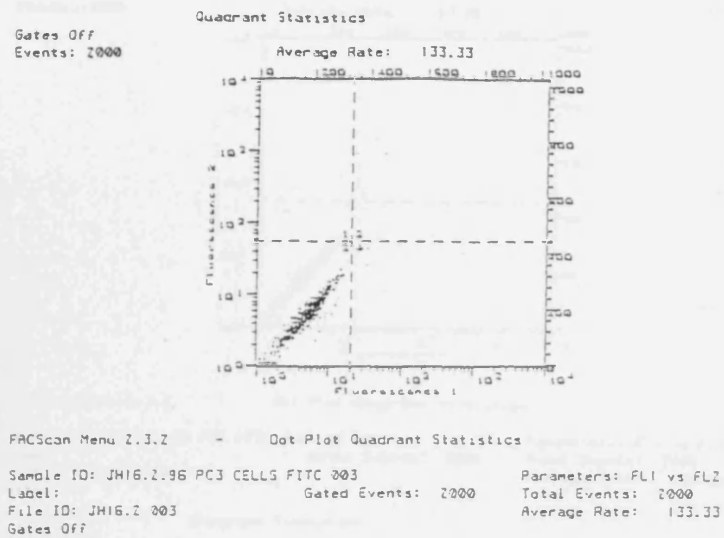
b) HUVECs
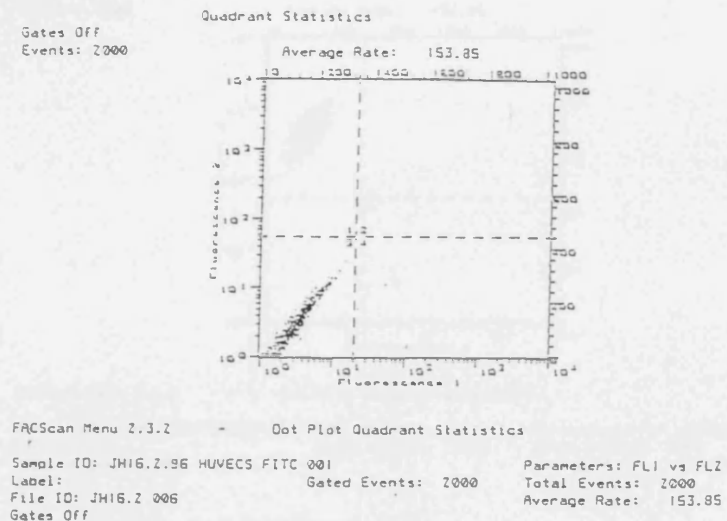
c) PC3 Cells And HUVECs
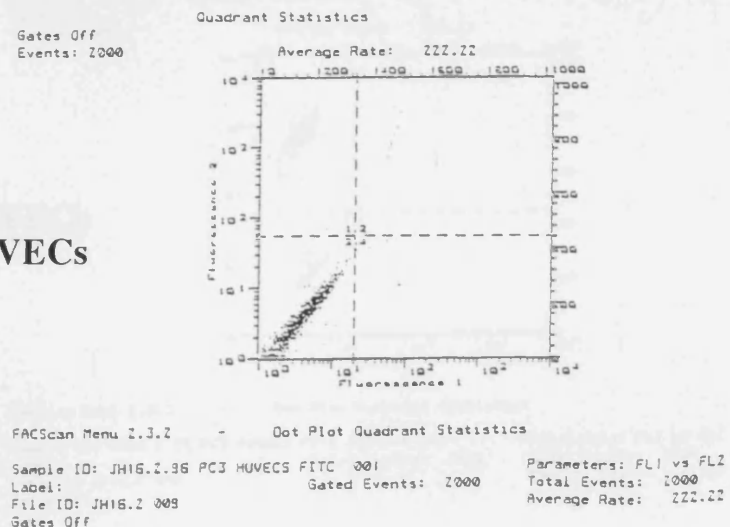
Figure 3.2.1.1 No Fluorescent Light Is Detected On PC3 Cells And HUVECs When Incubated With Goat Anti-mouse Immunoglobulin Conjugated to FITC. PC3 cells and HUVECs were incubated with goat anti-mouse immunoglobulin-FITC conjugate and analysed on the FACScan, as described in the text. PC3 cells alone, HUVECs alone and cells combined were analysed on the FACScan in a), b), and c) respectively.

a) PC3 cells
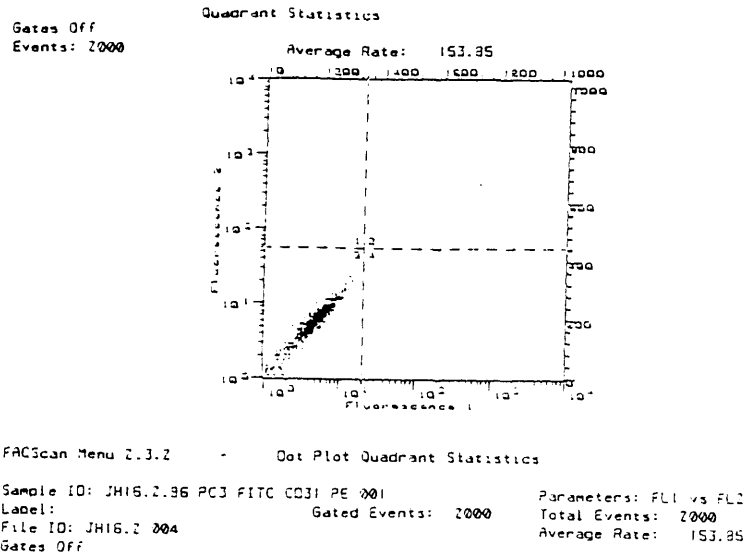
b) HUVECs
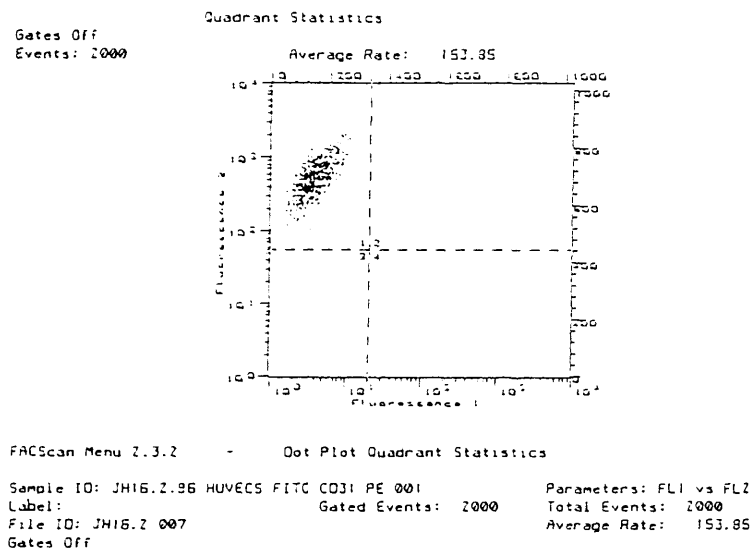
c) PC3 Cells And HUVECs
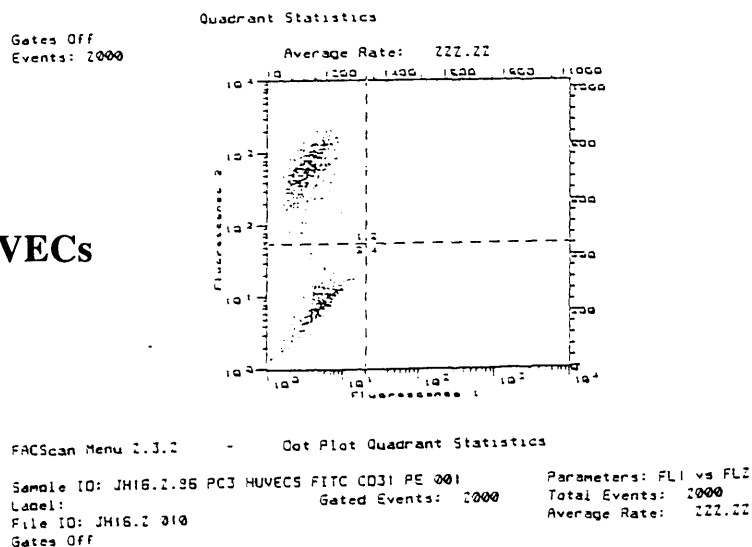
Figure 3.2.1.2 PC3 Cells And HUVECs Can Be Distinguished Using Anti-human CD31 Monoclonal Antibodies Conjugated To Phycoerythrin. PC3 cells and HUVECs were incubated with mouse anti-human CD31 directly conjugated to phycoerythrin (PE) and goat anti-mouse Immunoglobulin-FITC, as described in the text. PC3 cells alone, HUVECs alone, and cells combined were then analysed on the FACScan in a), b), and c) above.

a) PC3 Cells
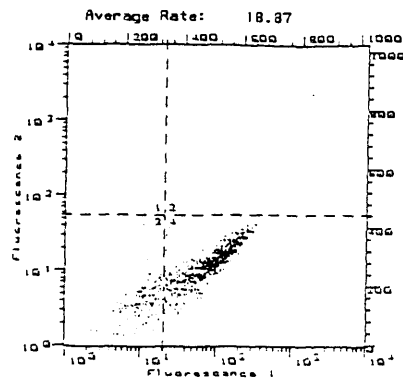

```
Quadrant Statistics
Gates Off
Events: 2000                Average Rate:    18.87

FACScan Menu 2.3.2      -    Dot Plot Quadrant Statistics

Sample ID: JH6.3.96 PC3 A CD44 002        Parameters: FL1 vs FL2
Label:                      Gated Events:  2000    Total Events:  2000
File ID: JH6.3 011                        Average Rate:    18.87
Gates Off
```

b) HUVECs
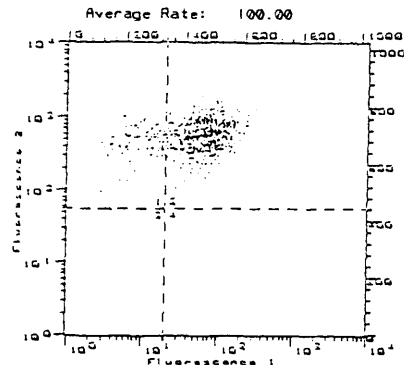

```
Quadrant Statistics
Gates Off
Events: 2000                Average Rate:   100.00

FACScan Menu 2.3.2      -    Dot Plot Quadrant Statistics

Sample ID: JH16.2.96 HUVECS FITC CD44 CD31 PE 001   Parameters: FL1 vs FL2
Label:                      Gated Events:  2000      Total Events:  2000
.. File ID: JH16.2 008                                Average Rate:   100.00
Gates Off              Quadrant Statistics
Gates Off
Events: 2000                Average Rate:   200.00
```

c) PC3 Cells And HUVECs
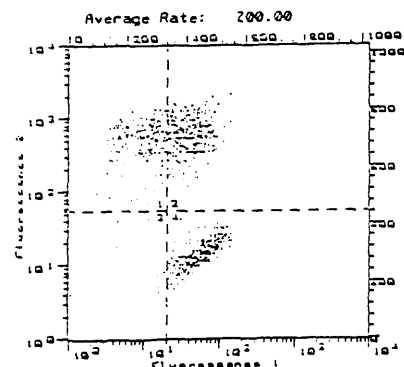

```
FACScan Menu 2.3.2      -    Dot Plot Quadrant Statistics

Sample ID: JH16.2.96 PC3 HUVECS FITC CD44 CD31 PE 001   Parameters: FL1 vs FL2
Label:                      Gated Events:  2000          Total Events:  2000
File ID: JH16.2 011                                       Average Rate:   200.00
Gates Off
```

Figure 3.2.1.3 The Expression Of CD44 By A Mixed Population Of PC3 Cells And HUVECs Can Be Investigated Using Anti-CD31 Monoclonal Antibodies Conjugated to Phycoerythrin. PC3 cells and HUVECs were incubated with mouse anti-human CD44, mouse anti-human PE-CD31, and goat anti-mouse Immunoglobulin-FITC, as described in the text. The expression of CD44 by a) PC3 cells, b) HUVECs, and c) PC3 and HUVECs combined was analysed on the FACScan.

Quadrant Statistics

Gates Off
Events: 5000
Average Rate: 250.00
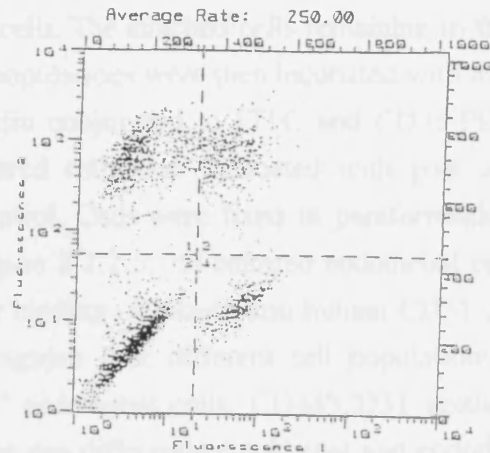
FACScan Menu 2.3.2   -   Dot Plot Quadrant Statistics

Sample ID: JH16.2.96 PC3 CELLS FITC 002
Label: Gated Events: 5000
File ID: JH16.2 002
Gates Off

Parameters: FL1 vs FL2
Total Events: 5000
Average Rate: 250.00

Figure 3.2.1.4 Fluorescein And Phycoerythrin Are Useful Fluorophores Enabling The Distinction Of PC3 And HUVECs. PC3 cells and HUVECs were incubated with either goat anti-mouse immunoglobulin conjugated to FITC, mouse anti-human CD44 and goat anti-mouse Immunoglobulin-FITC, or mouse anti-human CD44, goat anti-mouse Immunoglobulin-FITC, and mouse anti-human CD31 directly conjugated to phycoerythrin (PE). All cell populations were combined and analysed on the FACScan.

Gates Off
Events: 2000
Average Rate: 19.61
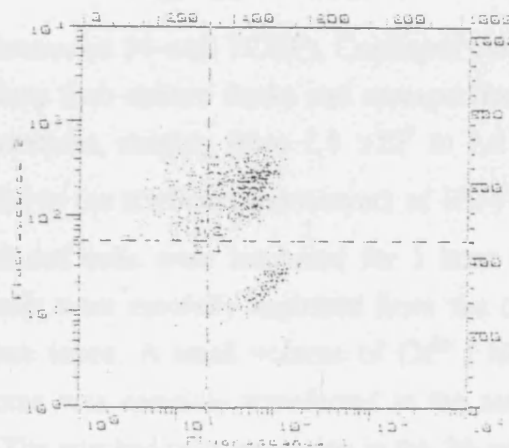
FACScan Menu 2.3.2   -   Dot Plot Quadrant Statistics

Sample ID: JH6.3.96 ENDO PC3 B CD44 CD31 PE 002
Label: Gated Events: 2000
File ID: JH6.3 018
Gates Off

Parameters: FL1 vs FL2
Total Events: 2000
Average Rate: 19.61

Figure 3.2.1.5 Fluorescein And Phycoerythrin Are Useful Fluorophores Enabling The Distinction Of PC3 Cells And HUVECs. PC3 cells and HUVECs were co-cultured in direct contact of each other for 1 hour. Unattached and attached cells were collected by aspiration and trypsinisation, respectively. Cell populations were incubated with either goat anti-mouse immunoglobulin conjugated to FITC, mouse anti-human CD44 and goat anti-mouse immunoglobulin conjugated to FITC, or mouse anti-human CD44, goat anti-mouse immunoglobulin conjugated to FITC and mouse anti-human CD31 conjugated to PE. All cell populations were combined and analysed on the FACScan.

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HBSS was swirled around each well and this volume was carefully transferred to the same polystyrene tube that contained the unattached cells. The attached cells remaining in the 24-well TCGP were trypsinised (Chapter 2.1.2). Cell populations were then incubated with mouse anti-human CD44, goat anti-mouse immunoglobulin conjugated to FITC and CD31-PE (Chapter 2.4.2.3.1). A control population of co-cultured cells was incubated with goat anti-mouse immunoglobulin-FITC only, as a negative control. Cells were fixed in paraformaldehyde and analysed on the FACScan. As illustrated in Figure 3.2.2.5, co-cultured endothelial cells can be clearly distinguished from Du145 cells by their binding of mouse anti-human CD31 conjugated to PE. Moreover, one can now clearly distinguish four different cell populations: namely, $CD44^+$, $CD31^+$ endothelial cells, $CD44^-CD31^+$ endothelial cells, $CD44^+CD31^-$ epithelial cells, and $CD44^-CD31^-$ epithelial cells. Therefore, one can differentiate epithelial and endothelial cells after 1 hour of co-culture and determine the level of CAM expression of the surface of each cell type.

3.2.2 Numerical Characteristics of Epithelial Cell Adherence to HUVECs

Chapter 3.2.1 demonstrated that co-cultured epithelial and endothelial cells can be distinguished by the use of monoclonal antibodies conjugated to fluorophores. Using these antibodies, the maximum number of epithelial cells that could attach to a monolayer of confluent endothelial cells was determined.

HUVECs were cultured to confluence on 24-well TCGPs. Confluent cultures of Du145 cells and A549 cells were trypsinised from their culture flasks and resuspended in endothelial cell medium (ECM) at varying concentrations, ranging from 1.8 $\times 10^5$ to 1.4 $\times 10^3$ cells/ml: 500µl of each cell suspension was added to the confluent monolayers of HUVECs in the 24-well TCGPs, in triplicate. These co-cultured cells were incubated for 1 hour under standard tissue culture conditions. Unattached cells were carefully aspirated from the culture plate by pipette and transferred to 5ml polystyrene tubes. A small volume of Ca^{2+} / Mg^{2+} HBSS was swirled around each well and this volume was carefully transferred to the same polystyrene tube that contained the unattached cells. The attached cells remaining in the 24-well TCGP were trypsinised (Chapter 2.1.2). Cell populations were incubated with mouse anti-human CD31-PE analysed on the FACScan. The percentage of $CD31^+$ HUVECs and $CD31^-$ Du145 / A549 cells in each population of cells was measured by the FACScan. Maximum Du145 cell and adherence to confluent monolayers of HUVECs occurs at a concentration of 4-5 $\times 10^4$ Du145 cells/well (Figure 3.2.2.1a). Maximum adherence of A549 cells to HUVECs occurs at concentrations in excess of 5 $\times 10^4$ cells/well (Figure 3.2.2.1b).

To determine absolute number of each cell population in an attached co-cultures viable cell counts were performed using trypan blue. Confluent Du145 cells were trypsinised and prepared to a concentration of 10^5 cells/ml in ECM. 500µl of this suspension was added to confluent monolayers of HUVECs in 24-well TCGPs, in quadruplicate. These co-cultures were

a)

Percentage Of Sample Which Were Du145 Cells (y-axis)

Concentration Of Du145 Added (x-axis)

b)

Percentage Of Sample Which Were A549 Cells (y-axis)

Concentration Of A549 Added (x-axis)
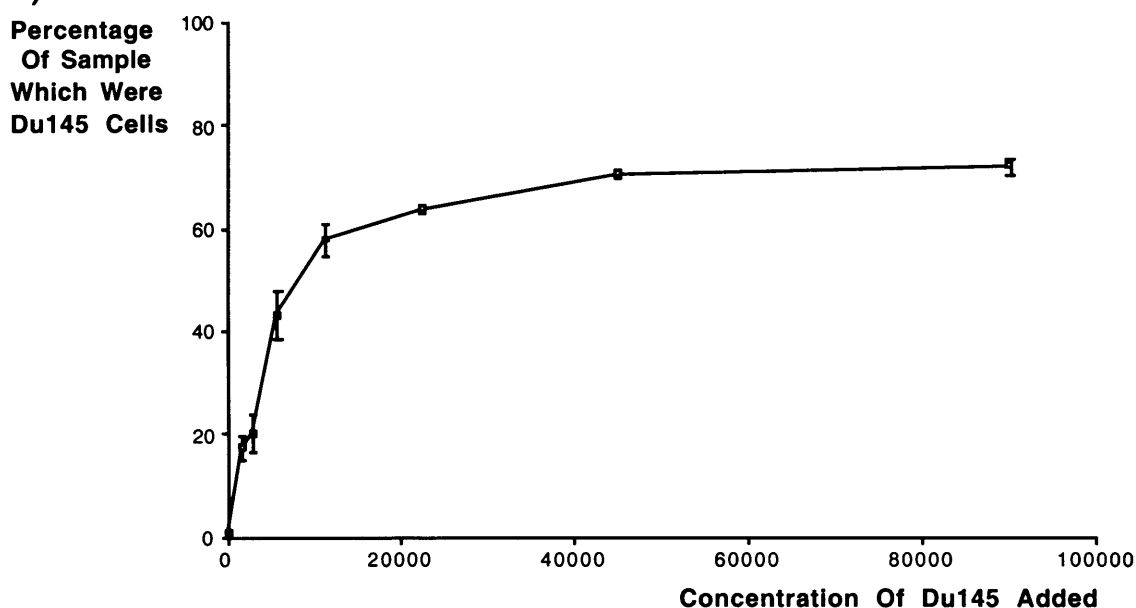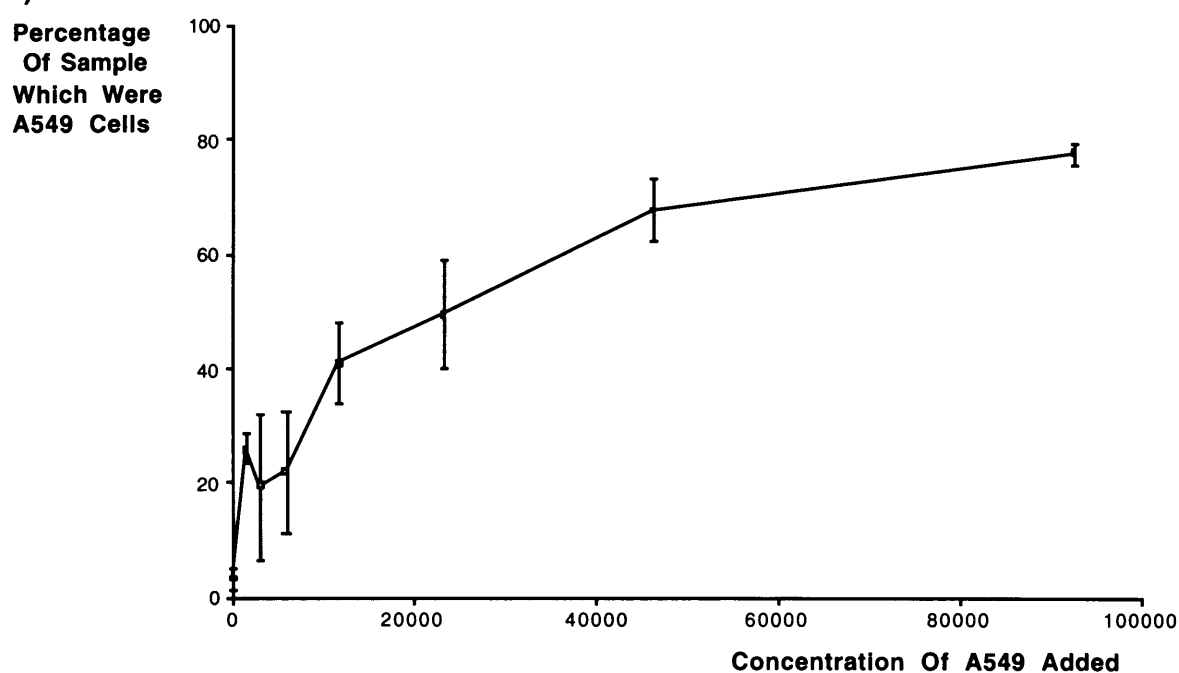
Figure 3.2.2.1 Saturation Of HUVECs With Du145 And A549 Cells. Increasing concentrations of a) Du145 and b) A549 cells were added to confluent monolayers of HUVECs in 24-well TCGPs for one hour. Attached cells were trypsinised and incubated with mouse anti-human CD31 conjugated to PE. Cells were then subjected to FACScan analysis, as detailed in the text. (Values quoted are the means of three measurements. Error bars represent the standard deviation of those means.)

incubated under standard tissue culture conditions for 1 hour. Unattached cells were aspirated off the HUVECs and attached cells were trypsinised (Chapter 2.1.2). Four control wells of HUVECs alone were also trypsinised. The number of viable cells in both populations of cells was determined by trypan blue exclusion (Appendix 1). A 24-well plate of co-cultured HUVECs and Du145 cells contains 15100 viable cells (Standard Deviation ± 6200, n=4). When 5 x10^4 Du145 cells are co-cultured with HUVECs for 1 hour 70% of the attached population are Du145 cells and 30% of the attached population are HUVECs (Figure 3.2.2.1). Therefore, if the total number of cells trypsinised from an identical co-culture of HUVECs and Du145 cells is 1.57 x10^4 cells, 1.05 x10^4 of these cells must be Du145 cells and 4.53 x10^3 must be HUVECs. Therefore, maximum attachment of Du145 cells to HUVECs occurs at a ratio of seven Du145 cells to three HUVECs, or 2.33 Du145 cells:1 HUVEC. Endothelial and epithelial cells are of a similar size, as discussed above. One would expect adhesion to occur on a one to one basis. However, it is highly likely that more than one Du145 cell can adhere to any one HUVEC. Regardless, while it appears that mouse anti-human CD31 conjugated to PE may be a useful tool for the distinction of epithelial and endothelial cells, a major problem was the expense. Each bottle of CD31-PE only would allow 3 assays to be conducted. Therefore, alternative methods were sought.

3.3 Double Staining with FITC and Acridine Orange

Acridine Orange (AO) is a metachromatic dye with an excitation maximum of 503nm. AO can bind the nucleic acids of double-stranded DNA and single-stranded RNA. When bound to DNA and RNA the emission maxima of AO are 530nm and 640nm, respectively. Therefore, AO can produce fluorescent light recognised by either the FL1 or FL3 detectors of the FACScan (Diagram 3.1).
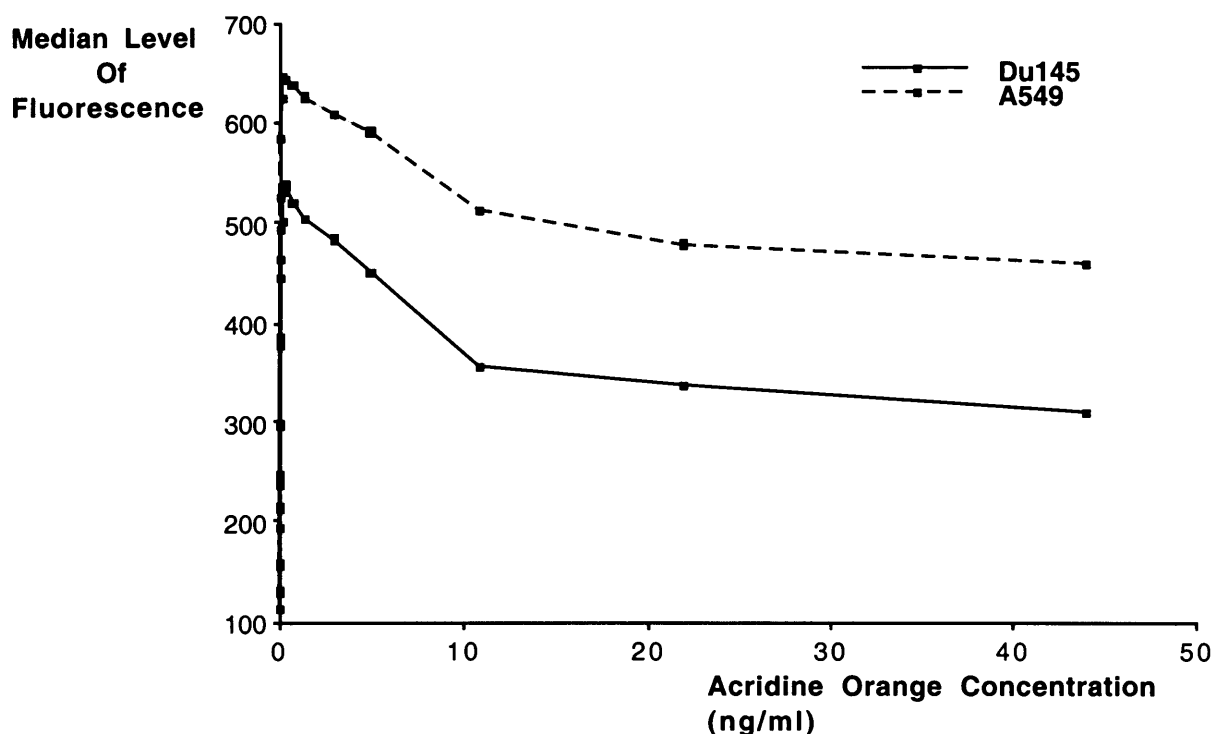
Figure 3.3.1 The Two-Phase Behaviour Of Acridine Orange Whilst Staining Du145 And A549 Cells. Cells were incubated with varying concentrations of acridine orange (AO), as described in the text. The level of fluorescence measured by the FL1 detectors of the FACScan was directly proportional to the concentration of AO at lower concentrations and inversely proportional at higher concentrations of AO.

To determine whether AO could be used to differentiate between HUVECs and epithelial cell lines, Du145 and A549 cells were incubated with AO and analysed on the FACScan. Confluent monolayers of Du145 and A549 cells were trypsinised and resuspended in Ca^{2+} / Mg^{2+} free-HBSS to a concentration of 4.5 x 10^5 cells/ml (Chapter 2.1.2). 100μl of each solution (i.e. 4.5 x 10^4 cells) was added to 18 appropriately labelled 5ml polystyrene tubes. Cells were washed once in phosphate buffered saline / Azide (PBS/Az) for seven minutes at 663xg. The supernatant was tipped out of the tube. AO solutions were prepared as in Appendix 5. Basically, serial dilutions of AO were prepared in PBS from 10mg/ml to 0.00015mg/ml. 100μl of each of the AO solutions was pipetted into the Du145 and A549 cells. Cells were vortexed and incubated for 10 minutes at room temperature in the dark. A control

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sample of cells was incubated with PBS alone. Cells were washed in PBS/Az as above. After discarding the supernatant cells were resuspended in 150μl FACSFlow, added with a Scocorex multi-stepper. The level of fluorescence due to AO present was detected on the FACScan.

The level of FL1 fluorescence emitted by the cells incubated with AO seemed to occur in two stages. Cells incubated with solutions of AO of concentrations less than 0.078125mg/ml or 0.039063mg/ml for Du145 and A549 cells, respectively, demonstrated a directly proportional relationship between AO concentration and the level of fluorescence emitted: however, cells labelled with AO solutions of concentrations greater than these levels displayed an inversely proportional relationship to the amount of AO present in the solution (Figure 3.3.1) (Appendix Table 3.2). This effect was most likely due to quenching. This phenomenon can occur in the presence of an excess of fluorophore. Excess AO molecules can interact with each other, or other substances in a solution, and the excitation energy is dissipated by non-radiative transitions; i.e. the emission of energy that occurs by the return of electrons to their ground state does not occur, or is reduced. This leads to the observation of false low or negative levels of fluorescence. This experiment was repeated and the lower concentrations of AO were examined in triplicate. Cells and AO were prepared as described above. Saturation levels of AO staining can be reached in the labelling of Du145 and A549 cells. Maximal staining is observed at 1.0ng AO / Du145 cell and 2.5ng AO / A549 cell (Figure 3.3.2) (Appendix Table 3.3).
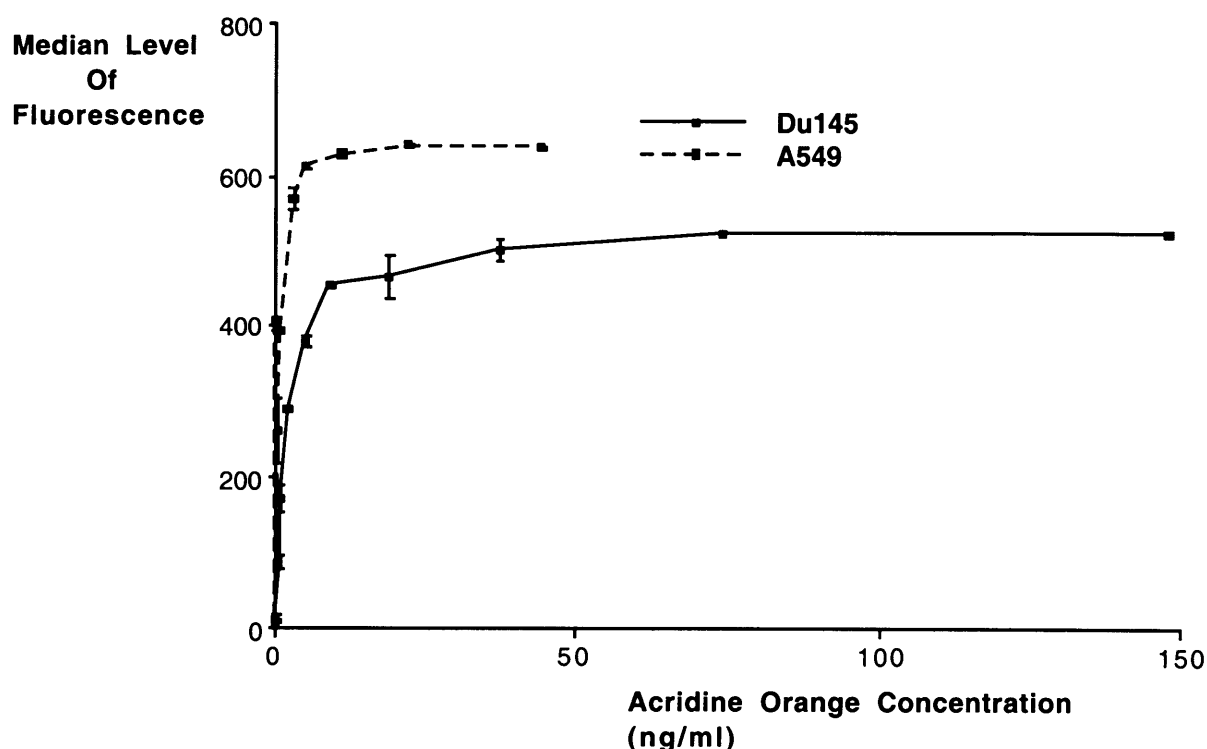
Figure 3.3.2 The Fluorescence Emitted By Lightly, Acridine Orange-Stained Du145 And A549 Cells Is Directly Proportional To The Concentration Of Acridine Orange Present. Cells were incubated with varying concentration of acridine orange (AO), as described in the text. This demonstrates that the optimal AO concentration, with respect to quantitatively labelling cells, was a) 1.0ng AO/ Du145 cell and b) 2.5ng AO/A549 cell. (Points plotted are the means of three measurements. Error bars represent the standard deviation of the mean.)

Having established that acridine orange could be used to stain prostatic epithelial cells, Du145 cells were labelled with AO and co-cultured with HUVECs for 1 hour. This was to investigate whether AO would allow the distinction between two populations of viable cells; namely, the labelled epithelial and the unlabelled endothelial cells. Du145 cells were trypsinised from the flask and resuspended in 1ml Ca^{2+} / Mg^{2+} free HBSS at a concentration of 4.48 x 10^5 cells/ml. From Figure 3.3.2, the optimal AO concentration for labelling Du145 cells was 1.0ng/cell. Therefore, for 4.48 x 10^5 Du145 cells 0.448μg of AO was required (1.0ng x 4.48 x10^5). The stock AO solution of 10mg/ml was serially diluted to give 0.448μg in 1ml PBS. The 1ml of Du145 cells was added to the 1ml of AO. This was left for 10 minutes in a dark 21^{0}C incubator. The solution was then washed in PBS at 663xg for seven minutes. The supernatant was discarded and the cell pellet was resuspended in ECM (Appendix 4.8) to a concentration of 1.12 x 10^5 cells/ml. 100μl of this suspension was combined with 100μl of a similar sample of Du145 cells that had not been stained with AO. This suspension was washed in PBS/Az at 663xg for seven minutes and resuspended in 150μl FACSFlow. The cells were analysed on the FACScan. Two populations of cells could be distinguished by their positive and negative FL1 emissions.

Therefore, the suspension of AO-stained Du145 cells were serially diluted in ECM to a minimum concentration of 1.75 x 10^4 cells/ml. 500μl of each cell suspension was added to confluent monolayers of HUVECs previously seeded in 24-well plates. The plates were incubated at 37^{0}C for 1 hour in an atmosphere of 5% CO_2. Following this period, unattached cells were carefully aspirated from the wells and pipetted into labelled 5ml polystyrene tubes. The attached cells were trypsinised (Chapter 2.1.2). All cells were washed in PBS/Az at 663xg for seven minutes. Cell pellets were resuspended in 150μl FACScan by Scocorex multi-stepper. Analysis was acquired on the Becton Dickinson FACScan.

Stained AO$^+$ Du145 cells can be clearly distinguished from AO$^-$ HUVECs at high cellular concentrations (Figure 3.3.3a). Figure 3.3.4 illustrates saturation levels of Du145 attachment occurs there are approximately 90% more Du145 cells than HUVECs (Appendix Table 3.4). This supports the theory that Du145 cells may also be attaching to each other as well as to the endothelial cells. However, the ratio of Du145 cells:HUVECs here is 9:1 compared to 2.33:1 described previously (Chapter 3.2.2).

The level of AO fluorescence, as well as the counts, appears to have been diluted as the cell number was serially diluted (Figure 3.3.3) The median level of fluorescence is the median of the fluorescence level per cell. Therefore, even as the cell number decreases the median level of fluorescence per cell should remain constant. It is most likely that the AO has leaked out from the cytoplasm of the original Du145 cell suspension and therefore, been diluted as the concentration of Du145 cells was diluted. To improve this procedure each cell dilution could be labelled with AO independently. However, the AO would still be capable of leaking out across

a) Co-culture of 5 x 10⁴ Du145 cells with A549 cells

```
        BECTON
    DICKINSON      FACScan Research Software   Version 2.1    3/89

Date: 10-OCT-97                                          Time: 15:57:32
Cytometer ID: FACScan

Sample ID: JH2.4.96 DU145 AO ATT A 001                   File Name: JH2.4690
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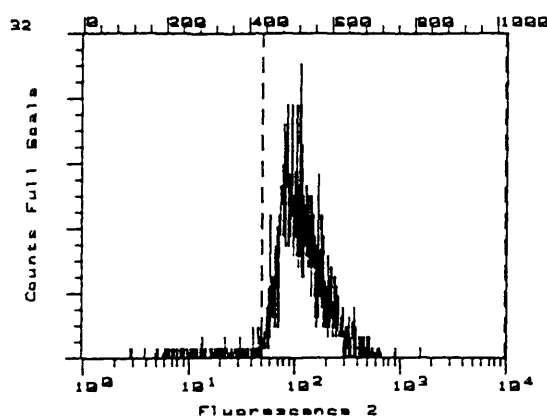


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    DICKINSON      FACScan Research Software   Version 2.1    3/89

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Cytometer ID: FACScan

Sample ID: JH2.4.96 DU145 ATT AO E 001                   File Name: JH2.4601
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b) Co-culture of 3.125 x10³ Du145 cells with A549 cells
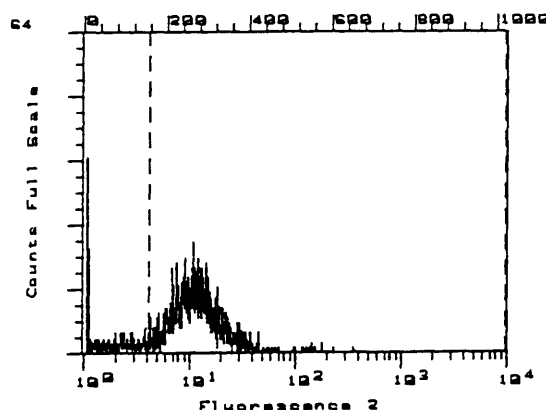
Figure 3.3.3 Dilution Of Du145 Cells Stained With Acridine Orange Dilutes The Fluorescence Emitted Due To Excitation Of Acridine Orange. A single cell suspension of Du145 cells was stained with acridine orange (AO) as described in the text. Serial dilutions of this cell preparation were made. Each dilution of cells was co-cultured with confluent monolayers of A549 cells for 1 hour. Resulting attached cells were analysed on the FACScan. A higher concentration of Du145 cells in a) gave higher levels of FL2 fluorescence and lower concentrations of Du145 cells in b) gave lower levels of fluorescence.
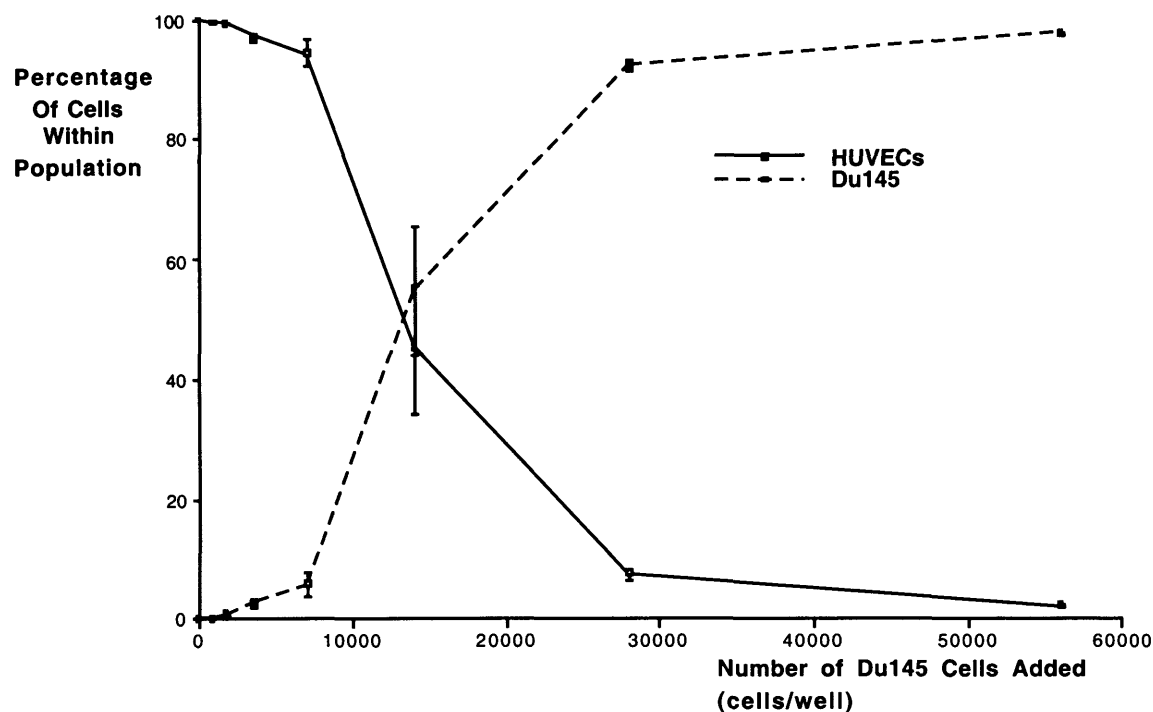
Figure 3.3.4 Saturation Of HUVECs With Acridine Orange- Stained Du145 Cells At A Concentration Of 4.5 x10⁴ Cell/well. Du145 cells were stained with acridine orange (AO), as described in the text. Serially diluted cells were then co-cultured with confluent monolayers of HUVECs for one hour. Attached and unattached cells were collected and analysed for fluorescent emission using a FACScan to determine the percentage of HUVECs and Du145 cells in each population.

the plasma membrane. It was concluded that Acridine Orange was not a suitable label for distinguishing the two cell types cultured together.

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3.4 Double Staining with FITC and The PKH26 Cell Linker Kit

PKH26-GL fluorescent cell linker uses patented Zynaxis technology to incorporate aliphatic reporter molecules into the lipid bilayer of the cytoplasmic membrane. Unlike acridine orange which, as seen in Chapter 3.3, leaches out of cells post-staining, the fluorescent probes of PKH26 remain incorporated into the membrane permanently, because of their inherent insolubility in aqueous environments (Horan and Slezak, 1989).

The fluorescent marker of PKH26 has excitation and emission wavelengths of 551nm and 567nm, respectively. Therefore, PKH26 was a suitable fluorochrome for use with the BD FACScan, with its emissions measured by the FL2 detectors. The manner of PKH26 incorporation into the membrane was examined. PKH26 was prepared to varying concentrations in a specific diluent supplied as part of the kit. Five suspensions of Du145 cells (10^6) were stained with 5 concentrations of PKH26, ranging from 0 to 10 x 10^{-6} M PKH26, as per the manufacturer's instructions described in 2.4.2.3.2. Briefly, 10^6 Du145 cells were prepared in exactly 1ml of diluent. 1ml PKH26 was added to cells for four minutes. The staining was stopped by the addition of 2ml FCS for one minute, after which 4ml of culture medium was added. The cell suspension was washed several times to remove excess dye. A proportion of cells was analysed immediately while the remainder was cultured in a TCGF for 3 days under standard tissue culture conditions.
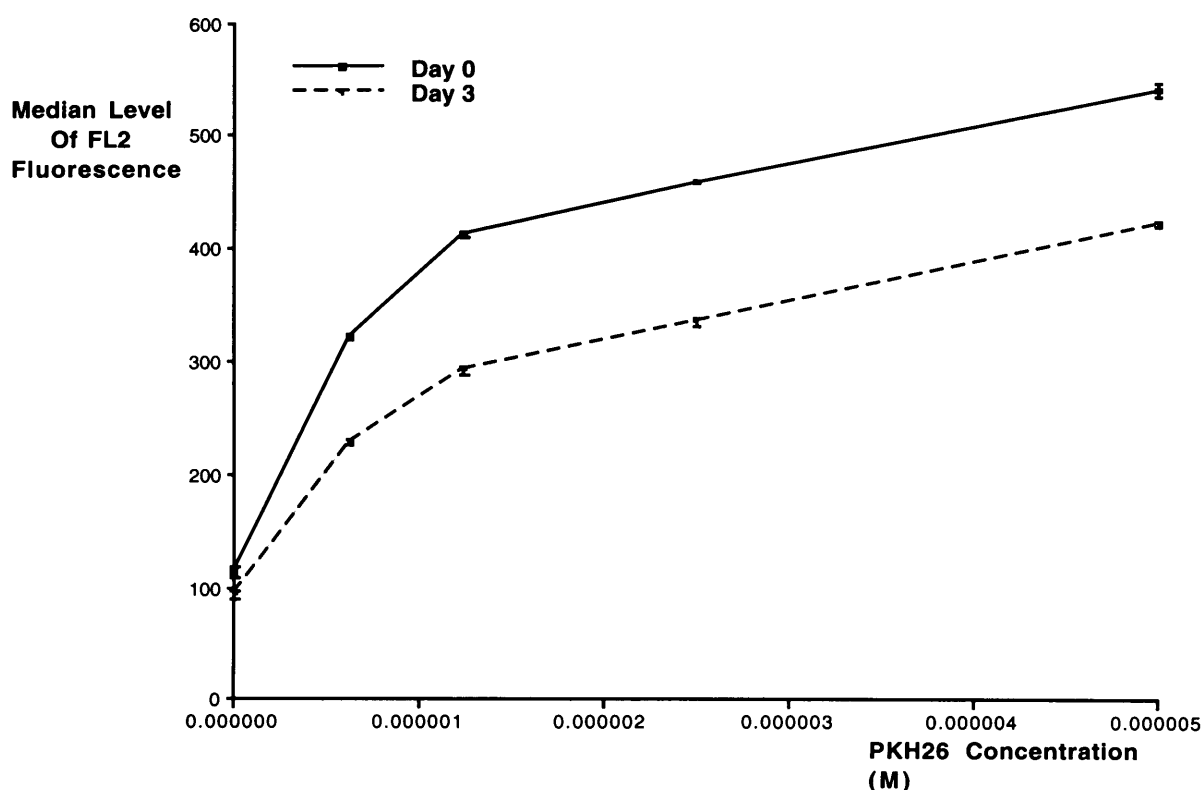
Figure 3.4.1 The Manner Of PKH26 Fluorescence Following Incorporation Into The Cytoplasmic Membrane Of Du145 Cells. Du145 cells (10^6) were stained with varying concentrations of PKH26, as detailed in the text. A PKH26 concentration of 5 x10^{-6} M produced Du145 cells that could be clearly distinguished from non-stained Du145 cells, and stained with lower concentration of PKH26. This pattern is also clearly demonstrated on cells that had been cultured for three days following staining.

One of the reported problems of PKH26 was overlabelling of cells. Figure 3.4.1 demonstrated that 5 x10^{-6} M PKH26 is a safe concentration for the labelling of Du145 cells: while it appears not to have reached maximum levels of staining, and thereby not reached toxic concentrations, it has reached sufficient concentrations that allow differentiation between stained and non-stained cells. One can also see that 5x10^{-6} M PKH26 is an optimal concentration to use for labelling cells to be further cultured. Three days following the staining procedure PKH26 remained within the cytoplasmic membrane of Du145 cells, and was incorporated into the membrane of daughter cells as demonstrated in Figure 3.4.1 (Appendix Table 3.5).

The effect of PKH26 on monoclonal antibody binding was investigated. Du145 cells (10^6) were dyed with 5 x10^{-6} M PKH26 as detailed above. A second population of Du145 cells (10^6) were incubated with the PKH26 diluent only (i.e. no dye). Both populations of cells were stained with mouse anti-human ICAM-1 and goat anti-mouse immunoglobulin conjugated to FITC as described in Chapter 2.4.2.3.2. Figure 3.4.2 demonstrates that the addition of PKH26 dye has no effect on the ability of monoclonal antibody to bind cell surface antigens. It can be concluded that the PKH26 used in this manner (i.e. Chapter 2.4.2.3.2) does not alter the cellular membrane integrity (Appendix Table 3.6).
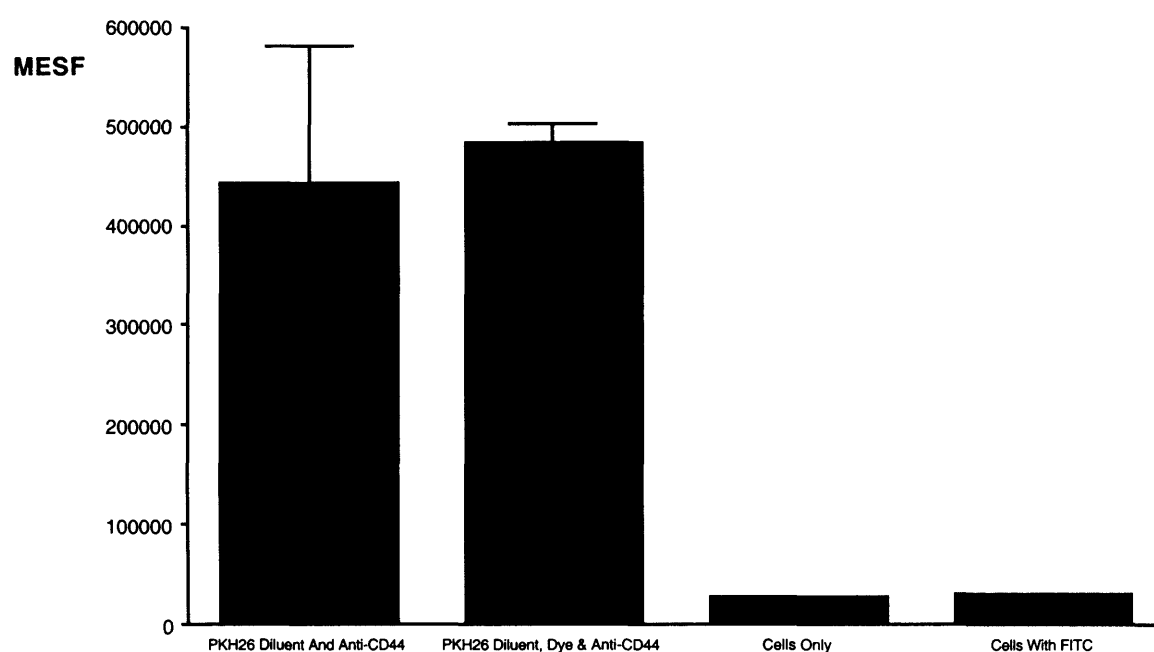
Figure 3.4.2 PKH26 Dye Does Not Interfere With The Interaction Of Monoclonal Antibodies And Cell Surface Antigens. Du145 cells were incubated with either PKH26 diluent only or PKH26 diluent and dye. Cells were then incubated with mouse anti-human intercellular cell adhesion molecule antibody and goat anti-mouse immunoglobulin conjugated to FITC. (Median levels of fluorescence were transformed to MESF values as described in Chapter 2.4.2.4. Values plotted are the means of three measurements. Error bars represent the standard deviation of those means.)

The next step was to examine the usefulness of PKH26 in a co-culture system with endothelial and epithelial cells, as was performed for acridine orange and PE previously. At this time point we were experiencing difficulties in propagating cultures of endothelial cells. Therefore, the lung epithelial cell line, A549, was employed. A549 are of a similar size to endothelial cells and were cultured as described in Chapter 2.3.1. Du145 cells were stained with PKH26 as detailed in Chapter 2.4.2.3.2 and prepared to a final concentration of 9 x10^4 cells/ml. A549 cells were cultured in 24-well plates and used at 100% confluence. Du145 cells (500µl) were added to the confluent monolayers of A549 cells. These cell co-cultures were incubated for 1 hour under standard tissue culture conditions. Attached and unattached cells were collected by trypsinisation and aspiration and stained with mouse anti-human CD44, as described in Chapter 2.4.2.3.2. Cell populations were then analysed for fluorescence on the BD FACScan.
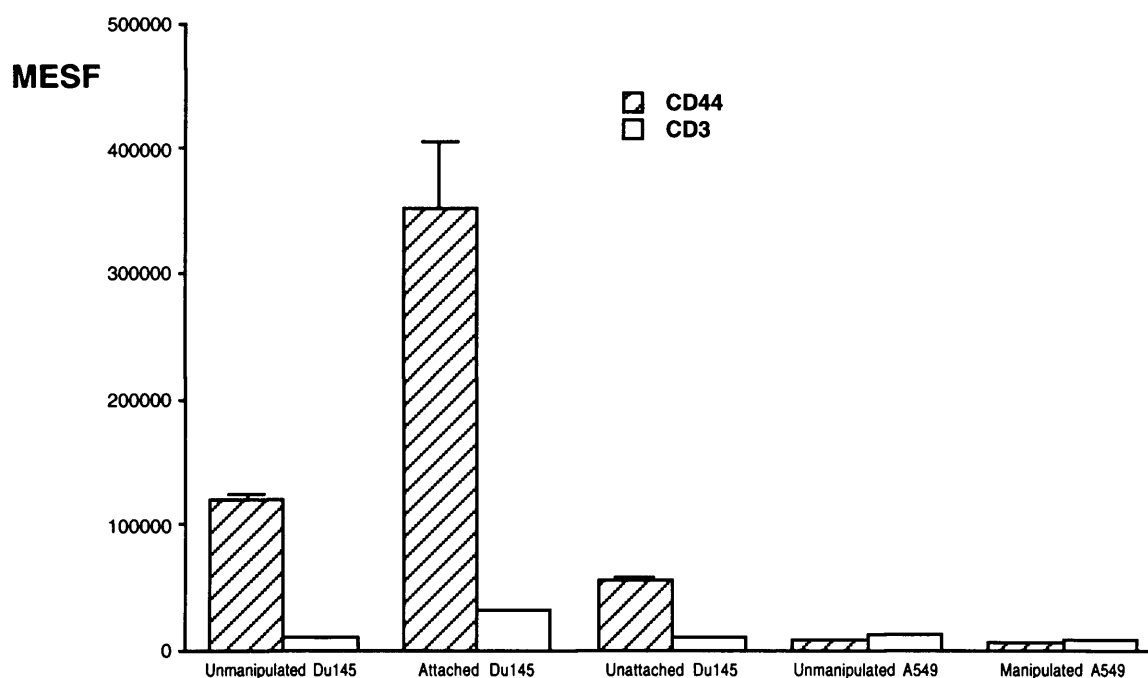
Figure 3.4.3 Fluorescence Emitted By PKH26$^+$ Du145 And PKH26$^-$ A549 Cells Following One Hour Of Co-culture. Du145 cells were stained with PKH26, as detailed in the text. These cells were then incubated with confluent monolayers of A549 cells for one hour in 24-well TCGPs. Attached, unattached, and unmanipulated cells were assayed separately for their surface expression of CD44 and CD3 by standard FACScan analysis. In mixed populations Du145 and A549 cells were separated by the level of FL2 fluorescence emitted after PKH26 excitation. (Columns represent the mean value of three measurements. Error bars represent the standard deviation of those means.)

PKH26$^+$ Du145 cells and PKH26$^-$ A549 cells were clearly distinguishable after one hour of co-culture. One can also see that monoclonal antibody linked indirectly to the FL1 fluorophore, FITC, can be used concurrently to PKH26 to observe cell surface activity. In this

assay one can observe quite clearly the different levels of expression of cell surface antigen by PKH26$^+$ Du145 cells and PKH26$^-$ A549 cells (Figure 3.4.3) (Appendix Table 3.7).

a)

b)
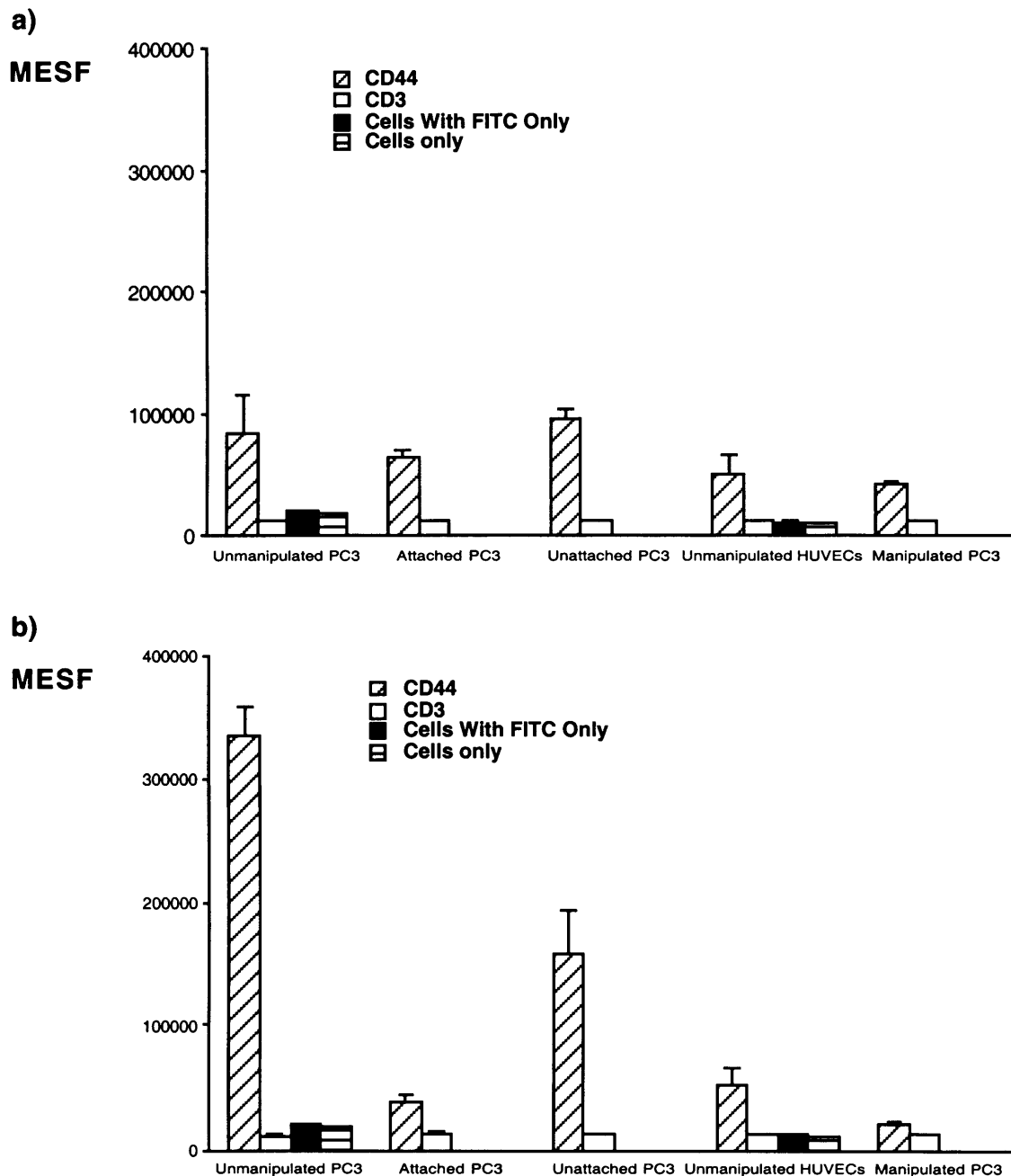
Figure 3.4.4 Fluorescence Emitted By PKH26$^+$ PC3 Cells And PKH26$^-$ HUVECs Following Co-culture. PC3 cells were stained with PKH26 as described in the text. Dyed cells were cultured in direct contact with confluent monolayers of HUVECs for 1 hour. Attached, unattached and unmanipulated cells were collected. A) cells were analysed immediately for surface expression of CD44 and CD3. B) cells were re-cultured for 24 hours before analysed for surface expression of CD44 and CD3. (Columns represent the mean MESF of three measurements. Median levels of fluorescence were converted to MESF values as described in Chapter 2.4.2.4. Error bars represent the standard deviation of the means.)

The next step was to ensure that PKH26 had no adverse effect during prolonged co-culture. Therefore, cells were cultured together for 1 hour and analysed as before. However, a second co-culture plate was established with the same cells. This plate was incubated for the initial 1 hour; cells were trypsinised and re-seeded in a fresh 24-well plate where they were cultured for a further 24 hours. On this occasion, PC3 cells were co-cultured with HUVECs. This was to ensure that staining of PC3 cells with PKH26 had no adverse effects on the experimental protocol.

PKH26 labelling of cells permitted the determination of cell surface antigen expression of a mixed population of two cell types (Appendix Table 3.8). Pre-staining PC3 cells with PKH26, and subsequent co-culture with confluent monolayers of HUVECs, allows the levels of CD44 and CD3 to be measured on both cell types individually. The measurements were reliable after both a short 1 hour and long 24 hour re-culture (Figure 3.4.4).

Price *et al* (1996) demonstrated that fluorophores could be used quantitatively to determine cell number using a Fluoroskan II plate reader. This laboratory only had access to a TiterTek multiscan plate reader. Although it was considered unlikely that PKH26 would be recognised by any of the filters of this machine, it was investigated whether PKH26 could be useful as a cell number determinant. A549 were prepared by trypsinisation as in Chapter 2.1.2. Cells (9.4×10^4 cells) were stained with PKH26 as described above. Serial dilutions were made to a final concentration of 3.67×10^2 cells/ml. 90µl of each dilution was pipetted into a flat bottomed 96-well plate, in duplicate, and left to adhere overnight. The plate was washed three times in PBS. Attached cells were lysed with 50µl of 10% SDS for 90 minutes at room temperature. The plate was vortexed and cell debris was collected by centrifugation. The optical density of the wells of the plate was measured on the TiterTek plate reader on all functional filters. (Filter number 2 was not functional.)

Figure 3.4.5 shows that the fluorophore PKH26 did not have an optical density that could be quantitatively measured on a TiterTek plate reader. It can be concluded that PKH26 could not be used as a method of calculating cell number in this co-culture system (Appendix Table 3.9).

To summarise, mouse anti-human CD31 conjugated to PE is a useful tool for the distinction of HUVECs and epithelial cells in suspension and following a direct co-culture. However, the expense of this antibody reduced its usefulness in this study. Pre-labelling of cells with Acridine Orange (AO) is not a satisfactory method for differentiating between two cell populations. Whilst AO is taken up by cells allowing them to be pictured on the FACScan, the AO leaks out from the cell and may by taken up by the second cell population over time. However, it can be concluded that PKH26 was a useful cell membrane dye in both short- and long-term co-culture systems, but could not be used quantitatively, as a measurement of cell number.
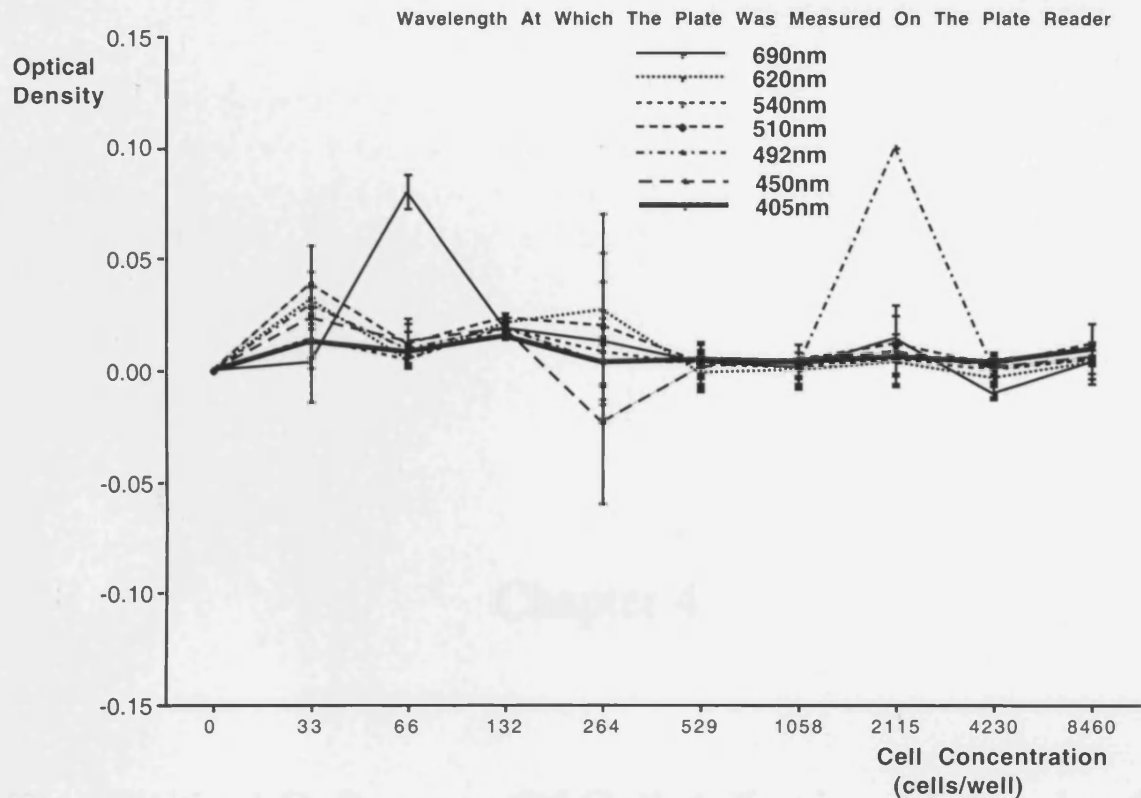
Figure 3.4.5 The Optical Density Of PKH26⁺ A549 Cells Measured By A TiterTek Plate Reader. A549 cells were prepared by trypsinisation. Cells were stained with PKH26 and serial dilutions were prepared, as describe in the text. Cells of each dilution were incubated overnight in a flat-bottomed 96-well plate. The plate was washed twice with PBS and the cells were lysed with 10% SDS. Optical density did not correlate to the level of PKH26 present and therefore the number of cells present. (Points plotted are the means of two measurements. Error bars represent the standard deviation of those means.)

Chapter 4

The Clinical Relevance Of Cell Adhesion Molecules On

The Progression Of Prostate Cancer

Contents

4.1 Introduction

It is the hypothesis of this study that the progression of prostate cancer is regulated by the expression of CAMs. Chapters 3 and 5 look at the role of these molecules at the site of tumour cell extravasation and intravasation. However, as discussed in Chapter 1.4, the progression of prostate cancer is a complex cascade of events and the interaction of tumour cells with vascular endothelial cells is only one of these events. Before a tumour cell can communicate with the vascular endothelium it must first escape from the primary tumour and invade through the basement membrane or extracellular matrix (ECM) and stroma of the surrounding tissue. In the case of prostate cancer, a tumour cell must first escape from the primary tumour within the glandular epithelium and invade through the prostatic stroma to the blood vessels. The first barrier that the tumour cell encounters is the basement membrane, which separates the glandular epithelial cells from the non-glandular stroma. The basement membrane contains extracellular proteins, including entactin, fibronectin, laminin, and collagen. Prostatic stroma is a generic term given to non-glandular prostatic tissue: this tissue is largely composed of fibroblastic cells and striated and smooth muscle cells. Therefore, for a prostatic carcinoma cell to extravasate, it must invade into and through the basement membrane, interacting with the aforementioned ECM proteins, and through the non-glandular stroma to a blood vessel. Once it reaches a blood vessel, the tumour cell must invade through the layer of smooth muscle that protects the vascular endothelial cells, before it can escape into the circulation. Similar events occur for the escape of a tumour cell into the lymphatic system.

As mentioned in Chapter 1.1, CAMs can interact with the ECM proteins. Therefore, it could be hypothesised that the expression of CAMs by primary cancer cells could influence their invasive character. Indeed, the expression of CAMs by many cancer cells, which their benign counterparts do not normally express or express at different levels, has been shown to control their invasive behaviour (discussed in detail in Chapter 1.5). Therefore, the expression of CAMs by primary prostatic cancer cells was immunohistochemically investigated in this study.

Prostatic tissue was obtained from patients undergoing radical prostatectomy or transurethral resection of the prostate (TURP) for clinical disease of the prostate. Tissue was snap frozen in liquid nitrogen and the expression of E-selectin, Intercellular Cell Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1, (VCAM-1), CD44, $\alpha 4$, $\alpha 5$, αL, and $\beta 1$ was examined on frozen sections by immunohistochemistry, using the alkaline phosphatase and anti-alkaline phosphatase (APAAP) method of detection, as described in Chapter 2.4.1. Briefly, tissue was incubated with mouse monoclonal antibodies against these CAMs, rabbit anti-mouse Haematoxylin and Eosin (H & E) section of each tissue sample was analysed histologically by a trained, consultant histopathologist (Dr. K O'Reilly of the Leicester Area Histopathology Service, Leicester General Hospital), to determine the state of differentiation of the tissue

microscopically. Patient notes were consulted to determine the clinical background of all patients, including the metastatic classification of all carcinoma patients (Table 4.1). The expression of CAMs was then compared between samples from prostatic carcinomas and benign prostatic hyperplasia (BPH).

Patient Study No.	Tissue Grade	Metastatic Status	Follow-up (Time)	*In Vivo* Treatment Regime	Sample Type
4	G5	No, Mo	No Change (30 months)	Orchiectomy (P) Anti-oestrogen (B)	TURP
7	G2-5	No, Mo	No Change (36 months)	Orchiectomy (P) Anti-oestrogen (B)	TURP
12	G5	Nx, Mo	No Change (36 months)	None	TURP
14	G3	Extracapsular invasion, No, Mo	No Change (36 months)	Anti-oestrogen (P)	Radical Prostatectomy
18	G3	Extracapsular invasion, No, Mo	No Change (24 months)	Anti-oestrogen (P)	Radical Prostatectomy
25	G5	No, Mo	No Change (12 months)	Anti-oestrogen (B & P)	TURP
27	G4	Extracapsular invasion, No, Mo	No Change (42 months)	Anti-oestrogen (B)	Radical Prostatectomy
28	G2	Mo	No Change (36 months)	None	Radical Prostatectomy
35	G2	Mx (bone)	No Change (24 months)	Anti-oestrogen (B)	TURP
49	G2/3/4	No, Mo	Mx (24 months)	Anti-oestrogen (P)	Radical Prostatectomy
51	G3/4	Mx	Hormone Resistance (18 months)	Anti-oestrogen (P)	TURP
70	G2	No, Mo	No Change (36 months)	None	TURP

Patient Study No.	Tissue Grade	Metastatic Status	Follow-up (Time)	*In Vivo* Treatment Regime	Sample Type
76	G2	No, Mo	No Change (36 months)	None	Radical Prostatectomy
80	G5	No, Mo	No Change (30 months)	None	TURP
94	G3	No, Mo	No Change (12 months)	Anti-oestrogen (B & P)	TURP
100	G2	No, Mo	No Change (24 months)	None	Radical Prostatectomy
102	G3/4/5	Mx	Death (2 months)	None	TURP
106	G2	No, Mo	No Change (36 months)	None	TURP
118	G2	No, Mo	PSA not detectable (18 months)	None	Radical Prostatectomy
123	G3	Mx	No Change (12 months)	Anti-oestrogen	TURP
126	G4	No, Mo	No Change (24 months)	Anti-oestrogen (P)	Radical Prostatectomy
134	G3	No, Mo	No Change (30 months)	None	Cystectomy

Table 4.1 Clinical Characteristics Of Malignant Prostatic Tissue Collected In This Study. Tissue grade indicates the Gleason grade of differentiation of the sample. No indicates no local lymph node involvement; Mo indicates no evidence of metastatic deposits; Mx indicates evidence of metastatic deposits; (B) indicates an event that occurred before the time of sample; (P) indicates an event that occurred after the time of sample. Extracapsular invasion indicates that the primary tumour has invaded into local tissue that is outside the prostatic capsule.

immunoglobulin and immune complexes of alkaline phosphatase and anti-alkaline phosphatase (APAAP) and the Vector Red Enzyme Substrate, which resulted in a red positive stain. The dilution at which each monoclonal antibody was used is described in Chapter 2.4.1. A

Sections were given an immunohistochemical score of 0, 1, 2, 3, 4, 5, or 6, where a score of 0 represents no expression and a score of 6 represents uniform expression by all nucleated cells (Table 4.2)

Immunohistochemical Score	Percentage Of Epithelial/Stromal Compartment Stained
0	0
1	<5
2	5-20
3	20-50
4	50-80
5	>80, but <100
6	100

Table 4.2 The Immunohistochemical Scoring System Adopted For Analysis Of Frozen Sections Of Solid Prostate.

4.2 Epithelial And Stromal Composition Of Prostatic Sections

Seventy-six prostatic samples were collected: 54 of these samples were classified as benign and 22 were malignant. Histological and clinical details of the malignant tissue are detailed in Table 4.1. The epithelial composition of the tissue was determined with monoclonal antibodies against cytokeratin (CK), Prostate Specific Antigen (PSA) and Prostatic Acid Phosphatase (PAP). Specifically, two cytokeratin antibodies were used: the first recognised CK-8, which is expressed in the membranes of glandular epithelial cells and the second recognised all cytokeratins and was denoted as CK-pan.

Of the 54 benign prostatic samples collected only 45 contained glandular epithelial structures that demonstrated staining with one or more of the epithelial cell markers. Therefore, only 45 benign prostatic samples were analysed for the epithelial expression of CAMs (Diagram 4.1). Of the 22 malignant prostatic samples collected, only 21 contained epithelial structures that expressed one or more of the epithelial cell markers. Therefore, only 21 malignant prostatic samples were available for the analysis for epithelial CAM expression (Diagram 4.2). The morphological differences between a benign hyperplastic prostatic gland and a malignant prostatic gland are highlighted in Diagram 4.3. The upper hyperplastic gland has the typical leaf-like structure of well differentiated prostatic epithelial cells. The lower photograph presents a poorly differentiated adenocarcinoma with a Gleason Grade of G5: i.e. the carcinoma is poorly defined and has a ragged appearance, with evidence of stromal infiltration. Vascular endothelial cells of blood vessels were histologically identifiable by eye (under light microscopy). Furthermore, the presence of endothelial cells was immunochemically determined by the presence of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1).

54 Benign Prostatic Samples

All Analysed For Epithelial Markers

(CK-8, CK-pan, PAP, & PSA)

13 CK-pan⁻

41 CK-pan⁺

9	3	1	25	4	4	4	1	1	2
PAP-	PAP-	PAP⁺	PAP⁺	PAP-	PAP⁺	PAP⁺	PAP-	PAP-	PAP-
PSA-	PSA-	PSA⁺	PSA⁺	PSA⁺	PSA-	PSA⁺	PSA-	PSA⁺	PSA-
CK-8-	CK-8⁺	CK-8⁺	CK-8⁺	CK-8⁺	CK-8⁺	CK-8-	CK-8⁺	CK-8-	CK-8-
GS-	GS⁺	GS⁺	GS⁺	GS⁺	GS⁺	GS⁺	GS⁺	GS⁺	GS

9

Not Analysed
For Epithelial
Expression
Of CAMs

45

Analysed For
Epithelial
Expression
Of CAMs
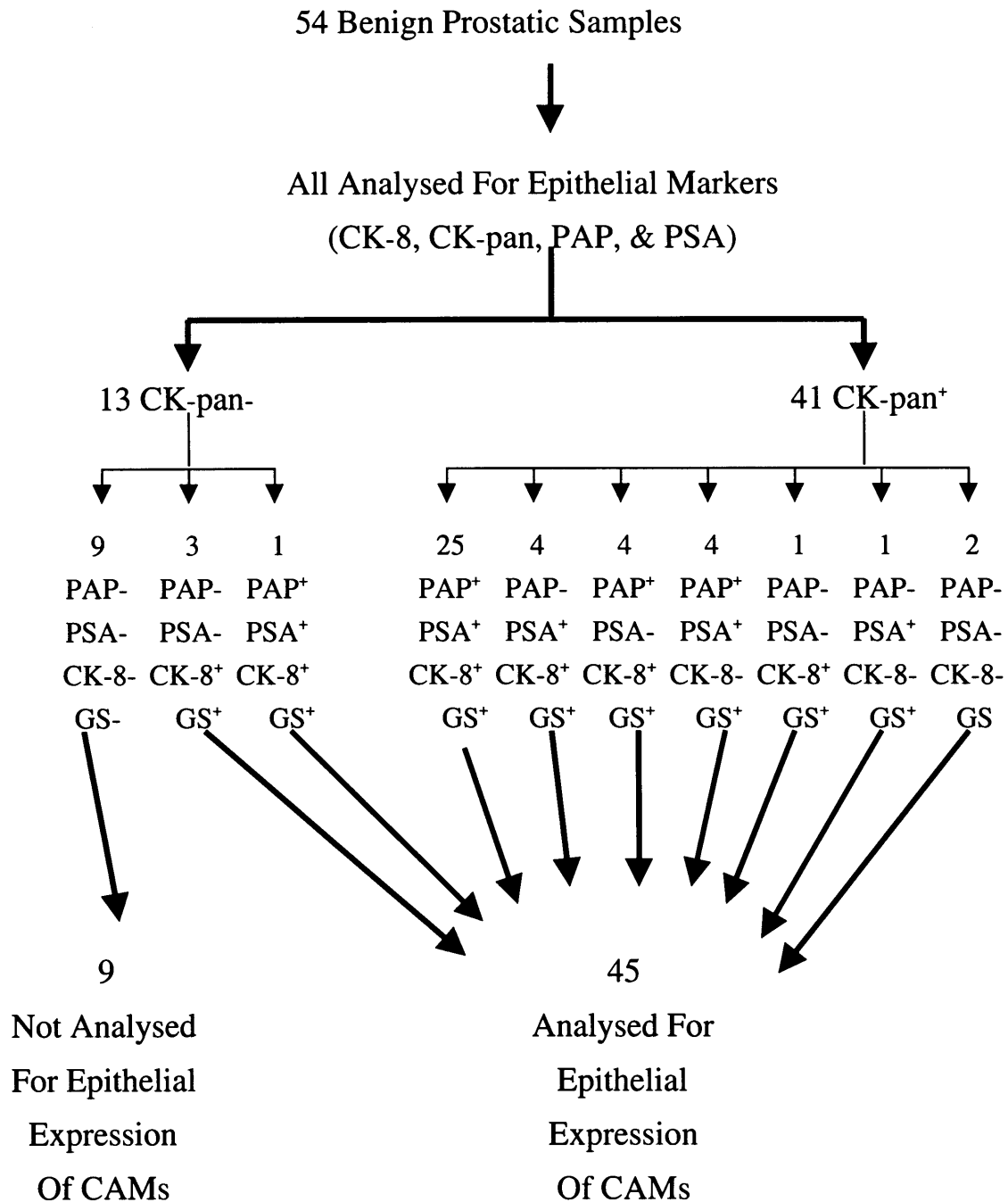
Diagram 4.1 Epithelial Distribution Of Benign Prostatic Samples. Fifty-four benign prostatic sections were immunohistochemically analysed using monoclonal antibodies against cytokeratin-8 (CK-8), cytokeratin-pan (CK-pan), prostatic acid phosphatase (PAP), and prostate specific antigen (PSA), as described in the text. Sections were analysed under light microscopy to determine their epithelial content. 45 samples were available for analysis of epithelial cell adhesion molecule (CAM) expression. (GS, glandular structure.)

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22 Malignant Prostate Samples

↓

All Analysed For Epithelial Markers

(CK-8, CK-pan, PAP, & PSA)

1	1	1	1	1	17
CK-pan-	CK-pan-	CK-pan-	CK-pan-	CK-pan$^+$	CK-pan$^+$
PAP-	PAP$^+$	PAP$^+$	PAP$^+$	PAP-	PAP$^+$
PSA-	PSA-	PSA$^+$	PSA$^+$	PSA$^+$	PSA$^+$
CK-8-	CK-8+	CK-8-	CK-8+	CK-8+	CK-8+
GS-	GS+	GS+	GS+	GS+	GS+

1

Not Analysed

For Epithelial

Expression

Of CAMs

21

Analysed For

Epithelial

Expression

Of CAMs
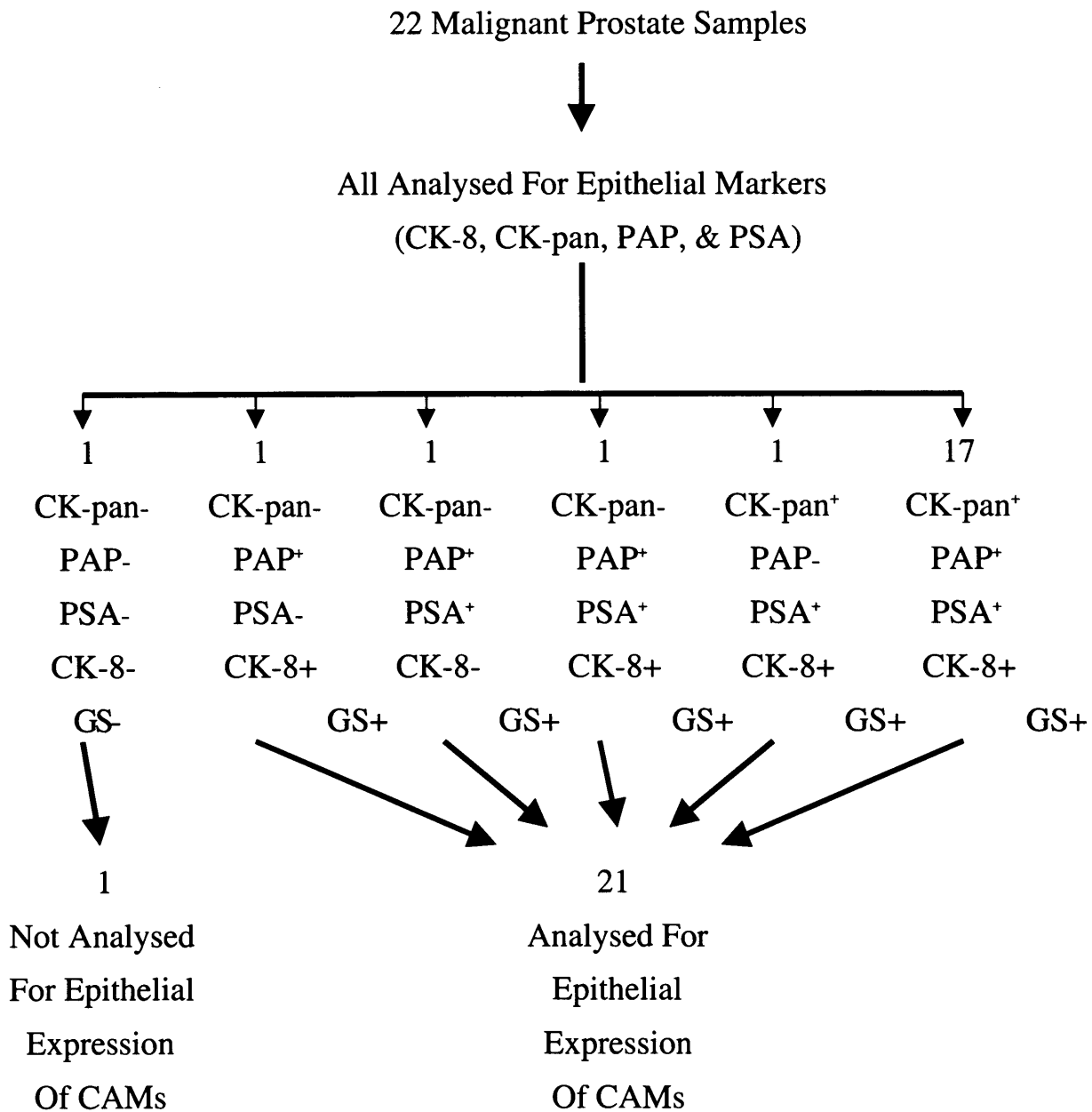
Diagram 4.2 The Epithelial Distribution Of Malignant Prostatic Samples. Twenty-two malignant prostatic samples were immunohistologically analysed with monoclonal antibodies against cytokeratin-8 (CK-8), cytokeratin-pan (CK-pan), prostatic acid phosphatase (PAP), and prostate specific antigen (PSA). Sections were examined under light microscopy to determine epithelial content. 21 samples were suitable for analysing epithelial cell adhesion molecule (CAM) expression. (GS, glandular structures.)

a)
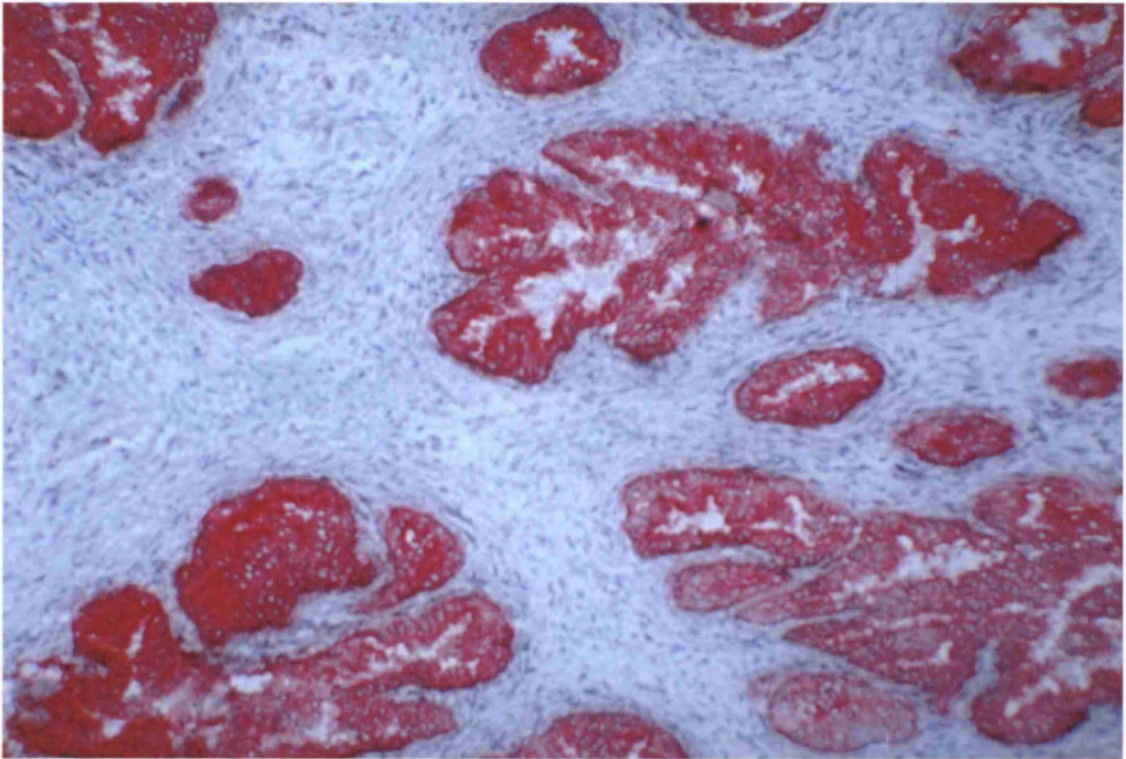
b)
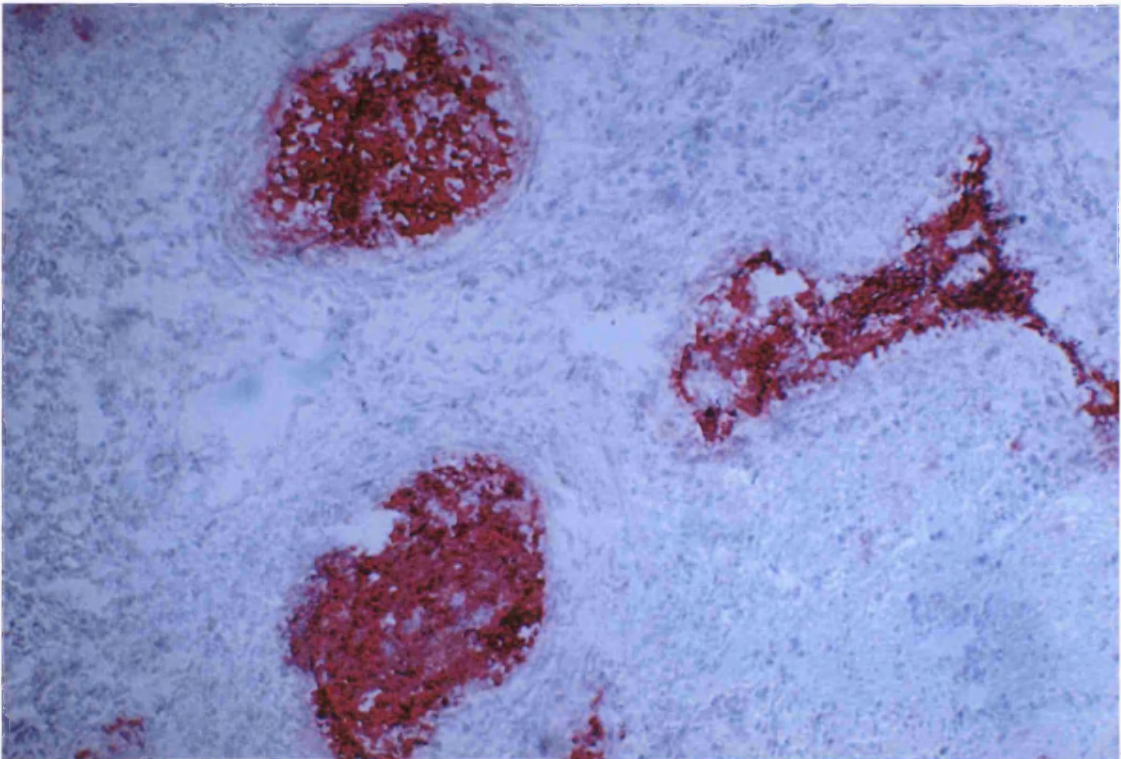
Diagram 4.3 The Glandular Structures Of Benign Prostatic Hyperplastic And Malignant Prostatic Tissue. Frozen section of a) a benign prostate and b) a Gleason Grade G5 prostatic adenocarcinoma were immunohistochemically stained monoclonal antibody against cytokeratin. (Magnification, x10.)

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4.3 The Distribution Of Cell Adhesion Molecules In Benign Hyperplastic Prostatic Tissue

Benign prostatic tissue, referred to as Benign Prostatic Hyperplasia or Hyperplastic (BPH) tissue from here on, was examined for the expression of E-selectin, Intercellular Cell Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), α4, α5, αL, β1, and CD44. CAM expression was not consistent on all tissue examined. ICAM-1 was expressed on the epithelium of 25 of the 45 samples examined and was the most frequently expressed CAM in benign prostatic epithelium. Alpha-4 was expressed in the epithelial cells of 17 of the 35 samples analysed. Epithelial CD44 expression could be demonstrated on 19 of the 41 samples investigated and αL expression was depicted in the epithelial cells of 15 of the 37 samples examined. Epithelial expression of E-selectin, VCAM-1, α5 and β1 could be demonstrated on approximately one third of the samples analysed (Table 4.3).

	E-selectin	ICAM-1	VCAM-1	PECAM-1	α4	α5	αL	β1	CD44
Number Of Sections Examined	45	45	38	42	35	38	37	38	41
Number That Did Not Express Marker	32	20	26	38	18	31	22	26	22
Number That Expressed Marker	14	25	12	4	17	7	15	12	19

Table 4.3 Numerical Details Of Benign Hyperplastic Tissues Examined For The Epithelial Expression Of Cell Adhesion Molecules.

E-selectin was expressed on the epithelial cells of 14 of the 45 samples examined. The E-selectin was randomly expressed in the epithelium and its distribution did not appear to polarise upon the cell membrane. E-selectin was not specifically expressed on basal or luminal epithelial cells. E-selectin was expressed in low levels within the epithelium, with an average immunohistochemical score of 0.4. Only one sample demonstrated high levels of E-selectin expression with an IS of 5 (Appendix Table 4.1). To summarise, E-selectin was expressed in low levels in the prostatic glands

a)
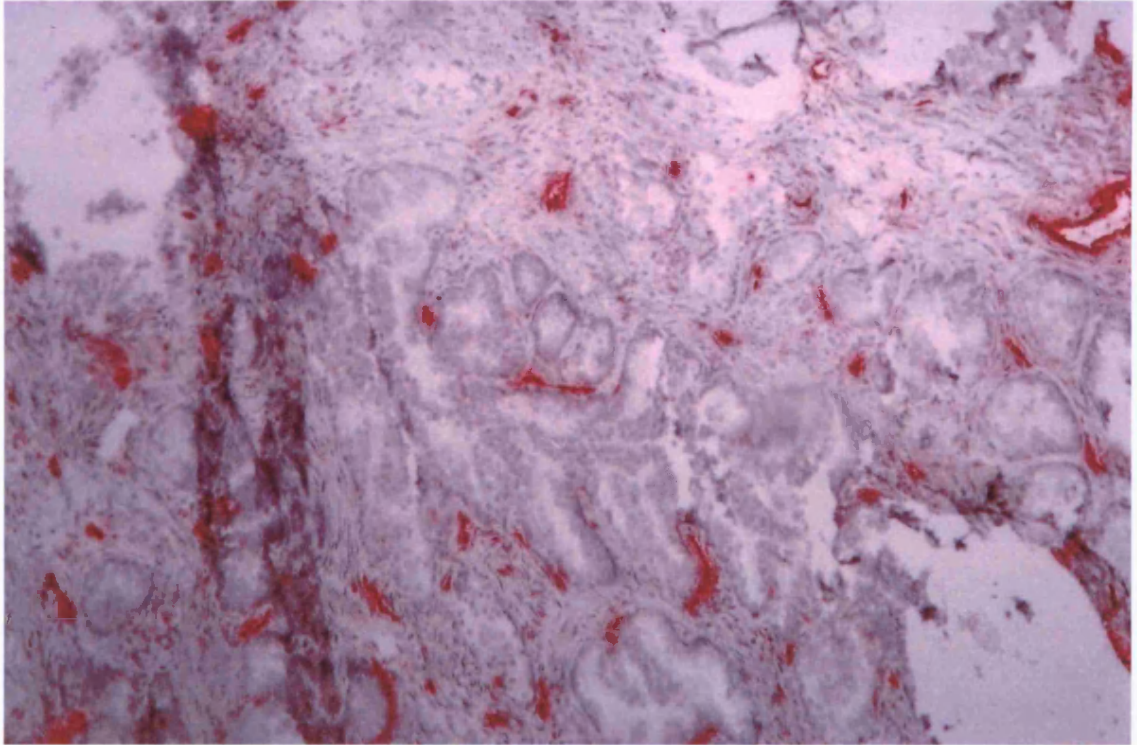
b)
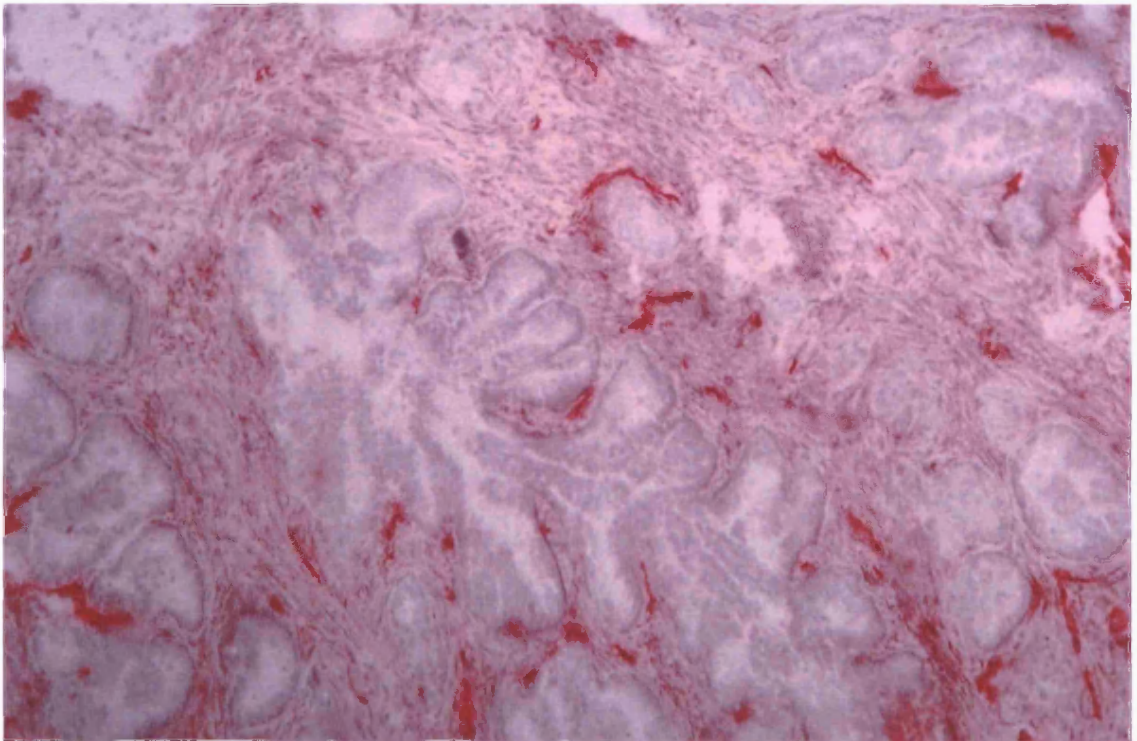
Diagram 4.4 The Expression Of PECAM-1 And ICAM-1 In The Stroma Adjacent To And Within Benign Prostatic Glands. Frozen sections of benign hyperplastic prostatic tissue were incubated with monoclonal antibodies against a) PECAM-1 and b) ICAM-1. The Vector Red Enzyme Substrate detection system was used. Arrows highlight PECAM-1$^+$ and ICAM-1$^+$ cells within the glandular structures. (Magnification, x10.)

ICAM-1 was expressed on the epithelial cells of 25 of the 45 samples analysed. Some of the ICAM-1$^+$ cells within the epithelium were PECAM-1$^+$ vascular endothelial cells; i.e. blood vessels were found within the glandular epithelium of benign prostatic tissue. However approximately one third (31%) of the samples examined contained ICAM-1$^+$ cells that were not PECAM-1$^+$ endothelial cells: some cells were glandular epithelial cells (Diagram 4.4). Nine samples contained ICAM-1$^+$/VCAM-1$^+$ epithelial cells. However, not all hyperplastic prostatic glands within these samples contained ICAM-1$^+$/VCAM-1$^+$ cells: three samples contained both double positive ICAM-1$^+$/VCAM-1$^+$ cells and ICAM-1$^+$ cells only. The epithelium of seven of the above nine samples contained ICAM-1$^+$/E-selectin$^+$ cells, although not all ICAM-1$^+$ epithelial cells were E-selectin$^+$. Four samples of benign hyperplastic prostatic tissue contained ICAM-1$^+$/VCAM-1$^+$/E-selectin$^+$ epithelial cells. Three samples demonstrated ICAM-1 expression on the basal epithelial cells: however, ICAM-1 was randomly distributed within the glandular epithelium in the remaining 22 samples. The majority of epithelia demonstrated low levels of ICAM-1 expression with ISs of 1.0; however, one epithelia expressed moderate levels of ICAM-1 with an IS of 3 and all the epithelial cells of a second sample expressed ICAM-1, having an IS of 6. These data gave a mean IS of 1 for the epithelial expression of ICAM-1 (Appendix Table 4.1).

Therefore, ICAM-1 expression could be demonstrated on glandular epithelial cells of 25 (56%) of the BPH samples analysed in low levels. ICAM-1$^+$/PECAM-1$^+$ cells, which appeared to form organised vascular structures, were observed within the prostatic glands of four samples.

VCAM-1 was expressed on the epithelial cells of 11 of the 38 benign hyperplastic prostatic samples examined. Twelve BPH samples displayed VCAM-1$^+$/PECAM-1$^+$ vascular endothelial cells within the glandular epithelium. As mentioned above, nine samples displayed ICAM-1$^+$/VCAM-1$^+$ double-positive epithelial cells. Six of these nine samples contained only VCAM-1$^+$/ICAM-1$^+$ cells, while the remaining three demonstrated the presence of both VCAM-1$^+$/ICAM-1$^+$ cells and VCAM-1$^+$/ICAM-1$^-$ cells. Three samples contained VCAM-1$^+$/E-selectin$^+$ cells within the epithelium and four samples contained VCAM-1$^+$/ICAM-1$^+$/E-selectin$^+$ cells within the glandular epithelium: not all of the VCAM-1$^+$ cells in these samples were double- and triple-positive, respectively. In general prostatic glandular epithelium expressed VCAM-1 in low levels, with eight samples having an IS of 1, two with an IS of 2 and one with an IS of 3. Those samples with higher ISs had corresponding high levels of expression of ICAM-1, but not of E-selectin or PECAM-1. The mean IS for VCAM-1 expression within the epithelium of benign hyperplastic prostatic tissue was 0.4 (Appendix Table 4.1).

Therefore, VCAM-1 was demonstrated on the epithelial cells of eleven (29%) samples of BPH tissue. The majority of these samples showed low levels of expression of VCAM-1. Those that displayed higher levels of expression of VCAM-1 also displayed higher levels of ICAM-1.

a)
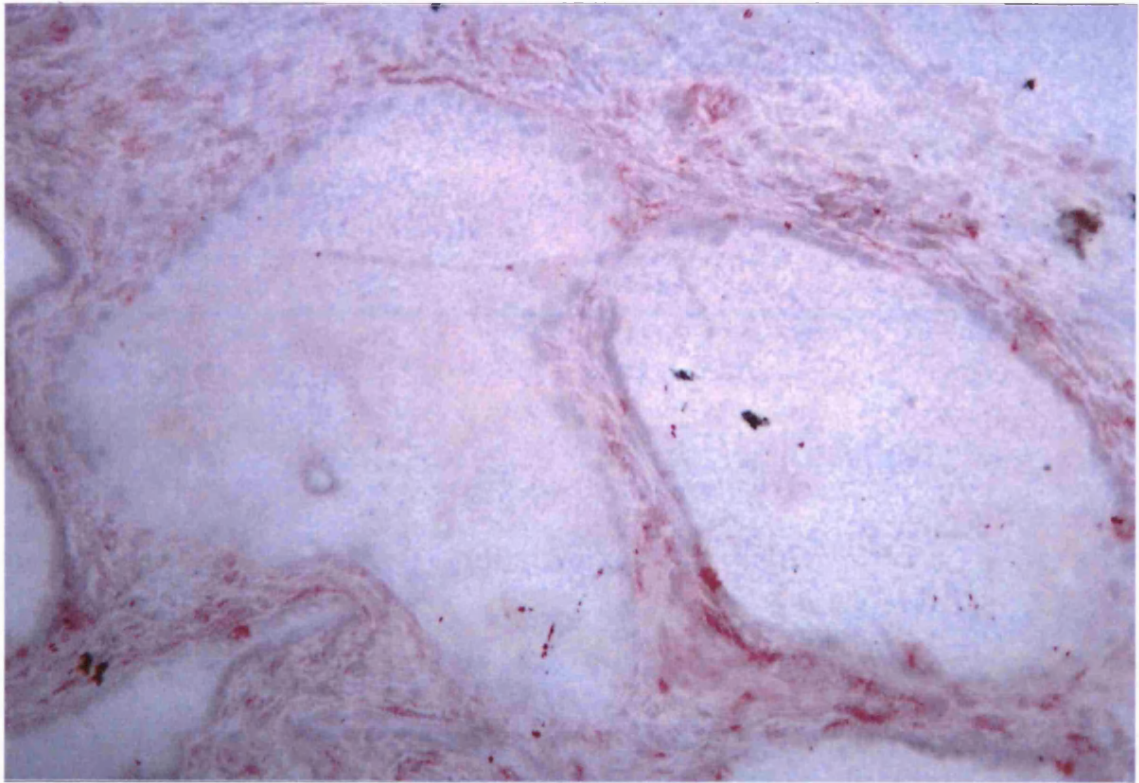
b)
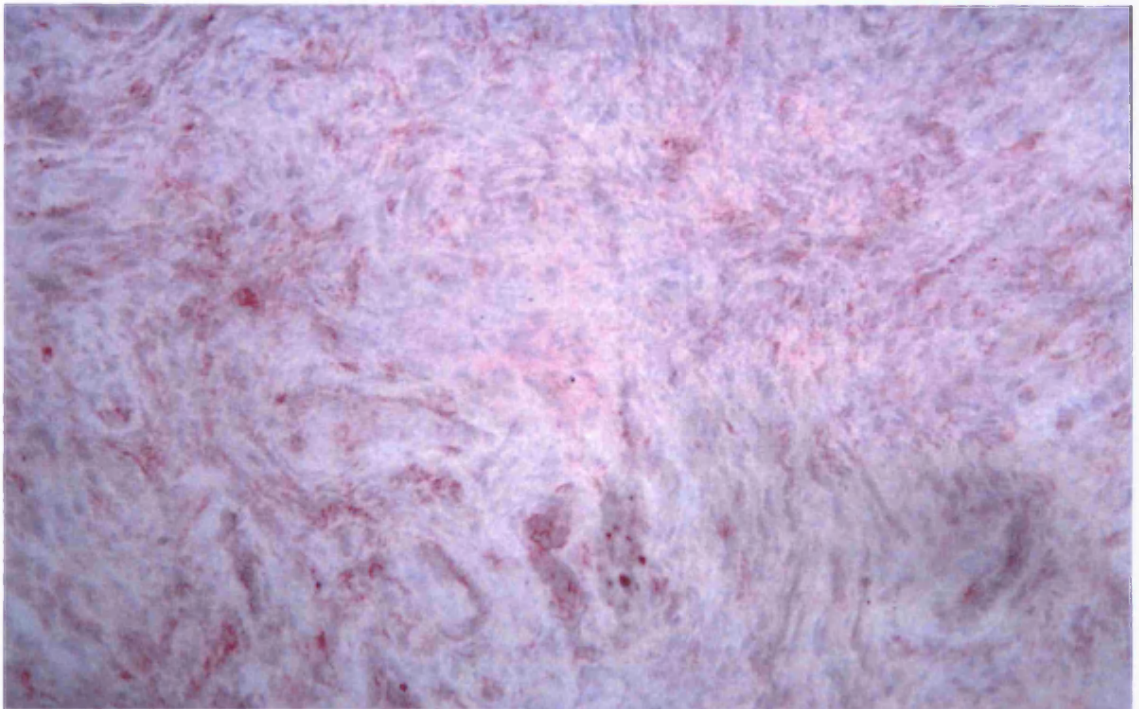
Diagram 4.5 The Expression Of Alpha-4 In Benign Hyperplastic Prostatic Tissue. Frozen sections of benign hyperplastic prostatic tissue were incubated with a monoclonal antibody against alpha-4. The Vector Red Enzyme Substrate detection system was used to highlight alpha-4[+] cells a) in and around prostatic glands and b) scattered throughout the stroma. (Magnification, x20.)

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a)
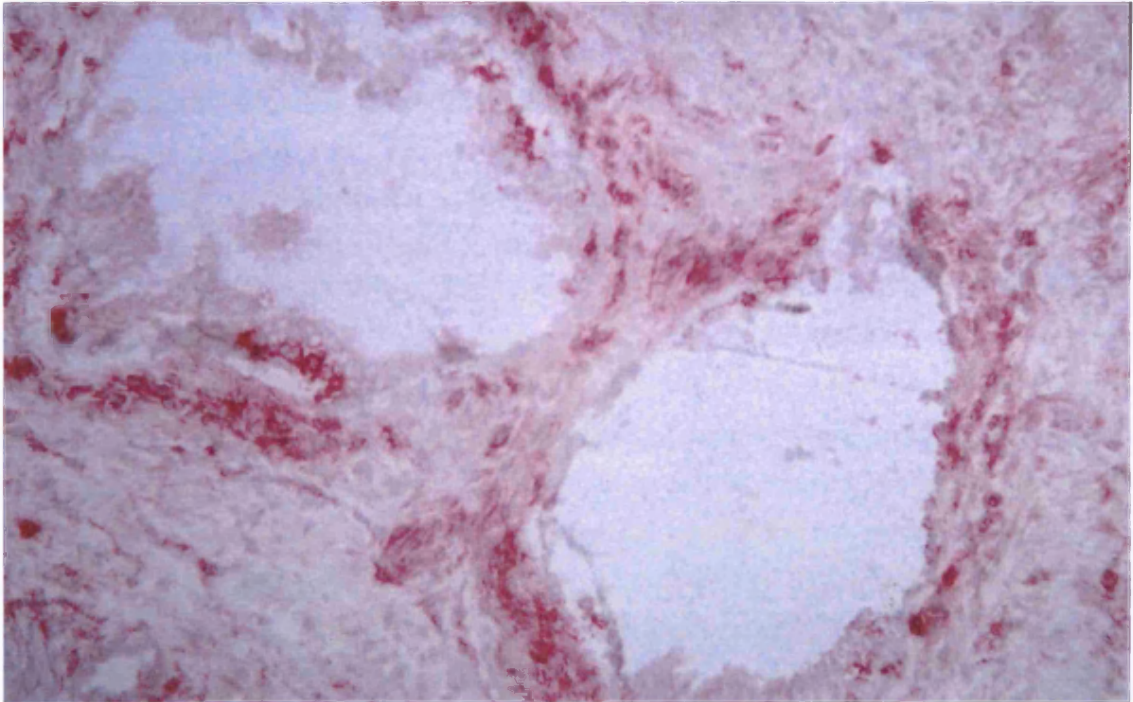
b)
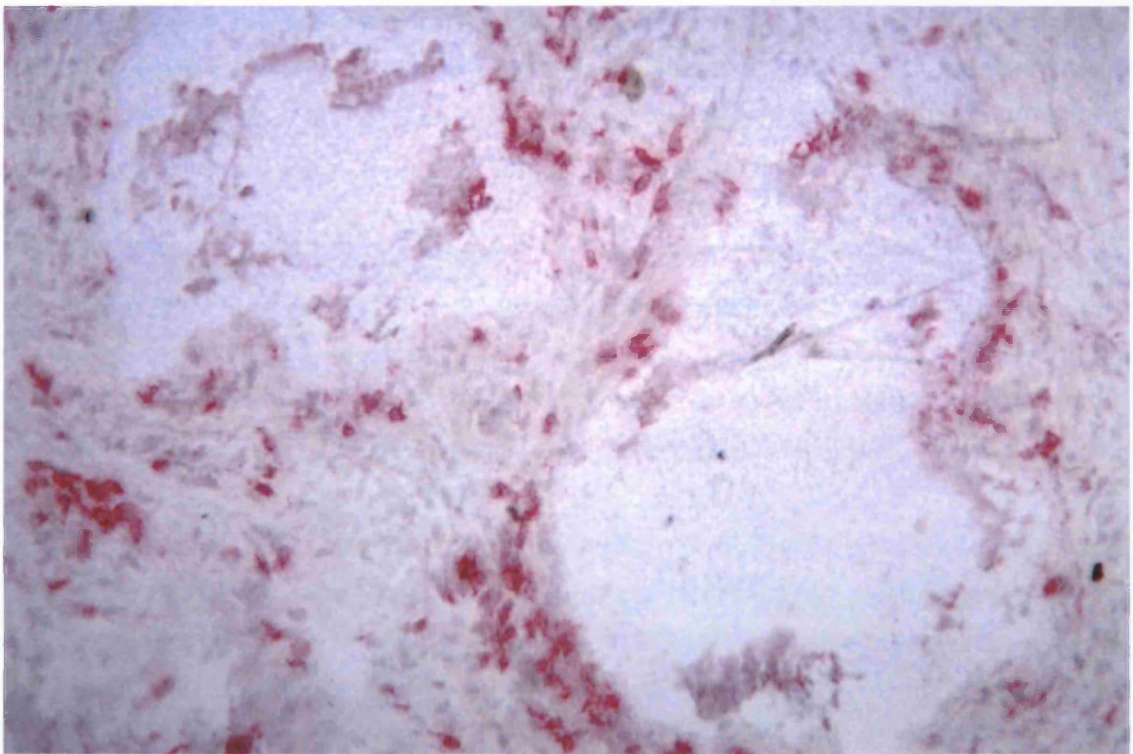
Diagram 4.6 Co-localisation Of Alpha-4 And Alpha-L In The Stroma And Glandular Epithelium Of Benign Hyperplastic Prostatic Tissue. Frozen sections of benign hyperplastic prostatic tissue were incubated with monoclonal antibodies against a) alpha-4 and b) alpha-L. The Vector Red Enzyme Substrate detection system was used to highlight co-localised alpha-4[+] and alpha-L [+] cells within and adjacent to the glandular epithelium. Not all of these cells were co-localised. (Magnification, x20.)

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VCAM-1$^+$/PECAM-1$^+$/ICAM-1$^+$ vascular endothelial cells were also present within the glandular epithelium of BPH tissue.

Alpha-4 was expressed in low levels on glandular epithelial cells of the majority (40%) of benign hyperplastic prostates examined (Diagram 4.5). Alpha-4$^+$ cells were localised in the same areas of epithelium as α5$^+$, αL$^+$, β1$^+$ and CD44$^+$ cells. Furthermore, cells expressing high levels of α4, α5, αL and β1 were co-localised.

Seven of the 38 BPH samples containing prostatic glands demonstrated expression of α5 within the glandular epithelium. These α5$^+$ epithelial cells were randomly scattered within the glandular structures and the level of expression was variable. Four samples displayed low levels of α5 within the glandular epithelium, with an IS of 1. A proportion of α5$^+$ cells co-localised with β^+ cells in one of these samples. The α5$^+$ cells of the second of these four BPH samples co-localised with similar levels of α4$^+$, αL$^+$, β1$^+$, CD44$^+$, and CD3$^+$ cells. A third sample contained α5$^+$ cells that appeared to co-localise with some, but not all, α4$^+$ cells and similar levels of αL$^+$ and CD44$^+$ cells. The fourth sample, which expressed low levels of α5, did not express α4, αL, β1 or CD44 within the glandular epithelium. One and two samples of BPH tissue contained higher levels of α5$^+$ cells within the glandular epithelium, having ISs of 3 and 4, respectively. All three of the samples contained α5$^+$ cells that co-localised with similar levels of α4$^+$, αL$^+$, β1$^+$, CD44$^+$ cells. Some of these α4$^+$/α5$^+$/αL$^+$/β1$^+$/CD44$^+$ cells co-localise with CD3$^+$ cells; however, the level of CD3$^+$ T cells found in the epithelium of these tissues could only account for a small proportion of these cells. This variable level of expression resulted in a mean IS for the epithelial expression of α5 in BPH tissue of 0.3: this relatively low IS is a result of only seven of the 35 BPH samples analysed expressing α5.

Therefore, α5 was not widely expressed on glandular epithelial cells of BPH tissue and was only expressed on seven of the 35 samples analysed (18%). Those samples that do demonstrate expression of α5 have variable levels of expression. The mean IS for epithelial expression of α5 was 0.3(Appendix Table 4.1). The majority of α5$^+$ cells co-localise with α4$^+$, αL$^+$, β1$^+$, CD44$^+$ cells or cells expressing a combination of these CAMs.

Alpha-L was expressed within the prostatic glands of 15 of the 37 BPH samples examined. All the epithelium of one of these samples expressed αL. Two samples contained high levels of αL$^+$ epithelial cells. High levels of α4$^+$/α5$^+$/β1$^+$/CD44$^+$ cells were also present in the glandular epithelium of these three samples. The remaining 12 samples expressed low levels of αL within the glandular epithelium of BPH tissue. The majority of αL present in these samples was found on cells scattered through the glandular epithelium. However, one sample demonstrated αL expression on the basal epithelial cells only. As discussed above, αL$^+$ cells frequently co-localise with α4$^+$ cells (eight samples) (Diagram 4.6). Indeed, the α4$^+$ epithelial cells of one of these samples were located basally. Alpha-L$^+$ cells also co-localise with CD44$^+$ cells (ten samples), β1$^+$ cells (six samples) and, less frequently to areas with α5$^+$ cells (two

samples). CD3$^+$ cells co-localised with a proportion of these αL^+ cells in six of the above 12 samples. However, not all αL^+ cells appeared in the same area of epithelium as the CD3$^+$ cells. The mean IS for expression of αL within the glandular epithelium of BPH tissue was 0.8 (Appendix Table 4.1). This low figure accounts for the 22 samples examined that did not express αL.

To summarise, α-L is not widely expressed on epithelial cells of benign hyperplastic prostatic glands. Fifteen of the 37 BPH samples investigated in this study expressed αL. The majority of these samples expressed low levels of αL, resulting in a mean IS score of 0.8. The majority of αL^+ cells co-localise with $\alpha 4^+$, $\beta 1^+$ or CD44$^+$ cells, or cells expressing a combination of these CAMs.

$\beta 1$ was expressed on the epithelial cells of 12 of the 38 BPH tissues examined. These $\beta 1^+$ cells were not clustered together within the glandular structure: all but one of these samples contained $\beta 1^+$ cells that were scattered through the prostatic glands. However, one sample, which expressed moderate levels of $\beta 1$, contained $\beta 1^+$ basal epithelial cells: this sample also contained high levels of basal epithelial cells expressing CD44 and some of these cells appeared to express αL also. The level of expression of $\beta 1$ was varied with the 12 BPH samples. The individual IS scores of these sections ranged from 0 to 4, with a mean IS of 38 samples of 0.9. Those with both low and high scores appeared to co-localise with $\alpha 4^+$, αL^+, CD44$^+$ and to a lesser extent $\alpha 5^+$ and CD3$^+$ cells: i.e. not all $\beta 1^+$ cells co-localised with CD3$^+$ cells.

Therefore, $\beta 1$ was not commonly expressed on the glandular epithelial cells of benign hyperplastic prostates. Twelve of the 38 BPH samples (32%) examined in this study expressed $\beta 1$. The level of expression was variable and gave a mean IS of 0.9 (Appendix Table 4.1). Most $\beta 1^+$ cells co-localised with cells expressing $\alpha 4$, αL, CD44 or combinations of these CAMs.

Approximately half of the BPH samples analysed expressed CD44 on glandular epithelial cells. Four of these 19 samples contained CD44$^+$ cells that were located at the basal apex of the prostatic glands. While these samples also contained $\alpha 4^+$ basal epithelial cells, the level of CD44$^+$ cells present is greater than that of $\alpha 4^+$ cells present. CD3$^+$ cells co-localised with the CD44$^+$ cells of two of these four samples, but not of the third and fourth samples. A proportion of the CD44$^+$ cells present in the 19 BPH tissues appear to localise in the same areas as $\alpha 4^+$, $\alpha 5^+$, αL^+ and $\beta 1^+$ cells: however, far more CD44$^+$ cells are present than cells expressing the integrin subunits. This was true for samples that contain both CD44$^+$ basal epithelial cells and those that contain CD44$^+$ cells scattered across the glandular epithelium, independent of the level of expression. The level of expression of CD44 within the glandular epithelium of benign hyperplastic prostatic tissue is variable. The IS of the 19 samples expressing CD44 ranged from 0 to 4, giving a mean IS of these 19 samples of 1.3 (Appendix Table 4.1).

Therefore, CD44 was expressed to varying levels in the glandular epithelium of approximately half of the benign hyperplastic prostatic tissue. Some CD44$^+$ cells are located

basally within the gland, while others were scattered throughout the contained glandular structures. Some CD44$^+$ cells co-localised with epithelial cells expressing the various integrin subunits examined in this study. However, not all CD44$^+$ appeared to co-express $\alpha 4$, $\alpha 5$, αL or $\beta 1$.

To summarise these data, the expression of E-selectin, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 within benign hyperplastic prostatic glands is not as widespread as that seen in the stroma surrounding these gland. Approximately half of the BPH samples examined expressed ICAM-1 on the epithelial cells within the glands. Approximately 10% of these sections contained vascularised glands, demonstrated by the presence of PECAM-1$^+$ vascular structures. The vascular endothelial cells expressed E-selectin, ICAM-1 and, in some cases, VCAM-1. These data suggest that the endothelial cells within the blood vessels were activated. Glandular epithelial cells were demonstrated to co-express E-selectin, ICAM-1 and VCAM-1. Some epithelial cells express ICAM-1 but not E-selectin and VCAM-1 and some express VCAM-1 but not ICAM-1 or E-selectin. The level of ICAM-1$^+$ cells present in these BPH samples was greater than that of VCAM-1$^+$ cells. The level of ICAM-1, VCAM-1 and E-selectin expression within the prostatic glands was relatively low with mean ISs of 0.8, 1.0 and 0.7, respectively. The mean IS for glandular PECAM-1 was 0.4.

$\alpha 5$ and $\beta 1$ integrin subunits were not expressed in the glands of many of the BPH samples examined. Only 18% and 22% of samples examined expressed $\alpha 5$ and $\beta 1$, respectively. The $\alpha 5^+$ and $\beta 1^+$ cells did co-localise with $\alpha 4^+$, αL^+ and CD44$^+$ cells, which were present in greater levels within the glands of benign hyperplastic prostatic tissues. Indeed, 49%, 41% and 46% of samples analysed demonstrated expression of $\alpha 4$, αL and CD44, respectively. Some of these cells co-localised with CD3$^+$ T cells and may form part of an inflammatory infiltrate, which is characteristic of benign prostatic hyperplasia. The level of expression of $\alpha 4$, $\alpha 5$, αL, $\beta 1$ and CD44 in these glands was low. With the exception of CD44, which had an IS of 1.3, the mean ISs for these integrin subunits were less than 1.0 (Appendix Table 4.1).

4.4 The Distribution Of Cell Adhesion Molecules In Malignant Prostatic Tissue

Malignant prostatic tissue was analysed for the expression of E-selectin, Intercellular Cell Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), $\alpha4$, $\alpha5$, αL, $\beta1$, and CD44. The expression of CAMs was not consistent, as seen previously in benign hyperplastic prostates. However, ICAM-1 expression was demonstrated in the majority of samples examined (Table 4.4)

	E-selectin	ICAM-1	VCAM-1	PECAM-1	$\alpha4$	$\alpha5$	αL	$\beta1$	CD44
Number Of Sections Examined	20	20	17	18	16	17	16	18	17
Number That Did Not Express Marker	14	2	7	14	6	15	8	13	6
Number That Expressed Marker	6	18	10	4	10	2	8	5	11

Table 4.4 Numerical Details Of Malignant Prostatic Tissues Examined Of The Epithelial Expression Of Cell Adhesion Molecules.

E-selectin was expressed within the glandular epithelium of 6 of the 20 malignant samples examined. In all cases the E-selectin$^+$ cells were randomly distributed within the epithelium and were not located at the basal or luminal boundaries of the prostatic glands (Diagram 4.7). Each of these six samples expressed very low levels of E-selectin with ISs of 1 (Appendix Table 4.2). Three samples were found to contain E-selectin$^+$/PECAM-1$^+$/ICAM-1$^+$/VCAM-1$^+$ vascular endothelial cells. Two further sections were found to contain E-selectin$^+$/ICAM-1$^+$/VCAM-1$^+$ cells that were not analysed for the expression of PECAM-1, but were contained within vascular structures. The sixth sample contained E-selectin$^+$/ICAM-1$^+$/VCAM-1$^-$/PECAM-1$^-$ cells. These cells did not appear to be associated with vascular structures and were located within the glandular epithelium. The tumour of this sample had a histological grade of G5 and was excised from a patient with local metastases in the pelvic lymph node. Four of the remaining tumours examined for the expression of E-selectin had histological grades of G2 and were non-metastatic and the fifth tumour had a mixed histological grade of G3/G4/G5 with clinical evidence of bony metastases.
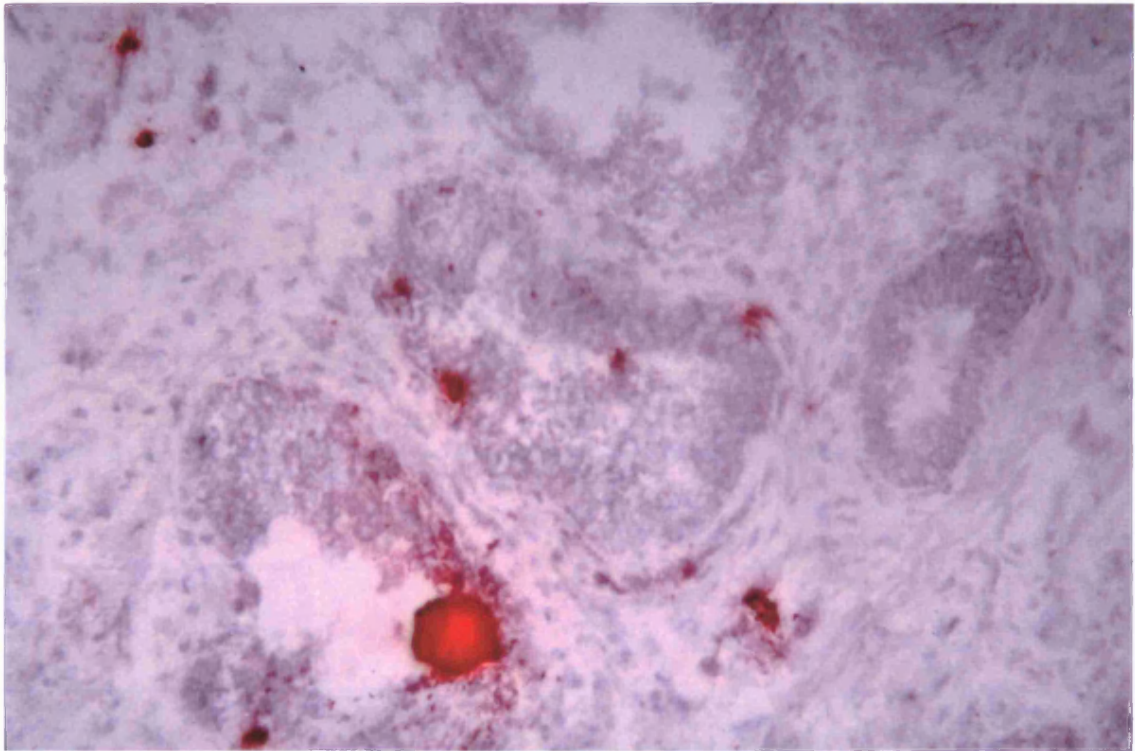
Diagram 4.7 Expression Of E-selectin In Malignant Prostatic Epithelial Cells. A frozen section of a G2, non-invasive prostatic tumour was incubated with an anti-E-selectin monoclonal antibody and highlighted with the Vector Red Enzyme Substrate. E-selectin$^+$ cells are clearly visible within the malignant glands of the tumour. (Magnification, x40.)

Therefore, E-selectin was expressed within the glandular epithelial, in low levels, in six of the 20 samples examined. In five of these six samples, the E-selectin$^+$ cells were activated endothelial cells, demonstrated by their co-expression of PECAM-1 and/or ICAM-1. The distribution and level of E-selectin expression within malignant prostatic lesions did not correlate with the histological grade or metastatic status of the tumours.

ICAM-1 was expressed in the glandular epithelium by 18 of the 20 samples of malignant prostatic tissue examined. The level of expression of ICAM-1 within the glandular epithelium of this malignant tissue was low to moderate with ISs ranging from 1 to 3, resulting in an average IS of 1.8 (Appendix Table 4.2). The ICAM-1$^+$ epithelial cells of two of these samples were located along the luminal edges of the prostatic glands and some co-expressed VCAM-1, although not all (Diagram 4.11). Both these samples had a histological grade of G2 and one had clinical evidence of bony metastases. The remaining samples had histological grades ranging from G2 to G5: some had evidence of metastatic deposits and varying levels of expression of epithelial ICAM-1. Four samples contained ICAM-1$^+$/PECAM-1$^+$/VCAM-1$^+$ vascular endothelial cells within the malignant epithelium (Diagram 4.8). Three of these tumours had a G2 histological grade and non-metastatic status, while the fourth had a varied histological grade of G3/G4/G5 with evidence of bony metastases. Four more samples contained ICAM-1$^+$/VCAM-1$^+$ cells that did not express PECAM-1. These tumours had histological grades ranging from G2 to G5; two had no

a)
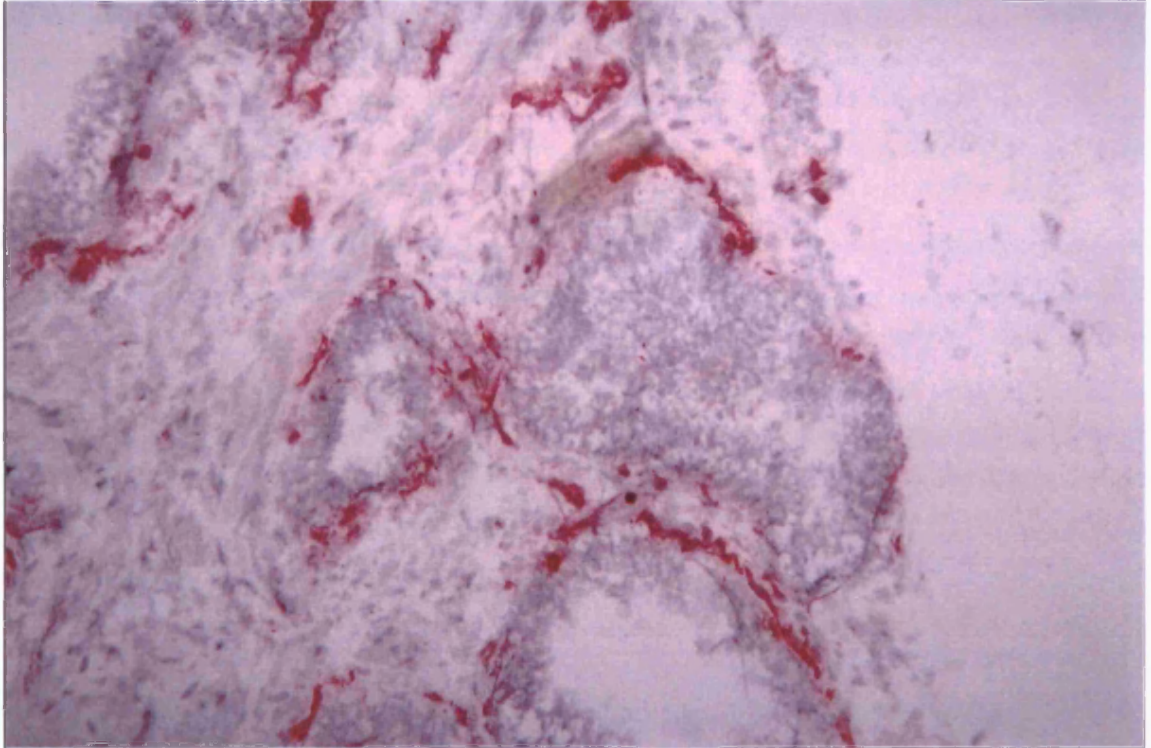
b)
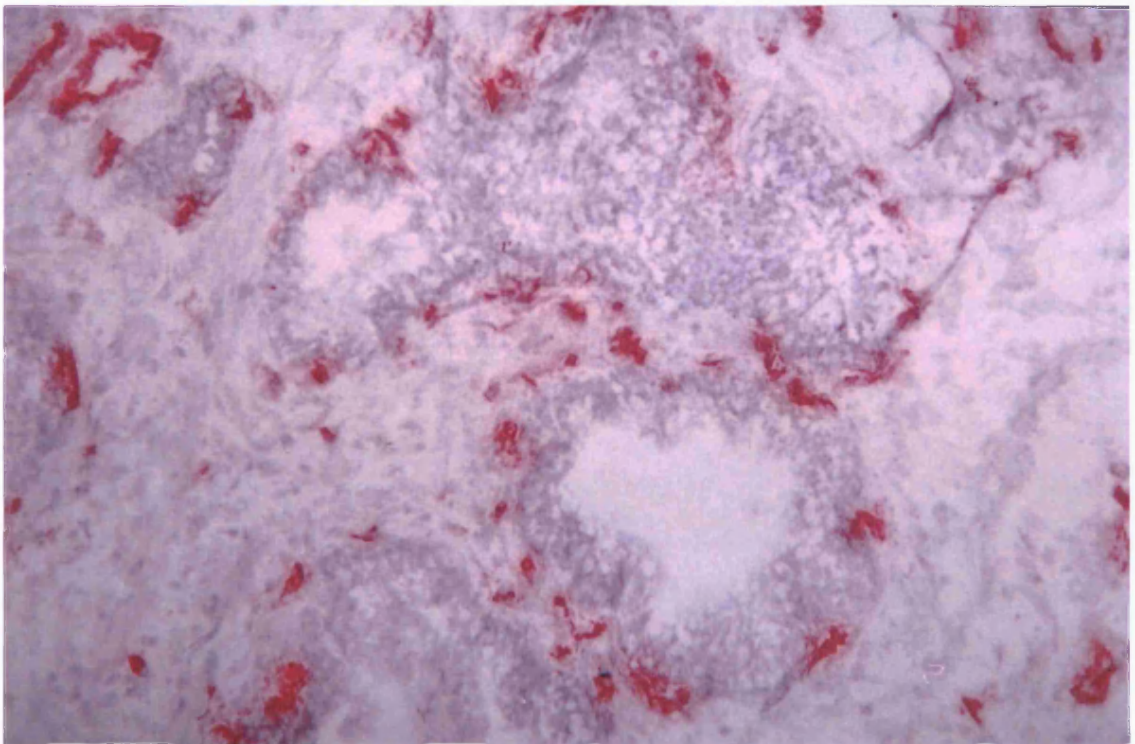
Diagram 4.8 Expression Of ICAM-1 And PECAM-1 By Malignant Prostatic Tumours. Frozen sections of G2, non-invasive, prostatic adenocarcinoma were incubated with monoclonal antibodies against a) ICAM-1 and b) PECAM-1. The Vector Red Enzyme Substrate detected PECAM-1$^+$/ICAM-1$^+$ and PECAM-1-/ICAM-1$^+$ cells within and in the stroma directly adjacent to the malignant prostatic glands. (Magnification, x20.)

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clinical evidence of metastases, one demonstrated invasion beyond the prostatic capsule and the fourth had bony metastases. ICAM-1$^+$/PECAM-1$^+$ cells were present in the stroma directly adjacent to the malignant prostatic glands (Diagram 4.9).

Therefore, ICAM-1 was expressed on the epithelial cells of the majority (90%) of samples of malignant prostatic glandular epithelium examined. ICAM-1 was also expressed on activated vascular endothelial cells located within the prostatic glands. While two of these samples expressed ICAM-1 on the luminal epithelial cells, the majority of ICAM-1$^+$ cells were located throughout the glandular structures (Diagram 4.11). However, no clinical correlation could be established between the level of expression and the distribution of expression of ICAM-1 within prostatic tumours and their histological grade and metastatic status.

VCAM-1 was expressed on the epithelial cells of ten of the 17 malignant prostatic samples examined. All of these samples expressed low levels of VCAM-1, with a mean IS for these 17 samples of 0.6 (Appendix Table 4.2). As described above, four of these ten samples contained cells within the glandular epithelium that expressed VCAM-1, ICAM-1 and PECAM-1: these cells were contained within vascular structures. Three of these four tumours had a G2 histological grade and non-metastatic status, while the fourth had a varied histological grade of G3/G4/G5 with evidence of bony metastases. Four more samples contained VCAM-1$^+$ cells that co-express ICAM-1, but not PECAM-1. These tumours had histological grades ranging from G2 to G5; two had no clinical evidence of metastases, one demonstrated invasion beyond the prostatic capsule and the fourth had bony metastases.

To summarise, VCAM-1 is expressed on epithelial cells, in low levels, by approximately half (59%) of the malignant prostatic samples analysed. Four of these samples contain VCAM-1$^+$/ICAM-1$^+$/PECAM-1$^+$ vascular endothelial cells. All VCAM-1$^+$ cells also expressed ICAM-1, but only a proportion expressed PECAM-1. Two samples demonstrated the presence of luminal VCAM-1$^+$/ICAM-1$^+$ epithelial cells; however, most VCAM-1$^+$ epithelial cells were located throughout the glandular epithelium. No correlation could be made between the level or distribution of VCAM-1 expression in malignant prostatic tumours and their histological grade and metastatic status.

Alpha-4 was expressed on the epithelial cells of ten of the 16 samples of malignant prostatic tissue examined. All of these samples expressed low levels of α4, with a mean IS of 0.8 (Appendix Table 4.2). These samples contained prostatic tumours with histological grades of differentiation ranging from G2 to G5. One of these ten samples contained a tumour with evidence of bony metastases: two samples contained tumours that had invaded through the prostatic capsule: the remaining seven samples contained tumours that had no clinical evidence of metastases. Alpha-4$^+$ cells frequently co-localised with αL$^+$ cells, but not all αL$^+$ cells co-localised with α4$^+$ cells. A proportion of β1 and CD44$^+$ cells also co-localised with the α4$^+$ cells (Diagram

a)
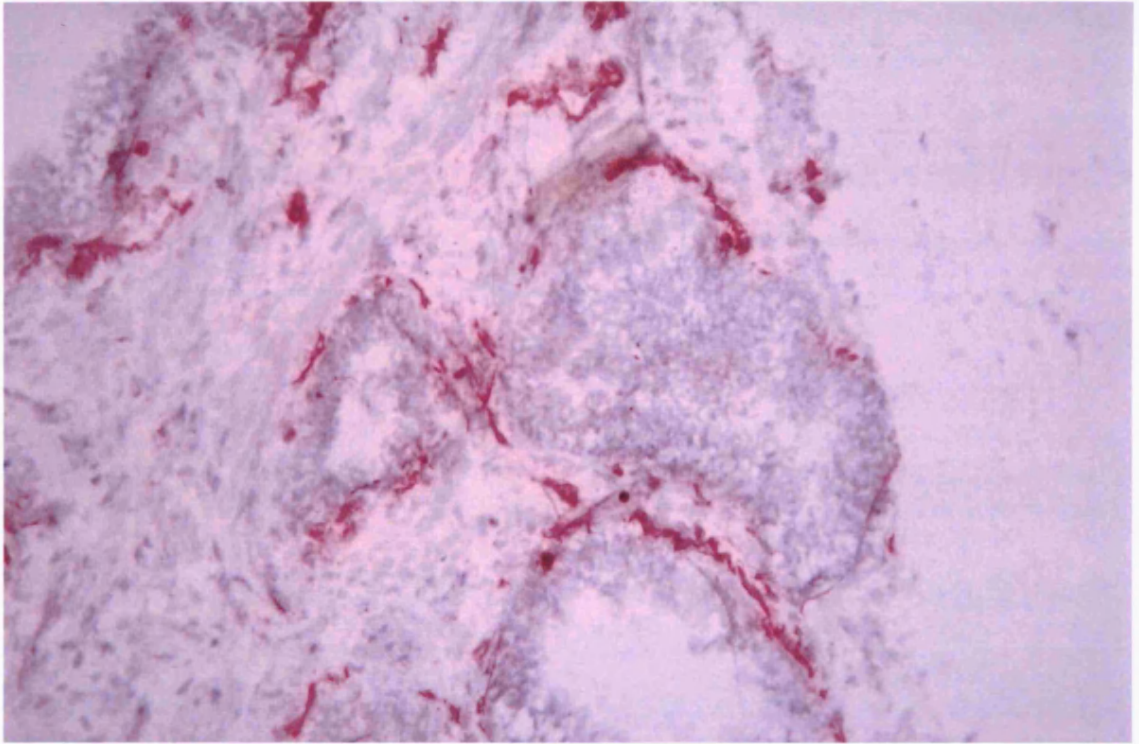
b)
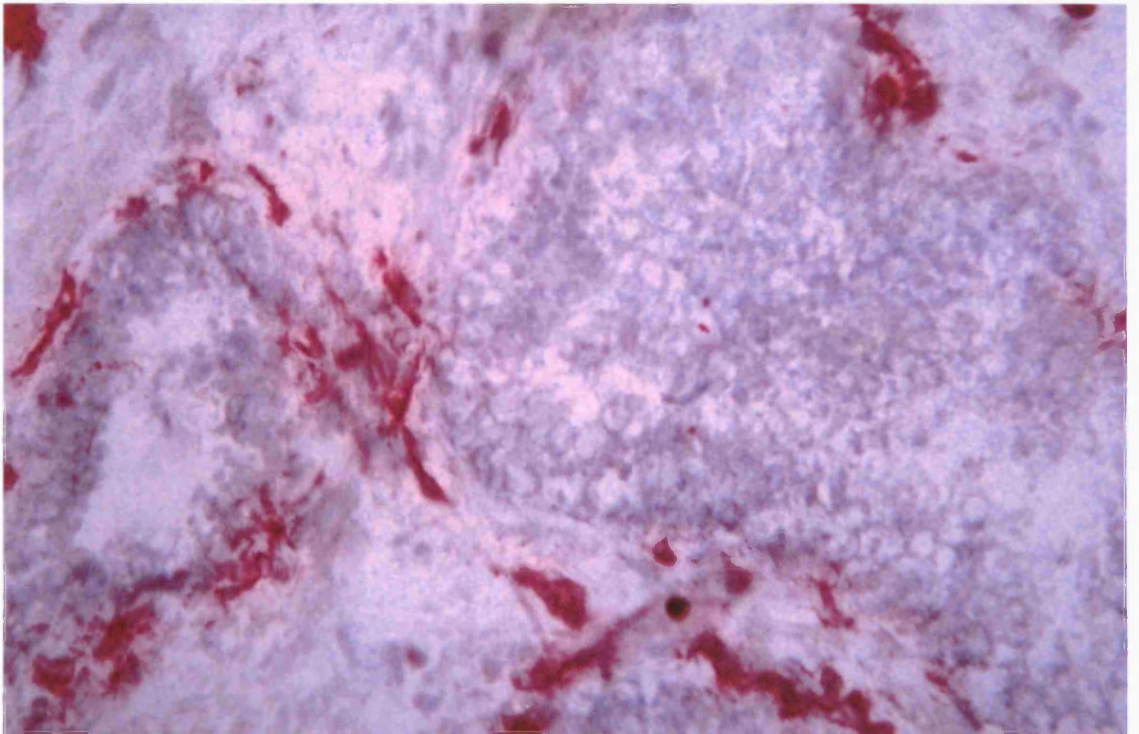
Diagram 4.9 The Expression Of ICAM-1 In The Stroma Adjacent To Malignant Prostatic Glands. Frozen sections of a G2, non-invasive prostatic tumour were incubated with anti-ICAM-1 monoclonal antibodies. The Vector Red Enzyme Substrate was used to highlight the ICAM-1[+] cells within the tissue. Magnification of a) x20 and b) x40.

a)
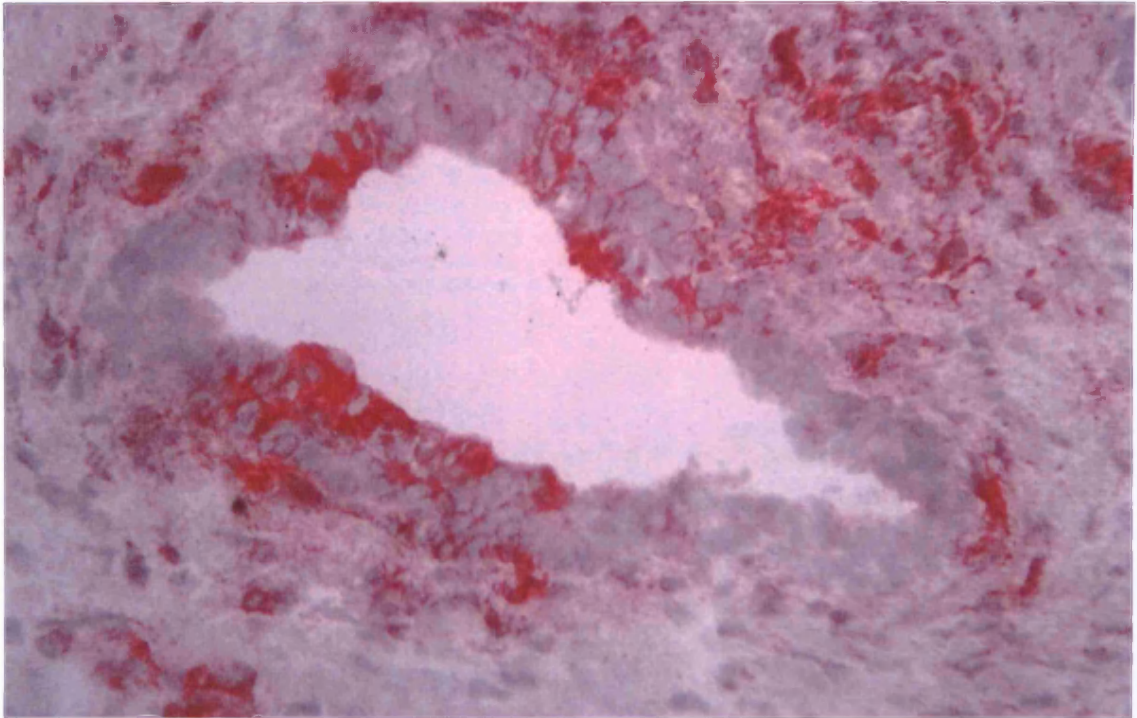
b)
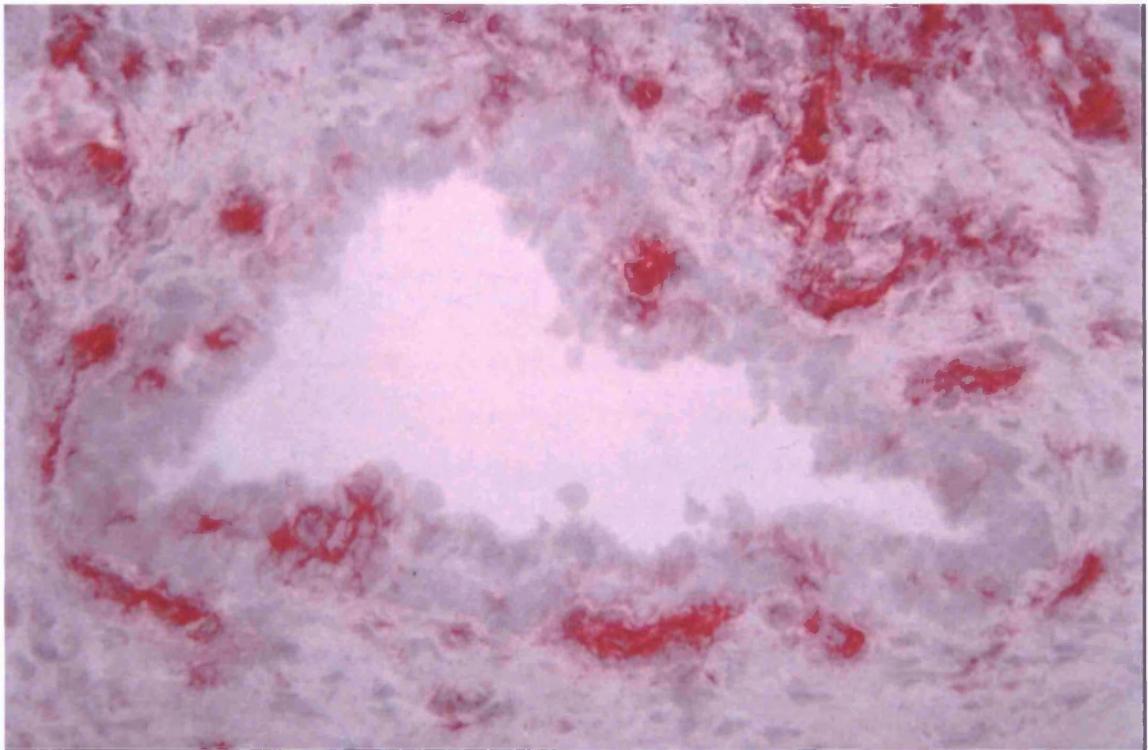
Diagram 4.10 The Expression Of ICAM-1 On Malignant Tumour Cells Located Throughout The Prostatic Gland. Frozen sections of a G2, non-invasive prostate tumour were incubated with monoclonal antibodies against a) ICAM-1 and b) PECAM-1. The Vector Red Enzyme Substrate detected the presence of ICAM-1[+] and PECAM-1[+] cells. Note that not all ICAM-1[+] cells are PECAM-1[+], but all PECAM-1[+] cells are ICAM-1[+]. (Magnification, x40.)
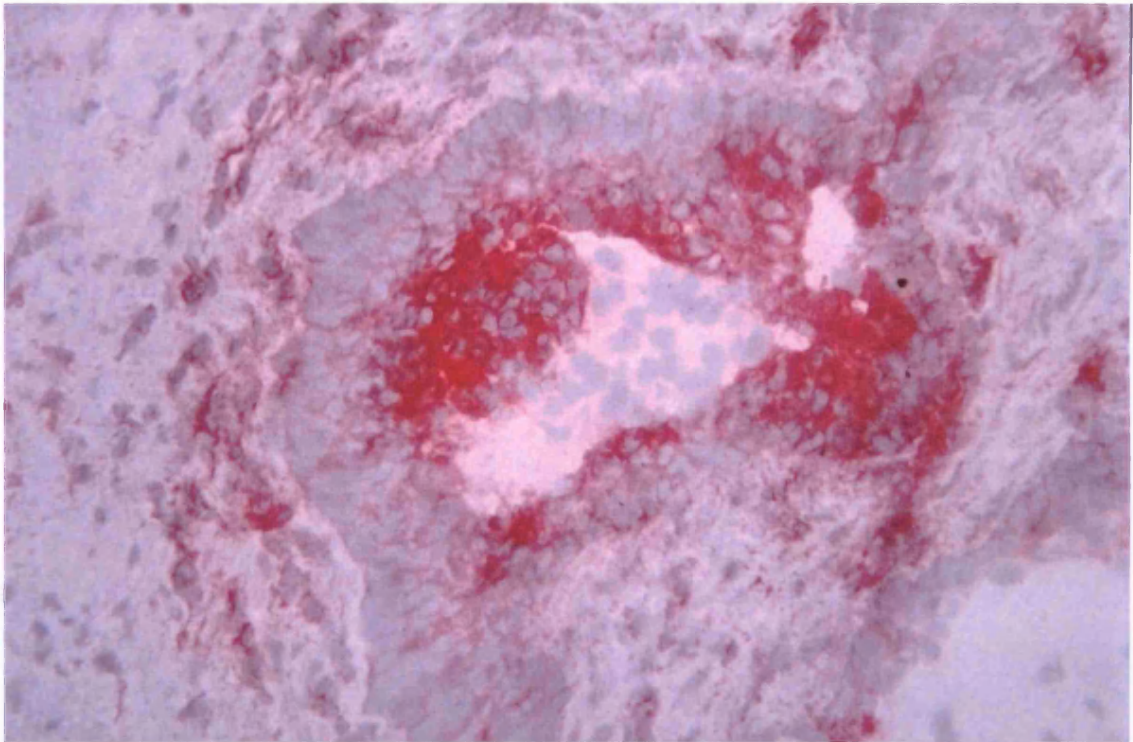
Diagram 4.11 Lumenal Expression Of ICAM-1 Within A Malignant Prostatic Tumour. A frozen section of a G2, non-invasive prostatic adenocarcinoma was incubated with an anti-ICAM-1 monoclonal antibody. The Vector Red Enzyme Substrate detected mostly luminal ICAM-1$^+$ prostatic tumour cells; however, basal ICAM-1$^+$ tumour cells were also present. (Magnification, x40.)

4.12) These cells were found throughout the glandular epithelium, some found basally, some lumenally and some in the centre of the prostatic glands.

To summarise, low levels of $\alpha4$ were expressed on some, but not all, epithelial cells of approximately half of the malignant prostatic samples examined. These $\alpha4^+$ cells appeared to co-localise with cells expressing αL, $\beta1$ and CD44 or combinations of these CAMs. Therefore, no correlation could be established between the level and distribution of $\alpha4$ expression in malignant prostatic glandular epithelium and the histological grade and metastatic status of the tumour.

Two of the 15 samples of malignant prostatic tissue examined expressed $\alpha5$ within the glandular epithelium. Low levels of expression were seen in the epithelial cells of these tumours, which had a histological grade of G5. One of these tumours had invaded through the prostatic capsule and the second had no clinical signs of metastases. The $\alpha5^+$ cells found within the invasive adenocarcinoma appeared to co-localise with αL^+ cells. The $\alpha5^+$ cells of the non-invasive adenocarcinoma also appeared to co-localise with $\alpha4^+$ cells. These cells were found scattered through the glandular epithelium and did not appear to specifically localise at either the basal or luminal boundaries of the prostatic glands. No correlation could be made between the level or distribution of $\alpha5$ expression by the malignant epithelial cells and the histological grade and metastatic status of the prostatic adenocarcinomas.

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a)
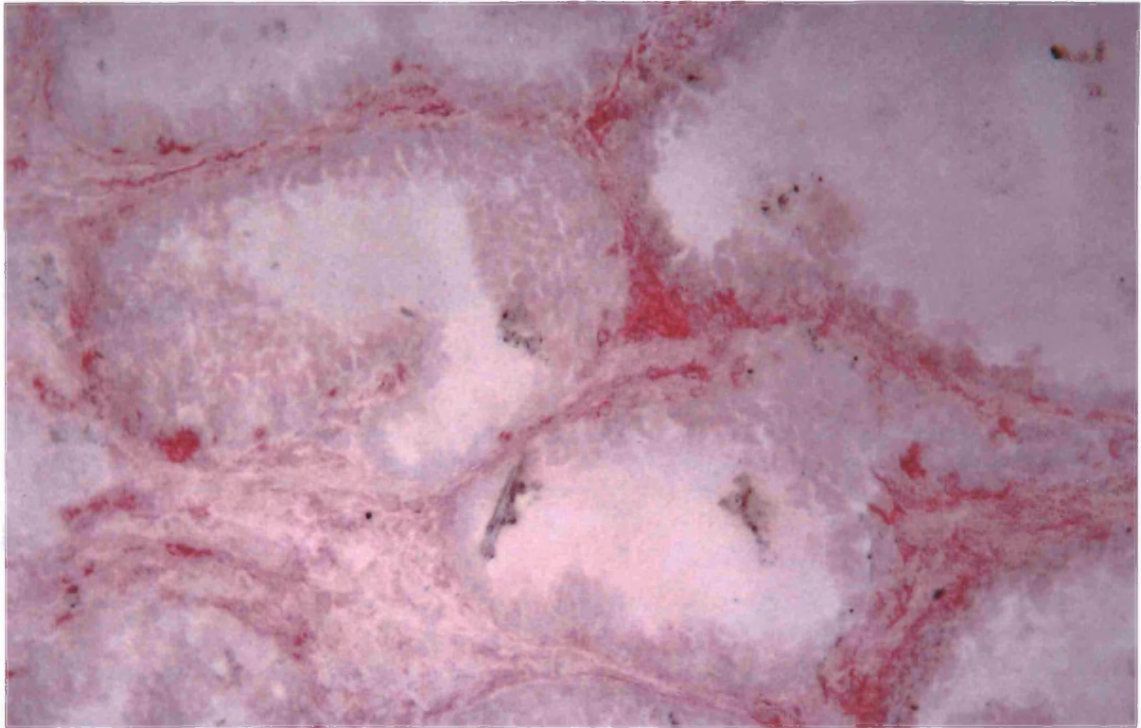
b)
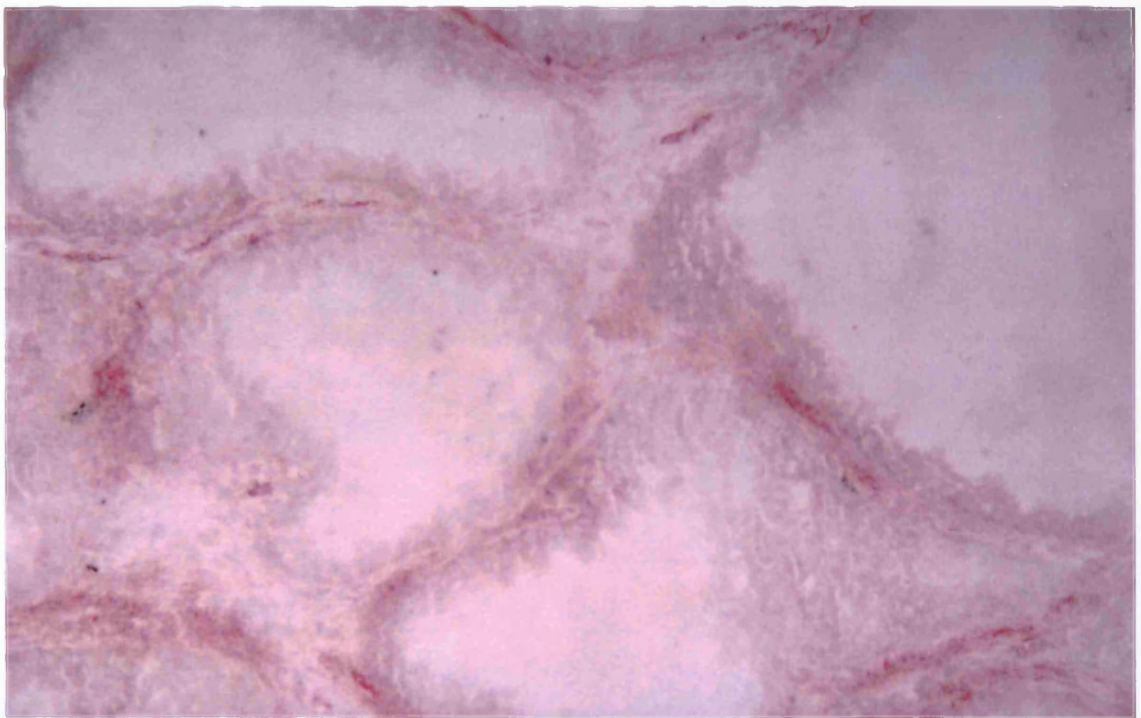
Diagram 4.12 Co-localisation Of Alpha-4 And Alpha-L In Malignant Prostatic Tumours. Frozen sections of a G5, non-invasive prostatic adenocarcinoma were incubated with monoclonal antibodies against a) alpha-4 and b) alpha-L. The Vector Red Enzyme Substrate highlighted alpha-4$^+$ and alpha-L$^+$ cells. Few alpha-L$^+$ cells are present, but those that are are also alpha-4$^+$. Almost all the tumour cells express alpha-4. (Magnification, x20.)

Eight of the 16 malignant prostatic samples analysed demonstrated expression of αL within the glandular epithelium. Five of these tumours had clinical evidence of a metastatic capacity; one tumour had metastases in the pelvic lymph nodes, one tumour had developed bony metastases and three tumours had evidence of invasion through the prostatic capsule. The tumours of three further samples were poorly differentiated with histological grades of G4 and G5. However, two tumours expressing αL had histological grades of G2 and had no clinical evidence of metastases. One tumour that did not express αL also had clinical evidence of metastases and the primary tumour was graded as a G3/G4/G5 tumour with areas of poor to moderately well differentiation. Alpha-L$^+$ cells were not localised specifically on basal or luminal surfaces, but were scattered across the glandular epithelium. The αL$^+$ cells commonly co-localised with CD44$^+$ cells and occasionally also with α4$^+$, α5$^+$ and β1$^+$ cells. Three prostatic tumours contained αL$^+$ cells, α4$^+$ cells, β1$^+$ cells and CD44$^+$ cells that appeared to co-localise: one of these tumours demonstrated clinical evidence of invasion through the prostatic capsule, the second was a poorly differentiated tumour and had no clinical evidence of metastases and the third was moderately well differentiated tumour with no clinical evidence of metastases. One tumour, which had clinical evidence of invasion through the prostatic capsule, demonstrated co-localisation of αL$^+$ cells and α5$^+$ cells. One tumour, which had a histological grade of G5 and no clinical signs of metastases, contained αL$^+$ cells that co-localised with α4$^+$ and CD44$^+$ cells. One tumour, which had a histological grade of G3 and clinical evidence of bony metastases, contained αL$^+$ cells that co-localised with α4. The αL$^+$ cells of one tumour with a histological grade of G5 and metastatic deposits in the pelvic lymph nodes co-localised only with CD44$^+$ cells. One further tumour, which had a histological grade of G4 with no clinical evidence of metastases, contained αL$^+$ cells that co-localised with only α4$^+$ cells. The mean IS score of the 16 samples was 0.7 (Appendix Table 4.2).

Therefore, half of the malignant samples analysed expressed αL on cells within the glandular epithelium. However, only low levels of αL were expressed in these prostatic glands. Alpha-L$^+$ cells co-localised frequently with CD44$^+$ cells and with cells expressing the integrin subunits α4 and β1. While the level of αL expression was low, there may be a correlation with the expression of αL by these epithelial cells and the metastatic status of the tumour. However, there was no correlation with the level and distribution of αL with the histological grade of the tumour.

The glandular epithelial cells of five of the 18 malignant prostatic samples examined expressed β1. The level of β1 expression varied from zero to three, giving an average IS of 0.6. The β1$^+$ cells of one of these five samples were located on the basal surfaces of the prostatic glands, which had a histological grade of G2 and no clinical evidence of metastatic spread. The remaining four samples of malignant prostatic tissue had ISs of 1, 2 or 3 for epithelial expression of β1. The histological grades of these four samples ranged from G2 to G5 and the level of expression was not related to the histological score. For example, two samples had an IS of three;

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these samples had histological grades of G5 and G2. Neither of these samples had clinical evidence of metastatic spread. One further sample had an IS of 1; the tumour of this tissue had a histological grade of G4 and clinical evidence of invasion through the prostatic capsule. These $\beta 1^+$ cells always co-localised with $CD44^+$ cells. Four of the five samples contained $\beta 1^+$ cells that also co-localised with $\alpha 4^+$ and αL^+ cells.

Therefore, $\beta 1$ was expressed on the epithelial cells of five (28%) of the malignant prostatic samples analysed. No correlation could be demonstrated between the level and distribution of $\beta 1^+$ cells and the histological grade and metastatic status of the tumour

Prostatic glands of 11 of the 17 malignant prostatic samples examined expressed CD44. The level of expression of CD44 varied and ISs of these 11 samples ranged from zero to four. One of these 11 samples, which contained a tumour with a histological grade of G2 and no evidence of metastases, expressed CD44 on the basal epithelial cells of the prostatic glands. The level of expression in this sample had an IS of 3. The remaining ten samples contained $CD44^+$ cells that were scattered throughout the glandular structures. Two samples had an IS of 4. These two samples contained tumours that had histological grades of G2/G3/G4 and G3/G4/G5 and neither had clinical evidence of metastatic disease. Malignant prostatic glands that did not express CD44 had histological grades that ranged from G2 to G5: three of these samples had no evidence of metastatic spread, one had evidence of invasion through the prostatic capsule and two had bony metastases. Therefore, the level of expression of CD44 does not correlate with the histological grade of prostatic tumours; nor does it reflect the metastatic status of the tumour. The $CD44^+$ cells of five of the eleven samples co-localised with $\alpha 4^+$ and αL^+ cells: $\beta 1^+$ cells co-localised with the $CD44^+$ cells of four of these samples. The $CD44^+$ cells of one of these eleven malignant prostatic glands co-localised with $\alpha 4^+$ cells only, one with αL^+ cells only and one with $\beta 1^+$ cells only. The $CD44^+$ cells within the prostatic gland of two samples did not co-localise with cells that expressed any of the CAMs investigated in this study.

To summarise, $CD44^+$ cells were found in the epithelium in 11 (65%) of the malignant prostatic samples analysed. The majority of these $CD44^+$ cells co-localised with epithelial cells expressing $\alpha 4$, αL and $\beta 1$. No correlation could be established between the level and distribution of CD44 expression by malignant prostatic tumours and the histological grade and metastatic status of those tumours.

To summarise these data, the expression of E-selectin, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 could not be demonstrated on all samples of malignant prostatic glands examined. PECAM-1 was expressed within the malignant prostatic glands of 22% of the samples examined. These $PECAM-1^+$ cells were located within vascular structures found in the malignant prostatic glands and expressed E-selectin, ICAM-1 and VCAM-1. ICAM-1 was expressed in the glandular

prostates of 90% of the samples examined. Many of these cells co-expressed VCAM-1. VCAM-1 expression could be demonstrated in 58% of the malignant samples analysed. E-selectin was expressed in the glandular epithelium of 30% of the samples surveyed. Therefore, not all ICAM-1^+ cells co-expressed VCAM-1 and E-selectin, but all VCAM-1^+ and E-selectin$^+$ cells co-expressed ICAM-1. Alpha-4, αL and CD44 expression was observed in the prostatic glands of 63%, 50% and 65% of samples analysed, respectively. Alpha-5 and β1 were not commonly expressed. Only 12% and 22% of malignant prostatic samples analysed expressed α5 and β1 within the prostatic glands, respectively. The $\beta1^+$ cells frequently co-localised with $\alpha4^+$, αL$^+$ and CD44$^+$ cells. These cells did not appear to localise specifically to the basal or luminal boundaries but were randomly scattered within the prostatic glands. The level of expression of these CAMs was low. The IS for E-selectin, VCAM-1, α4, α5, αL, and β1 were all below 1.0. The ISs for ICAM-1 and C44 were 1.8 and 1.4, respectively. No correlation could be established between the level or distribution of expression of E-selectin, ICAM-1, VCAM-1, α4, α5, β1 and CD44 in the malignant glands and the histological grade and metastatic status of the prostatic tumours. However, all but one of the prostatic tumours that express αL within the glandular epithelium have evidence of metastatic and / or invasive disease. Unfortunately, the numbers in this study were low. Therefore, it cannot be concluded that expression of αL by malignant prostatic tumours conveys a metastatic phenotype.

4.4 Comparisons Of The Expression Of Cell Adhesion Molecules In Benign Hyperplastic And Malignant Prostatic Tissues

The data presented above describes the expression of E-selectin, ICAM-1, VCAM-1, α4, α5, αL, β1, and CD44 in benign hyperplastic and malignant prostatic tissues. ICAM-1 expression was demonstrated within the glandular compartment of benign and malignant prostatic tissue. The level of expression was low with average ISs for ICAM-1 expression of 0.8 and 1.8 for benign and malignant prostatic glands, respectively. Alpha-4, αL and CD44 were commonly expressed, at low levels, within prostatic glands. E-selectin, VCAM-1, α5 and β1 were infrequently expressed in the glandular compartments prostatic tissue. Prostatic glands contained vascular structures that consisted partially of endothelial cells expressing PECAM-1, E-selectin, ICAM-1 and VCAM-1. None of the eight CAMs studied were constitutively expressed in both benign and malignant prostatic: in those samples that did express one or more of these CAMs not all prostatic glands within each sample expressed the particular CAM (Appendix Tables 4.1 and 4.2).

ICAM-1, VCAM-1 and E-selectin were expressed on vascular endothelial cells within the glandular areas of benign and malignant prostatic tissues and on single cells that did not appear to be associated with any glandular or vascular structures and malignant epithelial cells. A proportion of vascular endothelial cells in both benign hyperplastic and malignant glands, appear to be activated, demonstrated by their expression of E-selectin, ICAM-1 and VCAM-1. This accounts for the presence of inflammatory cells within the tissues. It is interesting that not all endothelial cells are activated. Moreover, not all vascular endothelial cells express all three of the aforementioned CAMs. This suggests that the endothelial cells present in these samples were at different levels of activation. It could be hypothesised that extravasation was actively occurring at the time of tissue sampling. Therefore, inflammatory cells (not necessarily T cells) are actively attaching to vascular endothelial cells expressing E-selectin, ICAM-1 and VCAM-1. These cells may be actively transmigrating into the site of prostatic disease, through the endothelial cells that only express ICAM-1.

The level and pattern of distribution of expression of E-selectin, ICAM-1, VCAM-1, α4, α5, αL, β1, and CD44 by malignant prostatic glands could not be correlated with the histological grade or metastatic phenotype of the prostatic adenocarcinomas. However, the level of expression of ICAM-1 was statistically greater in malignant than in benign hyperplastic prostatic glands

In conclusion, the level of expression of and the number of samples expressing ICAM-1 within the glandular epithelium appears to be significantly greater in malignant prostatic tissues than in BPH tissues (p< 0.005). However, while prostatic carcinoma cells express higher levels of

ICAM-1 than benign hyperplastic prostatic epithelial cells, no correlation could be made between the level of ICAM-1 expression and the histological grade or metastatic phenotype of the tumour.

Chapter 5

In Vitro Manipulation of Prostatic Cancer Cell Lines

Contents

5.1 Introduction

Approximately 50% of patients with cancer of the prostate have metastatic disease at the time of presentation (Rinker-Schaeffer *et al*, 1994). Second only to local lymph nodes, the most frequent site of metastatic prostate disease is the bone and bone marrow, as discussed in Chapter 1.4.5. Indeed, 70% of patients with prostate cancer will develop bone metastases (Haq *et al*, 1992). Why should prostate cancer cells metastasise to the bone marrow? As discussed in Chapter 1.4.5, the bone marrow is a rich source of growth factors. Many tumour cells, including prostatic cells, have increased growth patterns when cultured in the presence of bone marrow stromal cells or bone marrow conditioned medium. This supports the theory that the bone marrow provides a favourable milieu for metastasising tumour cells.

Functional adhesive studies investigating the molecular interactions of tumour cells with the bone marrow have centred around bone marrow-produced, immunologically associated cytokines, including members of the interleukin (IL), and Colony Stimulating Factor (CSF) families and their effect on tumour cell -endothelial cell interactions. Contact between myeloma tumour cells and bone marrow stromal cells augments the secretion of IL-6, which promotes the *in vitro* growth of myeloma cells, by the stromal cells. Lack of cell contact resulted in a lack of IL-6 secretion augmentation (Uchiyama *et al*, 1993). This data suggests that the tumour cell itself may serve to promote the existence of a favourable milieu. Subsequent attachment of tumour cells to stromal cells was due, in part, to the $\beta1$ and $\beta2$ integrins.

GM-CSF is known to enhance expression of ICAM-1 and, to a lesser extent, $\beta2$ integrins by blood monocytes and ovarian tumour-associated macrophages (Bernasconi *et al*, 1995). ICAM-1 and LFA-3 expression by acute myeloid leukaemia cells is upregulated by GM-CSF (Bendall *et al*, 1995). Incubation of neutrophils with GM-CSF increases their adhesion to and migration through endothelial cell monolayers. This can be inhibited by pre-treatment of the neutrophils with monoclonal antibodies against the $\beta2$ integrin subunit and, to a lesser extent, L-selectin. Adhesion of resting neutrophils can also be inhibited with $\beta2$ blocking antibodies, but not by L-selectin antibodies (Yong and Linch, 1993). GM-CSF increases the motility of differentiating myeloid cells from the bone marrow: this is thought to be due to an increase in FAK and subsequent downstream events (Kume *et al*, 1997). Moreover, PC3 and Du145 cell lines secrete GM-CSF and their growth is promoted when cultured in its presence (Lang *et al*, 1994).

5.2 The Effect of GM-CSF on the Expression of Seven Cell Adhesion Molecules by Prostate Cancer Cell Lines

GM-CSF was initially characterised by its ability to inhibit neutrophil migration in an agarose assay. However, it has since been shown to act as a chemo-attractant for neutrophils into inflamed tissue (Yong *et al*, 1993). The motogenic and CAM expression-inducing properties of GM-CSF, together with the evidence that prostatic carcinoma cell lines both secrete and respond to exogenous GM-CSF, led to the hypothesis that GM-CSF may influence the expression of CAMs on the surface of prostate cancer cells.

Experiments were carried out in this study to investigate the CAM expression by PC3 and Du145 cell lines in the absence and presence of GM-CSF. As discussed previously the LNCaP cell line differs in many ways from PC3 and Du145 cell lines. LNCaP was derived from a pelvic lymph node metastasis of a prostate cancer patient: thus, the cells from this lineage are not as clinically advanced as those from PC3 and Du145, isolated from the brain and bone deposits, respectively. Secondly, while LNCaP cells are not hormone-dependent, they are hormone-sensitive. The expression of some CAMs are known to be under hormonal control. Bone metastatic deposits of prostate cancer are not generally under the control of sex hormones. Therefore, the exclusion of LNCaP cells from this study removed the complication of hormonal effects.

Experiments were designed to investigate the cell surface expression of seven CAMs associated with the various stages of leucocyte extravasation. Chapter 1.5 discusses cell transmigration in detail. To summarise, CD44 is involved in leukocyte 'rolling', VCAM-1, $\alpha 4$, and $\beta 1$ are involved in leukocyte activation and ICAM-1, $\alpha 5$, and αL are involved in both leucocyte activation and transendothelium migration.

GM-CSF-supplemented-established cell line medium (ECLM, Appendix 5.7) was prepared to concentrations 0, 0.001, 0.01, 0.1, and 1.0ng/ml GM-CSF, where the EC_{50} value of the stock GM-CSF ranged from 0.02 to 0.2ng/ml. Du145 and PC3 cells were cultured as described in Chapter 2.1. Du145 and PC3 cells were incubated with GM-CSF as illustrated in Diagram 5.1. Differences In CAM expression between cells cultured in different concentrations of GM-CSF were analysed statistically with the Student's T-test.

PC3, but not Du145 cells expressed cell surface CD44. There was no difference in CD44 expression if cells were cultured with or without GM-CSF. Although the CD44 levels of PC3 cell surfaces varied throughout the 24 hour culture period this variation was not significant and was seen with all GM-CSF concentrations. Therefore, GM-CSF had no effect on PC3 or Du145 cell surface expression of CD44 (Appendix Table 5.2.1).

Both PC3 and Du145 cells show cell surface expression of ICAM-1. Du145 cells have fluorescence levels for ICAM-1 (MESF ICAM-1) which are approximately three times greater

Freshly Trypsinised PC3 / Du145 Cells

↓

Cultured Until 90-100% Confluent

↓

Addition Of GM-CSF Conditioned Medium

| 0ng/ml
GM-CSF | 0.001ng/ml
GM-CSF | 0.01ng/ml
GM-CSF | 0.1ng/ml
GM-CSF | 1.0ng/ml
GM-CSF |

Samples Incubated For 2, 4, 8, 12, and 24 Hours

↓

Examined Under Light Microscopy
(Check adherent characteristics)

↓

Trypsinisation Of Cells

↓

FACScan Analysis

CAM Antibody   Control Antibody
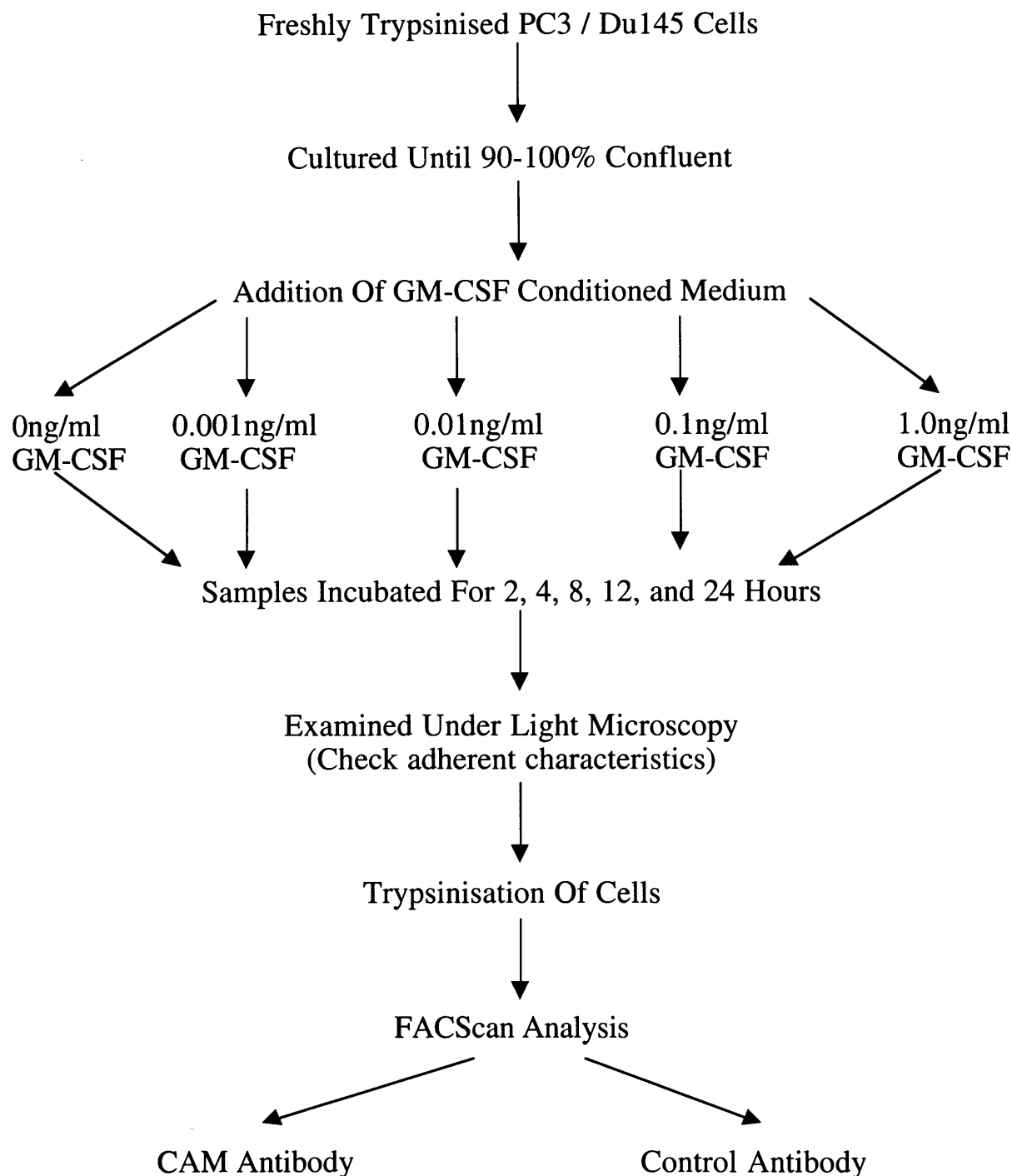
Diagram 5.1 Design Of Experiments Investigating The Effect Of GM-CSF On The Expression Of Cell Adhesion Molecules By PC3 And Du145 Cells. Freshly trypsinised cells were seeded in 24-well plates and cultured until 70-90% confluent. Cells were then incubated with varying concentrations of GM-CSF supplemented established cell line medium for 2, 4, 8, 12, and 24 hours. The adherent characteristics of the cells were examined under light microscopy at the end of each incubation period. Cells were then trypsinised from the plate and subjected to flow cytometric analysis with antibodies against the relevant cell adhesion molecule. Control FACScan analysis included incubation of cells without any antibody, with secondary antibody only, or with irrelevant primary antibody and secondary antibody. (GM-CSF, granulocyte, monocyte-colony stimulating factor.)
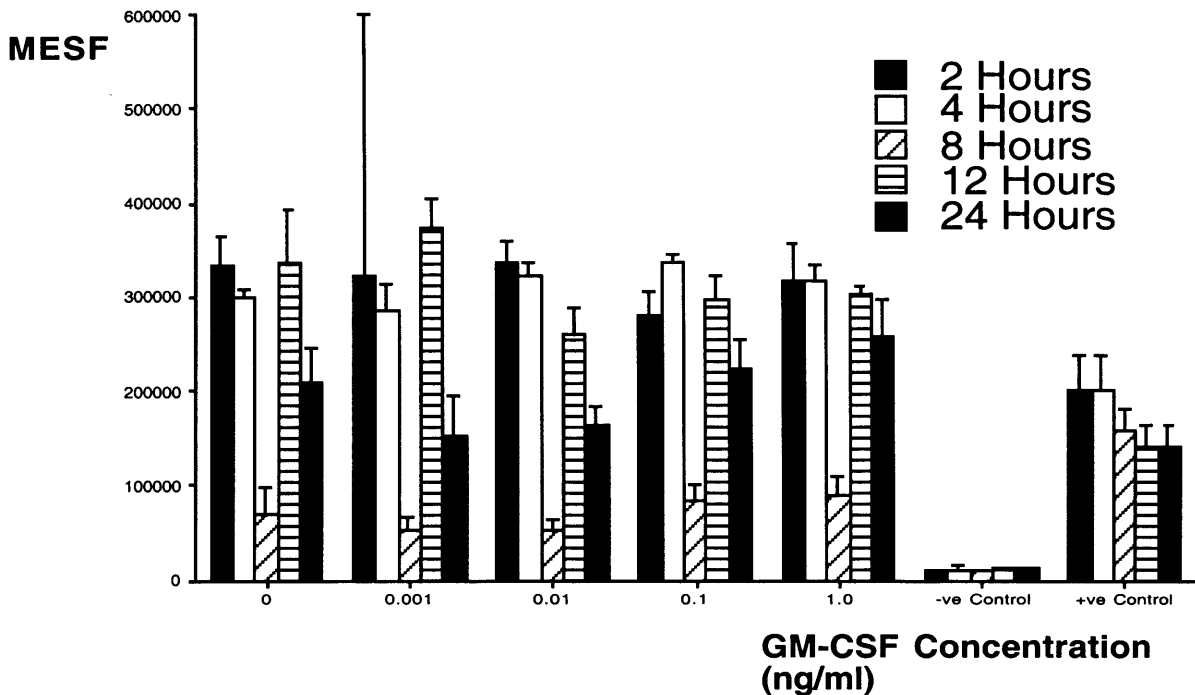
Figure 5.2.1 GM-CSF Had No Effect On The Expression Of ICAM-1 By Du145 Prostatic Adenocarcinoma Cells. 200μl GM-CSF-supplemented-ECLM was added to confluent monolayers of cells grown in 24-well TCGPs. Cells were incubated, under standard tissue culture conditions, for a further 2, 4, 8, 12, or 24 hours. Cell surface expression of CD44 was determined by FACScan analysis, as detailed in the text. (Median levels of fluorescence were converted to MESF as described in 2.4.2.4. Error bars represent the standard deviation of the mean MESF, n=3.)

those of PC3 cells. GM-CSF had no effect on the MESF ICAM-1 values for PC3 cells (Appendix Table 5.2.2). Although Du145 MESF ICAM-1 values troughs after 8 hours of incubation, the values had risen to normal four hours later. Moreover, this dip in ICAM-1 expression showed no correlation with GM-CSF expression: indeed, cells cultured without GM-CSF also demonstrated this decrease. Therefore GM-CSF had no effect on cell surface expression of ICAM-1 expression by PC3 and Du145 cells (Figure 5.2.1).

Both PC3 and Du145 cells showed cell surface α5 expression. Du145 cells demonstrated approximately four times greater levels of α5 than PC3 cells, with an MESF α5 value averaging 228434 over the 24-hour culture period. The addition of GM-CSF to PC3 and Du145 cells produced no changes in α5 cell surface expression over a 24-hour period (Appendix Table 5.2.3).

PC3 and Du145 cells expressed similar levels of β1, with MESF β1 values averaging 198643 and 225200, respectively, over the 24-hour incubation period. The addition of GM-CSF did not alter the expression of β1 by either cell line (Appendix Table 5.2.4).

Neither PC3 nor Du145 cells demonstrated surface expression of VCAM-1, α4, or αL. The addition of GM-CSF did not induce the expression of these CAMs by either cell line (Appendix Tables 5.2.5, 5.2.6 and 5.2.7).

To summarise, GM-CSF did not alter the cell surface level of CD44, ICAM-1, VCAM-1, α4, α5, αL, or β1 CAM expression by either PC3 or Du145 prostatic carcinoma cell lines.

5.3 The Effect of HUVEC-Conditioned Medium on the Expression of Seven Cell Adhesion Molecules by Prostate Cancer Cell Lines

Numerous other HGFs could have been tested in Chapter 5.2. However, it was hypothesised that the endothelial cell barrier between blood borne tumour cells and the bone marrow must first 'activate' the tumour cells rendering them capable of interacting with the endothelial cells themselves. During leucocyte extravasation, as discussed in Chapter 1.5, the endothelial cell surface is rich in leucocyte activating and chemo-attracting signals. Response to such stimuli induces rapid and dramatic changes in the cells' activity, including changes in CAM expression (Butcher, 1991). This evidence posed the question whether endothelial cell secretory compounds could induce changes in CAM by prostate cancer cell lines.

Experiments were designed to investigate the cell surface expression of seven CAMs, CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, and $\beta 1$. Human umbilical cords were readily available from the Maternity Unit of the Leicester General Hospital. Human Umbilical Vein Endothelial Cells (HUVECs) were prepared and cultured as described in Chapter 2.2. The spent medium from confluent HUVEC cultures was removed and prepared as in Chapter 2.2.5. This HUVEC supernatant, or HUVEC-Conditioned Medium (HUVEC-CM), was considered to be a more complete medium than established cell line culture medium (ECLM), and as such was used neat in this series of experiments. Du145 and PC3 cells were incubated with HUVEC-CM as illustrated in Diagram 5.2. Differences in the expression of each CAM by cells cultured with ECLM and HUVEC-CM were statistically analysed with the Student's T-test.

Similar basal levels of cell surface CD44, VCAM-1, $\alpha 5$, and $\beta 1$ CAMs were expressed by PC3 and Du145 cells as seen in Chapter 5.2. Likewise, the lack of cell surface expression of VCAM-1, $\alpha 4$ and αL by Du145 cells was consistent with that seen previously (Chapter 5.2). However, the cell surface levels of VCAM-1, $\alpha 4$ and αL of PC3 were higher than those seen in earlier experiments. The increased MESF values for these CAMs were constant throughout the 24 hour culture period.

In vitro culture of these PC3 and Du145 cells in HUVEC-conditioned medium did not alter the cell surface levels of CD44, VCAM-1, $\alpha 4$, $\alpha 5$, αL, or $\beta 1$ over a 24 hour period (Appendix Tables 5.3.1, 5.3.2, 5.3.3, 5.3.4, 5.3.5, and 5.3.6).

In vitro culture of Du145 cells with HUVEC-conditioned medium for 12 and 24 hours decreased their cell surface expression of ICAM-1 from levels seen for cells cultured in ECLM. However, these lower MESF ICAM-1 values were not statistically significant different to cells cultured in ECLM (Student's T-test). Cell surface levels of ICAM-1 were similar for both HUVEC-CM-treated- and ECLM-treated-PC3 cells (Figure 5.3.1).

Freshly Trypsinised PC3 / Du145 Seeded In 24-well Plates

Cultured Until 70-90% Confluent

Addition Of HUVEC-Conditioned Medium

2 Hours   4 Hours   12 Hours   24 Hours

Examined Under Light Microscopy
(Check adherent characteristics)

Trypsinisation Of Cells

FACScan Analysis

CAM Antibody   Control Antibody
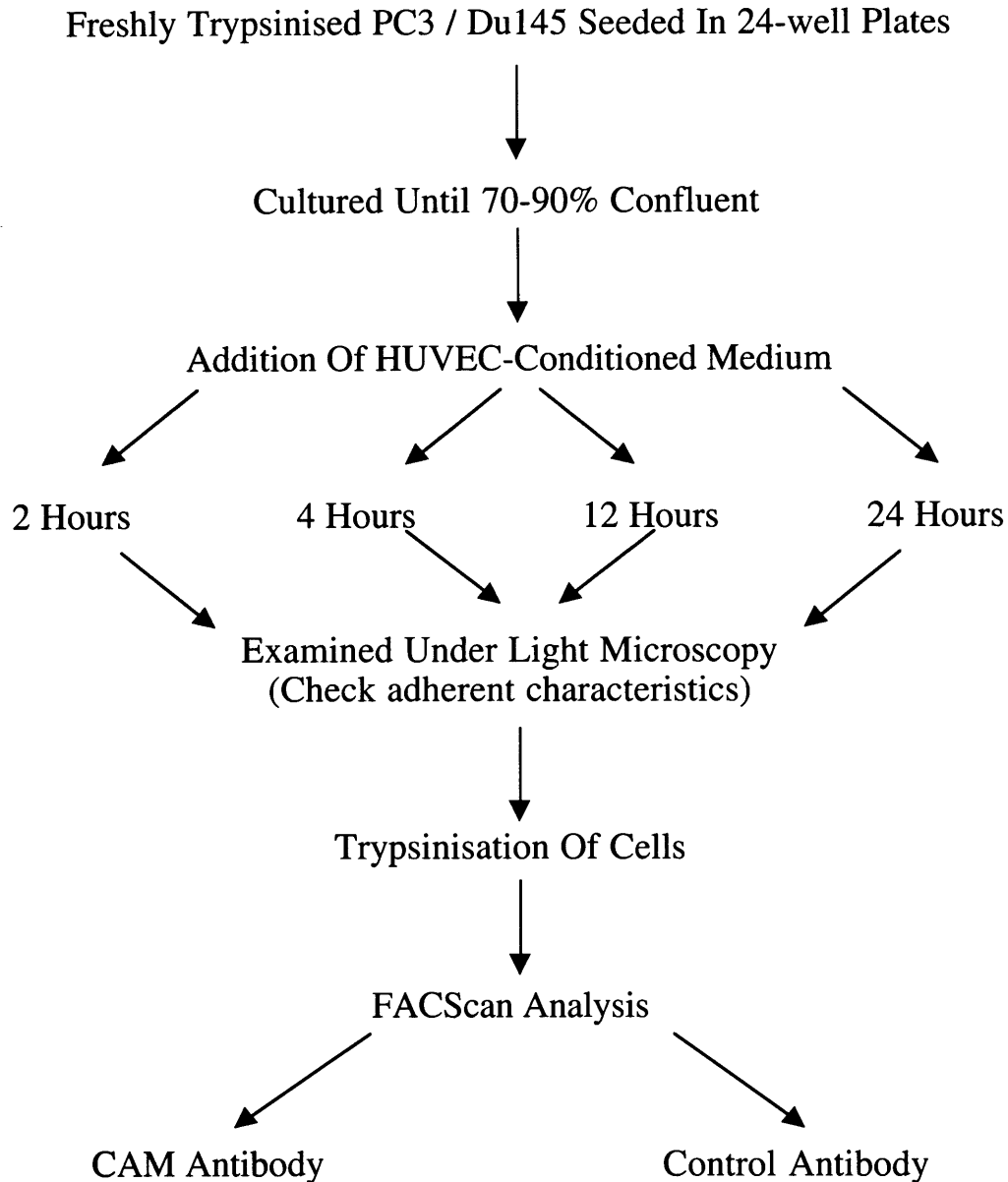
Diagram 5.2 Design Of Experiments Investigating The Effect Of HUVEC-Conditioned Medium On The Expression Of Cell Adhesion Molecules By Du145 And PC3 Cells. Freshly trypsinised cells were seed in 24-well tissue culture plates and cultured until 70-90% confluent. Cells were then incubated with HUVEC-conditioned medium for 2, 4, 12, and 24 hours. The adherent characteristics of the cells were examined under light microscopy. Cells were trypsinised from the plate and subjected to flow cytometric analysis with antibodies against cell adhesion molecules or control antibodies. Control FACScan analysis included incubation of cells without any antibody, with secondary antibody only, or with irrelevant primary antibody and secondary antibody. (HUVEC, human umbilical vein endothelial cell)
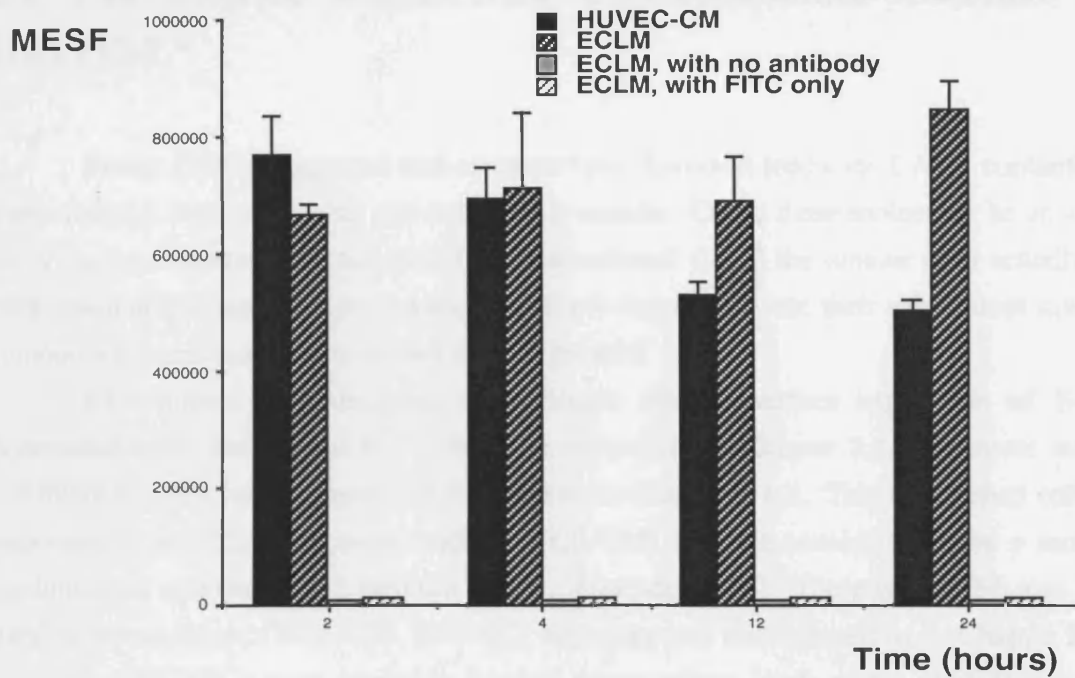
Figure 5.3.1 HUVEC-Conditioned Medium Had No Effect On The Cell Surface Expression Of ICAM-1 By Du145 Prostatic Adenocarcinoma Cells. 200µl of HUVEC-Conditioned Medium (HUVEC-CM) or standard Established Cell Line Medium (ECLM) was added to confluent monolayers of cells grown in 24-well TCGPs. Cells were then incubated for a further 2, 4, 12, or 24 hours. Cell surface expression of ICAM-1 was determined by FACScan analysis, as detailed in the text. (Median levels of fluorescence were converted to MESF values as described in 2.4.2.4. Error bars represent the standard deviation of the mean MESF, n=3).

Therefore, the expression of CD44, ICAM-1, VCAM-1, $\alpha4$, $\alpha5$, αL and $\beta1$ by PC3 and Du145 cells was not influenced by HUVEC-CM medium.

5.4 Can Prostate Cancer Cell Line-conditioned Medium Activate HUVECs?

Picker (1992) suggested that constitutively expressed leucocyte CAMs containing the sLe^x motif interact with endothelial cell-inducible E-selectin. Could these molecules be involved in the early stages of tumour cell-endothelial cell interactions? Could the tumour cells actually induce the expression of E-selectin by the endothelial cell and thereby activate their subsequent involvement in tumour cell extravasation into distant sites of growth?

Experiments were designed to investigate the cell surface expression of E-selectin by endothelial cells. Du145 and PC3 cells were cultured as in Chapter 2.1. The spent medium from confluent cultures was removed and prepared as in Chapter 2.1.4. This established cell line (ECL) supernatant, or ECL-conditioned medium (ECL-CM) was not considered to be a more complete medium than endothelial cell medium (ECM, Appendix 5.11). Therefore, ECM was prepared to varying percentages of ECL-CM. HUVECs were prepared and cultured as in Chapter 2.2. Freshly trypsinised HUVECs were seeded in 24-well tissue culture grade plates (TCGPs) and incubated with experimental media, as illustrated in Diagram 5.3. Cell surface expression of E-selectin was analysed by flow cytometric analysis.

Endothelial cells constitutively express PECAM-1 (Pigott and Power, 1992). HUVECs show strong surface expression of PECAM-1, with MESF PECAM-1 values in the range of 600000 to almost 3000000. Culturing these cells with ECL-CM for 2 or 4 hours did not induce their surface expression of E-selectin (Figure 5.4.1). Maximum activation (and E-selectin expression) of HUVECs is inducible by IL-1 after 4 hours of incubation (Rosen and Bertozzi, 1994). However, in the absence of E-selectin expression in these experiments, HUVECs were cultured with ECL-CM for 8, 12, 24, and 48 hours. No expression of E-selectin was detected (Appendix Tables 5.4.1 and 5.4.2).

Therefore, PC3- and Du145-CM does not influence the expression of E-selectin, and therefore the activation status, of vascular endothelial cells, HUVECs

Freshly Trypsinised HUVECs Seeded In 24-well Plates

Cultured Until 70-90% Confluent

Addition Of Experimental Medium (200μl)

| 100% ECM | 100% ECLM | 25% ECL-CM: 75% ECM | 50% ECL-CM: 50% ECM | 75% ECL-CM: 25% ECM | 100% ECL-CM |

Each Set Incubated Under Standard Tissue Culture Conditions

2 Hours   4 Hours

Examined Under Light Microscopy
(Check adherent characteristics)

Trypsinisation Of Cells

FACScan Analysis

PECAM Antibody   Control Antibody

Diagram 5.3 Experimental Design For Investigation Of Activation Status Of HUVECs When Incubated With Established Cell Line-Conditioned Medium. Freshly trypsinised HUVECs were seeded in 24-well culture plates and cultured to confluence. Cells were then incubated with ECM, ECLM, and mixtures of ECL-CM and ECM, from 25% ECL-CM to 100% ECL-CM. Cells were incubated for 2 or 4 hours. After examination under light microscope, cells were trypsinised and subjected to FACScan analysis with PECAM-1 and E-selectin antibodies. Control FACScan analysis included incubation of cells without any antibody, with secondary antibody only. (ECL, established cell line (PC3 and Du145); ECLM, established cell line medium; ECM, endothelial cell medium; HUVEC, human umbilical vein endothelial cell.)

a) PC3-CM
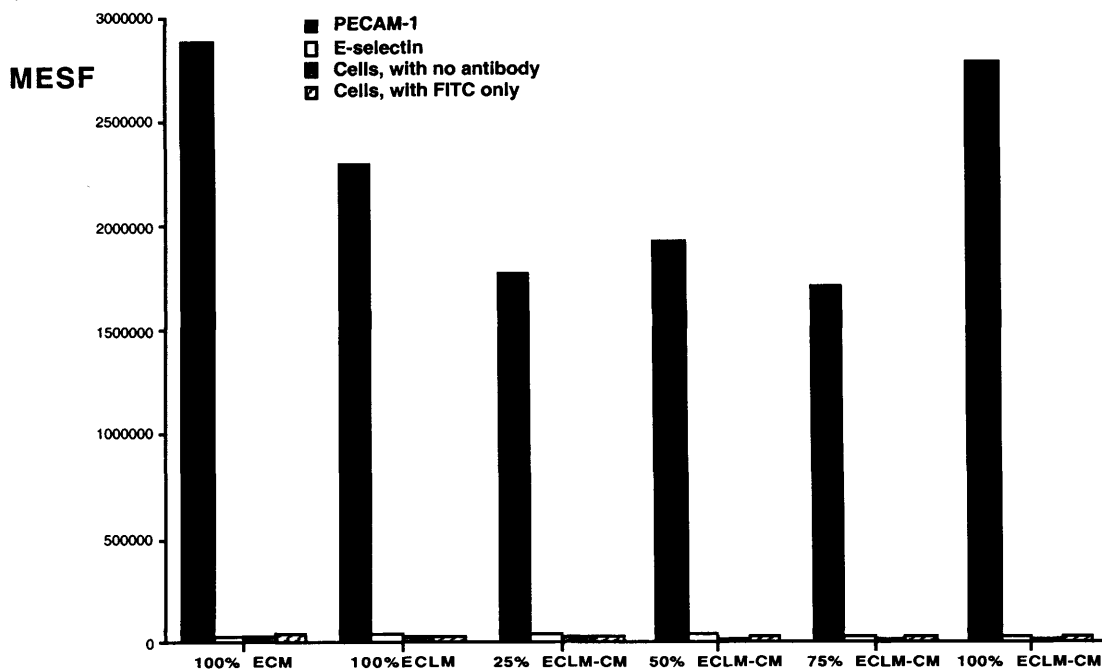
b) Du145-CM
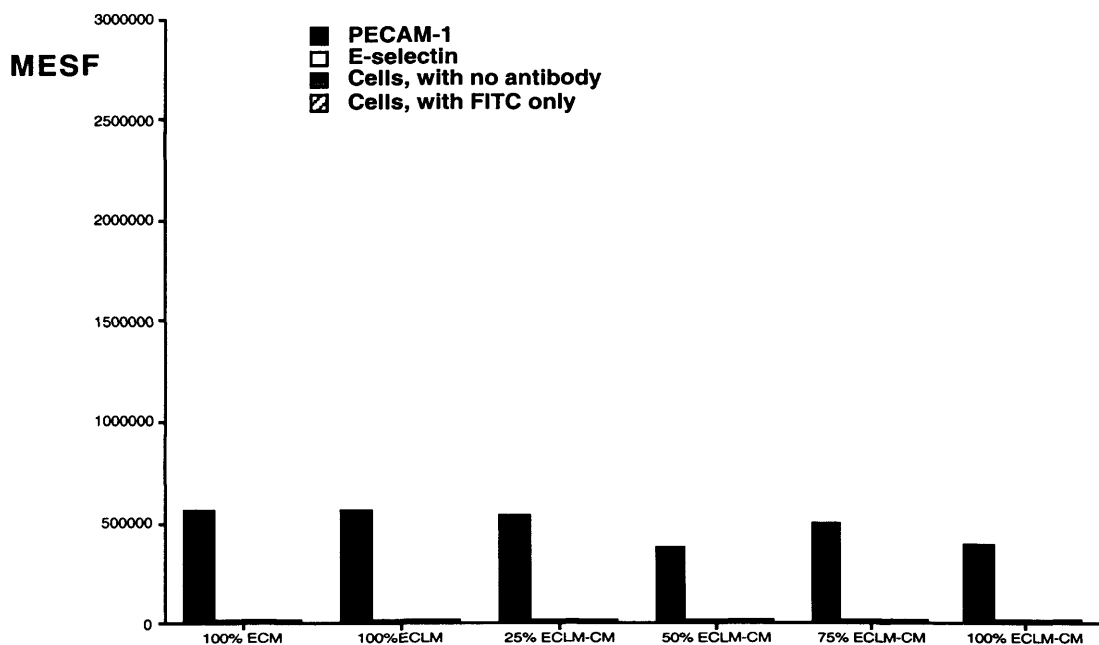
Figure 5.4.1 PC3- And Du145-Conditioned Medium Did Not Activate Endothelial Cells. 200μl of a) PC3-Conditioned Medium (PC3-CM) and b) Du145-Conditioned Medium (Du145-CM), at concentrations of either 25%, 50%, 75%, or 100% was added to monolayers of Human Umbilical Vein Endothelial Cells (HUVECs) at 70-100% confluence. These cells were further cultured for 2 hours, under otherwise standard tissue culture conditions. Activation of HUVECs was taken as the surface expression of E-selectin, and was measured by FACScan analysis, as detailed in the text. (Median levels of fluorescence were converted to MESF values as described in 2.4.2.4.)

5.5 The Effect of Co-Culturing HUVECs and Prostate Cancer Cells on the Expression of Six Cell Adhesion Molecules by Both Cell Types

GM-CSF and endothelial cell-conditioned medium did not change the surface expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, and $\beta 1$ by prostatic adenocarcinoma cell lines, PC3 and Du145 (Chapters 5.2 and 5.3). PC3-and Du145-conditioned medium did not alter the surface expression of the seven CAMs by human umbilical vein endothelial cells (Chapter 5.4). It appears that the process of tumour cell metastasis could not be paralleled with that of leucocyte extravasation. However, one basic difference exists between the experimental model designed in Chapters 5.2, 5.3, and 5.4 and that of leucocyte extravasation: there was no actual cell contact in the former. It was hypothesised that contact between tumour cells and endothelial cells is required to initiate changes in CAM expression.

5.5.1 Co-culture Studies with HUVECs and PC3 and Du145 Cells

In Chapter 3 experiments were conducted to design a co-culture system for prostatic epithelial cells and human umbilical vein endothelial cells (HUVECs), based upon the method used by Brodt (Brodt, *et al* 1997 and Leppens, et al 1996). Briefly, freshly trypsinised HUVECs were seeded in 24-well TCGPs and cultured to confluence. Confluent cultures of PC3 or Du145 cells were trypsinised and labelled with the membrane dye PKH26 (Chapter 2.5.3.2). These stained cells were adjusted to a concentration of 9×10^4 cells/ml in endothelial cell medium (ECM); 500µl was added to the confluent monolayer of HUVECs in the TCGPs. Cells were co-cultured for one hour and analysed for CAM expression, as illustrated in Diagram 5.4. CAM expression was detected with primary monoclonal antibodies raised in mice and a secondary antibody conjugated to fluorescein isothiocyanate (FITC) as detailed in Chapter 2.4.2.2. The FITC fluorophore emits light detected by the FL1 detector of the FACScan. PKH26 is a fluorophore that emits light detected by the FL2 detector of the FACScan. The optical layout of the Becton Dickinson FACScan is described in detail in Chapter 3. Therefore, firstly, Du145 / PC3 PKH26$^+$ cells could be distinguished from PKH26$^-$ HUVECs. Cells and secondly, the CAM expression by both cell populations could be analysed individually on the FACScan. Four populations of cells were produced by this analysis; namely, attached HUVECs; attached PC3 / Du145 cells; unattached HUVECs; and unattached PC3 / Du145 cells. Two control populations of cells were also included in this analysis – Du145 / PC3 cells and HUVECs cultured independently of each other in their own respective media. The specificity of monoclonal antibodies employed in these experiments are described in Table 7.1 (Appendix 6).

Unmanipulated Du145 cells demonstrated cell surface expression of CD44, ICAM-1 and α5 as previously seen in Chapter 5.2. ICAM-1 expression was greater than that of CD44 and α5: Du145 cells had MESF values for ICAM-1 approximately 10 and 100 times greater than those for α5 and CD44, respectively. Indeed, this MESF ICAM-1 was approximately double that seen in previous experiments. However, these MESF values were constant when the experiment was repeated (Table 5.1). Unmanipulated Du145 cells do not express VCAM-1, α4 or αL.

PC3 cells demonstrated cell surface expression of CD44, ICAM-1, α5 and low levels of αL, but lacked VCAM-1 and α4 expression. The MESF values for PC3 CD44 was similar to that for PC3 ICAM-1 (approximately 100000) and double that seen for Du145 CD44 expression. With the exception of αL, these MESF values for PC3 CAM cell surface expression were consistent with those seen in previous experiments. However, higher PC3 αL expression has been demonstrated previously in this study (Chapter 5.2), but, as for Du145 ICAM-1 above, this reduced level of αL expression was maintained when the experiment was repeated (Table 5.1).

Resting HUVECs expressed CD44 and α5, while no cell surface expression of ICAM-1, VCAM-1, α4, or αL was detected. HUVECs expressed similar levels of CD44 as PC3 and Du145 cells. The level of HUVEC α5 was similar to that of Du145 cells and double that of PC3 cells. The MESF values for CD44, ICAM-1, VCAM-1, α4, α5, and αL are summarised in Table 5.1 and compared to those of the cells alone and when incubated with irrelevant antibodies.

	Du145	**PC3**	**HUVECs**
CD44	54643	83268	51161
ICAM-1	1518127	102346	17639
VCAM-1	11111	7731	8414
α4	13992	9323	8998
α5	107671	69321	146889
αL	9489	21468	10445
MHC Class I	227811	12994	281769
Anti-mouse FITC-conjugated Ab	15207	12069	18573
Cells Only	9108	12406	13004

Table 5.1 The Cell Surface Expression Of Cell Adhesion Molecules By Du145, PC3 And Human Umbilical Vein Endothelial Cells. Cells were seeded in 24-well TCGPs and grown to confluence. Cells were trypsinised and incubated with mouse anti-human McAb's against the relevant cell adhesion molecule (or MHC Class I molecule) and a secondary anti-mouse antibody conjugated to FITC. Median levels of fluorescence were converted to MESF values as described in 2.4.2.4. Values quoted are the means of three MESF measurements and represent the results from one of three experiments. (FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; TCGP, tissue culture grade plate.)

Monolayer of PKH26⁻ HUVECs

↓

PKH26⁺ Du145 / PC3 Cells Added To HUVECs

↓

1 Hour Co-culture

↓

Examined Under Light Microscopy

(Check adherent characteristics)

Unattached Cells Aspirated Off   Attached Cells Trypsinised Off

↓   ↓

Incubation With Relevant Experimental Or Control Antibodies

↓

FACScan Analysis

PKH26⁺   PKH26⁻   PKH26⁺   PKH26⁻

Attached   Attached   Unattached   Unattached
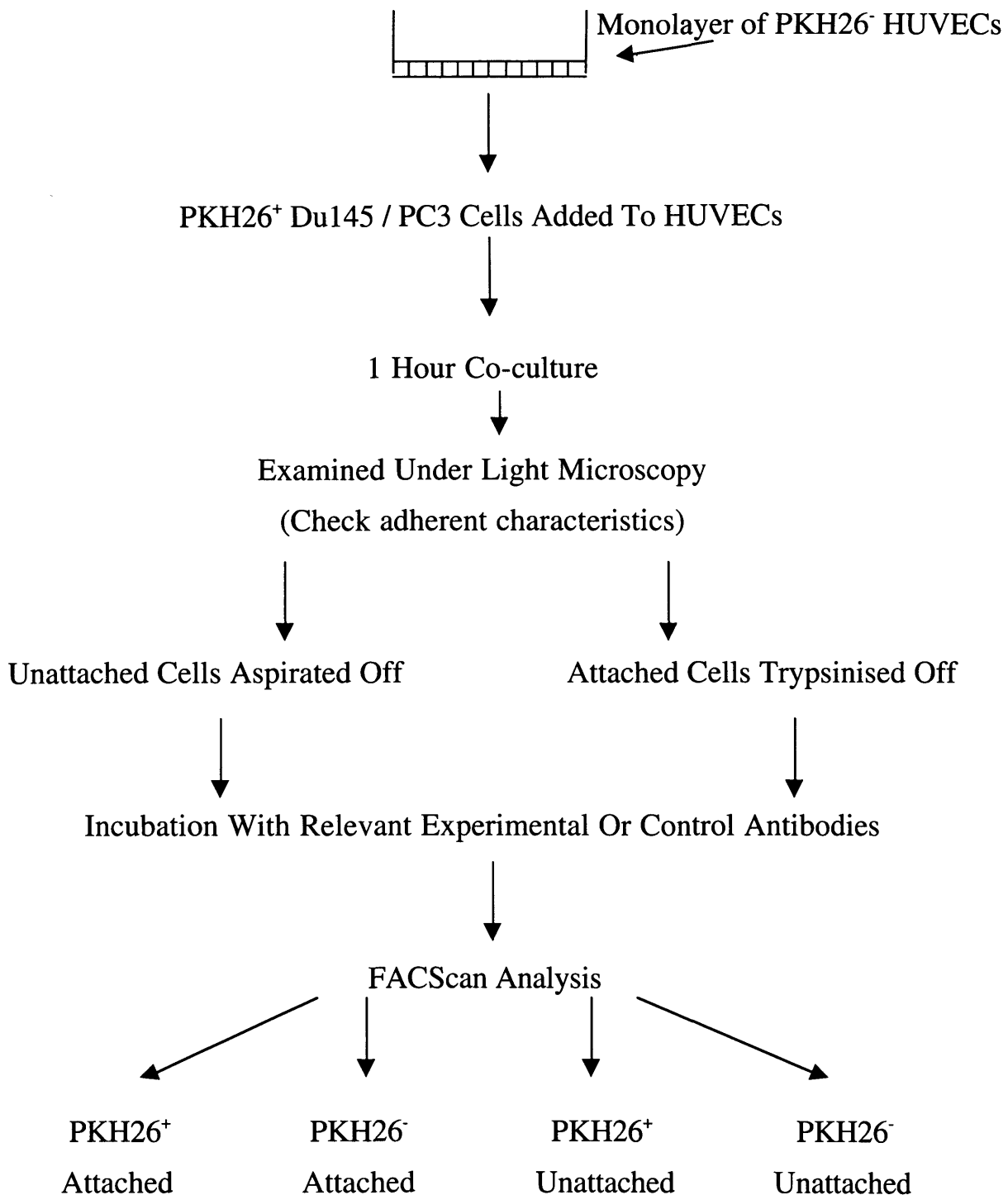
Diagram 5.4 The Co-culture System Used To Investigate Cell Adhesion Molecule Expression By HUVECs, PC3, And Du145 Cells. PKH26⁺ Du145 / PC3 cells were pipetted onto monolayers of confluent HUVECs. Cells were incubated for 1 hour. Unattached cells were aspirated off and attached cells were trypsinised. Cells were incubated with antibodies against the relevant cell adhesion molecule or control molecule. PKH26 emits fluorescence detected by the FL2 detector of the FACScan. Therefore, PC3 and Du145 cells could be differentiated from HUVECs. The level of fluorescence detected by the FL1 detector of the FACScan is due to the level of cell adhesion molecule expression by HUVECs, PC3, and Du145 cells.

Co-culture of Du145 prostate cancer cells with HUVECs for 1 hour did not induce the expression of VCAM-1 or αL by Du145 cells (Appendix Tables 5.5.1a and 5.5.2a). This co-culture did induce marginal increases in Du145 ICAM-1, α4 and α5 cell surface expression on both the cells that attached and those that remained unattached to the HUVECs (Appendix Tables 5.5.3a, 5.5.4a and 5.5.5a). However, when the MESF values of manipulated Du145 ICAM-1, α4 and α5 were compared statistically to those of unmanipulated Du145 cells the increases were not significant (Student's T-test). A marginal decrease in the expression of CD44 was measured on co-cultured Du145 cells (Appendix Table 5.5.6a). However, this decreased MESF CD44 value was not significantly lower than that of unmanipulated Du145 cells (Student's T-test).
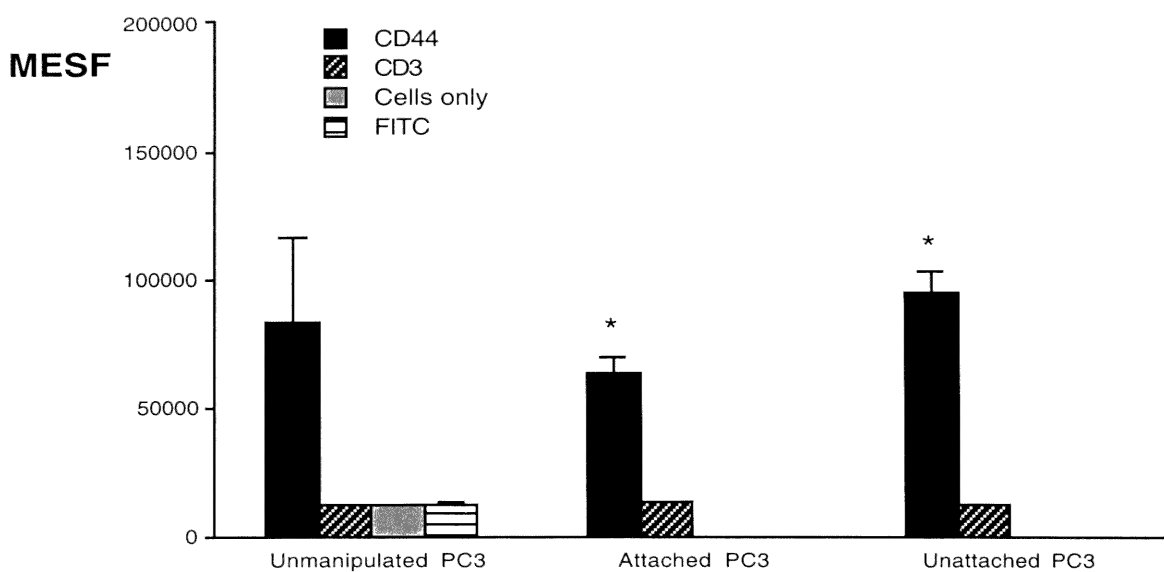
Figure 5.5.1 Co-culture Of PC3 Cells With HUVECs Decreases The PC3 Surface Expression Of CD44. PC3 prostatic adenocarcinoma cells were stained with the membrane dye PKH26 and incubated with confluent monolayers of Human Umbilical Vein Endothelial Cells (HUVECs). Cells were trypsinised after 1 hour of incubation and either a) analysed for surface CD44 expression immediately, or b) re-cultured for 24 hours before analysis, as detailed in the text. Error bars represent the standard deviation of those means, n=3. (MESF, molecular equivalent of soluble fluorochrome.) * indicates a significant difference, where $p < 0.005$.

Similar co-cultures with HUVECs and PC3 cells for 1 hour did not induce cell surface expression of VCAM-1, α4, or αL by PC3 cells (Appendix Tables 5.5.7a, 5.5.8a and 5.5.9a). Similar increases were seen for PC3 ICAM-1 and α5 expression as for co-cultured Du145 cells (Appendix Tables 5.5.10a and 5.5.11a). Again, the increased MESF ICAM-1 and MESF α5 of co-cultured cells (both attached and unattached to HUVECs) were not significantly greater than those of unmanipulated PC3 cells. As discussed in Chapter 3, not all co-cultured PC3 cells adhered to the HUVECs. It could be postulated that those cells that adhered to the HUVECs had different CAM

expression. Indeed, PC3 cells that adhered to HUVECs expressed less cell surface CD44 than both unmanipulated and unattached PC3 cells (Figure 5.5.1). Interestingly, unattached PC3 cells demonstrated higher MESF CD44 values than, not only the unmanipulated cells, but also the attached PC3 cells. The MESF CD44 values for co-cultured PC3 cells that adhered to HUVECs were significantly lower to those of PC3 cells that did not adhere (p<0.005, Student's T-test).

The co-culture of either Du145 or PC3 cells with HUVECs for 1 hour induced very little change in the CAM expression by the endothelial cells. There appeared to be marginal decreases in the level of CD44 and α5 surface expression by both PC3- and Du145-co-cultured HUVECs (Appendix Tables 5.5.12a, 5.5.11a, 5.5.6a, and 5.5.5a). However none of these decreases were to MESF values significantly lower than those for unmanipulated HUVECs (Student's T-test). Co-culture of HUVECs with either PC3 or Du145 cells did not induce the cell surface expression of ICAM-1, VCAM-1, α4, or αL (Appendix Tables 5.5.3a, 5.5.1a, 5.5.4a, 5.5.10a, 5.5.7a, 5.5.8a, and 5.5.9a).

To summarise, the co-culture of Du145 prostate cancer cells with HUVECs for 1 hour did not alter the expression of ICAM-1, VCAM-1, α4, α5, or αL by either cell line. Co-culture of PC3 cells with HUVECs for 1 hour did not change the HUVEC expression of the aforementioned CAMs. However, PC3-HUVEC co-culture for one hour did induce increases in the cell surface expression of CD44 by unattached PC3 cells, but not Du145 cells.

Since cells co-cultured for 1 hour saw so little change in CAM expression, it was postulated that perhaps a longer period of co-culture was required. Therefore, co-cultures were established as above and incubated for 24 hours under standard tissue culture conditions.

When Du145 cells were co-cultured with HUVECs for 24 hours continuously, their expression of ICAM-1 was significantly greater than that of unmanipulated Du145 cells (Figure 5.5.2). MESF ICAM-1 levels averaged 998104, 1046510 and 434577 for attached, unattached and unmanipulated Du145 cells (Appendix Table 5.5.3c). Similar increases were demonstrated when the experiment was repeated twice. The levels of ICAM-1 expression demonstrated by these attached and unattached Du145 cells were significantly greater than that of unmanipulated Du145 cells (p<0.01 and p>0.005, Student's T-test). Manipulated Du145 cells demonstrated lower levels of surface CD44 expression than unmanipulated cells: however, this difference was not statistically significant. No VCAM-1, α4 or αL expression could be induced on Du145 cells by their co-culture with HUVECs for 24 hours (Appendix Tables 5.5.1c, 5.5.4c and 5.5.2c).

PC3 αL surface expression was not induced by co-culture of cells with HUVECs for 24 hours (Appendix Table 5.5.9c). Both attached and unattached, co-cultured PC3 cells demonstrated slightly increased MESF values for α4 (Appendix Table 5.5.8c). PC3 cells attached to HUVECs after a 24 hour co-culture also showed marginal increases in MESF values for VCAM-1 (Appendix Table 5.5.7c). However, none of these increased MESF values correlated to significant induction

of α4 or VCAM-1 cell surface expression (Student's T-test). The expression of α5 by PC3 cells was greater after a 24 hour co-culture with HUVECs than when left unmanipulated (Appendix Table 5.5.11c). This increased α5 cell surface expression, although seen on both attached and unattached PC3 cells, was significantly higher than that of unmanipulated PC3 cells for unattached cells (Student's T-test, Figure 5.5.3).
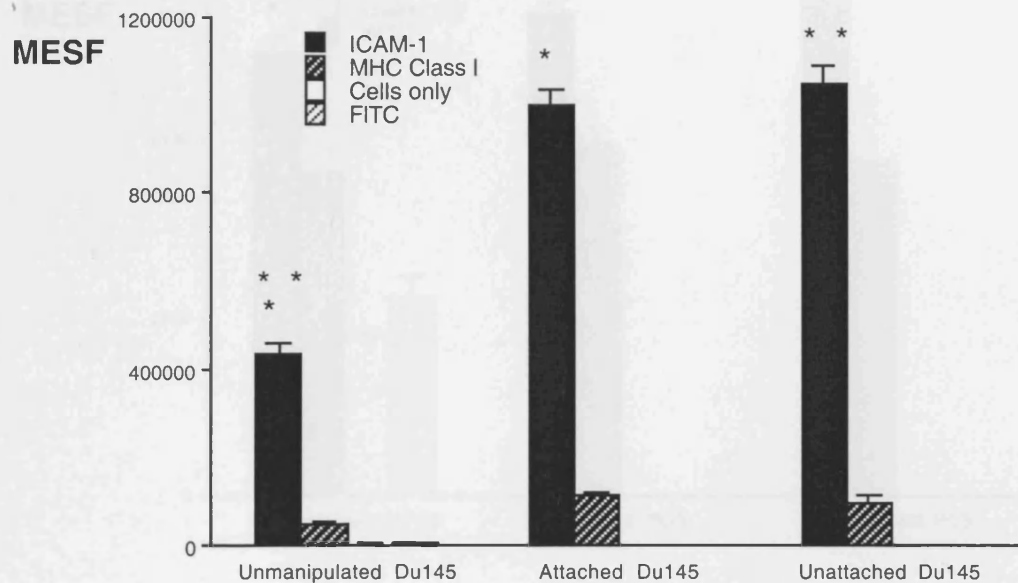
Figure 5.5.2 Prolonged Co-culture Of Du145 Cells With HUVECs Increased The Surface Expression Of ICAM-1 By Du145 Cells. Prostatic adenocarcinoma cells, Du145 cells, were stained with the membrane dye, PKH26, and incubated in direct contact with confluent monolayers of HUVECs for 24 hours. Columns represent the mean of three values: error bars represent the standard deviation of those means. (FITC, fluorescein isothiocyanate; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule-1; MESF, molecular equivalent of soluble fluorochrome.) * and ** indicate that the increases over unmanipulated MESF values are significant, where $p<0.01$ and $p<0.0005$, respectively.

Neither VCAM-1 nor ICAM-1 expression was induced in HUVECs when co-cultured with Du145 or PC3 cells for 24 hours (Appendix Tables 5.5.1c, 5.5.3c, 5.5.7c, and 5.5.10c). When co-cultured with Du145 cells for 24 hours there was a slight increase in the MESF values for α4 and a slight decrease in the values for α5 of HUVECs. However, neither of these MESF values were significantly different from those of unmanipulated HUVECs (Appendix Table 5.5.4c and 5.5.5c). Likewise, when HUVECs were co-cultured with PC3 cells for 24 hours the MESF values for HUVEC αL expression were slightly higher, although not significantly, than those of unmanipulated HUVECs (Appendix Table 5.5.9c). HUVECs co-cultured with Du145 cells for 24

140

hours showed reduced levels of surface CD44 than unmanipulated HUVECs (Figure 5.5.4). With average MESF CD44 values of approximately 15000 and 50000 for manipulated and unmanipulated HUVECs, co-cultured HUVECs expressed significantly less CD44 than unmanipulated HUVECs (p<0.005, Student's T-test, Appendix Table 5.5.6c)

To summarise, the co-culture of Du145 cells with HUVECs for 24 hours induced
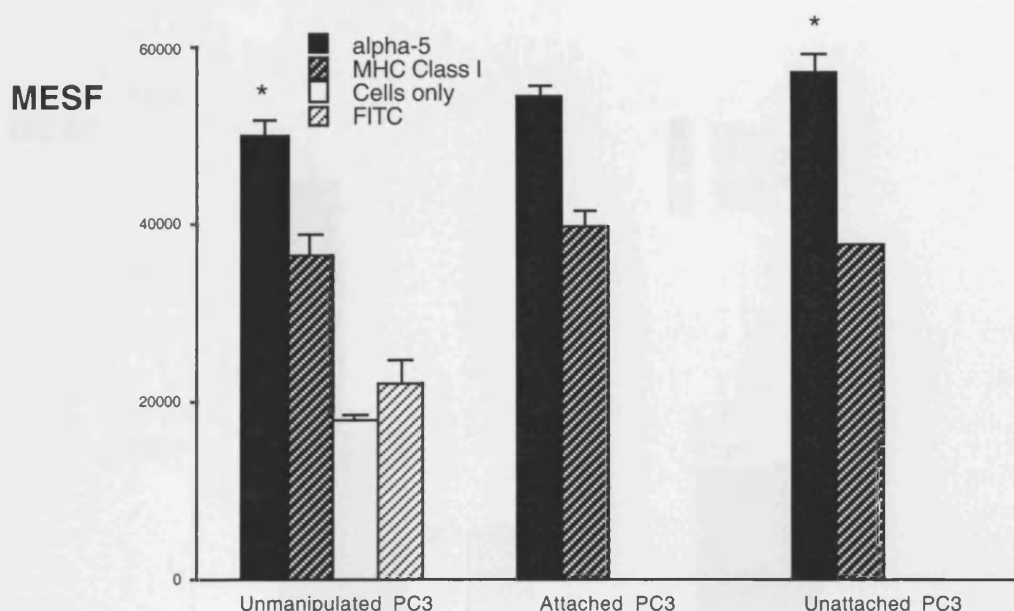
Figure 5.5.3 Prolonged Co-culture Of PC3 Cells With HUVECs Induced Up-regulation Of PC3 Cell Surface Expression Of α5. Prostatic adenocarcinoma cells, PC3, were co-cultured in direct contact with confluent monolayers of human umbilical vein endothelial cells (HUVECs) for 24 hours. Columns plotted represent the mean MESF value for α5 expression of three measurements: error bars represent the standard deviation of those means. (MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; FITC, fluorescein isothiocyanate.) * indicates that the MESF was significantly greater than that of the unmanipulated cells, p<0.05.

increased cell surface ICAM-1 by Du145 cells and reduced levels of surface CD44 by HUVECs. The co-culture of PC3 cells with HUVECs for 24 hours increased cell surface expression of α5 by PC3 cells, but no changes in HUVEC CAM expression. These changes in CAM expression differed to those seen after a one hour co-culture, when only increased CD44 expression was seen on PC3 cells.

It could be argued that neither a 1 hour or a 24 hour co-culture was truly representative of the *in vivo* process of leucocyte or tumour cell extravasation. Chapter 1 distinguishes three separate steps in extravasation; namely (i) leucocyte rolling, (ii) leucocyte activation and (iii) leucocyte transendothelial migration. Step (i) occurs relatively quickly while steps (ii) and (iii) require up-

regulation of several CAMs. To mimic this situation *in vitro* a third set of co-cultures were established. Du145 / PC3 cells were co-cultured with HUVECs for 1 hour to represent the first step of leucocyte rolling. Unattached cells were aspirated off the HUVECs and attached cells were trypsinised. Cells were then re-cultured separately for 24 hours and analysed for their CAM expression as described in Diagram 5.4.

Co-cultures of Du145 cells with HUVECs for 1 hour induced no changes in CAM
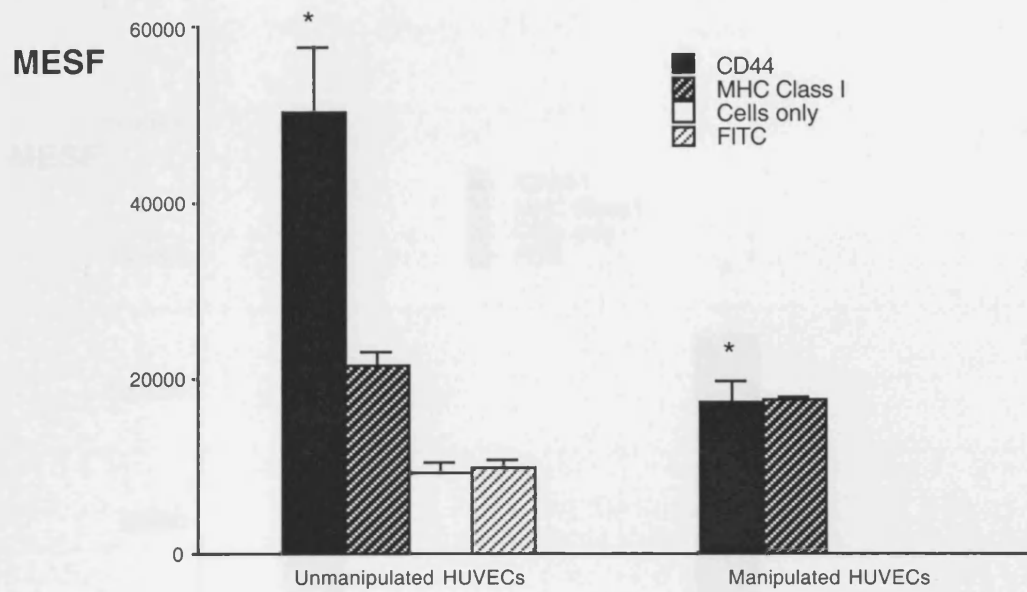
Figure 5.5.4 Prolonged Co-culture Of HUVECs With Du145 Cells Induced A Decreased Surface Expression Of CD44 By HUVECs. Prostatic adenocarcinoma cells, Du145, were stained with the membrane dye, PKH26, and co-cultured in direct contact with confluent monolayers of human umbilical vein endothelial cells (HUVECs) for 24 hours. Cells were trypsinised and analysed for CD44 expression as detailed in Diagram 5.4. Columns represent the mean MESF values for CD44 expression of three measurements: error bars represent the standard deviation of those means. (FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex.) * indicates that the MESF values are significantly different, $p < 0.005$.

expression by either cell line. Co-culture of Du145 cells with HUVECs for 24 hours increased Du145 ICAM-1 levels and reduced HUVEC CD44 levels. These changes remained when unattached Du145 cells were removed and attached Du145 cells and HUVECs were re-cultured for 24 hours (Figure 5.5.5 and Table 5.2). Both the increased Du145 ICAM-1 and decreased HUVEC CD44 expression were significantly different to those of unmanipulated, re-cultured cells (Appendix Tables 5.5.3b and 5.5.6b).

There was a slight increase in the MESF values of HUVECs re-cultured with Du145 cells for VCAM-1, α4 and α5. However, these levels were not significantly greater than those of unmanipulated, re-cultured HUVECs (Appendix Tables 5.5.1b, 5.5.4b and 5.5.5b). No induction of Du145 αL cell surface expression was seen after 24 hours of re-culture with HUVECs (Appendix Table 5.5.2b). These results were similar to those observed after a simple 24 hour co-culture: i.e. the removal of unattached Du145 cells after 1 hour of co-culture with HUVECs did not affect the CAM expression of Du145 cells. However, this re-culture in the absence of unattached Du145 cells induced expression of ICAM-1 by the HUVECs (Table 5.2). This increased level
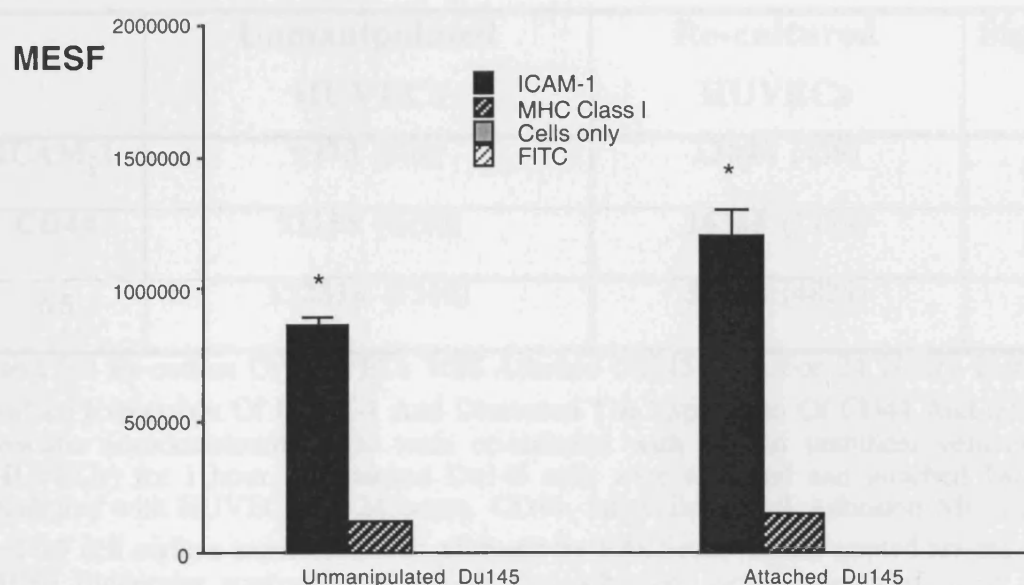
Figure 5.5.5 Re-culture Of Du145 Cells With HUVECs For 24 Hours In The Absence Of Du145 Cells That Remained Unattached After A 1 Hour Co-culture With HUVECs Expressed Increased Levels Of ICAM-1. Prostatic adenocarcinoma cells, Du145, were stained with the membrane dye PKH26 and co-cultured with confluent monolayers of human umbilical vein endothelial cells (HUVECs) for 1 hour. Unattached cells were removed and attached Du145 cells were re-cultured with the HUVECs for 24 hours. The surface expression of Intercellular Cell Adhesion Molecule-1 (ICAM-1) was analysed by FACScan. Columns represent the mean MESF of three measurements: error bars represent the standard deviation of those means. (FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability.) * indicates that the MESFs are significantly different, p<0.005.

of HUVEC ICAM-1 was statistically greater than that of unmanipulated, re-cultured HUVECs (Appendix Table 5.5.3b: p<0.001, Student's T-test). This change was not observed after a simple 24 hour co-culture. Therefore, it appears that the removal of unattached Du145 cells from a co-culture of Du145 cells and HUVECs induces up-regulation of ICAM-1 by HUVECs. This removal

of unattached Du145 cells also induced a decrease in the surface expression of α5 by HUVECs to levels that were significantly higher than those of unmanipulated, re-cultured HUVECs (Table 5.2). Decreased HUVEC α5 expression was observed after a simple 24 hour co-culture with Du145 cells, but this decrease was not demonstrated to be significant (Student's T-test). The reduction in CD44 expression on HUVECs co-cultured continuously for 24 hours with Du145 cells was also seen when unattached Du145 cells were removed and the HUVECs and attached Du145 cells were re-cultured for 24 hours (p is less than 0.001, Student's T-test, Table 5.2).

Co-culture of PC3 cells with HUVECs for 1 hour induced increased CD44 expression by PC3 cells. This change was not observed after 24 hours of co-culture: however, expression of α5 by

	Unmanipulated HUVECs	Re-cultured HUVECs	Significance
ICAM-1	9973 (305)	13044 (459)	p<0.001
CD44	51158 (6390)	16365 (1429)	p<0.001
α5	122316 (7316)	59524 (4825)	p<0.05

Table 5.2 Re-culture Of HUVECs With Attached Du145 Cells For 24 Hours Increased The Cell Surface Expression Of ICAM-1 And Decreased The Expression Of CD44 And α5 By HUVECs. Prostatic adenocarcinoma cells were co-cultured with human umbilical vein endothelial cells (HUVECs) for 1 hour. Unattached Du145 cells were removed and attached Du145 cells were recultured with HUVECs for 24 hours. CD44, Intercellular Cell Adhesion Molecule-1 (ICAM-1) and α5 cell surface expression was analysed by FACScan. Values quoted are the means of three MESF (molecular equivalent of soluble fluorochrome) measurements of 1 of 3 experiments. (Values in brackets represent the standard deviation of those means.) p values quoted represent the significance of differences between MESF values for unmanipulated and re-cultured HUVECs.

these PC3 cells was significantly increased above that of unmanipulated PC3 cells. Increased α5 expression by PC3 cells was also observed following a 24 hour re-culture of PC3 cells with HUVECs in the absence of unattached PC3 cells: however, this increase was not to levels significantly greater than that seen for unmanipulated, re-cultured PC3 cells (Appendix Table 5.5.11b). Although increased MESF values were observed for re-cultured PC3 ICAM-1 and α4 expression these levels were not significantly different to those of unmanipulated, re-cultured PC3 cells (Appendix Tables 5.5.10b and 5.5.8b). PC3 αL expression was not induced by re-culture of cells with HUVECs for 24 hours in the absence of unattached PC3 cells (Appendix Table 5.5.9b). While decreased levels of PC3 CD44 were demonstrated following re-culture with HUVECs, these levels were not significantly different to those of unmanipulated, re-cultured PC3 cells (Appendix Table 5.5.12b).

The co-culture of HUVECs with PC3 for both 1 and 24 hours of co-culture induced no change in the CAM expression of HUVECs. Similarly, when HUVECs were co-cultured with PC3 cells for 1 hour and re-cultured with the attached cells in the absence of unattached cells, no changes in HUVEC CAM expression was observed (Appendix Tables 5.5.7b, 5.5.8b. 5.59b, 5.5.10b, 5.5.11b, and 5.5.12b).

To summarise, Du145 cells that adhered to HUVECs after a 1 hour co-culture were re-cultured with these HUVECs: these Du145 cells expressed greater cell surface levels of ICAM-1 than unmanipulated cells. HUVECs re-cultured with attached Du145 cells expressed increased levels of ICAM-1 and decreased levels of CD44 and $\alpha 5$ than unmanipulated HUVECs. No changes in CAM expression were observed when PC3 cells and HUVECs were similarly re-cultured.

5.5.2 Co-culture Studies with Du145 Cells and LLC PK1 Cells

It could be argued that the changes in expression seen above were non-specific and simply general changes that occur after any cell-cell contact; that is, that these CAMs do not play a role in prostate tumour cell metastasis. Therefore, a co-culture experiment was performed with Du145 cells and cells from the porcine kidney tubular epithelial cell line, LLC PK1 (Chapter 2.3.2). Before co-culture was initiated, the inability of the mouse anti-human CAM monoclonal antibodies to recognise the LLC PK1 cell surface epitopes was ensured (Table 5.3).

LLC PK1 cells, acting as the HUVECs used in previous co-cultures, were co-cultured with Du145 cells. At the end of the 1 hour co-culture few Du145 cells were attached to the LLC PK1

	MESF
E-selectin	7115 (358)
CD44	8360 (531)
ICAM-1	8158 (144)
VCAM-1	6764 (285)
α4	7040 (215)
α5	8725 (362)
αL	7281 (297)
LLC PK1 Cells Only	5247 (296)
LLC PK1 Cells With FITC	6719 (278)

Table 5.3 Porcine Kidney Epithelial Cells, LLC PK1, Did Not Express Human Cell Adhesion Molecules. Freshly trypsinised cells were incubated with mouse anti-human antibodies reactive against several cell adhesion molecules, as described in Chapter 2.4.2. Cells were analysed on a FACScan. Median levels of fluorescence were converted into MESF values as described in Chapter 2.4.2.4.

cells when examined by light microscopy. These unattached cells were re-cultured for 24 hours. The few attached Du145 cells and LLC PK1 cells were trypsinised and re-cultured for 24 hours. Cells were trypsinised and analysed for ICAM-1 expression by FACScan (Chapter 2.4.2). Only 9% of the attached re-cultured cell population were PKH26 positive (PKH26[+]) Du145 cells. Throughout these co-culture and re-culture experiments where Du145 cells did not attach to LLC PK1 cells, there was no statistically significant change in Du145 ICAM-1 surface expression (Figure 5.5.6).

5.5.3 Co-culture Studies with HUVECs and A549 Cells

The next question that arose was whether the adherence of PC3 and Du145 cells to HUVECs was specific to prostate cancer cells. Therefore, the adherence of a lung
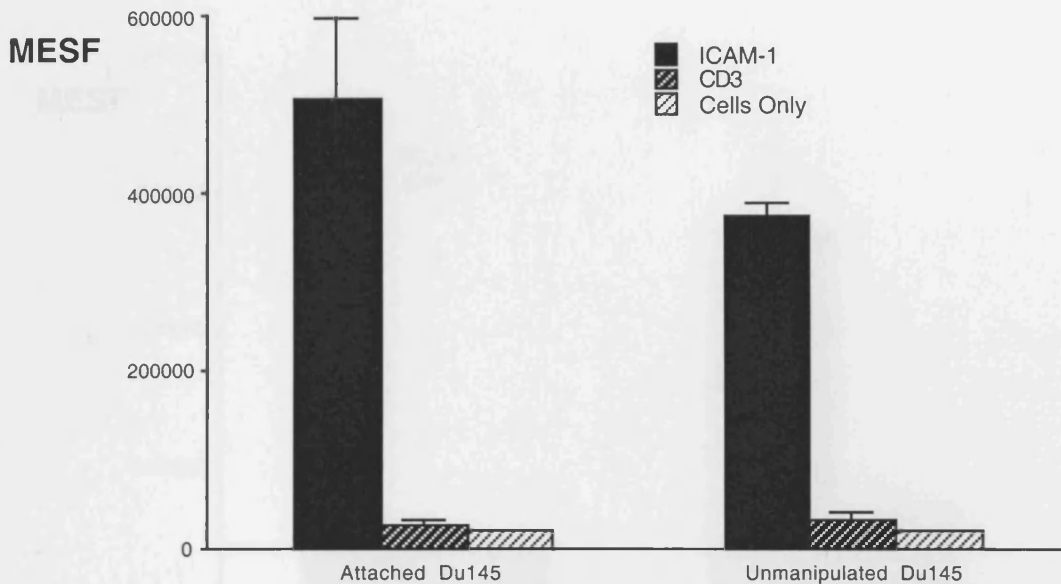
Figure 5.5.6 Du145 Co-culture With LLC PK1 Cells Did Not Change ICAM-1 Expression By Du145 Cells. Du145 cells were co-cultured with porcine kidney epithelial cells, LLC PK1, for 1 hour. Unattached Du145 cells were removed and recultured for 24 hours. ICAM-1 cell surface expression was analysed by FACScan. Columns represent the means of three MESF (molecular equivalent of soluble fluorochrome) measurements. Error bars represent the standard deviation of those means)

adenocarcinoma cell line, A549, to HUVECs was examined. HUVECs were prepared in 24-well TCGPs as before. A549 cells were labelled with PKH26 and co-cultured with HUVECs for 1 hour. Attached and unattached cells were collected and analysed for CD44 surface expression. A parallel group of co-cultured cells were separated and re-cultured for 24 hours as before.

After the initial 1 hour of co-culture, A549 cells had attached to the HUVECs when examined under light microscopy. A549 cells and HUVECs expressed large amounts of CD44 on their surfaces, with MESF CD44 values of 694008 and 417805, respectively. Co-culture of these two cell types for 1 hour induced a decrease in the surface expression of CD44 by those A549 cells that attached to the HUVECs. However, this decreased MESF level was not significantly lower than that of unmanipulated A549 cells (Student's T-test). There was no change in unattached A549 cell CD44 expression. The CD44 levels of expression by HUVECs were marginally increased

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when cultured with A549 cells, but this increase was to levels that were not significantly differently to that of unmanipulated HUVECs (Appendix Table 5.5.15a). Following a 24-hour re-culture of the mixed population of initially attached A549 cells (i.e. attached after 1 hour of co-culture) and HUVECs, the majority of cells were no longer attached to the TCGP: the cells were in suspension within the medium (i.e. re-culture of attached A549 cells and HUVECs for 24 hours caused
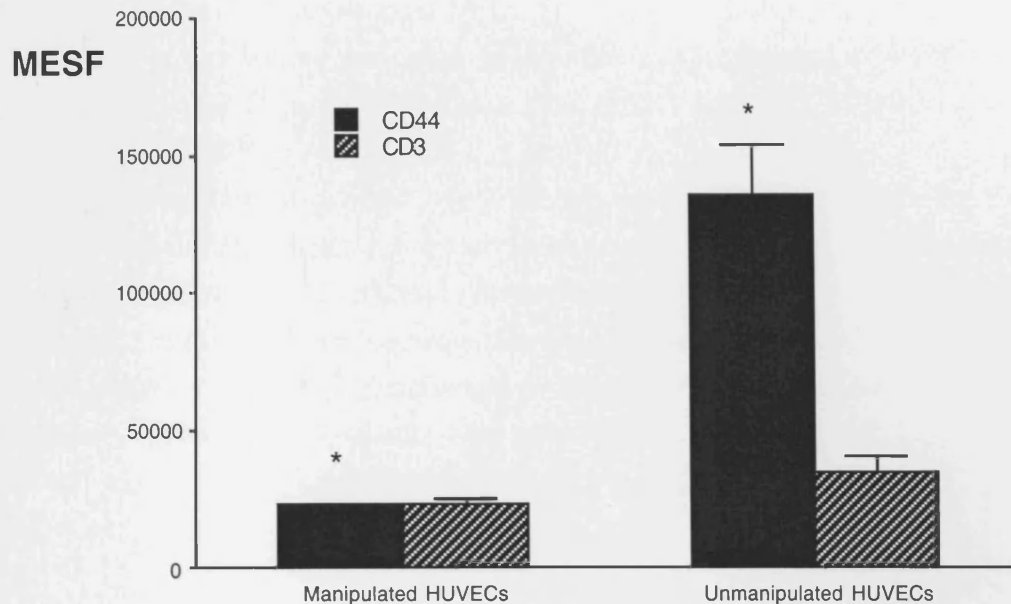
Figure 5.5.7 Prolonged Re-culture Of HUVECs With A549 Cells Induced A Decrease In The Cell Surface Expression Of CD44 By HUVECs. A549 lung adenocarcinoma cells were stained with PKH26 and incubated in direct contact with confluent monolayers of HUVECs for 1 hour. Attached cells were trypsinised and unattached cells were aspirated from the culture plate. These two cell populations were re-cultured separately for a further 24 hours. The initially attached cells had become unattached and were collected for flow cytometric analysis of CD44 surface expression. Columns plotted represent the mean MESF of three measurements: error bars represent the standard deviation of those means. *, indicate that the differences in MESF values are significantly different, p<0.01.

detachment of the majority of cells, not only from each other, but also from the TCGP. This was observed under light microscopy. Therefore, only unattached cells were collected for FACScan analysis. The CD44 surface expression by these unattached HUVECs was significantly reduced compared to that of unmanipulated HUVECs (p<0.01, Student's T-test, Figure 5.5.7). There was no significant difference in the A549 CD44 surface expression (Appendix Table 5.5.15b).

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This re-culture was repeated. Both the unattached and attached populations were collected after the 24 hours of re-culture. The majority of unattached cells were again HUVECs. The CD44 surface expression of these cells was significantly reduced over both unmanipulated and attached HUVECs. The majority of attached cells were A549 cells. There was no significant change in their CD44 surface expression (Appendix Table 5.5.16).

5.5.4 Adherent Patterns of HUVECs and Prostate Cancer Cell Lines During Co-culture

Upon the realisation that prolonged contact between cells appeared to induce detachment of HUVECs from the plate, the previous co-culture experiments with HUVECs and PC3 / Du145 cells were analysed further. The FACScan refers to each cell detected by the FACScan as an event. The total number of events collected by the FACScan, as well as the number of events that were PKH26$^+$ and PKH26$^-$, were recorded by the FACScan. Therefore, the percentage of PKH26$^+$ and PKH26$^-$ cells in any one sample could be determined. The percentage of PKH26$^-$ HUVECs and PKH26$^+$ PC3 / Du145 cells was calculated for each 1 hour and 24 hour co-culture (Appendix Tables 5.5.17 and 5.5.18).

PC3 cells were co-cultured with confluent monolayers of HUVECs for 1 or 24 hours. At the end of both culture periods an unattached and attached population of cells were identified. After a short 1 hour co-culture the attached population of cells was 35% PKH26$^-$ HUVECs and 53% PKH26$^+$ PC3 cells: the unattached suspension of cells was comprised of 26% PKH26$^-$ HUVECs and 66% PKH26$^+$ PC3 cells. Following a prolonged 24 hour co-culture of HUVECs with PC3 cells the attached population of cells was 10% PKH26$^-$ HUVECs and 79% PKH26$^+$ PC3 cells, while the unattached cell suspension was 14% PKH26$^-$ HUVECs and 72% PKH26$^+$ PC3 cells (Table 5.4).

	1 Hour of Co-culture			
	Attached Population		**Unattached Population**	
	PKH26$^+$ PC3 Cells	PKH26$^-$ HUVECs	PKH26$^+$ PC3 Cells	PKH26$^-$ HUVECs
Mean Percentage	53	35	66	26
SD of Mean Percentage	14	16	22	28
Number in Sample	102	102	102	102
	24 Hours of Co-culture			
	Attached Population		**Unattached Population**	
	PKH26$^+$ PC3 Cells	PKH26$^-$ HUVECs	PKH26$^+$ PC3 Cells	PKH26$^-$ HUVECs
Mean Percentage	79	10	73	14
SD of Mean Percentage	10	5	12	8
Number in Sample	72	72	72	72

Table 5.4 The Distribution Of PC3 Prostatic Adenocarcinoma Cells And Human Umbilical Vein Endothelial Cells In The Attached And Unattached Cell Suspensions Generated By Direct Contact Co-cultures. PHK26$^+$ PC3 cells were incubated in direct contact with confluent monolayers of human umbilical vein endothelial cells (HUVECs) for either 1 or 24 hours. Unattached cells were collected by aspiration and attached cells by trypsinisation from the tissue culture plate. All cells were analysed on a FACScan, as described in the text.

Du145 cells were co-cultured with HUVECs as PC3 above. After a 1 hour co-culture the attached population of cells was 58% Du145 cells and 40% HUVECs, while the unattached population was 75% Du145 cells and 21% HUVECs. After a 24 hour co-culture the ratios of

Du145 cells:HUVECs in the attached and unattached populations were 79%:19% and 47%:47%, respectively (Table 5.5).

1 Hour of Co-culture				
	Attached Population		**Unattached Population**	
	PKH26$^+$ Du145 Cell	PKH26⁻ HUVECs	PKH26$^+$ Du145 Cell	PKH26⁻ HUVECs
Mean Percentage	58	40	75	21
SD of Mean Percentage	17	17	19	19
Number in Sample	108	105	93	106
24 Hours of Co-culture				
	Attached Population		**Unattached Population**	
	PKH26$^+$ Du145 Cell	PKH26⁻ HUVECs	PKH26$^+$ Du145 Cell	PKH26⁻ HUVECs
Mean Percentage	79	19	47	47
SD of Mean Percentage	11	10	20	18
Number in Sample	102	108	104	104

Table 5.5 The Distribution Of Du145 Prostatic Adenocarcinoma Cells And Human Umbilical Vein Endothelial Cells In The Attached And Unattached Cell Suspensions Generated By Direct Contact Co-cultures. PHK26$^+$ Du145 cells were incubated in direct contact with confluent monolayers of human umbilical vein endothelial cells (HUVECs) for either 1 or 24 hours. Unattached cells were collected by aspiration and attached cells by trypsinisation from the tissue culture plate. All cells were analysed on a FACScan, as described in the text.

The ratio of attached PC3 / Du145 cells to attached HUVECs after co-culture for 1 hour was approximately 2:1. However, following a 24 hour co-culture the ratios of attached PC3 cells:attached HUVECs and Du145 cells:attached HUVECs were now approximately 8:1 and 4:1, respectively. This suggests that both PC3 and Du145 cells continued to attach to the HUVECs after 1 hour of co-culture. The ratio of unattached PC3 cells:unattached HUVECs and unattached Du145 cells:unattached HUVECs after 1 hour of co-culture were 5:2 and 7:2, respectively. These ratios changed following 24 hours of co-culture to 5:1 and 1:1 for PC3 and Du145 cells, respectively. This relative increase in HUVECs and decrease in PC3 / Du145 cells in the unattached cell suspension following pro-longed co-culture indicates that HUVECs are detaching from the tissue culture plate. This effect was more striking when Du145 cells were co-cultured with HUVECs. One could postulate that as more Du145 cells adhere to the culture plate, more HUVECs detach from the plate; i.e. that the interaction of Du145 cells with the HUVECs may have induced the detachment of HUVECs from the culture plate. The detachment of HUVECs from the culture plate was paralleled by a decrease in their surface CD44 and α5 expression (Table 5.2).

To summarise, prolonged co-culture of PC3 and Du145 cells with HUVECs induced detachment of HUVECs from the culture plate. This effect was more pronounced when cells were co-cultured with Du145 cells.

5.6 Summary Of *In Vitro* Manipulations Of Prostate Cancer Cell Lines

To summarise, neither GM-CSF, nor HUVEC-conditioned medium altered the cell surface level of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, or $\beta 1$ CAM expression by either PC3 or Du145 prostatic carcinoma cell lines. Conditioned medium from PC3 and Du145 cell cultures did not induce expression of E-selectin by HUVECs, a marker of their activation.

Direct contact of Du145 prostate cancer cells with HUVECs for 1 hour induced no alterations in the expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, or αL by HUVECs or Du145 cells. Nor did co-culture of PC3 cells with HUVECs for 1 hour induce changes in the HUVEC expression of the aforementioned CAMs. However, PC3-HUVEC co-culture for one hour did induce increases in the cell surface expression of CD44 by PC3 cells.

Prolonged co-culture (for 24 hours) of Du145 cells with HUVECs induced increased cell surface ICAM-1 by Du145 cells and reduced levels of surface CD44 by HUVECs. The co-culture of PC3 cells with HUVECs increased cell surface expression of $\alpha 5$ by PC3 cells, but no changes in HUVEC CAM expression. These changes in CAM expression differed to those seen after a one hour co-culture, when only increased CD44 expression was seen on PC3 cells.

Du145 cells that adhered to HUVECs after a 1 hour co-culture and were then re-cultured for 24 hours in the presence of the HUVECs to which they had initially attached, expressed greater cell surface levels of ICAM-1 than unmanipulated cells: these re-cultured HUVECs expressed increased levels of ICAM-1 also and decreased levels of CD44 and $\alpha 5$ than unmanipulated HUVECs. No changes in CAM expression were observed when PC3 cells and HUVECs were similarly re-cultured. Such prolonged co-culture of PC3 and Du145 cells with HUVECs induced detachment of HUVECs from the culture plate. This effect was more pronounced when cells were co-cultured with Du145 cells.

Chapter 6

Discussion

Contents

6.1 Introduction

Cell adhesion molecules (CAMs) regulate cell – cell interactions by binding cell surface ligands and cell – extracellular matrix (ECM) interactions by binding proteins of the ECM. Ligation of CAMs can activate intracellular signaling pathways that result in gene expression and subsequent differentiation and / or proliferation of cells. Therefore, CAMs play a pivotal role in the organisation of cells in differentiated organs, in embryonic development and haematopoiesis. There are five major CAM families; namely, the cadherins, the cartilage link protein family (CD44), the immunoglobulin superfamily, the integrins, and the selectins.

The cadherins, which form homodimeric complexes, are considered one of the most important groups of CAMs that participates in the formation of cell-cell associations. Mice with genetic deletion of Neural-cadherin die in mid-embryogenesis with heart malformations (Albelda, 1993). The cytoplasmic domain of cadherins is intimately associated with the cytoplasmic catenin family of proteins and the actin-based microfilament network.

CD44 was first described as a lymphocyte homing receptor and thought to play a pivotal role in leucocyte trafficking around the body (Jalkenen *et al*, 1987). CD44 is now known to participate in lymphocyte activation (including the up-regulation of other CAMs) and differentiation, inflammation, tissue regeneration and pattern formation in embryogenesis (Denning *et al*, 1989, Koopman *et al*, 1990, Stauder and Gunthert, 1995). The major ligand for CD44 is the ECM protein hyaluronan (or hyaluronate), but CD44 can also bind fibronectin, laminin and type IV collagen (Knudson *et al*, 1993, Jalkenen and Jalkenen, 1992, Ishii *et al*, 1993).

Most members of the immunoglobulin superfamily of CAMs are involved in cell – cell interactions. The most well known members of this family are the Intracellular Cell Adhesion Molecules (ICAMs), the Vascular Cell Adhesion Molecules (VCAMs), and the Neural Cell Adhesion Molecules (NCAMs). Most of these molecules are immune regulators and mediate a wide range of leucocyte interactions including leucocyte extravasation (Springer, 1990).

The integrins are a family of heterodimeric CAMs composed of α and β subunits (Fawcett, 1992). Most integrins bind ECM proteins, including fibronectin, collagen and vitronectin. Some integrins bind cell surface ligands. For example, Lymphocyte Function-associated Antigen-1 (LFA-1), or $\alpha L \beta 2$, binds ICAM-1 and Very Late Antigen-4 (VLA-4), or $\alpha 4 \beta 1$, binds VCAM-1 (Springer, 1990). Many of the interactions of integrins with ECM proteins is thought to involve a tri-peptide motif on the proteins, Arg-Gly-Asp, known as the RGD sequence based on the biochemical abbreviations of the three amino acids, detailed in Appendix 8 (Dedhar *et al*, 1987)

The selectins are a family of highly homologous glycoproteins whose physiological function appears to be uniquely restricted to the vascular system, where they mediate the

interactions of white blood cells with the endothelial cells (Pignatelli and Vessy, 1994). The prototype ligand for Endothelial-selectin (E-selectin) and Platelet-selectin (P-selectin) is the tetrasaccharide sialyl Lewis x antigen (sLe^x) or CD155. Other ligands include sLe^a, and CD34 (Rosen and Bertozzi, 1994).

As mentioned briefly above and in detail in Chapter 1.3.6, many of these CAMs participate in the multi-step process of leucocyte extravasation. Leucocytes interact loosely with the endothelial cells, 'rolling' along the vessel wall. L-selectin presents leucocyte carbohydrate ligands such as the sLe^x antigen to endothelial E- and P-selectin, mediating this temporary rolling along the venule wall (Picker, 1992). CD44 and CD31 (Platelet Endothelial Cell Adhesion Molecule, PECAM-1) have been implicated in this primary adhesion (Butcher, 1991, Degrendele *et al*, 1996, Estess *et al*, 1999). Activation of leucocytes induces L-selectin shedding and the functional expression of several integrin CAMs, including VLA-4 and LFA-1, which mediate stabilised leucocyte binding to endothelial cells via their endothelial counterreceptors VCAM-1 and ICAM-1. Many other CAMs have been implicated in stabilised binding, including ICAM-2, ICAM-3, CLA (Cutaneous Lymphocyte Antigen), VAP-1 (Vascular Adhesion Protein-1), LFA-2, CD2, CD48, CD58, and CD59. ICAM-1 and LFA-1, independent of the activation state of endothelial cells, mediate the transendothelial migration of T cells. Binding of CAMs may induce signals that regulate transendothelial migration. LFA-1 itself can transmit a stimulatory signal to T cells that results in enhanced activation (Oppenheimer-Marks *et al*, 1990).

Cancer is defined clinically as the breakdown of tissue organisation and the acquisition of invasiveness and as such is a complex cascade of events. One of the prominent morphological changes in malignant adenocarcinomas is a loosening of intercellular adhesion. This is a consequence of a functional disturbance of the cell-cell contacts described above. In particular, the metastatic progression of carcinomas involves the escape of the tumour cells from the primary deposit, invasion through the basement membrane and extracellular matrix, gaining access to the lymphatics and / or vasculature, extravasation at distant sites, and invasion through the basement membrane of the site of metastatic deposit. With the knowledge that CAMs are crucially responsible for maintaining intercellular adhesion, along with their role in leucocyte extravasation, their role in the progression of metastatic prostatic carcinoma becomes an obvious line of investigation

It is the hypothesis of this study that invasive prostate cancer cells employ cell adhesion molecules to facilitate their progression. Prostate cancer is the most commonly diagnosed cancer in men. Although it has been said that most men will die with, rather than of, their (prostatic) cancer, most patients who do die from their cancer have bone metastases (Waltregny and Castronovo, 1996).

6.2 Establishment Of An *In Vitro* Co-culture System of HUVECs and Prostatic Cancer Cell Lines

During this study, a co-culture system was established where Human Umbilical Vein Endothelial Cells (HUVECs) were grown in direct contact with PC3 and Du145 prostatic adenocarcinoma cells. The co-culture system was designed in order to investigate the expression of CAMs by both endothelial cells and prostatic adenocarcinoma cells when cultured in direct contact with each other. The level of expression was to be determined using a Fluorescence Activated Cell Scanning (FACScan) machine and fluorescently labelled antibodies. The FACScan machine can also distinguish two different cell populations if their size and granularity are different. However, endothelial and epithelial cells are of similar size and granularity: therefore, alternative mechanisms for their identification were needed.

As mentioned above, the FACScan measures fluorescent emission and therefore, it was hypothesised that the two cell populations could be distinguished with antibodies against different cell surface markers that were conjugated to fluorophores. To this effect, a mouse anti-human CD31 (PECAM-1) monoclonal antibody conjugated to phycoerythrin (CD31-PE) was used to fluorescently label endothelial cells. PE has two excitation wavelengths of 564nm and 495nm. The fluorescent light emitted by excited PE is measured by the FL2 detector of the FACScan. In the initial experiments of Chapter 3.2.1, endothelial and epithelial cells were incubated with CD31-PE. When analysed on the FACScan, only the endothelial cells demonstrated detectable levels of fluorescence. When these two cell populations were mixed together, the CD31-PE$^+$ endothelial cells could be distinguished from the CD31-PE$^-$ epithelial cells (Figure 3.2.1.2). Endothelial and epithelial cells were then incubated with a mouse anti-human monoclonal antibody against the CAM, CD44, which was then linked to goat anti-mouse immunoglobulins conjugated to Fluorescein Isothiocyante (FITC), followed by the CD31-PE monoclonal antibody. The FL1 detector of the FACScan detects the fluorescent light emitted by FITC. Therefore, the fluorescence emitted by FITC and PE are measured on two different detectors within the FACScan, as described in detail in Chapter 3. Therefore, fluorescent light due to PE excitation should not interfere electronically with that due to FITC excitation. Indeed, the level of FITC-derived fluorescence detected on CD44$^+$ CD31-PE$^-$ epithelial cells was not affected by the presence of PE-derived fluorescence of CD31$^+$ endothelial cells, the majority of which were also CD44$^+$. Therefore, fluorescent labelling of endothelial cells via the cell surface molecule CD31 was an effective method of distinguishing endothelial cells from epithelial cells in a mixed cell population (Figures 3.2.1.3 and 3.2.1.4).

A second fluorophore that was used in this study to differentiate endothelial cells from epithelial cells was Acridine Orange (AO). As described in Chapter 3, AO is excited by the LASER of the FACScan and emits fluorescent light in the same wavelength of PE. Therefore, in

theory, AO could be used simultaneously with FITC in a similar manner as PE was above. Epithelial cells labelled with AO emitted detectable levels of fluorescence when excited by the FACScan LASER. Furthermore, these AO$^+$ epithelial cells could be distinguished from the AO$^-$ endothelial cells (Figures 3.3.1 and 3.3.2).

Having established that a mixed populations of endothelial and epithelial cells could be distinguished with both CD31-PE and Acridine Orange, co-cultures were established with HUVECs and prostatic adenocarcinoma cells lines, PC3 and Du145 and the lung adenocarcinoma cell line, A549. Varying concentrations of AO-stained Du145 cells were incubated in direct contact with confluent monolayers of HUVECs for one hour under standard tissue culture conditions. AO$^+$ Du145 cells could be distinguished from AO$^-$ HUVECs, but only when large numbers of Du145 cells were co-cultured with HUVECs. Conversely, when lower numbers of AO$^+$ Du145 cells were co-cultured with confluent monolayers of AO$^-$ HUVECs, the two cell populations could not be differentiated in terms of the amount of fluorescence detected by the FACScan. Indeed, the peak level of fluorescence emitted upon excitation of AO$^+$ Du145 cells appears to be lower when fewer Du145 cells are added to the co-cultures (Figure 3.3.3). These findings were unexpected. When the FACScan analyses a cell suspension, it regards each cell as a single event; i.e. the level of fluorescence emitted by each cell in any one sample is measured. In the above experiments the FACScan measured 2000 events in each co-culture sample. The FACScan calculates the median of these 2000 events or measurements. This value is referred to as the median level of fluorescence. Therefore, all AO$^+$ Du145 cell populations in these experiments should have the same median level of fluorescence. However, the Du145 cells from more concentrated populations have higher median levels of fluorescence and those from less concentrated populations have lower median levels of fluorescence. For these experiments, Du145 cells were stained with AO before the serial dilutions were prepared. Therefore, the level of AO appears to have been serially diluted with the dilution of the cell number. It could be hypothesised that as the Du145 cells bind to the HUVECs the AO is released through the desmosomes and tight junctions from the Du145 cell to the HUVEC. This is unlikely for three reasons. Firstly, no corresponding increase was seen in the number of AO$^+$ cells / events, suggesting that there was no corresponding increase in the fluorescence emitted by the endothelial cells. Indeed, the total number of AO$^+$ cells in the suspension was also lower. Secondly, the incubation time of the co-culture was one hour. Therefore, there was insufficient time for the formation of tight junctions between cells; probably only antigen / receptor – antibody / ligand complexes would have been formed. Thirdly, the same phenomenon was observed in AO$^+$ Du145 cells that were unattached to the HUVECs. More reasonably, it is postulated that the AO leaked out from the cytoplasm of the Du145 cells into the surrounding fluid. Subsequently, as the concentration of Du145 cells was diluted, the level of AO was also diluted. To improve the use of AO in this co-culture system each dilution of cells should be

incubated individually with AO. However, the AO would still be capable of leaking out across the plasma membrane.

Co-cultures with varying concentrations of unstained Du145 cells and A549 cells were established with HUVECs in a similar manner to those with AO-stained Du145 cells above. These co-cultures were incubated for one hour under standard tissue culture conditions. The resulting cell populations were treated with mouse anti-human antibodies against CD44, goat anti-mouse immunoglobulin conjugated to FITC and CD31-PE. A control population of co-cultured cells was treated with goat anti-mouse FITC-conjugated immunoglobulins only. Not only could the CD31$^+$ HUVECs be differentiated from the CD31$^-$ Du145 / A549 cells, but the level of CD44 present on both cell populations could be identified by the level of FITC-induced fluorescence (Figure 3.2.1.4). Therefore, unlike AO, PE-conjugated antibody against endothelial cell CD31 is a reliable tool for distinguishing HUVECs from epithelial cells. However, it needs to be remembered that the hypothesis of this thesis is that CAMs play a crucial role in the progression of prostatic adenocarcinoma. CD31 is also known as Platelet Endothelial Cell Adhesion Molecule-1. PECAM-1 is a member of the immunoglobulin superfamily of CAMs. It is conceivable that endothelial PECAM-1 expression may be altered during co-culture with adenocarcinoma cells. It is also conceivable, more importantly, that PECAM-1 expression may be induced upon the prostatic adenocarcinoma cells, PC3 and Du145, upon co-culture with vascular endothelial cells. Therefore, the two cell populations would no longer be distinguishable. More importantly, if induction of PECAM-1 was not consistent on all cells, it may not be acknowledged. Consequently, cells would be classified as PECAM-1$^+$ HUVECs, but actually be PECAM-1$^+$ adenocarcinoma cells. Therefore, PE-conjugated CD31 as a marker for endothelial cells in a co-culture system of HUVECs with prostatic adenocarcinoma cells was not considered a viable system.

While AO and PE-CD31 were not considered to be useful tools for a co-culture system of HUVECs with PC3 and Du145 cells, they were useful for the semi-quantitative analysis of epithelial cell adherence to endothelial cells. Both fluorophores demonstrated maximal saturation of confluent monolayers of HUVECs, pre-plated in 24-well Tissue Culture Grade Plates (TCGPs), by the addition of $4 - 5 \times 10^4$ epithelial cells / well (Figures 3.2.2.1 and 3.3.4). When 5 $\times 10^4$ Du145 cells are co-cultured with a confluent monolayer of HUVECs for 1 hour, 70% of the attached cells are Du145 cells and 30% of the cells are HUVECs. Therefore, maximum saturation of the HUVECs by Du145 cells occurs at a ratio of approximately 2 Du145 cells:1 HUVEC. Endothelial and epithelial cells are of a similar size, as discussed above. One would expect adhesion to occur on a one to one basis. However, adhesion appears to occur at an approximate ratio of 2 Du145 cells:1 HUVEC. It could be argued that HUVECs lose their expression of PECAM upon co-culture with Du145 cells. This theory would explain a ratio of 2 Du145 cells:1 HUVEC, in that cells which appear to be CD31-PE$^-$ and therefore considered to be Du145 cells

are actually HUVECs that have lost their cell surface expression of CD31. However, it may be possible for more than one Du145 cell to adhere to any one HUVEC.

Acridine Orange and CD31-PE were not considered useful tools for the differentiation of endothelial and epithelial cells in the co-culture system used in this study. PKH26-GL (PKH26) is a fluorescent cell marker that is incorporated into the lipid bilayer of the cytoplasmic membrane. Patented technology renders this molecule insoluble when it taken up by the membrane. PKH26 has an excitation wavelength that should be excited by the LASER of the FACScan. Excited PKH26 emits light that can be measured by the FL2 detector of the FASCcan. PKH26 should remain with the cell membrane and not leak out of the cell as AO did. PKH26 does not bind to any cell surface molecules and therefore could be more useful than CD31-PE.

Du145 cells were incubated with varying concentrations of PKH26, as described in Chapter 3.4. When cells were incubated with PKH26 at a concentration of $5 \times 10^{-6}M$, stained cells were clearly distinguishable from those that were incubated with medium alone (i.e. in the absence of PKH26). This was also true when stained and non-stained cells were cultured for a further three days, under standard tissue culture conditions (Figure 3.4.2). Therefore PKH26 is not only taken up permanently into the cell, but it also appears to be transferred onto daughter cells. This provides a great advantage over the cell leakage seen with Acridine Orange. This suggests that PKH26 may be useful as a long term cell tracker.

The fluorescent light emitted by excited PKH26 does not electronically interfere with that of FITC. Nor does the presence of PKH26 in the cell membrane interfere with the interaction of monoclonal antibodies and their antigenic cell surface epitopes (Figure 3.4.2). PKH26-stained and non-stained Du145 cells were incubated with mouse anti-human monoclonal antibodies against CD44 and goat anti-mouse immunoglobulin conjugated to FITC. The level of fluorescent light emitted upon the excitation of FITC, a representation of the level of CD44 expression on the cell surface, was the same for both PKH26$^+$ Du145 cells and PKH26$^-$ Du145 cells. When PKH26$^+$ Du145 cells were co-cultured with confluent monolayers of PKH26$^-$ A549 epithelial cells for one or 24 hours, the two cell populations were distinguishable by FACScan analysis as a result of different levels of FL2 fluorescence. The level of cell surface CD44 on both cell types could be measured as above(Figures 3.4.3 and 3.4.4).

The fluorescent dye PKH26 can be used to label cells fluorescently, emitting light when excited by a LASER that can be quantified by the FL2 detector of a FACScan machine. Cells can be labelled and subjected to further manipulations over a period of days: the fluorescent label is not only maintained within the plasma membrane of these cells, but it is also transported into that of daughter cells. While it is present in these daughter cells at a lower intensity than that seen in the progenitor cells, as demonstrated by lower median levels of fluorescence, PKH26-stained and PKH26-non stained cells can still be distinguished. Therefore, PKH26 labelling is preferable to Acridine Orange labelling, where the stain can leak out from the cells over a relatively short

period of time. Labelling cells with PKH26 does not rely on the expression of any cell surface molecules as CD31-PE labelling of endothelial cells does. Therefore, changes in surface expression of molecules, such as the CAMs, will not interfere with the effectiveness of PKH26 as a fluorescent cell marker. The detection of CAM surface expression is not affected by the presence of PKH26. No electronic interference occurs between fluorescent light emitted by excited PKH26 and that by excited FITC (used to detect monoclonal antibodies that recognise cell surface CAMs). The interaction of the monoclonal antibody with its epitope on the cell surface is not affected by the presence of PKH26 within the cell membrane.

In conclusion, while Acridine Orange and CD31-PE proved to be poor tools for the identification of endothelial or epithelial cells in a mixed suspension of the two cell types, PKH26 is a very useful tool. PKH26 can be used within a co-culture system to identify one of two cell types.

6.3 The Influence Of GM-CSF On The Expression Of Cell Adhesion Molecules By Prostatic Cancer Cell Lines

The major site of prostatic carcinoma metastatic deposits is the bone and bone marrow. Indeed, 70% of patients with cancer of the prostate will develop bony metastases (Haq *et al*, 1992). The bone marrow is the main site of haematopoietic growth factor (HGF) production, including the colony stimulating factor (CSF) family (Nicola, 1989).

Granulocyte Macrophage – CSF (GM-CSF) was first identified by its effect on neutrophil migration (Yong *et al*, 1993). GM-CSF has since been shown to influence the expression of many CAMs by many different cells, including neutrophils, differentiating myeloid cells and blood monocytes (Yong and Linch, 1993, Bendall *et al*, 1995, Bernasconi *et al*, 1995). Prostatic carcinoma cell lines, PC3 and Du145, secrete GM-CSF. The addition of exogenous GM-CSF to cultures of PC3 and Du145 cells promotes their growth *in vitro* (Lang *et al*, 1994).

Neutrophils and leucocytes employ different CAMs during extravasation into the circulation. However, three pieces of evidence suggest that GM-CSF may have an effect of the expression of CAMs by prostatic carcinoma cell lines and therefore, effect the metastatic progression of prostate cancer. Firstly, GM-CSF is actively secreted by PC3 and Du145 cells. Secondly, GM-CSF manipulates the CAM expression by white blood cells. Thirdly, GM-CSF is present in the bone marrow. Therefore, it was hypothesised that GM-CSF may have effects on the expression of CAMs by prostatic carcinoma cells and thereby controls their invasive and migratory phenotype.

This study hypothesis that the process of prostate tumour cell metastasis can be paralleled with that of leucocyte extravasation. The process of leucocyte extravasation is a three-step process. The CAMs involved in this process include CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL and $\beta 1$. Therefore, Du145 and PC3 cells were treated with varying concentration of GM-CSF for 2, 4, 8, 12, and 24 hours under standard tissue culture conditions. Two questions were asked. Firstly, is GM-CSF involved in the process of tumour cell intravasation and / or extravasation and invasion through the bone marrow extracellular matrix (ECM)? The process of leucocyte extravasation is a relatively quick event. Therefore, to answer this question PC3 and Du145 cells were treated with GM-CSF for relatively short periods of time – two or four hours. Secondly, if GM-CSF is present in the bone marrow and effects the growth patterns of PC3 and Du145 cells, what role do CAMs play here? For example, signaling through CAMs have been shown to promote the growth of many carcinoma cells (Zutter *et al*, 1993, Miyake *et al*, 1998, Petitclerc *et al*, 1999, Schroder *et al*, 1999, Wang *et al*, 1999). Therefore, cells were treated with GM-CSF for relatively long periods – 8, 12 and 24 hours. Following each period of treatment, cells were incubated with monoclonal antibodies against the relevant molecule and linked to immunoglobulins conjugated

to FITC and the FACScan detected the level of fluorescent light emitted. The level of fluorescent light detected represents the level of surface expression of a particular CAM.

Treatment of PC3 and Du145 cells with GM-CSF did not alter their expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, or $\beta 1$. This was true when cells were incubated with GM-CSF for 2, 4, 8, 12, and 24 hours. It could be argued that the concentration of GM-CSF added to the cultures was too low to influence the expression of CAMs. Indeed, the increase in neutrophil adhesion to vascular endothelial cells demonstrated by Yong and Linch (1993) employed concentrations of GM-CSF in excess of those used in this study. However, Yong and Linch agree that the concentrations of GM-CSF used in their study were in excess of physiological levels. The EC_{50} (i.e. the dose that produces a response that is half of the maximum response) of the GM-CSF and the concentration range used in the experiments of this study ranged from 0.02 to 0.2 ng/ml and zero to 1.0ng/ml, respectively. GM-CSF is secreted by Du145 and PC3 cells; thus the actual concentration of GM-CSF in the culture medium will be greater than that added exogenously. Therefore, the maximum concentration of GM-CSF used in the experiments discussed above is more than double that of the EC_{50}.

It is the nature of carcinoma cells to mutate. Indeed PC3 and Du145 cells do not express the androgen receptor found on non-malignant prostatic epithelial cells. These two cell lines do not express or secrete Prostate Specific Antigen (PSA) or Prostatic Acid Phosphatase (PAP). Therefore, it could be argued that the clones of PC3 and Du145 used in this study have lost their cell surface expression of the GM-CSF receptor. However, GM-CSF is secreted by PC3 and Du145 cells and it promotes their growth when exogenously added. GM-CSF is also present in the bone marrow, the major site of prostate cancer metastases. Therefore, it is unlikely that the expression of a receptor for such a tumour cell growth promoter would be lost.

GM-CSF may act on endothelial cells or bone marrow stromal cells to promote up-regulation or activation of certain CAMs that could then interact with constitutively expressed CAMs of PC3 and Du145 cells. GM-CSF may also act in conjunction with other cytokines present in the bone marrow or at sites of intravasation and / or extravasation, but that were absent in the experiments described above. Therefore, while GM-CSF does not alter the expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, and $\beta 1$ by PC3 and Du145 prostatic carcinoma cells, a role in the progression of prostatic carcinoma should not be excluded.

6.4 The Influence Of Vascular Endothelial Cells On The Expression Of Cell Adhesion Molecules By Prostate Cancer Cell Lines

If the process of prostatic tumour cell metastasis is paralleled with that of leucocyte extravasation, the effect of the vascular endothelial cell must be considered. The vascular endothelial cell is rich in chemo-attractant and activating signals that influence the expression and activation of CAMs by the leucocyte. Therefore, it was postulated that the expression of CAMs by PC3 and Du145 prostate cancer cells might be influenced by vascular endothelial cell secretory factors.

Human Umbilical Vein Endothelial Cells (HUVECs) were used as a source of vascular endothelial cells. The supernatant of confluent cultures was removed and prepared as described in Chapter 5.3. Treatment of PC3 and Du145 cells with this HUVEC-conditioned medium (HUVEC-CM) for two or four hours, under standard tissue culture conditions, did not alter the expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL or $\beta 1$ by either cell line. Although the interaction of leucocytes with the endothelial cells in the process of extravasation occurs over a relatively short period of time, the PC3 and Du145 cells were further incubated with HUVEC-CM for 12 and 24 hours. The interaction of migratory tumour cells with vascular endothelial cells is not physiologically normal: while parallels are drawn between this process and that of leucocyte extravasation, the two processes may not be identical. However, incubation of PC3 and Du145 cells with HUVEC-CM for 12 and 24 hours did not alter their expression of CD44, ICAM, VCAM-1, $\alpha 4$, $\alpha 5$, αL, or $\beta 1$. Therefore, HUVECs do not secrete compounds that induce or up-regulate the expression of these CAMs by prostatic carcinoma cells. These data suggest that the initial interaction of prostatic tumour cells is not influenced by secretory factors produced by the endothelial cells. Initial cellular connections may occur between constitutively expressed CAMs on the prostate cancer cells. Alternatively, the secretory factors of the prostate cancer cell may influence the expression or activation of CAMs upon the endothelial cells (see below). A regulatory feedback loop may exist, where the prostatic carcinoma cell produces secretory factors that influence the CAM expression and / or function of the endothelial cells. Activation of certain CAMs on the endothelial cell induces intracellular signaling through second messenger systems, including the activation of Focal Adhesion Kinase (FAK), which in turn can induce transcription and secretion of many soluble factors, one of which may influence the expression of CAMs by the prostate carcinoma cell itself.

HUVECs are endothelial cells derived from the umbilical vein. It could be argued that foetal vascular endothelial cells are not the same as adult vascular endothelial cells and as such are not an appropriate tool for the investigation of prostate cancer metastases development.

Various immortal endothelial cell lines are available, which have been established from adult endothelium. However, immortalisation of cell lines usually involves the transformation of some cellular characteristics. Most immortal cell lines established from prostatic carcinomas have been transfected with the SV40 antigen, for example. Therefore, the use of a primary cell line, is more representative of the *in vivo* parallels that are being made in this study. Access to adult vascular endothelial cells is very difficult. Since access to human umbilical cords is relatively easy, HUVECs have been accepted as a suitable model for investigating the activity of human endothelial cells *in vitro*. HUVECs have been shown to lose their surface expression of endothelial cell markers over time, such as von Willebrand Factor and clotting Factor VIII. To avoid such issues, the HUVECs used in this study were of low passage number.

In the model of leucocyte extravasation, after endothelial cells have attracted leucocytes to sites of extravasation, the two cells make contact and molecular interactions occur via the CAMs expressed by both cells. This initial interaction then up-regulates the expression of and / or activates the surface CAMs by both cell types. Perhaps in the model of tumour cell migration and invasion, direct cell to cell contact is required for changes in CAM expression by either cell type. Therefore, attempts were made to mimic the *in vivo* rolling of leucocytes along the vascular wall. Chapter 3 describes the establishment of a co-culture system and this is discussed above (Chapter 6.2). This system enables the culture of endothelial and epithelial cells in direct contact with each other and the subsequent analysis of CAM expression by each cell type. Briefly, Du145 and PC3 prostate cancer cells were fluorescently labelled with the dye PKH26. These cells were incubated with confluent monolayers of HUVECs for one hour, under standard tissue culture conditions. Unattached cells were collected by careful aspiration and attached cells were collected by trypsinisation. The surface expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, and αL were measured with monoclonal antibodies against the relevant molecule and linked to immunoglobulins conjugated to FITC. The FACScan detected the level of fluorescent light emitted by FITC.

Unmanipulated Du145 cells express relatively high levels of ICAM-1, moderate levels of $\alpha 5$ and low levels of CD44. These Du145 cells do not express VCAM-1, $\alpha 4$ or αL. Unmanipulated PC3 cells express moderate levels of CD44 and ICAM-1, moderate to low levels of $\alpha 5$ and low levels of αL. PC3 cells do not express VCAM-1 or $\alpha 4$ (Table 5.1).

Co-culture of Du145 cells with HUVECs for 1 hour did not induce the expression of VCAM-1, $\alpha 4$ or αL, nor was the level of expression of CD44, ICAM-1 or $\alpha 5$ altered. Co-culture of PC3 cells with HUVECs for 1 hour did not induce the expression of VCAM-1 or $\alpha 4$, nor was the level of expression of ICAM-1, $\alpha 5$ or αL altered. However, co-cultured PC3 cells showed altered CD44 expression (Figure 5.5.1). PC3 cells that adhered to HUVECs during a one hour co-culture demonstrated lower levels of CD44 expression than control, unmanipulated PC3 cells. PC3 cells that remained unattached from HUVECs after the same one hour co-culture showed

higher levels of CD44 than both the unmanipulated and attached PC3 cells. The level of CD44 expressed by the unattached PC3 cells was not significantly greater than the unmanipulated PC3 cells, but was significantly greater than that of the manipulated, attached PC3 cells (compared by the Student's T-test, $p < 0.005$). These data support the theory discussed above that the initial interaction of prostatic carcinoma cells with vascular endothelial cells involves constitutively expressed CAMs. Unmanipulated PC3 cells express moderate levels of CD44. Upon attachment to endothelial cells the level of CD44 expressed by PC3 is reduced. However, those PC3 cells that remain unattached express higher levels of CD44. It could be postulated that CD44 is utilised in the initial attachment of PC3 cells to HUVECs and that those cells attempting to attach demonstrate a rapid upregulated expression. Once attached the expression of CD44 is rapidly down-regulated, as it is no longer required. As detailed in Chapter 1.3.6, it is thought that CD44 is involved in the initial rolling of leucocytes long the venule wall. Therefore, these data support the theory that the metastatic spread of prostate cancer may be paralleled with leucocyte extravasation.

The fact that a one hour co-culture of Du145 cells with HUVECs did not influence the expression of CD44, ICAM-1 or α5 CAMs does not exclude these CAMs from being involved in the initial attachment of these cells to each other. Any of these three CAMs may be involved in the adherence of Du145 cells to HUVECs. Likewise, ICAM-1, α5 and αL expressed on the PC3 cells may also be involved in the initial attachment of the cells to HUVECs. The data above do not prove or disprove a role for CD44, ICAM-1 and α5 or ICAM-1, α5 and αL in the initial attachment of prostatic cancer cells, PC3 or Du145, respectively to HUVECs. The data presented here does support the role of CD44 in the initial attachment of PC3 cells to HUVECs.

The second stage of leucocyte extravasation involves stabilised binding of the leucocyte to the endothelium. This process involves the upregulation of several CAMs by both the leucocyte and the endothelial cells and therefore, occurs over a longer time than the initial rolling. To mimic this process *in vitro*, Du145 and PC3 cells were co-cultured with HUVECs for 24 hours, as described in Chapter 5.5.1. Briefly, Du145 and PC3 cells were fluorescently labelled with the dye PKH26. Labelled cells were incubated in direct contact with confluent monolayers of HUVECs for 24 hours. Unattached cells were collected by aspiration and attached cells were collected by trypsinisation. The surface expression of CD44, ICAM-1, VCAM-1, α4, α5, and αL were measured with monoclonal antibodies against the relevant molecule and linked to immunoglobulins conjugated to FITC. The FACScan detected the level of fluorescent light emitted by FITC.

Co-culture of Du145 cells with HUVECs for 24 hours did not induce their expression of VCAM-1, α4 or αL. Furthermore, no significant changes in the expression of CD44 and α5 was demonstrated by Du145 cells after this period of co-culture. However, co-cultured Du145 cells, both those that attached and those that did not attach to HUVECs, expressed significantly higher

levels of surface ICAM-1 than unmanipulated Du145 cells ($p < 0.01$ and $p < 0.005$, for attached and unattached cells, respectively when compared by the Student's T-test). These data suggest that prostatic carcinoma cells may use their ICAM-1 to attach to vascular endothelial cells in the process of metastatic spread. While the involvement of ICAM-1 in tumour cell attachment to vascular endothelial cells highlights similarities with the process of stabilised binding of leucocytes to vascular endothelium, the two processes cannot be directly compared. ICAM-1 is involved in stabilised binding of leucocytes, but is expressed on the endothelial cell. Endothelial ICAM-1 binds to LFA-1($\alpha L\beta 2$) on the leucocyte. However, in this co-culture system of prostatic carcinoma cells and vascular endothelial cells, the ICAM-1 of the carcinoma cells is upregulated. It seems unlikely the expression of a CAM that is constitutively expressed at high levels on a cell would be increased further without being functionally active. Therefore, an important question that arises here is whether endothelial cells express $\alpha L\beta 2$ or whether their expression of this CAM can be induced by a co-culture with Du145 cells (see below).

Co-cultures of PC3 cells with HUVECs for 24 hours did not induce their expression of VCAM-1 or $\alpha 4$. Co-culture of these two cell types did not influence the expression of CD44, ICAM-1 or αL by the PC3 cells. However, co-cultured PC3 cells demonstrated higher surface expression of $\alpha 5$ than unmanipulated cells. While, the level of αL was higher upon both attached and unattached PC3 cells, only the increase seen in the unattached cells was to levels that were significantly higher than those of unmanipulated PC3 cells ($p < 0.05$, Students T-test). While these data suggest a role for $\alpha 5$ in the interaction of PC3 cells with HUVECs, they do not provide conclusive evidence to support a role for PC3 $\alpha 5$ in their attachment to HUVECs. The increase in $\alpha 5$ may act as CD44 after one hour of co-culture. For example, $\alpha 5$ may be involved in the initiation of stabilised binding of PC3 cells to HUVECs. When $\alpha 5$ on the PC3 cell interacts with its ligand on the HUVEC it may induce stabilised binding. Ligation of many of the integrins has been shown to trigger second messenger systems that induce downstream effects. One of these effects may be the upregulation of other CAMs involved in stabilised binding of the PC3 cells to HUVECs, but not investigated in this study. Upon upregulated and / or induced expression of these CAMs, the upregulated expression of $\alpha 5$ becomes redundant and therefore, returns to normal levels. Therefore, $\alpha 5$ expressed by PC3 cells may play a role in the adhesion of these prostatic carcinoma cells to vascular endothelial cells, HUVECs. Therefore, $\alpha 5$ may promote the metastatic spread of prostate cancer.

As discussed previously, there are three distinct steps in the *in vivo* process of leucocyte extravasation. The first step, where leucocytes roll along the vessel wall under the influence of chemo-attractants, is represented in the experiments detailed here by a one hour co-culture of PC3 and Du145 cells with HUVECs. The second step, where leucocytes become activated and form stable complexes with the vascular endothelium, occurs over a longer period of time: this is represented in the experiments described above by a 24 hour co-culture of prostatic carcinoma

cells with vascular endothelial cells. However, *in vivo* leucocytes that are not loosely attached and therefore do not receive stimulatory signals are swept away from the endothelial cell surfaces by the flow of blood. Therefore, the stationary co-culture of prostatic adenocarcinoma cells with HUVECs for 24 hours is not truly representative of the *in vivo* parallels that are being drawn in this thesis. Therefore, experiments were designed to remove those Du145 and PC3 cells that were unattached following one hour of co-culture, as described in Chapter 5.5.1. Briefly, PKH26$^+$ PC3 and Du145 cells were co-cultured with confluent monolayers of HUVECs in 24-well tissue culture grade plates (TCGPs) for one hour. The resulting unattached cells were removed by aspiration. Attached cells were collected by trypsinisation and transferred. Both cell populations were transferred to fresh TCGPs and re-cultured for 24 hours, under standard tissue culture conditions. All cell populations were then collected by trypsinisation. The surface expression of CD44, ICAM1-, VCAM-1, $\alpha 4$, $\alpha 5$, and αL were measured with monoclonal antibodies against the relevant molecule and linked to immunoglobulins conjugated to FITC. The FACScan detected the level of fluorescent light emitted by FITC.

A one hour co-culture of Du145 cells with HUVECs and re-culture for 24 hours in the absence of unattached Du145 cells did not induce the expression of VCAM-1, $\alpha 4$ or αL by the Du145 cells. No changes in the expression of CD44 and $\alpha 5$ were seen on the Du145 cells. These finding were true for both unattached and attached Du145 cells. However, this period of co-culture and re-culture of attached Du145 cells with HUVECs in the absence of unattached Du145 induced increased expression of ICAM-1 by the attached Du145 cells. This increase was to levels significantly greater than Du145 cells that were cultured and re-cultured in the absence of HUVECs ($p < 0.005$, Student's T-test). The level of ICAM-1 expressed by these co-cultured Du145 cells was similar to that expressed by attached Du145 cells that had been co-cultured with HUVECs continuously for 24 hours. These data provide strong evidence to support a role for ICAM-1 in the development of metastatic carcinoma, as discussed above. Specifically, it could be postulated that the interaction of invasive prostatic carcinoma cells with the vascular endothelium is mediated, in part, by ICAM-1. Do endothelial cells express a suitable ligand to support the functional expression of ICAM-1 by Du145 and PC3 cells (see below)?

Similar co-cultures of PC3 cells with HUVECs for one hour and subsequent re-culture for 24 hours in the absence of unattached PC3 cells did not induce the expression of VCAM-1 or $\alpha 4$ by the PC3 cells. This co-culture and re-culture did not influence the expression of CD44, ICAM-1 or αL by these manipulated PC3 cells. However, co-culture of PC3 with HUVECs for one hour and re-culture in the absence of unattached PC3 cells for 24 hours induced increased expression of $\alpha 5$ by both attached and unattached PC3 cells. However, neither of these increases were to levels that were significantly higher that those seen for PC3 cells that had been cultured for one hour and re-cultured for 24 hours in the absence of HUVECs. These data differ to those seen after a continuous co-culture of PC3 cells with HUVECs for 24 hours. PC3 cells that were both

attached and unattached to HUVECs after a continuous co-culture for 24 hours, demonstrated increased surface expression of $\alpha 5$. The increased expression seen on unattached PC3 cells was significantly higher than unmanipulated PC3 cells. These data suggest that PC3 cells that are exposed to HUVECs for a prolonged period, but that do not actually attach to the cells express increased levels of $\alpha 5$. When these unattached cells are removed from the HUVECs their increased expression of $\alpha 5$ is lost. Therefore, one could postulate that HUVECs provide a signal to PC3 cells that stimulated the upregulation of $\alpha 5$: upon removal from the HUVECs the stimulatory signal is lost and the expression of $\alpha 5$ returns to normal levels. Alternatively, upon the attachment of PC3 cells to the HUVECs, the stimulatory signal is no longer required. The interaction of $\alpha 5$ on the PC3 cells with its ligand on the endothelial cells may turn off the activating signal, via the activation of Focal Adhesion Kinase (FAK) and other second messenger systems. Therefore, this data suggests a role for $\alpha 5$ in the interaction of PC3 cells with the vascular endothelium.

To summarise these data, co-culture of Du145 cells with HUVECs induced upregulation of ICAM-1 after 24 hours. This increase remained when unattached Du145 cells were removed from the culture. Therefore, ICAM-1 may be involved in the metastatic spread of cancer of the prostate to the brain. Co-culture of PC3 cells with HUVECs induced an initial upregulation of CD44 by those cells that adhered to the HUVECs and a subsequent increase in $\alpha 5$ by those PC3 cells that were not attached to the HUVECs. These data suggest a role for CD44 and $\alpha 5$ in the development of bony metastases of prostatic carcinoma.

6.5 The Influence Of Prostatic Cancer Lines On The Expression Of Cell Adhesion Molecules By Vascular Endothelial Cells

The vascular endothelial cell is intimately involved in the control of leucocyte extravasation and therefore, possibly of tumour cell vascular dissemination. Indeed, it has been shown above that HUVECs can induce changes in the level of expression of CD44, α5 and ICAM-1 by prostatic carcinoma cell lines, PC3 and Du145. In the process of stabilised binding of leucocytes during their extravasation, endothelial cells express ICAM-1 and VCAM-1. Is the attachment of prostatic carcinoma cells to HUVECs mediated through the endothelial expression of these same molecules? More importantly, based on the data presented above, does the expression of the ligands for CD44, ICAM-1 and α5 increase on HUVECs when co-cultured in direct contact with prostate cancer cell lines, PC3 and Du145? Picker (1992) suggested that E-selectin is induced upon the endothelial cell at the early stages of extravasation and mediates the initial rolling of the leucocyte. The expression of E-selectin by endothelial cells is a sign of endothelial cell activation. Does the prostate cancer cell secrete products that induce the expression of E-selectin, and therefore activation, of vascular endothelial cell initiating the process of tumour cell extravasation?

To answer these questions conditioned medium was collected from confluent monolayers of PC3 and Du145 cells, as described in Chapter 2.1.4. Maximum activation of HUVECs, demonstrated by E-selectin expression is inducible by Interleukin-1 after four hours. Treatment of HUVECs with PC3- and Du145-conditioned medium did not induce their expression of E-selectin. Although the initial interaction of leucocytes and vascular endothelial cells is rapid, it could be argued that tumour cell – endothelial cell association is not physiologically normal and therefore, may not occur in an identical manner as leucocyte extravasation. Therefore, HUVECs were further treated with prostate cancer cell line conditioned medium for 8, 12, 24, and 48 hours. However, no induction of E-selectin could be demonstrated. Therefore, PC3 and Du145 cells do not secrete compounds that induce the expression of E-selectin, and therefore activation of vascular endothelial cells. These data suggest that E-selectin does not play a role in the initial loose adhesion of PC3 and Du145 cells to HUVECs. PC3 and Du145 cells may secrete factors that regulated the expression of other CAMs that have not been investigated here. Alternatively, as discussed above, initial cellular connections may occur between constitutively expressed CAMs on the endothelial cells. The advantages and disadvantages of using HUVECs, a foetal endothelial cell line, has been discussed previously.

As discussed previously, in Chapter 6.4, direct cell to cell contact is required for changes in CAM expression by PC3 and Du145 cells. Therefore, the expression of CAMs by the HUVECs co-cultured with PC3 and Du145 cells was examined. As a reminder, Du145 and PC3

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prostate cancer cells were fluorescently labelled with the dye PKH26. These cells were incubated with confluent monolayers of HUVECs for one hour, under standard tissue culture conditions. Unattached cells were collected by careful aspiration and attached cells were collected by trypsinisation. The surface expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, and αL were measured with monoclonal antibodies against the relevant molecule, linked to immunoglobulins conjugated to FITC. The FACScan detected the level of fluorescence light emitted by FITC. HUVECs could be distinguished from PC3 and Du145 cells by their lack of PKH26 stain and therefore, lack of FL2 fluorescent light.

Unmanipulated HUVECs express high levels of $\alpha 5$ and moderate levels of CD44. Unstimulated HUVECs do not normally express ICAM-1, VCAM-1, $\alpha 4$ or αL. The expression of CAM expression by HUVECs was not influenced by co-culture with PC3 cells. ICAM-1, VCAM-1, $\alpha 4$, and αL expression by HUVECs was not induced by co-culture with PC3 cells for one or 24 hours of co-culture or one hour of co-culture and 24 hours of re-culture in the absence of unattached PC3 cells. Likewise, there was no change in the level of expression of CD44 or $\alpha 5$ by HUVECs during co-culture with PC3 cells. These data suggest firstly, that PC3 cells attach to HUVECs via constitutively expressed CD44 or $\alpha 5$ on the HUVECs and that upregulation of these molecules is not required to maintain adhesion over a prolonged period. Secondly, PC3 cells may interact with other CAMs not examined in this thesis. Moreover, the interaction of PC3 cells with HUVECs may induce the upregulation of these CAMs to promote their attachment. However, these data do not suggest a role for ICAM-1, VCAM-1, $\alpha 4$, or αL in the attachment of PC3 cells to HUVECs.

When Du145 cells are co-cultured with HUVECs for one hour, no changes in HUVEC expression of CD44, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, or αL is observed. These data suggest that the initial attachment of Du145 cells to HUVECs must be either through constitutively expressed CD44 and / or $\alpha 5$ on the HUVECs, or through other CAMs not investigated in this study. However, following 24 hours of continuous co-culture with Du145 cells, HUVECs express lower levels of CD44 than unmanipulated HUVECs. This is also demonstrated when unattached Du145 cells are removed and the HUVECs are re-cultured with the attached Du145 cells for 24 hours. These data support the theory that the initial attachment of Du145 cells to HUVECs occurs through constitutively expressed CD44 on the HUVEC. This could induce up-regulation of other CAMs. The interaction of these up-regulated CAMs with their ligands could induce the down-regulation of CD44, as it is no longer required. When unattached Du145 cells are removed and the attached Du145 cells are re-cultured with HUVECs for 24 hours, HUVECs express significantly higher levels of ICAM-1 and significantly lower levels of $\alpha 5$ than unmanipulated HUVECs ($p < 0.001$ and $p < 0.05$, respectively, Student's T-test). Therefore, the removal of unattached cells from the co-cultures induces upregulation of ICAM-1 and down-regulation of $\alpha 5$ by the HUVECs.

Initial binding of Du145 cells to HUVECs may occur through constitutively expressed CD44 and $\alpha 5$ on the HUVECs. This binding may induce secretion of factors, by either the HUVECs or the Du145 cells that promote the down-regulation of CD44 by HUVECs. However, the mechanisms that regulate the expression of $\alpha 5$ may be different to those that regulate CD44 expression. Indeed, when HUVECs and attached Du145 cells are co-cultured in the absence or presence of unattached Du145 cells, their expression of CD44 is lower than that of unmanipulated HUVECs: however, co-cultured HUVECs express lower $\alpha 5$ than unmanipulated HUVECs only in the absence of unattached cells. Therefore, perhaps the unattached Du145 cells, which are not in direct contact with the HUVECs but may be under the influence of factors secreted by them, themselves secrete factors that maintain the expression of $\alpha 5$ by the HUVECs. One could postulate that the Du145 cells secrete factors that act upon the HUVECs, which in turn maintain the expression of $\alpha 5$. HUVECs treated with Du145-conditioned medium showed no changes in CAM expression. It was concluded that this medium had no effect on the CAM expression by the HUVECs; it could, however, act to maintain the expression of $\alpha 5$ in the presence of attached Du145 cells when unattached Du145 cells are also present. Therefore, in the absence of unattached cells, i.e. when all Du145 cells present in the co-culture are attached, the expression of $\alpha 5$ by the HUVECs, which is now no longer required to mediate the initial loose interaction between Du145 cells and HUVECs, is down-regulated. This theory would also explain why increased ICAM-1 expression by HUVECs is only seen in the absence of unattached Du145 cells. If the HUVECs are under the influence of secretory factors from unattached cells, i.e. they believe that the Du145 cells are still in the process of initial attachment, ICAM-1 expression is not upregulated. Again, perhaps the unattached cells themselves secrete factors that inhibit the up-regulation of ICAM-1. When unattached cells are removed from the co-culture and, therefore, all Du145 cells present are attached, the expression of ICAM-1 is upregulated. Activation and subsequent stabilised binding of leucocytes to vascular endothelial cells is thought to be mediated, in part, through endothelial ICAM-1. Therefore, these data support the theory that tumour cell metastasis can be paralleled with the process of leucocyte extravasation.

6.6 Intra-prostatic Invasion By Prostatic Epithelial Cells And Their Cell Adhesion Molecule Expression

The process of tumour metastasis is a complex cascade of events. The initial stage requires the tumour to grow and acquire an invasive phenotype. These events result in the release of neoplastic cells from the primary tumour. As discussed in Chapter 1, the loss of intercellular adhesion is a crucial step in the acquirement of a metastatic phenotype. Prostatic epithelial cells form into tightly packed glands. Tight junctions, adherens junctions and desmosomes maintain these tight intercellular contacts. E-cadherin and associated cytoplasmic proteins maintain the integrity of the adherens junctions. Dysfunction of the E-cadherin – cytoskeletal protein network is associated with the metastatic spread of many solid cancers, including prostatic adenocarcinoma. Loss of E-cadherin-mediated homotypic adhesion of prostatic epithelial cells can confer an invasive phenotype to these cells. These epithelial cells can migrate to the basal surfaces of the prostatic glands. At these basal surfaces, invasive tumour cells encounter the basement membrane, through which they must invade into the prostatic stroma or non-glandular compartment. The basement membrane contains many extracellular proteins, including fibronectin and laminin. Once in the prostatic stroma, the tumour cells must navigate to the vascular or lymphatic vessels, which they must invade through into the vascular or lymphatic circulation. These tumour cells can then circulate around the body and metastasise into organs that provide a favourable milieu.

It is the hypothesis of this study that the invasive and / or metastatic phenotype of prostatic tumours are regulated by the expression of CAMs. That is, that the CAMs expressed by prostatic tumour cells mediate the invasion of the basement membrane and blood vessel wall by interactions with extracellular or cell surface ligands.

Therefore, the expression of CAMs by *in situ* prostatic carcinomas was immunohistochemically investigated and compared to that of benign hyperplastic tissue. Briefly, prostatic tissue was obtained at the time of transurethral resection of the prostate or radical prostatectomy. The expression of E-selectin, Intercellular Cell Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 was examined on frozen sections of these tissues by immunohistochemistry, using the alkaline phosphatase method of detection, as described in detail in Chapter 2.4.1. The level of stain was given an Immunohistochemical Score (IS) of 0, 1, 2, 3, 4, 5, or 6, where a score of 0 represented no expression and a score of 6 represents uniform expression of all nucleated cells. A Haematoxylin and Eosin-stained section of each sample was analysed histologically by a trained consultant histopathologist. Prostatic glands were highlighted with monoclonal antibodies against Prostatic Acid Phosphatase (PAP), Prostate Specific Antigen (PSA), and cytokeratin, as described in

Chapter 4.2. Blood vessels were highlighted with a monoclonal antibody against Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), which depicts vascular endothelial cells.

The expression of E-selectin, ICAM-1, VCAM-1, $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 was demonstrated in the prostatic glands of both benign hyperplastic and malignant tissue (Chapters 4.3 and 4.4). However, the expression of these CAMs was not consistent in the samples examined in this study. Alpha-4, $\alpha 5$, αL, $\beta 1$, and CD44 were expressed in the prostatic glands of 49%, 18%, 41%, 32%, and 46%, respectively, of BPH samples and 63%, 12%, 50%, 28%, and 35%, respectively, of malignant samples (Tables 4.3 and 4.4). The ISs of glandular $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 in BPH tissues were 0.9, 0.4, 0.8, 0.9, and 1.3, respectively (Appendix Table 4.1). The ISs of glandular $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 in malignant prostatic glands were 0.8, 0.1, 0.7, 0.6, and 1.4, respectively (Appendix Table 4.2). Therefore, the level of $\alpha 4$, $\alpha 5$, αL, $\beta 1$, and CD44 expression in malignant prostatic glands was not significantly different to that observed in the glands of BPH tissue.

Cells within the glandular epithelium of BPH and malignant prostatic tissues also expressed E-selectin, ICAM-1 and VCAM-1. However, as above, the expression of these CAMs was not consistent on all samples examined. E-selectin, ICAM-1 and VCAM-1 were expressed on cells within the glandular epithelium of 31%, 55% and 32% of BPH tissues and 30%, 90%, and 59% of malignant tissues examined, respectively (Tables 4.4 and 4.7). The average ISs for glandular E-selectin, ICAM-1 and VCAM-1 were 0.4, 0.8 and 0.4 for BPH tissue and 0.3, 1.8 and 0.6 for malignant prostatic tissue, respectively (Appendix Tables 4.3 and 4.6). Therefore, the level of expression of and the number of samples expressing ICAM-1 within the glandular epithelium appears to be significantly greater in malignant prostatic tissues than in BPH tissues ($p < 0.005$). However, while prostatic carcinoma cells express higher levels of ICAM-1 than benign hyperplastic prostatic epithelial cells, no correlation could be made between the level of ICAM-1 expression and the histological grade or metastatic phenotype of the tumour.

It could be postulated that a second event is required to confer an invasive and / or metastatic phenotype to the prostatic tumour cell. For example, although there was no difference in the level of αL expression between BPH and malignant tissues, the majority of metastatic prostatic tumours expressed αL. Therefore, it could be hypothesised that a combination of ICAM-1 and αL expression confers an invasive phenotype to prostatic tumour cells. This theory fits with the hypothesis that for a normal cell to transform into to malignant, metastatic tumour cell, it must pass through a series of steps.

However, these data support the hypothesis that CAMs control the progression of carcinoma of the prostate.

6.7 The Role Of Cell Adhesion Molecules In The Progression Of Prostatic Carcinoma

The hypothesis of this study is that cell adhesion molecules (CAMs) play a role in the progression of cancer of the prostate. Particularly, the aim of this thesis was to investigate the role of CAMs in the invasive and metastatic spread of prostate cancer. The process of prostate cancer cell metastases is a complex cascade of events. Two of these events, which play a critical role in the development of invasive and metastatic prostate cancer, are firstly, tumour growth, invasion and release of neoplastic cells from the primary tumour and secondly, arrest of the tumour cells at distant sites via interactions with the vascular and / or lymphatic endothelium,. To understand the role of CAMs in the release of neoplastic cells from the primary tumour the expression of CAMs by *in situ* prostatic carcinomas, with and without evidence of invasive or metastatic disease, was examined. This thesis aims to draw parallels between the process of leucocyte extravasation and the interaction of disseminated prostatic tumour cells with the vascular endothelium. To this effect, experiments were designed to investigate the interaction of prostate cancer cell lines, PC3 and Du145, with vascular endothelial cells, HUVECs.

In summary of the data presented in this thesis, the prevalence and level of expression of ICAM-1 in prostate tumours appear to be significantly greater than in their benign counterparts (BPH tissue). Furthermore, the expression of ICAM-1 by Du145 metastatic prostate cancer cells may be involved in the stabilised attachment of Du145 cells to HUVECs. The expression of CD44 by HUVECs may play a role in the initial attachment of Du145 cells to HUVECs. The expression of CD44 by PC3 prostate cancer cells may be important in the initial attachment of PC3 cells to HUVECs, while α5 may play a role in the stabilised binding and / or transendothelial migration of PC3 cells.

What advantage does ICAM-1 expression confer to malignant prostate cancer cells? ICAM-1 is a member of the immunoglobulin superfamily of CAMs. ICAM-1 is expressed on resting leucocytes at low levels and activated endothelial cells. ICAM-1 expression can be induced on a wide range of nucleated cells, including epithelial cells. The interaction of ICAM-1 with its ligand Lymphocyte Function-associated Antigen-1 (LFA-1), or αLβ2, mediates a wide range of leucocyte interactions, including those with vascular endothelial cells. ICAM-1 mediates the stabilised binding and transendothelial migration of leucocytes to vascular endothelial cells during leucocyte extravasation. ICAM-1 expression has been detected in malignant melanoma cells and pancreatic cancer cell and its expression has been correlated with the metastatic potential of melanoma cells (Tang and Honn, 1994-1995). Therefore, the upregulation of ICAM-1 on Du145 prostatic cancer cells and *in situ* carcinoma cells supports the hypothesis that the process of tumour cell metastases can be paralleled to that of leucocyte extravasation.

Attachment

Tumour Cell

Epithelim / Endothelium

Stroma

Proteolysis

Migration
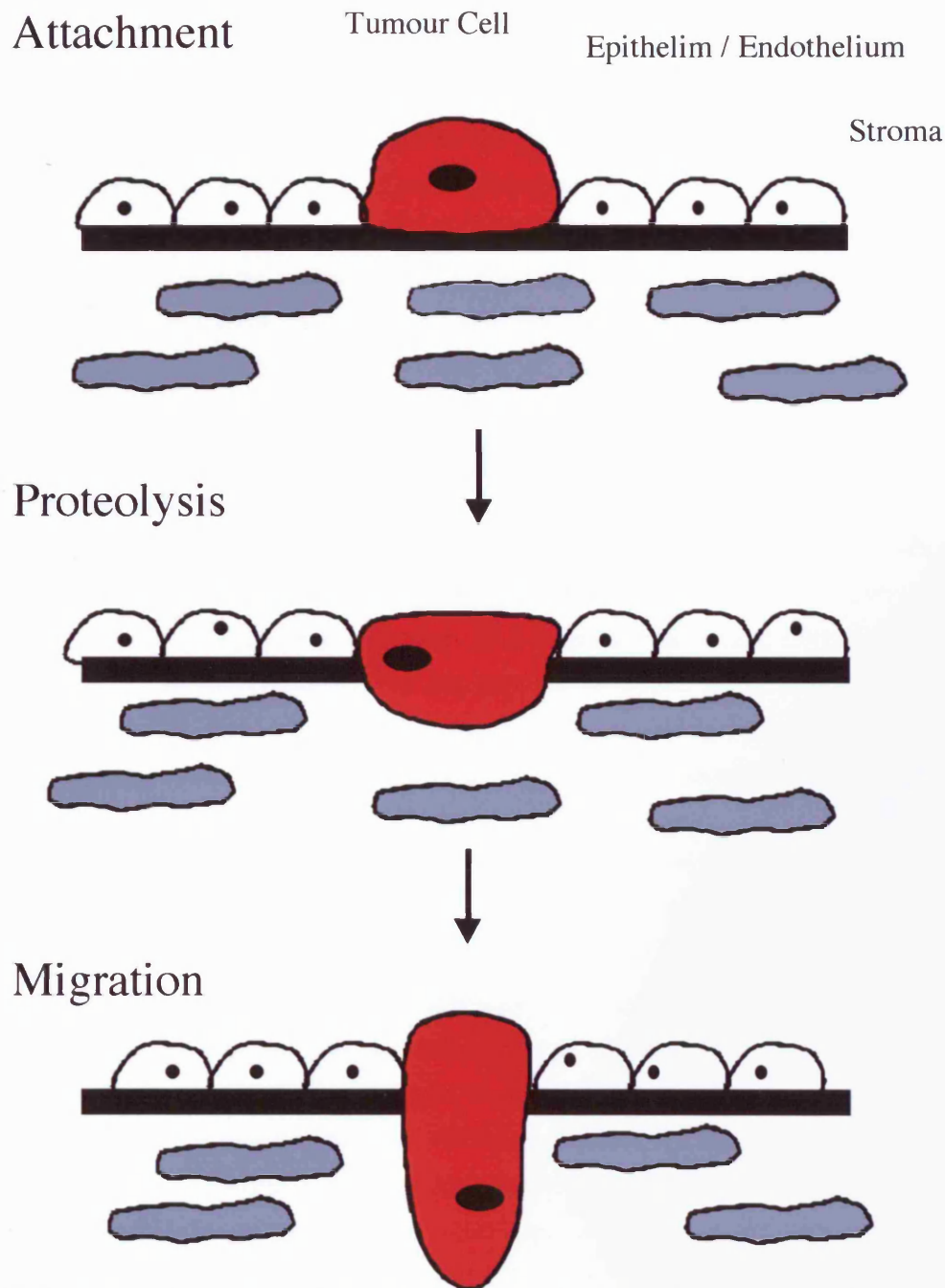
Figure 6.1 Schematic Representation of the Biochemical Events that Occur During Tumour Cell Invasion of the Extracellular Matrix. The first step is tumour cell attachment to extracellular matrix or basement membrane by tumour cell receptors that may parallel those used by leucocytes during extravasation. The second step is local degradation of the basement membrane by tumour associated proteolytic enzymes. The third step is tumour cell migration through the degraded basement membrane and extracellular matrix.

What role does ICAM-1 play when expressed in higher levels by *in situ* prostatic carcinoma cells? The first step in the metastatic spread of a cancer is the escape from the primary tumour. Therefore, it could be postulated that upregulated expression of ICAM-1 by *in situ* prostatic carcinoma cells might serve to promote their escape from the prostatic gland into the

non-glandular components. Prostatic glands are separated from the stromal or mesenchymal compartment of the prostate by the basement membrane. Characteristic constituents of the basement membrane are extracellular matrix proteins, including fibronectin, laminin and collagen and elastin. One of the rate limiting steps in the progression of carcinoma is the invasion of this basement membrane (Diagram 6.1). Cross-linking of ICAM-1 by its ligand transduces signals that stimulate second messenger systems intra-cellularly and can activate the expression of surface proteins and secretion of soluble proteins by T cells. ICAM-1 expressed on the tumour cell may interact with one or more of the extracellular matrix proteins. ICAM-1 has been shown to bind fibrinogen and hyaluronan (Mc Court *et al*, 1994, Gardiner and D'Souza, 1995). Cross-linking of ICAM-1 in this manner could activate intra-cellular signaling pathways, resulting in the release of proteases that break down these extracellular matrix proteins. Alternatively, the prostatic tumour cell itself may secrete these proteases.

Indeed, urinary Plasminogen Activator (uPA) is secreted in great abundance by highly aggressive prostate cancers, when compared to their normal counterparts (Goltzman *et al*, 1992). Metastatic PC3 cells secrete ten times more uPA than non-metastatic LNCaP cells (Hollas *et al*, 1992). UPA catalyses the conversion of the inactive zymogen plasminogen to the active serine protease plasmin. Plasmin cleaves, thereby degrading, many extracellular matrix proteins, including laminin, fibronectin and collagen. Therefore, increased levels of uPA promote the invasion of these tumour cells through the basement membrane.

The expression of matrix Metalloproteinases (MMPs) is increased in many cancers. MMP-9 activity appears to be increased in malignant prostatic tissue with an aggressive and metastatic phenotype (Hamdy *et al*, 1994). Physiologically, the MMPs are believed to play a role in female reproductive tract biology, including menstruation and cervical dilatation in pregnancy. The MMP family includes interstitial collagenases, gelatinases and stromelysins. MMPs are secreted as an inactive zymogen and require an activation step to become catalytically active. The binding of extracellular matrix proteins, including laminin, by many tumour cells stimulates the production of several MMPs. This has been shown to increase the invasive behaviour of these tumour cells. Therefore, interaction of ICAM-1 on prostatic tumour cells with the extracellular matrix proteins may induce the expression and / or activation of one or more MMPs. Indeed, the overexpression of the MMP matrilysin by transfection of Du145 cells dramatically increases their invasive behaviour *in vivo*. It has also been suggested that tumour cells can produce factors that stimulate the production of MMPs by the adjacent stromal cells. Indeed, MMP-11 is expressed by stromal cells surrounding malignant epithelial cells of invasive breast tumours (Basset *et al*, 1990).

An elevated level of the serine protease, elastase, which is involved in the breakdown of elastin, has been reported in prostatic tumour systems (Lowe and Isaacs, 1984). Du145 cells secrete increased levels of cathepsin B to that of the less aggressive cell line, LNCaP (Weiss *et al*,

1994). It has been suggested that cathepsin B acts directly and indirectly, via activation of other proteases, to promote the degradation of the basement membrane of prostatic tumours. Cathepsin D had been investigated in prostate cancer, but its definite role is still unclear.

Therefore, ICAM-1 expression by prostatic tumour cells may serve to induce the secretion and activation of uPA, members of the MMP family, cathepsin B or other unknown proteinases. The source of the degradative proteins is unclear. The ICAM-1 expressed by tumour cell may interact with the extracellular matrix proteins, which subsequently induces the cell to secrete these degradative proteins. Alternatively, the tumour cell, possibly through an interaction involving ICAM-1, may induce the production of these proteinases by the surrounding stromal cells. The tumour cell may by itself produce these proteins independent of its ICAM-1 expression. That is, ICAM-1 may not function to promote the production of extracellular proteinases.

The stroma surrounding the prostatic tumours expressing higher levels of ICAM-1 were well vascularised, as demonstrated by the presence of PECAM-1$^+$ cells within organised vascular structures. Therefore, ICAM-1 expressed by prostatic tumour cells may serve as a true cell adhesion molecule. That is, these tumour cells may attach and transmigrate through the endothelial cells of these local blood vessels. The data presented above from co-culture experiments with Du145 cells and HUVECs would support this theory. The co-culture of Du145 cells in direct contact with HUVECs for 24 hours resulted in an increased ICAM-1 expression by Du145 cells. These data provide evidence to support the hypothesis that CAMs play a role in the progression of prostate cancer. However, the ligand for ICAM-1 is LFA-1 or αLβ2. If ICAM-1 expression is increased by the intravasating and / or extravasating tumour cell, the vascular endothelial cell must express LFA-1. No corresponding increase in the levels of αL expressed by the HUVEC could be demonstrated. The expression of β2 was not investigated. However, cell adhesion molecules can bind to different ligands when expressed on different cells. For example, α2β1 is a collagen receptor when expressed on platelets and a collagen / laminin receptor when expressed on endothelial cells (Kirchhofer *et al*, 1990). It is possible that ICAM-1 binds to ligands other than LFA-1 when expressed on prostatic tumour cells. For example, of the CAMs investigated in this study HUVECs constitutively express α5. Although no increase in α5 expression by the HUVECs could be demonstrated, no decrease was observed either. Therefore, ICAM-1 may interact with integrin molecules incorporating the α5 subunit. Alternatively, ICAM-1 could bind to integrins expressed on the HUVECs that were not examined in this study. Although the data presented in this thesis supports a role for ICAM-1 in the progression of metastatic prostate cancer, recently published proposals suggest a tumour suppressive role for ICAM-1. Chromosome transfer studies of the TSU-pr1 prostatic adenocarcinoma cell lines with genomic ICAM-1 appears to suppress their tumourigenicity when injected into athymic nude mice (Gao *et al*, 1999). However, the genomic material transfected, 19p13.1-13.2, encodes other

putative tumour suppressor genes. Therefore, the suppression seen by Gao may not be a result of functional ICAM-1 expression. Indeed, ICAM-1 is expressed on the bone marrow metastatic deposits of breast, colon and prostatic cancers (Putz *et al*, 1999). ICAM-1 expression has also been demonstrated in many solid and lymphoid cancers, as described in detail in Chapter 1.5.3. In conclusion, the data above suggest a role for ICAM-1 in the progression of prostate cancer, both within the prostate itself and at distant sites of metastases.

What role does CD44 play in the metastatic spread of prostate cancer? Co-culture of Du145 cells with HUVECs for 24 hours induced down-regulation in the expression of CD44 by the HUVECs. As discussed in Chapter 6.5, CD44 may be involved in the initial stages of Du145 cell attachment to the HUVECs. As stabilised binding occurs over a prolonged period the expression of CD44 is no longer required and is therefore, down-regulated. CD44 was first described as a lymphocyte homing receptor, mediating the attachment of circulating lymphocytes to high endothelial venules. CD44 is thought to be involved in the early rolling stage of leucocyte extravasation. Therefore, if Du145 cells attached to HUVEC CD44 as suggested, the parallels drawn in this thesis between tumour cell metastasis and leucocyte extravasation would be realistic. However, it has also been proposed by Jalkenen *et al* (1987) that leucocyte CD44 does not actually bind ligands on the endothelial cell surface during extravasation. Instead the early interaction between CD44 and hyaluronate, or other unknown ligands, on the endothelial cell exposes or activates the expression of other CAMs involved in leucocyte extravasation. For example, this interaction may induce upregulation of ICAM-1 ligands on the endothelial cell or upregulation of the ICAM-1 itself.

The co-culture of PC3 metastatic prostate cancer cells for 1 hour with HUVECs induced the upregulation of CD44 by PC3 cells. These data again suggest a role for CD44 in the early rolling stage of extravasation; however, on this occasion expression is seen on the tumour cell and not the endothelial cell. However, both theories described above would apply to the PC3 cells and HUVECs. As discussed above, the ligation of the same CAM on two different cells can produce two different results. Therefore, if the interaction of HUVEC CD44 with an unknown Du145 cell ligand induced the upregulation of ICAM-1 by the Du145 cell, then the interaction of PC3 CD44 with unknown ligand on the endothelial cell could induce the upregulated expression of β5 by the PC3 cells.

The expression of CD44 in *in situ* prostatic carcinomas was not significantly different to that seen in benign hyperplastic prostatic tissue. Therefore, the *in vitro* data suggesting a role of CD44 in the early attachment of prostate cancer cells to vascular endothelial cells is not supported by the *in vivo* data. However, it could be argued that the process of leucocyte rolling, to which that early interaction between tumour cell and vascular endothelial cell has been paralleled, is a relatively quick process and that the increased CD44 may only occur for a very brief period of time. Alternatively, it must be remembered that both Du145 and PC3 were

established from metastatic deposits of prostate cancer. The PC3 cell line was established from a bony metastasis of prostate cancer. Therefore, these cells have specifically metastasised to the bone marrow. It could be postulated that PC3 cells specifically utilise CD44 to extravasate from the blood vessel to the bone and bone marrow. Tumour cells found examined in *in situ* primary prostatic carcinoma will undergo intravasation. The process of extravasation involves interactions between the tumour cell and the vascular endothelial cell. The process of intravasation involves interactions of the tumour cell with the smooth muscle and the vascular endothelial cells that the muscle cells protect. Therefore, these two processes, although similar, may use different CAMs to navigate a pathway through the blood vessel. Therefore, while a specific role for CD44 in the progression of prostate cancer cannot be determined, these data do suggest that CD44 may be involved in the early stages in intravasation and / extravasation, either indirectly or directly.

These data contradict the bulk of the literature depicting the role of CD44 in the metastatic pathway of prostate cancer. Artificial hypermethylation of the 5' regulatory sequence of genomic CD44 induces its down-regulation, which is associated with the acquisition of a high metastatic capacity within the Dunning rat model of metastatic prostate cancer (Verkaik *et al*, 1999, Gao *et al*, 1998). However, the majority of studies investigating the role of CD44 in non-prostatic cancers support the hypothesis that the expression of CD44 and / or its isoforms promotes the progression of carcinoma (Chapter 1.5.2). In conclusion, the data presented in this thesis suggest a role for CD44 as a tumour oncogene or, more specifically, a metastasis-promoting gene.

The different roles of CD44 in PC3 and Du145 cell attachment to HUVECs emphasise the complexity of tumour biology, with regard to the multiple pathways through which a cancer cell can progress. Prostatic carcinoma is highly heterogeneous and more than one tumour can occur in the prostate with different histological and metastatic characteristics at any one time. Paget's 'seed and soil' theory states that a metastasis arose from a proliferation of tumour cells (the 'seeds') in the favourable milieus provided by certain organs (the 'soil'). Therefore, every tumour cell has a favourable milieu or optimal requirement and has its own preferential site of metastasis. Indeed, PC3 and Du145 cell lines were both derived from metastatic deposits of prostatic carcinoma. However, PC3 cells were isolated from a bone marrow metastasis and Du145 cells were isolated from a brain metastasis. Histologically, cytologically and genetically, PC3 and Du145 cells are similar. However, these two cell lines have different favourable milieus. Therefore, it is not altogether surprising that the two cell lines employ different CAMs in the process of vascular cell adhesion. Alternatively, it could be that these two cells metastasised to different sites because of the adhesion molecules that they expressed. For example, both cell lines express CD44, ICAM-1 and α5; however, the level of expression is different. Moreover, PC3 cells express low levels of αL, while Du145 cells do not express αL. Conversely, PC3 cells do not express MHC Class I, while Du145 cells express high levels of MHC Class I (Table 5.1).

To conclude, the aim of this thesis was to investigate the role of cell adhesion molecules in the progression of prostate cancer. The hypothesis proposed suggests that the process of tumour cell metastasis could be paralleled to that of leucocyte extravasation. The data presented in this thesis supports that hypothesis and proposes a role for ICAM-1 in the extravasation and intravasation of prostatic tumour cells. The data presented here suggests a role for CD44, by both vascular endothelial cells and tumour cells and for α5 by the tumour cell. These data are summarised in Diagram 6.2.
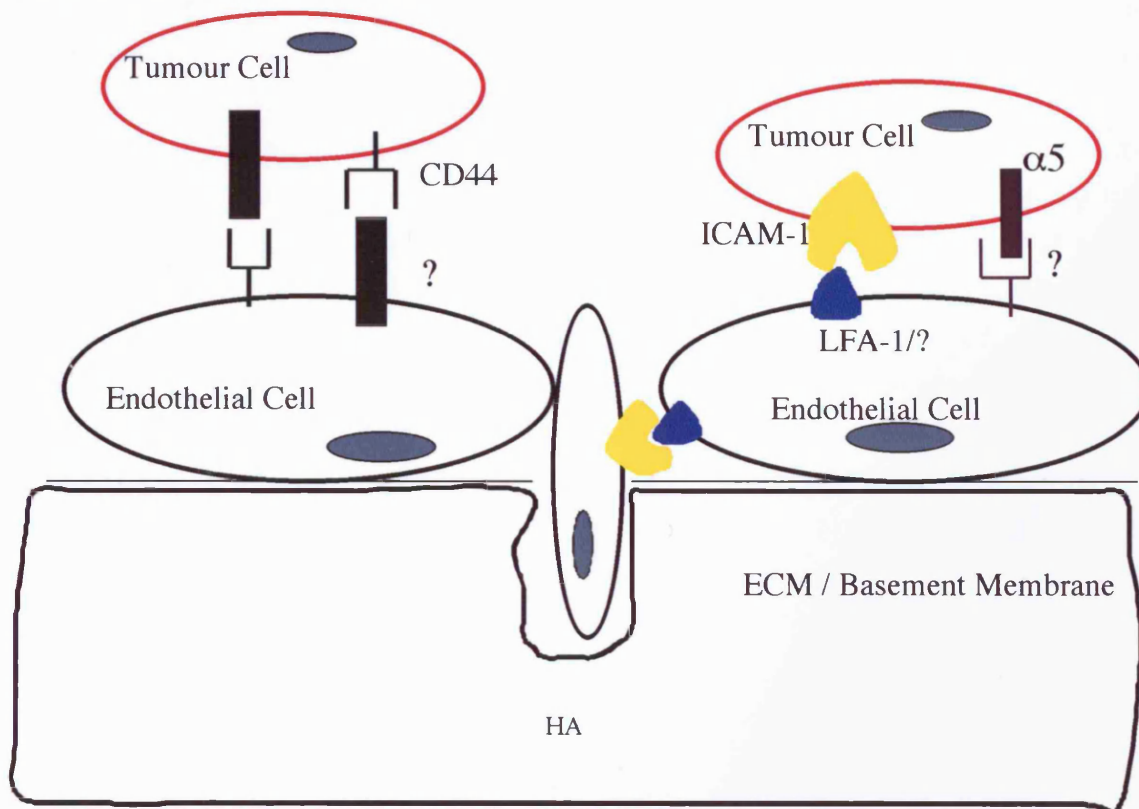
Diagram 6.2 Schematic Representation Of The Proposed Interactions Of Tumour Cell Intravasation And Extravasation. This model is based on the multi-step process of leucocyte extravasation. Initial interactions of tumour cells with the endothelium may be mediated by CD44 on both the tumour cell and the endothelial cell. Stabilised attachment may occur through ICAM-1 and α5 on the tumour cell and unidentified ligands on the endothelial cell. Transendothelial migration may be mediated by ICAM-1 on the tumour cell. (ICAM-1, intercellular cell adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1).

6.8 Is There A Future For Cell Adhesion Molecules In Prostate Cancer Progression

The data presented in this thesis supports the hypothesis that cell adhesion molecules play a role in the progression of prostate cancer. Specifically, ICAM-1 appears to be involved in the attachment of prostatic tumour cells to vascular endothelial cells. Prostatic tumour cells express elevated levels of ICAM-1 *in situ*. Therefore, ICAM-1 appears to be involved in the extravasation and intravasation of malignant prostatic tumour cells. CD44 and α5 have been implicated in the process of extravasation, although the precise role of these CAMs is unclear.

To elaborate on the molecular interactions between prostate cancer cells and vascular endothelial cells better time studies should be performed. Although the doubling time of an *in vivo* prostatic cancer cell may be as long as a few months, cells from the PC3 and Du145 cell lines proliferate more than once in 24 hours. Therefore, as with experiments described in Chapters 5.2, 5.3 and 5.4, co-culture assays should be conducted for 2, 4, 8, and 12 hours. Attachment of PC3 and Du145 cells occurs within 1 hour. In the pro-longed co-culture experiments, unattached cells should be removed following the initial attachment: this is more representative of the *in vivo* model of leucocyte extravasation. Alternatively, co-culture experiments could be conducted under flow conditions, which impersonate the effect of the physiological flow of blood. These co-cultures should be performed in the presence of blocking monoclonal antibodies. Physiologically leucocyte extravasation occurs to attract leucocytes into inflamed tissue for wound healing purposes. Therefore, the vascular endothelium is usually activated. These co-cultures should be conducted with activated HUVECs. This could be achieved by the addition of IL-1 before the addition of the prostate cancer cells. These co-culture experiments should be performed with bone marrow stromal cells, which are the cells that PC3 and metastatic prostate cancer cell encounter when they metastasis to the bone.

The results presented in Chapter 5.5.4 describe that PC3 and Du145 cells may have invaded through the monolayer of endothelial cells during the pro-longed co-cultures with HUVECs. Therefore, invasion assays should be conducted. Firstly, the ability of prostatic tumour cells to invade through re-constituted membranes should be investigated. The ability of blocking monoclonal antibodies against ICAM-1, CD44 and α5 to inhibit any invasion should be investigated. Secondly, co-culture experiments should be conducted in Transwell-COL chambers. This system provides a microporous membrane between the prostatic tumour cells and the endothelial cells (Quinn *et al*, 1996).

A wider panel of CAMs should be investigated, including the β2 integrins. The expression of CAMs in *in situ* carcinomas should be fully investigated. In particular, the expression of CAMS by the vascular bed in *in situ* prostatic carcinomas should be investigated. The ability of

PC3 and Du145 cells to invade through a reconstituted basement membrane should be investigated. The expression of the CAMs should be investigated at the leading edge of invasive prostatic tumours. Particularly, the role of $\alpha v \beta 3$, which has been shown by other authors to be mediate interactions between invasive prostatic carcinoma cells and the extracellular matrix, should be investigated.

In vivo experiment should be conducted to investigate the metastatic capabilities of these PC3 and Du145 cells in the presence of blocking monoclonal antibodies against ICAM-1, VCAM-1 and $\alpha 5$. Do antibodies against particular CAMs inhibit the development of bone marrow or bony metastases, but promote the development of metastatic deposits in the brain, for example?

A more difficult issue to investigate, but one that needs to be answered, is what the ligands are for ICAM-1, $\alpha 5$ and CD44 on the endothelial cell in this co-culture system

If these experiments were conducted the role of ICAM-1, CD44 and $\beta 5$ in the progression of cancer of the prostate would be clarified.

Appendices

Contents

Appendix 1 Viable Cell Count Studies With Trypan Blue

Viable cell count studies were performed with trypan blue dye. Trypan blue powder was resuspended to give a 5% solution with Elga-purified H_2O. Trypan blue penetrated the lipid bi-layer of cellular membranes of dead cells, but not of living cells. Therefore, trypan blue caused a cytoplasmic exclusion effect in viable cells. Viable and non-viable cells can be distinguished by this method.

Cell suspensions to be counted were suspended to a volume of 10ml. A 1/20 dilution of the cells was prepared in trypan blue. This solution was immediately loaded onto the haemocytometer. The number of cells in the inner grid of the haemocytometer with no cytoplasmic dye was counted with the use of a Leitz HM-LUX light microscope. Dividing that number by the number of squares in which the cells were counted and then multiplying that number by the dilution factor gives the number of cells per ml.

Appendix 2 Gelatinisation Of Glass Microscope Slides

1 gram of gelatin was weighed on a bench top balance. This was placed in a 500ml glass conical flask with 250ml of Elga-purified H_2O. The flask was placed on a Revotherm heater-mixer to aid the dissolution of the gelatin. Once this was achieved 0.1 g of chrome alum, weighed as above, was added to the gelatin solution. This solution was dispensed into a small tub: racked microscope slides were submerged in the gelatin for a couple of seconds. The slides and racks were drained and left, covered with tissue, overnight to dry at room temperature. The slides were stored, racked, in a covered plastic tub.

Appendix 3 Acetone Fixation

Microscope slides with tissue sections or single cell cytospin preparations were placed in plastic racks. The tub now contains acetone. Tissue sections were submerged in acetone for 10 minutes: cytospin preparations were submerged for 3 minutes. Slides were removed from the acetone and racks and left to air dry on the bench top. Cytospin preparations were always fixed immediately prior to use: therefore, these slides were placed into a tub of PBS and APAAP staining was performed as described in 2.4.1.4. Slides with tissue sections were wrapped together with 3 layers of autoclave tape. These slides were placed into appropriately labelled specimen bags containing silica gel as a dehydrating agent. These bags were stored at - 20^0C until required.

Appendix 4 Reagents

Reagents 4.1, 4.2, 4.3, 4.4, 4.5, 4.7, 4.8, 4.9, 4.10 were prepared aseptically. Ingredients of these reagents were also kept sterile. Excess reagents not consumed within one month of preparation were discarded.

4.1 HBSS

Stock solutions of 1x HBSS were buffered with HEPES (N- (2-Hydroxyethyl)piperazine - N' - (2-ethanesulphonic acid)) buffer to a concentration of 10mM HEPES buffer. HBSS was stored at 4^0C.

4.2 Roswell Park Memorial Institute (RPMI) 1640 Medium

Three different RPMI 1640 media were used during this study. All media were kept at 4^0C.

4.2.1 Imperial Laboratories RPMI 1640

In the earlier part of the study RPMI 1640 was received from Imperial Laboratories. 136ml was removed from a litre bottle of sterile H_2O: this was replaced by 100ml of a 10x RPMI 1640 solution, 10ml of 1M HEPES buffer, and 26ml of $NaHCO_3$ (stock 7.5% solution).

4.2.2 10x Sigma Chemical Co. Ltd. RPMI 1640.

140.5ml was removed from a litre bottle of sterile H_2O. This volume was replaced by 100ml of a 10x RPMI 1640 solution, 10ml of 1M HEPES buffer, 27ml $NaHCO_3$ (7.5% stock solution), and 3.5ml of 2M NaOH.

4.2.3 1x Sigma Chemical Co. Ltd. RPMI 1640

Sigma Chemicals Ltd. stopped adding folic acid to their 10x RPMI 1640 medium. Therefore, 1x RPMI 1640 that contained folic acid was used. 5ml of medium was removed and replaced by 5ml of 1M HEPES buffer. This RPMI 1640 also contained $NaHCO_3$ and L-glutamine: therefore, the make-up of Established Cell Line Medium (Appendix 4.7) and Endothelial Culture Medium (Appendix 4.8) was altered.

4.3 Foetal Calf Serum (FCS)

All FCS used was first heat-inactivated by incubation at 56^0C for 45 minutes in a Grant W14 waterbath. Stores of FCS were stored at -20^0C

4.4 Established Cell Line Medium (ECLM)

ECLM was used for the growth of PC3, Du145, and A549. A 200ml solution of ECLM was composed of either 176ml or 178ml of RPMI 1640 medium, 20ml FCS, 2ml L-glutamine (stock concentration of 200mM), if required, and 2ml of a penicillin (10,000U/ml), streptomycin (10mg/ml), and amphotericin B (25mg/ml) solution. ECLM was kept at 4^0C.

4.5 Trypsin / EDTA

The trypsin/EDTA stock solution had a concentration of 50mg/ml trypsin and 20mg/ml EDTA. Stock solutions of trypsin/EDTA were prepared to a 1/20 dilution with Ca^{2+} / Mg^{2+} free HBSS. This solution was warmed to 37^0C in a waterbath before use. Trypsin / EDTA solutions were always used fresh. 0.05ml, 0.2ml, 2.5ml, 5ml, and 10ml of this diluted solution was

used to trypsinise a 96-well plate well, a 24-well plate well, a 25cm^2 TCGF, an 80cm^2 TCGF, and a 175cm^2 TCGF, respectively.

4.6 Propan-2-ol

Propan-2-ol was diluted to 70% of its stock concentration with sterile H$_2$O. Propan-2-ol was stored at room temperature.

4.7 Sigma Collagenase

5mg of collagenase powder was reconstituted in sterile H$_2$O to give a final volume of 10ml and a concentration of 1mg/ml. This solution was passed through a 0.2 micron filter. The solution was warmed to 37^{0}C before use and always used fresh.

4.8 Endothelial Cell Culture Medium (ECCM)

A 200ml solution of ECM was composed of:

> 152.5ml of 1x RPMI (Appendix 5.2),
>
> 2ml of penicillin (10,000IU/ml), streptomycin (10mg/ml) and amphotericin B (25mg/ml),
>
> 1ml of Endothelial Cell Growth Supplement (stock concentration of 3mg/ml),
>
> 2ml of l-glutamine (stock concentration of 200mM), if required, and
>
> 0.4 ml of sodium pyruvate (stock concentration of 100mM).

ECM was kept at 4^{0}C.

4.9 LLC PK1 Culture Medium

DMEM / HAM's F12 medium was used for the culture of LLC PK1 cells: a 200ml solution was composed of 176ml DMEM / HAMS F12 (Appendix 4.10), 20ml heat-inactivated FCS, 2ml L-glutamine (stock concentration of 200mM), and 2ml of a penicillin (10,000U/ml), streptomycin (10mg/ml), and amphotericin B (25mg/ml) solution. This medium was stored at 4^{0}C.

4.10 DMEM / HAM's F12

1x DMEM / HAM's F12 was used: 5ml of medium was removed from a 500ml bottle of medium and replaced by 5ml of 1M HEPES. This medium was stored at 4^{0}C.

4.11 Phosphate Buffered Saline (PBS)

10x stock PBS solution was diluted with Elga-purified H$_2$O to 1x. The Elga Micromeg Water Purifying System used a MC:ES cartridge producing pure, de-ionised H$_2$O. PBS was stored at room temperature.

4.12 Tris HCl

15.76g of TrisHCl was re-suspended in Elga-purified H$_2$O to give a 1 litre, 100mM solution. The pH was adjusted, if required, to 8.2 with 2M hydrochloric acid (HCl). Tris HCl was kept at 4^{0}C.

4.13 PBS-sodium azide (PBS/Az)

1g of sodium azide was dissolved in 1x PBS (Appendix 4.11). The solution was made up to a final volume of 1L with PBS and kept at 4^{0}C.

4.14 PBS/Az- normal goat serum (PBS/Az/NGS)

2ml of NGS was added to PBS/Az (Appendix 4.13) to give a final volume of 200ml. The solution was sterile-filtered through a 0.2 micron filter and stored at 4^0C.

4.15 Paraformaldehyde

Five 1g tablets of paraformaldehyde were added to 450ml of PBS (Appendix 4.11) in a conical flask. The flask was placed on a Revotherm and left until the tablets had fully dissolved. The volume was made up to 500ml with PBS and this solution was left to cool. The paraformaldehyde was filtered through a 0.2 micron filter and separated into two 250ml aliquots. The solution was kept at 4^0C.

4.16 PBS/Az/NGS-normal Mouse Serum (PBS/Az/NGS/NMS)

5ml of NGS and NMS were dissolved in PBS/Az (Appendix 4.13) in a conical flask. This solution was made up to 500ml with PBS/Az. The resulting solution was sterile-filtered through a 0.2 micron filter and aliquots were stored at 4^0C in 25ml universals.

Appendix 5 Preparation Of Acridine Orange Solution

8mg of AO was weighed using a bench top balance. AO was placed in a 1ml plastic centrifuge tube using a spatula. This tube was sealed before being weighed on the bench top. 800μl PBS was pipetted into the tube. The resulting solution was aspirated by pipette to ensure complete dissolution of the powder. This 10mg/ml was serially diluted with PBS by 50% 16 times to give a solution of AO concentration 0.00015mg/ml. These solutions were stored in 1ml centrifuge tubes.

Appendices

Appendix 6 Monoclonal Antibodies

The monoclonal antibodies used in this study are detailed in Table 7.1 below. The working concentrations that these antibodies were used at are described in Table 7.2.

Monoclonal Antibody	Specificity	Distribution	Supplier	Cat. No.	IgG Isotype
HLA-ABC	MHC Class I	All nucleated cells	Serotec	MCA 673	IgG1
CD3	T cell-associated CD3ε chain	T lymphocytes	Dako	M756	IgG1
CK-pan	All Cytokeratin	Epithelial cells, from simple glandular to stratified squamous epithelia	Dako	M 717	IgG1
CD62E	E-selectin	Activated endothelial cells and some T lymphocytes	R&D Systems	BBA1 6	IgG1
CD31	PECAM-1	Endothelial cells, platelets, T lymphocytes, monocytes, and granulocytes	R&D Systems	BBA 7	IgG1
CD106	VCAM-1	Activated endothelial cells	R&D Systems	BBA 5	IgG1
CD54	ICAM-1	Activated and non-activated endothelial cells	R&D Systems	BBA3	IgG1
CK-8	52.5kDa protein, Cytokeratin-8	Epithelium of liver, intestine, pancreas, urinary bladder, salivary gland, thyroid, prostate, and placenta	Sigma	C5301	IgG1
PAP	Prostatic Acid Phosphatase	Normal and neoplastic prostatic epithelium	Sigma	P9808	IgG2a
PSA	Prostate Specific Antigen	Prostatic epithelium and prostatic carcinoma cells	Euro-Diagnostica (Euro-Path, Ltd)	2222 MPA	IgG1

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Monoclonal Antibody	Specificity	Distribution	Supplier	Cat. No.	IgG Isotype
CD44	All CD44 isoforms	Peripheral blood leucocytes, liver Kupffer cells, fibroblasts, epidermal keratinocytes, some pancreatic acinar cells, and brain cells	Sigma	C7923	IgG1
CD49d	Alpha chain of VLA-4	Monocytes, T and B lymphocytes, thymocytes, and Langherhans cells.	Serotec	MCA 697	IgG1
CD49e	Alpha chain of VLA-5	T lymphocytes, granulocytes, platelets, some melanoma cells	Serotec	MCA 698	IgG1
CD11a	Alpha chain of LFA-10	Various cells	R&D Systems	BCA 1	IgG2a
CD29	Beta 1	Various cells			
CD31-PE	PECAM-1	Endothelial cells, platelets, T lymphocytes, monocytes, and granulocytes	Becton Dickinson	34029 7	IgG1

Table 7.1 The Specificity, Distribution And Supplier Details Of Monoclonal Antibodies Employed In This Study.

Monoclonal Antibody	Immunohistochemistry Dilution Factor	Flow Cytometry Dilution Factor
HLA-ABC	100	100
CD3	100	10
CK-pan	100	Not Applicable
CD62E	500	100
CD31	1000	1000
CD106	1000	100
CD54	500	100
CK-8	250	Not Applicable
PAP	400	Not Applicable
PSA	50	Not Applicable
CD44	900	50
CD49d	500	50
CD49e	1000	10
CD11a	500	50
CD29	1000	100
CD31-PE	Not Applicable	Neat

Table 7.2 Working Concentrations of Monoclonal Antibodies Employed In This Study

Appendix 7 Reagent Suppliers

Product	Supplier	Cat. No.
AB Serum	Quest Biomedical	3GT041B
Acetone	BDH	27023
Acridine Orange	BDH	34001
APAAP Complexes	Dako	D651
Calcium/Magnesium Free HBSS	Sigma Chemical Co. Ltd.	H9394
Carbon Dioxide	BOC Ltd.	NA
Chrome Alum	Aldrich Chemicals Co Ltd.	24,336-1
Dexamethasone	Sigma Chemical Co. Ltd.	D8893
DiMethylSulphOxide	Sigma Chemical Co. Ltd.	D05879
DMEM	Sigma Chemical Co. Ltd.	D6546
DMEM/Nutrient Mix F12 (1:1)	GIBCO BRL	21331-020
Endothelial Cell Growth Supplement	Sigma Chemical Co. Ltd.	E2759
Ethanol	Hayman Ltd.	SIN1170
FACS Flow	Becton Dickinson	342003
Foetal Calf Serum	Sigma Chemical Co. Ltd.	F7524
Gelatin	BDH	44075
Glycerol Gelatin	Sigma Chemical Co. Ltd.	GG1
Goat Anti-mouse Immunoglobulin FITC Conjugate	Sigma Chemical Co. Ltd.	F8264
Granulocyte Monocyte Colony Stimulating Factor	Sigma Chemical Co. Ltd.	G0532
Heparin	Sigma Chemical Co. Ltd.	H3149
HEPES Buffer	Sigma Chemical Co. Ltd.	H0887
L-glutamine	Sigma Chemical Co. Ltd.	G7513
Levamisole	Sigma Chemical Co. Ltd.	L9756
Liquid Nitrogen	BOC Ltd.	NA
Mayer's Haemalum	BDH	650604T
MTT	Sigma Chemical Co. Ltd.	M5655
Normal Goat Serum	Sigma Chemical Co. Ltd.	S6898
Normal Mouse Serum	Sigma Chemical Co. Ltd.	M5905
OCT Tissue Tek Compound	Raymond A Lamb	4583
Paraformaldehyde	BDH	33233
Penicillin/Streptomycin/ Amphotericin B	Sigma Chemical Co. Ltd.	A9909
Phosphate Buffered Saline	Microgen	M34A
PKH26 Cell Linker Kit	Sigma Chemical Co. Ltd.	PKH26-GL
Propan-2-ol	Merck	1022400
Propidium Iodide	Sigma Chemical Co. Ltd.	P4170
Rabbit Anti-mouse Immunoglobulin	Dako	Z0259
RPMI 1640	Imperial Laboratories	2-540-07
RPMI 1640 (10x)	Sigma Chemical Co. Ltd.	R1145
RPMI 1640 (1x)	Sigma Chemical Co. Ltd.	R8758
Sigma Collagenase	Sigma Chemical Co. Ltd.	C8051
Silica Gel Type III	Sigma Chemical Co. Ltd.	S7625
Sodium Azide	Sigma Chemical Co. Ltd.	S2002
Sodium Bicarbonate	Sigma Chemical Co. Ltd.	S8761
Sodium Hydroxide	BDH	10252
Sodium Pyruvate	Sigma Chemical Co. Ltd.	S8636

Product	Supplier	Cat. No.
Sterile Water	Parkfields	L6A
Tris HCl	Sigma Chemical Co. Ltd.	T7149
Trypan Blue	BDH	34078
Trypsin/EDTA	Sigma Chemicals Ltd.	T4174
Vector Red Enzyme Substrate Kit	Vector Labs Ltd.	SK-5100

Table 7.3 Supplier Details For Products Used In This Study

Appendix 8 One Letter And Three Letter Codes For Amino Acids

One Letter Code	Three Letter Code	Amino Acid
A	Ala	Alanine
B	Asx	Asparagine or Aspartic Acid
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Trytophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or Glutamic Acid
X		Amino acid questionable

Table 7.4 The One And Three Letter Codes For Amino Acids

Number Of Du145	Attached Population		Unattached Population	
Cells Added Per Well	Endothelial Cells	Epithelial Cells	Endothelial Cells	Epithelial Cells
0	0.55	99.45	NA	NA
	0.90	99.10	NA	NA
	1.30	98.70	NA	NA
1406	19.65	80.35	NA	NA
	15.70	84.30	NA	NA
	16.30	83.70	NA	NA
2813	26.95	73.05	39.39	61.11
	22.25	77.75	45.40	54.60
	29.05	70.95	62.16	37.86
5625	41.35	58.65	60.15	39.85
	39.60	60.40	69.00	31.00
	48.45	51.55	67.15	32.85
11250	58.85	41.15	76.20	23.80
	60.35	39.65	76.00	24.00
	54.25	45.75	75.65	24.35
22500	63.30	36.70	81.40	18.60
	64.75	35.25	80.40	19.60
	63.45	36.55	85.35	14.65
45000	70.80	29.20	92.50	7.50
	70.50	29.50	90.80	9.20
	69.90	30.10	91.90	8.10
90000	73.35	26.65	94.85	5.15
	70.10	29.91	94.40	5.60
	72.40	27.60	94.50	5.50

Appendix Table 3.1A The Distribution Of Endothelial And Epithelial Cells Following 1 Hour Of Co-culture. Varying concentrations of Du145 cells were added to confluent monolayers of HUVECs and incubated for 1 hour. Attached and unattached cells were subjected to FACScan analysis with mouse anti-human PECAM-1 conjugated to phycoerythrin. (NA, not applicable.)

Number Of A549	Attached Population		Unattached Population	
Cells Added Per Well	Endothelial Cells	Epithelial Cells	Endothelial Cells	Epithelial Cells
0	5.40	94.60	NA	NA
	2.75	97.25	NA	NA
	2.05	97.95	NA	NA
1445	25.20	74.80	NA	NA
	29.20	70.80	NA	NA
	23.90	76.10	NA	NA
2890	34.20	65.80	NA	NA
	10.85	89.15	NA	NA
	13.35	88.65	NA	NA
5781	16.00	84.00	76.61	23.38
	15.55	84.45	60.85	39.15
	34.00	66.00	50.63	24.74
11562	40.25	86.75	85.20	14.80
	48.55	51.45	77.65	21.35
	34.40	65.60	87.60	12.40
23125	56.60	43.40	85.65	14.35
	45.65	54.35	90.10	9.90
	46.10	53.90	81.00	19.00
46250	63.85	36.15	92.20	7.80
	65.20	34.80	92.05	7.95
	73.85	26.15	90.70	9.30
92500	75.15	24.85	97.15	2.85
	79.20	20.20	96.75	3.25
	77.70	22.30	97.45	2.55

Appendix Table 3.1B The Distribution Of Endothelial And Epithelial Cells Following 1 Hour Of Co-culture. Varying concentrations of A549 cells were added to confluent monolayers of HUVECs and incubated for 1 hour. Attached and unattached cells were subjected to FACScan analysis with mouse anti-human PECAM-1 conjugated to phycoerythrin. (NA, not applicable.)

Du145 Cells		A549 Cells	
Acridine Orange Concentration (ng/ml)	Median Level Of Fluorescence	Acridine Orange Concentration (ng/cell)	Median Level Of Fluorescence
0.0000	113	0.0000	238
0.0007	130	0.0002	193
0.0010	155	0.0004	246
0.0030	158	0.0010	213
0.0050	297	0.0020	377
0.0100	385	0.0030	462
0.0300	444	0.0060	524
0.0400	492	0.0100	584
0.1000	500	0.0200	624
0.2000	532	0.0500	646
0.3000	536	0.1000	643
0.7000	519	0.2000	638
1.3000	503	0.4000	626
3.0000	482	1.0000	607
5.0000	450	2.0000	591
11.0000	355	3.0000	511
22.0000	336	6.0000	478
44.0000	308	13.0000	456

Appendix Table 3.2 Fluorescent Light emitted By Acridine Orange-stained Cells Has A Biophasic Behaviour. Cells were incubated with varying concentration s of acridine orange (AO) for 10 minutes, as described in the text. At low levels of AO concentrations the amount of fluorescence measured on the FACScan was directly proportional to the number of cells present. The reverse was seen at higher concentration of AO.

Du145 Cells				A549 Cells			
Acridine Orange Concentration (pg/ml)	Median Level Of Fluorescence			Acridine Orange Concentration (pg/ml)	Median Level Of Fluorescence		
0.00	10	9	10	0.00	21	12	12
0.50	79	92	96	0.20	185	196	180
1.00	192	169	158	0.30	290	285	213
2.00	292	292	286	0.70	396	412	402
5.00	375	377	389	1.00	494	492	497
9.00	454	457	454	3.00	567	585	556
19.00	477	486	432	5.00	609	610	616
37.00	511	506	485	11.00	627	626	631
74.00	522	520	523	22.00	638	637	640
148.00	518	522	524	44.00	634	637	637

Appendix Table 3.3 The Fluorescent Light Emitted By Lightly Acridine Orange-stained Cells. Du145 cells (50000) and A549 cells (100000) were incubated with varying concnetrations of acridine orange, as described in the text.

Number Of Du145 Cells Added Per Well	Percentage Of Unattached Population						Percentage Of Attached Population					
	Endothelial Cells			Epithelial Cells			Endothelial Cells			Epithelial Cells		
56000	1.50	1.80	1.75	98.50	98.20	98.25	2.80	2.45	2.20	97.20	97.55	97.80
28000	2.00	2.70	3.40	98.00	97.30	96.60	6.80	7.20	8.45	93.20	92.80	91.55
14000	18.55	14.50	8.45	81.45	85.50	91.55	38.90	57.55	38.65	61.10	42.45	61.35
7000	62.30	62.75	58.25	67.30	37.25	41.75	96.75	93.95	92.90	4.25	6.05	7.10
3500	93.95	89.05	87.60	6.05	10.95	12.40	96.40	97.00	97.85	3.60	3.00	2.15
1750	98.25	98.25	99.40	1.75	1.75	0.60	99.30	99.45	99.20	0.70	0.55	0.80
875	99.70	99.65	99.65	0.30	0.35	0.35	99.65	99.40	99.85	0.35	0.60	0.15
0	NA	NA	NA	NA	NA	NA	99.95	99.90	ND	0.05	0.10	ND

Appendix Table 3.4 The Distribution Of Acridine Orange Stained-Du145 (epithelial) Cells And HUVECs In Attached And Unattached Populations Of Cells Following 1 Hour Of Co-culture. All cells were subjected to FACScan analysis. Endothelial cells were distinguished from epithelial cells by their FL1 fluorescence. (NA, not applicable: ND, not done.)

Day	PKH26 Concentration (Molar)	Median Level Of FL2 Fluorescence		
0	0	110	109	119
0	6.25 x10(-6)	323	320	323
0	1.25 x 10(-6)	411	411	414
0	2.5 x10 (-6)	459	460	457
0	5 x 10(-6)	539	535	547
3	0	92	93	95
3	6.25 x10(-6)	230	231	227
3	1.25 x 10(-6)	297	291	289
3	2.5 x10 (-6)	338	336	332
3	5 x 10(-6)	425	421	420

Appendix Table 3.5 The Level Of FL2 Fluorescence Emitted By Du145 Cells After Staining With PKH26. Du145 cells were stained with PKH26, as detailed in the text. FL2 fluorescence was measured immediately or 2 days later, after cells had been cultured under standard tiisue culture conditions.

Cell Preparation	Median Level Of FL1 Fluorescence			Corresponding MESF Values		
Du145 With PKH26 Diluent Only	469	510	453	395263	597149	336477
Du145 With PKH26 Dye And Diluent	486	488	493	469015	478551	503247
Du145 Cells Only	199	201	197	26110	26641	25590
Du145 Cells With FITC	213	210	212	30061	29167	29760

Appendix Table 3.6 The Levels Of FL1 Fluorescence Emitted By Du145 Cells Incubated With PKH26 Dye And Diluent. Du145 cells were incubated with PKH26 diluent only or PKH26 dye and diluent. The levels of FL1 flourescence was measured by a BD FACScan. Median levels of fluorescence were converted to MESF values as described in Chapter

Cell Preparation	Median Level Of Flourescence For CD44			MESF For CD44		
Attached Du145	463	493	489	288552	390046	374678
Unattached Du145	296	298	304	53905	54999	58416
Unmanipulated Du145	377	378	369	121624	122852	112232
Manipulated A549	101	100	91	7601	7525	6875
Unmanipulated A549	108	112	103	8155	8489	7755
	Median Level Of Fluorescence For CD3			MESF For CD3		
Attached Du145	239	241	245	30405	31022	32294
Unattached Du145	134	137	137	10859	10913	10913
Unmanipulated Du145	134	137	133	10589	10913	10483
Manipulated A549	125	113	123	9674	8575	9481
Unmanipulated A549	145	148	148	11826	12188	12188

Appendix Table 3.7 The Level Of Fluorescence Emitted By PKH26 Positive Du145 And PKH26 Negatice A549 Cells When Incubated With Antibodies Against CD44 And CD3. Du145 cells were satined with PKH26, as described in the text. These cells were then incubated with A549 cells for 1 hour. Attached, unattached, and unmanipulated cells were then subjected to FACScan analysis with mouse anti-human CD44 and CD3 and goat anti-mouse immunoglobulin conjugated to FITC.

Cell Preparation	Median Level Of Fluorescence For CD44			MESF For CD44		
1 hour attached PC3	278	287	297	57822	63303	70006
1 hour unattached PC3	324	324	338	91863	91863	105762
1 hour unmanipulated PC3	350	303	275	119338	74363	56102
1 hour manipulated HUVECs	240	251	251	39446	44064	44064
1 hour unmanipulated HUVECs	293	235	261	67244	37510	48729
24 hours attached PC3	222	224	249	32910	41067	43186
24 hours unattached PC3	393	349	385	183957	118143	169727
24 hours unmanipulated PC3	459	453	445	357420	336477	310449
24 hours manipulated HUVECs	179	180	182	21350	21566	22004
24 hours unmanipulated HUVECs	293	235	261	67244	37510	48729
Cell Preparation	Median Level Of Fluorescence For CD3			MESF For CD3		
1 hour attached PC3	133	132	132	13438	13304	13304
1 hour unattached PC3	126	121	124	12524	11910	12275
1 hour unmanipulated PC3	120	128	127	11790	12779	12651
1 hour manipulated HUVECs	126	129	130	12524	12908	13039
1 hour unmanipulated HUVECs	132	130	123	13304	13039	12152
24 hours attached PC3	138	244	249	32910	41067	43186
24 hours unattached PC3	123	125	121	12152	12399	11910
24 hours unmanipulated PC3	117	118	122	11440	11555	12030
24 hours manipulated HUVECs	116	118	126	11325	11555	12524
24 hours unmanipulated HUVECs	132	130	123	13304	13039	12152
Cell Preparation	Median Level Of Fluorescence For FITC			MESF For FITC		
1 hour unmanipulated PC3	176	177	177	20715	20924	20924
1 hour unmanipulated HUVECs	115	111	124	11212	10769	12275
24 hours unmanipulated PC3	178	179	174	21136	21350	20302
24 hours unmanipulated HUVECs	119	128	124	11672	12779	12275
Cell Preparation	Median Level Of Fluorescence For Cells			MESF For Cells		
1 hour unmanipulated PC3	167	167	166	18921	18921	18732
1 hour unmanipulated HUVECs	101	105	97	9738	10138	9354
24 hours unmanipulated PC3	167	167	166	18921	18921	18732
24 hours unmanipulated HUVECs	104	98	100	10037	9449	9641

Appendix Table 3.8 The Level Of Fluorescence Emitted By PKH26 Positive PC3 Cells And PKH26 Negative HUVECs Following Co-culture. Pc3 cells were stained with PKH26, as described in the text. These cells were co-cultured with confluent monolayers of HUVECs for 1 hour. Attached, unattached, and unmanipulated cells were collected. Some cells of each population were analysed immediately for their surface expression of CD44 and CD3 by standard FACS analysis. The remaining cells were washed and re-seeded for a further 24 hours before FACS analysis.

Cell Concentration (cells/ml)	Optical Density At 690nm		Optical Density At 620nm		Optical Density At 540nm		Optical Density At 510nm		Optical Density At 492nm		Optical Density At 450nm		Optical Density At 405nm	
0	0.026	0.026	0.028	0.034	0.032	0.037	0.033	0.034	0.030	0.034	0.031	0.037	0.035	0.037
33	0.073	0.048	0.069	0.057	0.056	0.042	0.084	0.060	0.062	0.061	0.055	0.059	0.054	0.045
66	0.029	0.039	0.030	0.040	0.035	0.043	0.037	0.052	0.034	0.049	0.035	0.058	0.040	0.049
132	0.046	0.450	0.047	0.058	0.048	0.059	0.056	0.059	0.048	0.054	0.052	0.053	0.050	0.052
264	0.021	0.058	0.025	0.092	0.029	0.056	0.030	0.077	0.027	0.043	0.028	0.086	0.031	0.049
529	0.340	0.250	0.033	0.027	0.042	0.032	0.042	0.032	0.040	0.030	0.035	0.032	0.038	0.035
1058	0.028	0.027	0.032	0.030	0.038	0.032	0.040	0.037	0.036	0.030	0.041	0.036	0.042	0.036
2115	0.051	0.029	0.038	0.031	0.045	0.033	0.054	0.036	0.040	0.044	0.042	0.043	0.046	0.038
4230	0.027	0.023	0.030	0.025	0.036	0.033	0.038	0.033	0.034	0.031	0.037	0.033	0.041	0.037
8460	0.032	0.026	0.038	0.031	0.042	0.036	0.051	0.039	0.035	0.038	0.041	0.038	0.047	0.044

Appendix Table 3.9 The Optical Density Of A549 Cells Stained With PKH26. Cells were stained with PKH26, as described in the text. Serial dilutions of this suspension were made and were cultured in a flat bottomed 96-well plate overnight. The cells were washed and then lysed with SDS. The optical density was then measured by the TiterTek plate reader at all functional wavelengths.

Patient Identification Number	Prostatic Acid Phosphatase	Prostate Specific Antigen	Cytokeratin-pan	Cytokeratin-8	E-selectin	ICAM-1	VCAM-1	PECAM-1	α4	α5	αL	β1	CD44	CD3
3	6	8	6	6	0	1	0	0	Not Done	Not Done	Not Done	Not Done	0	1
9	4	4	0	6	0	0	0	0	0	0	0	0	0	0
10	6	Not Done	6	6	0	0	0	0	0	0	0	0	0	0
15	6	5	6	6	0	1	0	0	Not Done	0	0	0	1	1
17	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
26	3	3	6	6	0	1	Not Done	1	0	0	0	0	0	1
29	6	2	6	6	0	1	0	0	2	0	0	4	0	0
30	0	Not Done	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
31	6	6	6	6	0	1	0	0	0	0	0	0	0	0
32	6	6	6	6	0	6	2	Not Done	Not Done	0	Not Done	0	0	0
33	6	6	6	6	0	0	0	0	0	0	1	0	4	1
39	6	2	6	6	1	0	1	0	0	1	0	3	0	0
40	6	3	6	5	5	1	1	0	1	0	0	3	3	1
42	6	Not Done	6	6	0	3	3	0	0	0	0	0	0	0
45	6	3	6	6	1	0	0	0	4	3	4	4	3	1
47	0	6	6	0	0	0	0	0	0	0	0	0	0	0
48	6	6	6	0	0	0	1	0	0	1	0	0	0	0
50	6	1	4	0	0	2	2	1	2	0	2	0	4	1
52	6	4	6	0	0	1	0	0	1	0	0	0	0	0
59	6	0	6	0	0	0	0	0	0	0	0	0	0	0
61	6	1	6	6	1	0	Not Done	0	0	0	0	4	0	Not Done
63	6	6	6	4	1	1	1	0	1	0	1	1	2	1
64	0	0	6	0	0	0	0	0	Not Done	Not Done	Not Done	Not Done	Not Done	0
66	5	2	5	5	0	1	0	0	1	0	1	0	0	0
72	6	4	6	6	1	0	1	0	1	0	1	0	3	0
75	6	Not Done	6	6	0	0	0	0	4	4	6	4	Not Done	1
79	0	0	6	3	0	0	0	0	Not Done	0	0	Not Done	0	0
84	6	4	5	6	0	0	0	0	0	0	0	0	0	0
86	6	0	6	0	6	1	0	0	0	Not Done	Not Done	Not Done	Not Done	0
89	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
92	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
93	Not Done	4	6	6	1	1	1	0	3	0	1	0	2	1
95	4	2	6	6	0	0	Not Done	0	0	0	0	0	0	0
96	0	6	6	6	0	0	0	0	1	0	1	0	4	0
97	6	Not Done	6	6	1	1	1	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done	1
98	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
101	0	5	3	6	0	0	0	0	0	0	0	0	0	0
103	4	1	6	6	0	0	0	0	2	0	2	4	0	0
105	6	3	6	6	1	2	1	0	Not Done	Not Done	Not Done	Not Done	0	1
107	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
109	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
110	0	0	0	0	Not Done	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
115	Not Done	6	6	6	1	0	0	0	1	0	0	0	3	1
117	6	4	6	6	Not Done	0	1	Not Done	0	0	0	0	0	0
119	6	2	6	6	Not Done	1	1	0	0	0	1	2	4	0
122	6	2	6	6	0	1	Not Done	0	0	0	0	0	0	2
124	6	4	6	6	0	1	Not Done	0	4	4	4	2	3	1
125	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
129	6	4	6	6	4	1	0	0	0	0	0	0	0	0
132	6	4	6	6	0	1	Not Done	0	Not Done	Not Done	Not Done	Not Done	0	1
133	6	4	6	0	1	1	0	0	1	0	1	2	2	0
135	6	6	6	6	Not Done	0	0	0	1	0	3	4	0	0
136	Not Done	Not Done	6	6	1	0	1	Not Done	1	1	1	1	1	1
141	6	4	6	6	0	2	1	1	2	1	1	0	1	1
Average	3.9216	2.9792	4.4259	4.3200	0.4000	0.7727	0.4211	0.0952	0.9167	0.3947	0.7568	0.8947	1.2927	0.4318
Standard Deviation	2.7484	2.2737	2.5669	2.6220	0.8367	1.0754	0.7215	0.2971	1.2277	1.0277	1.3418	1.4849	1.6162	0.5455
Total Number Examined	51	48	54	50	54	54	47	50	44	47	46	47	50	53
Number = 0	16	12	13	13	31	20	26	38	18	31	22	26	22	26
Number =1	0	3	0	0	13	19	9	4	10	4	10	2	4	17
Number =2	0	6	0	0	0	3	2	0	4	0	2	3	4	17
Number =3	1	4	1	0	0	1	1	0	1	1	0	2	3	1
Number =4	3	10	1	2	0	0	0	0	2	2	2	5	7	0
Number =5	1	2	2	2	1	0	0	0	0	0	0	0	0	0
Number =6	30	11	37	33	0	1	0	0	0	0	1	0	0	0
Number Which Are "Not Applicable"	0	0	0	0	9	9	9	9	9	9	9	9	9	9

Appendix Table 4.1 The Distribution Of Cell Adhesion Molecules In The Epithelial Compartment Of Benign Hyperplastic Prostatic Tissue. Frozen sections of tissue were immunohistochemically analysed with monoclonal antibodies against prostatic acid phosphatase (PAP), prostate specific antigen (PSA), cytokeratin-pan (CK-pan), CK-8, E-selectin, ICAM-1, VCAM-1, PECAM-1, α4, α5, αL, β1, CD44, CD3. Sections were given a score of o to 6, where 0 represented no staining for the relevant antigen and 6 indicated that the entire epithelium was positive.

Patient Identification Number	Prostatic Acid Phosphatase	Prostate Specific Antigen	Cytokeratin-pan	Cytokeratin 8	E-selectin	ICAM-1	VCAM-1	PECAM-1	α4	α5	αL	β1	CD44	CD3
4	6	4	6	6	0	3	0	0	1	0	1	3	2	1
7	3	Not Done	6	6	0	2	1	0	Not Done	0	0	0	4	0
12	4	4	2	2	1	1	0	0	0	0	1	0	2	0
14	2	2	2	6	0	2	1	0	1	0	1	0	1	1
18	6	2	4	6	0	0		0	0	1	1	0	0	1
25	6	3	4	6	0	1	1	Not Done	1	0	0	0	1	0
27	6	Not Done	0	6	0	2	Not Done	0	1	0	1	1	2	0
28	5	6	6	6	1	2	1	1/0	2	0	2	1	1	0
35	6	6	6	6	0	2	1	0	0	Not Done	Not Done	0	0	1
49	6	Not Done	0	0	0	3	0	0	0	0	0	0	4	0
51	6	6	4	6	0	1	Not Done	0	Not Done	Not Done	Not Done	Not Done	Not Done	0
70	0	0	0	0	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable
76	6	Not Done	5	6	1	2	1	1	1	0	0	0	0	0
80	Not Done	6	6	6	0	1	0	0	1	1	0	0	0	0
94	6	1	6	0	0	3	1	0	0	0	0	0	0	1
100	6	3	Not Done	4	1	3	1	Not Done	Not Done	0	Not Done	0	Not Done	0
102	6	1	3	4	1	2	1	1	0	0	0	2	2	0
106	6	1	6	6	1	2	1	1	2	0	2	3	3	1
116	Not Done	Not Done	6	6	0	0	Not Done	0	Not Done	Not Done	Not Done	Not Done	Not Done	2
123	Not Done	2	6	5			0	0	1	0	2	0	0	0
126	6	2	2	5	0	2	0	0	0	0	0	0	0	0
134	6	6	6	6	0	2	0	0	Not Done	Not Done	Not Done	Not Done	Not Done	0
Average	5.1579	3.2353	4.0476	4.7273	0.3000	1.8000	0.5882	0.1667	0.7500	0.1176	0.6875	0.5556	1.3529	0.4286
Standard Deviation	1.7083	2.1074	2.2243	2.1643	0.4702	0.8944	0.5073	0.3835	0.6831	0.3321	0.7932	1.0416	1.3666	0.5976
Total Number Examined	19	17	21	22	20	20	17	18	16	17	16	18	17	21
Number = 0	1	1	3	3	14	2	7	15	6	15	8	16	17	21
Number =1	0	3	0	0	6	4	10	3	8	2	5	2	4	13
Number =2	1	4	3	1	0	10	0	0	2	0	3	1	4	7
Number =3	1	2	1	0	0	4	0	0	0	0	0	2	1	0
Number =4	1	2	3	2	0	0	0	0	0	0	0	0	2	0
Number =5	1	0	2	2	0	0	0	0	0	0	0	0	0	0
Number =6	14	5	9	14	0	0	0	0	0	0	0	0	0	0

Appendix Table 4.2 The Distribution Of Cell Adhesion Molecules in The Epithelial Compartment Of Malignant Prostatic Tissue. Frozen sections of malignant prostatic tissue were immunohistochemically stained with monoclonal antibodies against prostatic acid phosphatase (PAP), prostate specific antigen (PSA), cytokeratin-pan (CK-pan), CK-8, E-selectin, ICAM-1,VCAM-1, α4, α5, αL, β1, CD44, and CD3. Sections were given a score of 0 to 6, depending upon The level of expression of the particular antigen. A score of 0 indicated that the section was negative for a particular antigen, while a score of 6 indicated that 100% of the epithelium was expressed the antigen.

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	242	247	257	40248	42325	46807	43127	3352
0.001	224	275	240	33580	56102	39446	43043	11684
0.01	287	283	297	63303	60806	70006	64705	4757
0.1	318	334	216	86480	101589	30982	73017	37179
1	370	300	263	145946	72151	49720	89272	50346
0, Cells With No Antibody	113	113	110	10988	10988	10662	10879	189
0, Cells With FITC Only	125	ND	ND	12399	NA	NA	12399	NA
0, Cells With MHC Class I Antibody	182	155	153	22004	16769	16435	18403	3124
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	322	342	346	90033	110106	114629	104923	13092
0.001	256	311	369	46338	80598	144484	90473	49813
0.01	419	346	322	238975	114629	90033	147879	79845
0.1	362	376	324	134656	155030	91863	127183	32240
1	249	288	285	43186	63944	62042	56390	11475
0, Cells With No Antibody	113	112	111	10988	10878	10769	10879	109
0, Cells With FITC Only	126	123	126	12524	12152	12524	12400	215
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674
8 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	254	310	311	45415	79791	80598	68601	20084
0.001	266	315	280	51244	83908	58997	64717	17067
0.01	218	267	326	31612	51762	93731	59035	31692
0.1	258	242	221	47280	40248	32581	40036	7352
1	209	237	240	28875	38273	39446	35531	5795
0, Cells With No Antibody	105	111	110	10138	10769	10662	10523	338
0, Cells With FITC Only	123	119	122	12152	11672	12030	11951	249
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674
12 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	231	248	253	36030	42753	44960	41248	4651
0.001	213	216	204	30061	30982	27458	29500	1828
0.01	189	219	221	23610	31932	32581	29374	5002
0.1	240	208	231	39446	28585	36030	34687	5554
1	175	214	219	20508	30365	31932	27601	6193
0, Cells With No Antibody	118	115	120	11555	11212	11790	11519	291
0, Cells With FITC Only	127	128	125	12651	12779	12399	12610	193
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Vaules**			**Mean MESF**	**SD Of Mean MESF**
0	189	178	209	23610	21136	28875	24540	3952
0.001	179	168	181	21350	19113	21784	20749	1434
0.01	195	197	223	25080	25590	33243	27971	4573
0.1	159	200	195	17458	26374	25080	22971	4818
1	201	217	223	26641	31295	33243	30393	3392
0, Cells With No Antibody	112	111	116	10878	10769	11325	10991	295
0, Cells With FITC Only	120	120	119	11790	11790	11672	11751	68
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

Appendix Table 5.2.1a The Expression Of CD44 By Prostatic Adenocarcinoma Cells, PC3, When Incubated With Varying Concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) for 2, 4, 8, 12, and 24 Hours.

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	208	181	186	28585	21784	22908	24426	3646
0.001	190	187	196	23849	23140	25334	24108	1119
0.01	195	196	188	25080	25334	23374	24596	1066
0.1	204	193	201	27458	24580	26641	26226	1483
1	191	178	178	24090	21136	21136	22121	1706
0, Cells With No Antibody	103	106	105	9936	10241	10138	10105	155
0, Cells With FITC Only	112	118	117	10878	11555	11440	11291	362
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	204	194	194	27458	24829	24829	25705	1518
0.001	195	181	197	25080	21784	25590	24151	2066
0.01	193	171	183	24580	19698	22227	22168	2441
0.1	181	174	171	21784	20302	19698	20595	1073
1	182	180	179	22004	21566	21350	21640	333
0, Cells With No Antibody	101	102	109	9738	9837	10555	10043	446
0, Cells With FITC Only	111	105	118	10769	10138	11555	10821	710
0, Cells With MHC Class 1 Antibody	396	386	420	189595	171444	241392	200810	36298
8 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	166	161	154	18732	17812	16601	17715	1069
0.001	147	154	147	15472	16601	15472	15848	652
0.01	148	151	151	15628	16107	16107	15947	277
0.1	157	153	154	17110	16435	16601	16715	352
1	141	131	148	14565	13171	15628	14455	1232
0, Cells With No Antibody	101	110	117	9738	10662	11440	10613	852
0, Cells With FITC Only	105	102	108	10138	9837	10449	10141	306
0, Cells With MHC Class I Antibody	393	373	366	183957	150419	140187	158188	22896
12 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	185	185	155	22679	22679	16769	20709	3412
0.001	178	168	187	21136	19113	23140	21130	2014
0.01	185	177	188	22679	20924	23374	22326	1262
0.1	180	179	175	21566	21350	20508	21141	559
1	175	163	175	20508	18175	20508	19730	1347
0, Cells With No Antibody	94	98	95	9076	9449	9168	9231	194
0, Cells With FITC Only	134	116	128	13574	11325	12779	12559	1140
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
0	218	222	189	31612	32910	23610	29378	5037
0.001	206	202	215	28016	26910	30672	28533	1933
0.01	175	209	211	20508	28875	29462	26281	5009
0.1	225	209	149	33919	28875	15786	26193	9359
1	147	194	220	15472	24829	32255	24185	8410
0, Cells With No Antibody	107	94	111	10344	9076	10769	10063	881
0, Cells With FITC Only	133	157	148	13438	17110	15628	15392	1847
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

Appendix Table 5.2.1b The Expression Of CD44 By Du145 When Incubated With Varying Concentrations of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 24 hours. Cells were seeded in 24-well TCGPs and cultured until 90-100% confluent. 200µl of the appropriate GM-CSF-supplemented ECLM was added to the wells in triplicate. Median levels of fluorescence were converted into MESF values as described in 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

Concentration Of GM-CSF Of ECLM (ng/ml)

2 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	320	333	301	88238	100572	72881	87231	13873
0.001	338	298	312	105762	70714	81413	85963	17962
0.01	277	318	314	57243	86480	83068	75597	15987
0.1	342	336	341	110106	103655	109004	107588	3451
1	312	332	305	81413	99565	75875	85618	12392
0, Cells With No Antibody	111	109	113	10769	10555	10988	10771	217
0, Cells With FITC	121	126	124	11910	12524	12275	12236	309
0, Cells With MHC Class I Antibody	182	155	153	22004	16769	16435	18403	3124

4 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	324	329	338	91863	96604	105762	98076	7066
0.001	333	333	315	100572	100572	83908	95017	9621
0.01	325	323	320	92792	90943	88238	90658	2290
0.1	319	305	317	87255	75875	85614	82948	6187
1	324	322	342	91863	90033	110106	97334	11099
0, Cells With No Antibody	114	109	114	11099	10555	11099	10918	314
0, Cells With FITC	125	125	123	12399	12399	12152	12316	143
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674

8 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	338	327	321	105762	94679	89131	96524	8468
0.001	334	343	343	101589	111220	111220	108010	5560
0.01	319	333	322	87355	100572	90033	92653	6987
0.1	257	276	280	46807	56669	58997	54158	3472
1	281	256	236	19594	46338	37890	47941	10940
0, Cells With No Antibody	112	112	113	10878	10878	10988	10915	64
0, Cells With FITC	124	123	122	12275	12152	12030	12152	122
0, Cells With MHC Class I Antibody	183	165	171	22227	18544	19698	20156	1884

12 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	316	266	328	84757	51244	95636	77212	23138
0.001	278	269	323	57822	52815	90943	67193	20720
0.01	301	272	288	72881	54434	63944	63753	9225
0.1	260	262	232	48241	49222	36395	44619	7140
1	264	308	298	50223	78201	70714	66379	14484
0, Cells With No Antibody	102	103	104	9837	9936	10037	9937	100
0, Cells With FITC	110	106	106	10662	10241	10241	10381	243
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

24 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	190	148	180	23849	15628	21566	20348	4244
0.001	170	151	161	19501	16107	17812	17807	1697
0.01	241	253	300	30365	44960	72151	49159	21207
0.1	175	154	314	20508	16601	83068	40059	37298
1	197	175	183	25590	20508	22227	22775	2585
0, Cells With No Antibody	100	99	103	9641	9544	9936	9707	204
0, Cells With FITC	105	104	100	10138	10037	9641	9939	263
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

Appendix Table 5.2.2a The Expression Of Intercellular Cell Adhesion Molecule-1 (ICAM-1) for Prostatic Adenocarcinoma Cells, PC3, When Incubated With varying concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) For 2, 4, 8, 12, and 24 Hours.

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	442	451	461	301216	329772	364687	331892	31789
0.001	457	440	447	350298	295214	316761	320758	27759
0.01	456	457	446	346790	350298	313589	336892	20258
0.1	450	439	433	326470	292258	275133	297953	26138
1	440	457	444	295214	350298	307340	317617	28945
0, Cells With No Antibody	100	100	106	9641	9641	10241	9841	346
0, Cells With FITC Only	116	122	122	11325	12030	12030	11795	407
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298

4 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	443	438	444	304263	289331	307340	300311	9633
0.001	428	436	446	261631	283566	313589	286262	26084
0.01	447	445	454	316761	310449	339880	322363	15495
0.1	456	451	453	346790	329772	336477	337680	8573
1	452	448	441	333108	319965	298200	317091	17630
0, Cells With No Antibody	105	104	109	10138	10037	10555	10243	274
0, Cells With FITC Only	111	142	121	10769	14712	11910	12464	2029
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298

8 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	335	267	273	102617	51762	54984	69788	28476
0.001	297	250	240	70006	43623	39446	51025	16570
0.01	253	265	288	44960	50731	63944	53211	9732
0.1	296	335	315	69305	102617	83908	85277	16698
1	335	291	329	102617	65904	96604	88375	19691
0, Cells With No Antibody	98	84	95	9449	8207	9168	8941	651
0, Cells With FITC Only	104	99	98	10037	9544	9449	9677	316
0, Cells With MHC Class I Antibody	393	373	366	183957	150419	140187	158188	22896

12 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	470	436	449	399260	283566	323201	335342	58795
0.001	438	439	418	289331	292258	236582	272724	31334
0.01	428	416	438	261631	231868	289331	260943	28738
0.1	430	447	443	266950	316761	304263	295991	25915
1	445	443	440	310449	304263	295214	303308	7662
0, Cells With No Antibody	96	100	81	9260	9641	7963	8955	880
0, Cells With FITC Only	134	137	135	13574	13990	13712	13759	212
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

24 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	394	424	397	185817	251308	191513	209546	36279
0.001	359	402	354	130651	201396	124240	152096	42816
0.01	382	391	368	164679	180291	143037	162669	18708
0.1	415	398	424	229546	193450	251308	224768	29223
1	442	417	418	301216	234213	236582	257337	38019
0, Cells With No Antibody	108	103	77	10449	9936	7649	9345	1491
0, Cells With FITC Only	133	126	137	13438	12524	13990	13318	740
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

Appendix Table 5.2.2b The Expression Of Intercellular Cell Adhesion Molecule-1 (ICAM-1) By Du145 When Incubated With Varying Concentrations of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 24 hours. Cells were seeded in 24-well TCGPs and cultures until 90-100% confluent. 200µl of the appropriate GM-CSF-supplemented ECLM was added to the wells in triplicate. Median levels of fluorescence were converted to MESF values as described in 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; SD, standard deviation; TCGP, tissue culture grade plate.)

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	273	206	274	54984	28016	55540	46180	15733
0.001	260	260	244	48241	48241	41067	45850	4142
0.01	255	236	250	45874	37890	43623	42462	4117
0.1	267	231	235	51762	36030	37510	41768	8687
1	242	236	233	40248	37890	36763	38300	1779
0, Cells With No Antibody	83	85	89	8125	8290	8631	8348	258
0, Cells With FITC only	111	108	104	10769	10449	10037	10418	367
0, Cells With MHC Class I Antibody	182	155	153	22004	16769	16435	18403	3124
4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	253	274	270	44960	55540	53349	51283	5585
0.001	244	235	257	41067	37510	46807	41795	4691
0.01	261	251	234	48729	44064	37135	43309	5834
0.1	254	254	252	45415	45415	44510	45113	522
1	252	240	241	44510	39446	39845	41267	2815
0, Cells With No Antibody	82	83	85	8043	8125	8290	8153	126
0, Cells With FITC only	107	105	89	10344	10138	8631	9704	936
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674
8 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	285	289	282	62042	64590	60197	62276	2206
0.001	247	267	265	42325	51762	50731	48273	5176
0.01	270	254	265	53349	45415	50731	49831	4043
0.1	270	242	263	53349	40248	49720	47772	6764
1	261	268	265	48729	52286	50731	50582	1783
0, Cells With No Antibody	84	84	86	8207	8207	8374	8263	96
0, Cells With FITC only	102	104	104	9837	10037	10037	9970	115
0, Cells With MHC Class I Antibody	183	165	171	22227	18544	19698	20156	1884
12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	262	240	276	49222	39446	56669	48446	8638
0.001	213	246	254	30061	41902	45415	39126	8045
0.01	232	229	225	36395	35313	33919	35209	1241
0.1	269	275	249	52815	56102	43186	50701	6713
1	221	237	347	32581	38273	115789	62214	46484
0, Cells With No Antibody	91	98	91	8806	9449	8806	9020	371
0, Cells With FITC only	105	103	104	10138	9936	10037	10037	101
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489
24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	258	257	263	47280	46807	49720	47936	1563
0.001	228	230	220	34959	35670	32255	34294	1802
0.01	225	226	223	33919	34262	33243	33808	518
0.1	282	242	276	60197	40248	56669	52372	10646
1	225	227	231	33919	34609	36030	34853	1077
0, Cells With No Antibody	81	81	81	7963	7963	7963	7963	0
0, Cells With FITC only	82	90	93	8043	8718	8985	8582	485
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

Appendix Table 5.2.3a The Expression Of Alpha-5 By Prostatic Adenocarcinoma Cells, PC3, When Incubated With varying concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) For 2, 4, 8, 12, and 24 Hours.

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	435	425	424	280726	253850	251308	261961	16301
0.001	414	406	403	227248	209669	203433	213450	12349
0.01	415	416	399	229546	231868	195407	218940	20414
0.1	394	415	407	185817	229546	211790	209051	21993
1	408	418	407	213932	236582	211790	220768	13737
0, Cells With No Antibody	106	101	116	10241	9738	11325	10435	811
0, Cells With FITC Only	202	157	153	26910	17110	16435	20152	5863
0, Cells With MHC Class I Antibody	329	335	332	96604	102617	99565	99595	3007
4 Hours	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	411	417	417	220489	234213	234213	229639	7924
0.001	382	381	403	164679	163030	203433	177048	22866
0.01	382	400	393	164679	197383	183957	182006	16439
0.1	385	400	406	169727	197383	209669	192260	20458
1	403	405	415	203433	207570	229546	213516	14035
0, Cells With No Antibody	163	107	105	18175	10344	10138	12886	4581
0, Cells With FITC Only	138	139	137	14132	14275	13990	14132	142
0, Cells With MHC Class I Antibody	370	315	325	145946	83908	92792	107549	33548
8 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	409	423	429	216096	248792	264277	243055	24598
0.001	400	414	418	197383	227248	236582	220404	20476
0.01	393	405	401	183957	207570	199380	196969	11990
0.1	411	423	414	220489	248792	227248	232176	14781
1	407	415	420	211790	229546	241392	227576	14899
0, Cells With No Antibody	104	104	104	10037	10037	10037	10037	0
0, Cells With FITC Only	127	105	117	12651	10138	11440	11410	1257
12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	436	426	428	283566	256417	261631	267205	14407
0.001	379	397	379	159782	191513	159782	170359	18320
0.01	387	369	411	173178	144484	220489	179384	38381
0.1	410	398	412	218282	193450	222720	211484	15774
1	413	420	420	224972	241392	241392	235919	9480
0, Cells With No Antibody	112	105	93	10878	10138	8985	10001	954
0, Cells With FITC Only	127	125	134	12651	12399	13574	12875	619
24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	423	426	423	248792	256417	248792	251333	4403
0.001	411	402	404	220489	201396	205491	209126	10052
0.01	418	411	399	236582	220489	195407	217493	20751
0.1	417	398	402	234213	193450	201396	209687	21609
1	391	389	409	180291	176699	216096	191029	21783
0, Cells With No Antibody	112	113	110	10878	10988	10662	10843	166
0, Cells With FITC Only	122	128	121	12030	12779	11910	12240	471
0, Cells With MHC Class I Antibody	315	341	337	83908	109004	104703	99205	13421

Appendix Table 5.2.3b The Expression Of Alpha-5 By Du145 When Incubated With Varying Concentrations of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 24 hours. Cells were seeded in 24-well TCGPs and cultured until 90-100% confluent. 200μl of GM-CSF-supplemented ECLM was added to the wells in triplicate. Median level of fluorescence were converted to MESF values as described in 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; SD, standard deviation; TCGP, tissue culture grade plate.)

GM-CSF Concentration Of ECLM (ng/ml)								
2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	425	416	425	253850	231868	253850	246523	12691
0.001	443	437	438	304263	286434	289331	293343	9567
0.01	433	417	442	275133	234213	301216	270187	33774
0.1	431	429	420	269650	264277	241392	258440	15006
1	442	415	442	301216	229546	301216	277326	41378
0, Cells With No Antibody	115	116	112	11212	11325	10878	11138	232
0, Cells With FITC Only	120	122	127	11790	12030	12651	12157	444
0, Cells With MHC Class I Antibody	182	155	153	22004	16769	16435	18403	3124
4 Hours	Median Level Of Fluorescence			Corresponding MESF Vaules			Mean MESF	SD Of Mean MESF
0	439	424	434	292258	251308	277915	273827	20779
0.001	435	446	432	280726	313589	272378	288898	21787
0.01	434	436	450	277915	283566	326470	295984	26553
0.1	452	453	448	333108	336477	319965	329850	8725
1	413	431	429	224972	269650	264277	252967	24392
0, Cells With No Antibody	116	112	115	11325	10878	11212	11138	232
0, Cells With FITC Only	118	113	119	11555	10988	11672	11405	366
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674
8 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	289	367	362	64590	141605	134656	113617	42600
0.001	280	289	325	58997	64590	92792	72127	18114
0.01	233	212	260	36763	29760	48241	38255	9331
0.1	308	269	226	78201	52815	34262	55093	22058
1	386	388	308	171444	174929	78201	141525	54868
0, Cells With No Antibody	103	104	107	9936	10037	10344	10106	213
0, Cells With FITC Only	110	109	116	10662	10555	11325	10847	417
0, Cells With MHC Class I Antibody	183	165	171	22227	18544	19698	20156	1884
12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	393	414	347	183957	227248	115789	175664	56190
0.001	369	428	403	144484	261631	203433	203183	58574
0.01	374	408	429	151940	213932	264277	210050	56269
0.1	420	374	374	241392	151940	151940	181758	51645
1	347	153	154	115789	16435	16601	49608	57314
0, Cells With No Antibody	113	113	118	10988	10988	11555	11177	327
0, Cells With FITC Only	123	148	124	12152	15628	12275	13352	1973
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489
24 Hours	Median Level Of Fluorescence			Corresponding MESF Vaules			Mean MESF	SD Of Mean MESF
0	373	410	357	150419	218282	128048	165583	46989
0.001	326	352	401	93731	121764	199380	138292	54729
0.01	411	370	414	220489	145946	227248	197894	45116
0.1	311	325	365	80598	92792	138783	104058	30685
1	404	298	412	205491	70714	222720	166308	83234
0, Cells With No Antibody	112	114	115	10878	11099	11212	11063	170
0, Cells With FITC Only	116	116	118	11325	11325	11555	11402	133
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

Appendix Table 5.2.4a The Expression Of Beta-1 By Prostatic Adenocarcinoma Cells, PC3, When Incubated With Varying Concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) For 2, 4, 8, 12, and 24 Hours.

GM-CSF Concentration Of ECLM (ng/ml)								
2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	350	417	354	119338	234213	124240	159264	64955
0.001	363	382	423	136018	164679	248792	183163	58615
0.01	380	370	404	161398	145946	205491	170945	30900
0.1	395	423	369	187697	248792	144484	193658	52409
1	395	420	411	187697	241392	220489	216526	27066
0, Cells with No Antibody	108	99	99	10449	9544	9544	9846	522
0, Cells With No FITC	118	119	109	11555	11672	10555	11261	614
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298
4 Hours	Median Level Of Fluorescence			Corresponding MESF Vaules			Mean MESF	SD Of Mean MESF
0	429	428	420	264277	261631	241392	255767	12519
0.001	443	373	407	304263	150419	211790	222157	77444
0.01	438	328	433	289331	95636	275133	220033	107965
0.1	437	431	433	286434	269650	275133	277072	8558
1	425	413	416	253850	224972	231868	236897	15081
0, Cells with No Antibody	108	99	99	10449	9544	9544	9846	522
0, Cells With No FITC	118	119	109	11555	11672	10555	11261	614
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298
8 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	358	325	427	129343	92792	259011	160382	87348
0.001	488	391	430	478551	180291	266950	308597	153429
0.01	442	393	403	301216	183957	203433	229535	62836
0.1	405	361	358	207570	133308	129343	156740	44064
1	321	298	387	89131	70714	173178	111007	54623
0, Cells with No Antibody	109	112	105	10555	10878	10138	10524	371
0, Cells With No FITC	127	120	126	12651	11790	12524	12322	465
0, Cells With MHC Class I Antibody	393	373	366	183957	150419	140187	158188	22896
12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	463	442	452	372102	301216	333108	335475	35502
0.001	465	412	317	379667	222720	85614	229334	147138
0.01	342	355	382	110106	125496	164679	133427	28138
0.1	424	390	326	251308	178486	93731	174508	78864
1	449	436	449	323566	283566	323201	309989	22883
0, Cells with No Antibody	77	91	99	7649	8806	9544	8666	955
0, Cells With No FITC	94	96	104	9076	9260	10037	9458	510
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816
24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	420	389	414	241392	176699	227248	215113	34011
0.001	459	452	441	357420	333108	298200	329576	29768
0.01	445	412	403	310449	222720	203433	245534	57039
0.1	462	431	443	368376	269650	304263	314096	50092
1	387	399	363	173178	195407	136018	168201	30006
0, Cells with No Antibody	77	91	99	7649	8806	9544	8666	955
0, Cells With No FITC	94	96	104	9076	9260	10037	9458	510
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

Appendix Table 5.2.4b The Expression Of Beta-1 By Du145 When Incubated With Varying Concentrations Of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 24 hours. Cells were seeded in 24-well TCGPs and cultured until 90-100% confluent. 200µl of the appropriate GM-CSF-supplemented CLM was added to the wells in triplicate. Median levels of fluorescence were converted to MESF values as described in 2.4.2.4 (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; MESF, moleclular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	141	143	151	14561	14857	16102	15173	818
0.001	142	138	137	14708	14128	13986	14274	382
0.01	136	130	132	13846	13035	13300	13394	414
0.1	137	132	133	13986	13300	13434	13573	364
1	131	132	130	13167	13300	13035	13167	133
0, Cells With No Antibody	121	118	122	11906	11552	12026	11828	247
0, Cells With FITC Only	130	130	131	13035	13035	13167	13079	76
0, Cells with MHC Class 1 Antibody	182	155	153	21998	16764	16430	18397	3123

4 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	135	131	133	13707	13167	13434	13436	270
0.001	131	133	131	13167	13434	13167	13256	155
0.01	132	129	133	13300	12904	13434	13213	276
0.1	154	131	135	16596	13167	13707	14490	1844
1	133	131	130	13434	13167	13035	13212	204
0, Cells With No Antibody	120	121	127	11787	11906	12647	12113	466
0, Cells With FITC Only	130	131	130	13035	13167	13035	13079	76
0, Cells with MHC Class 1 Antibody	164	157	163	18353	17104	18169	17876	674

8 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	132	131	133	13300	13167	13434	13300	134
0.001	126	131	130	12520	13167	13035	12907	341
0.01	134	133	133	13570	13434	13434	13480	78
0.1	135	135	130	13707	13707	13035	13483	388
1	134	133	130	13570	13434	13035	13346	278
0, Cells With No Antibody	118	120	120	11552	11787	11787	11709	136
0, Cells With FITC Only	135	133	132	13707	13434	13300	13480	208
0, Cells with MHC Class 1 Antibody	183	165	171	22220	18539	19692	20150	1883

24 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	127	128	129	12647	12775	12904	12775	129
0.001	125	127	126	12395	12647	12520	12521	126
0.01	128	124	124	12775	12271	12271	12439	291
0.1	128	129	127	12775	12904	12647	12775	129
1	127	131	125	12647	13167	12395	12736	393
0, Cells With No Antibody	119	119	116	11669	11669	11322	11553	200
0, Cells With FITC Only	130	127	129	13035	12647	12904	12862	197
0, Cells with MHC Class 1 Antibody	193	164	166	24573	18353	18726	20551	3488

Appendix Table 5.2.5a The Expression Of Vascular Cell Adhesion Molecule-1 (VCAM-1) By Prostatic Adenocarcinoma Cells, PC3, When Incubated With Varying Concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) For 2, 4, 8, and 24 Hours

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	116	117	113	11325	11440	10988	11251	235
0.001	120	118	117	11790	11555	11440	11595	179
0.01	116	120	116	11325	11790	11325	11480	269
0.1	119	123	117	11672	12152	11440	11755	363
1	111	107	110	10769	10344	10662	10592	221
0, Cells With No Antibody	103	106	105	9936	10241	10138	10105	155
0, Cells With FITC Only	112	114	108	10878	11099	10449	10809	331
0, Cells with MHC Class 1 Antibody	396	386	420	189595	171444	241392	200810	36298

4 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	112	115	109	10878	11212	10555	10882	328
0.001	106	109	108	10241	10555	10449	10415	160
0.01	105	110	106	10138	10662	10241	10347	277
0.1	110	111	115	10662	10769	11212	10881	292
1	111	108	106	10769	10449	10241	10486	266
0, Cells With No Antibody	103	106	105	9936	10241	10138	10105	155
0, Cells With FITC Only	102	97	106	9837	9354	10241	9811	444
0, Cells with MHC Class 1 Antibody	396	386	420	189595	171444	241392	200810	36298

8 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	119	119	121	11672	11672	11910	11751	137
0.001	117	121	107	11440	11910	10344	11231	803
0.01	116	116	110	11325	11325	10662	11104	383
0.1	104	116	111	10037	11325	10769	10710	646
1	112	117	118	10878	11440	11555	11291	362
0, Cells With No Antibody	103	106	105	9936	10241	10138	10105	155
0, Cells With FITC Only	103	112	112	9936	10878	10878	10564	544
0, Cells with MHC Class 1 Antibody	393	373	366	183957	150419	140187	158188	22896

12 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	135	130	136	13712	13039	13850	13533	434
0.001	127	130	131	12651	13039	13171	12953	270
0.01	126	129	125	12524	12908	12399	12610	265
0.1	132	129	137	13304	12908	13990	13401	548
1	127	122	124	12651	12030	12275	12319	313
0, Cells With No Antibody	103	106	105	9936	10241	10138	10105	155
0, Cells With FITC Only	135	128	121	13712	12779	11910	12800	901
0, Cells with MHC Class 1 Antibody	356	383	359	126766	166345	130651	141254	21816

24 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	137	139	145	13990	14275	15163	14476	612
0.001	125	129	130	12399	12908	13039	12782	338
0.01	126	128	135	12524	12779	13712	13005	625
0.1	114	129	110	11099	12908	10662	11556	1191
1	132	127	132	13304	12651	13304	13086	377
0, Cells With No Antibody	103	106	105	9936	10241	10138	10105	155
0, Cells With FITC Only	133	120	130	13438	11790	13039	12756	860
0, Cells with MHC Class 1 Antibody	356	383	359	126766	166345	130651	141254	21816

Appendix Table 5.2.5b The Expression Of Vascular Cell Adhesion Molecule-1 (VCAM-1) By Du145 When Incubated With Varying Concentrations of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 4 hours. Cells were seeded in 24-well TCGPs and cultured imtil 90-100% confluent. 200μl of the appropriate GM-CSF-supplemented ECLM was added to the wells in triplicate. Median Levels of fluorescence were converted to MESF values as described in 2.4.2.4. (ECLM, established cell lone medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatablilty complex; SD, standard deviation; TCGP, tissue culture grade plate.)

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	135	138	139	13712	14132	14275	14039	293
0.001	136	137	138	13850	13990	14132	13991	141
0.01	142	141	141	14712	14565	14565	14614	85
0.1	142	143	141	14712	14861	14565	14713	148
1	134	136	134	13574	13850	13574	13666	159
0, Cells With No Antibody	115	120	121	11212	11790	11910	11637	373
0, Cells With FITC Only	134	131	133	13574	13171	13438	13394	205
0, Cells With MHC Class I Antibody	182	155	153	22004	16769	16435	18403	3124

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	135	136	134	13712	13850	13574	13712	138
0.001	137	133	135	13990	13438	13712	13713	276
0.01	136	135	133	13850	13712	13438	13667	210
0.1	136	136	141	13850	13850	14565	14089	413
1	132	130	131	13304	13039	13171	13171	133
0, Cells With No Antibody	121	118	118	11910	11555	11555	11673	204
0, Cells With FITC Only	132	133	132	13304	13438	13304	13349	78
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674

8 Hours	Median Level Of Fluorescence			Corresponding MESF Valuse			Mean MESF	SD Of Mean MESF
0	129	130	134	12908	13039	13574	13174	353
0.001	131	133	135	13171	13438	13712	13440	271
0.01	137	132	133	13990	13304	13438	13577	364
0.1	138	138	135	14132	14132	13712	13992	243
1	129	130	128	12908	13039	12779	12909	130
0, Cells With No Antibody	119	121	118	11672	11910	11555	11712	180
0, Cells With FITC Only	131	141	143	13171	14565	14861	14199	903
0, Cells With MHC Class I Antibody	183	165	171	22227	18544	19698	20156	1884

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	135	126	129	13712	12524	12908	13048	606
0.001	128	128	129	12779	12779	12908	12822	75
0.01	127	127	128	12651	12651	12779	12694	74
0.1	128	130	131	12779	13039	13171	12996	199
1	128	129	128	12779	12908	12779	12822	75
0, Cells With No Antibody	119	119	117	11672	11672	11440	11595	134
0, Cells With FITC Only	123	123	127	12152	12152	12651	12318	288
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

Appendix Table 5.2.6a The Expression Of Alpha-4 By Prostatic Adenocarcinoma Cells, PC3, When Incubated With Varying Concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) For 2, 4, 8, and 24 Hours.

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	157	157	160	17110	17110	17634	17284	303
0.001	151	157	158	16107	17110	17283	16833	635
0.01	158	156	159	17283	16938	17458	17226	264
0.1	160	158	153	17634	17283	16435	17117	617
1	162	161	163	17993	17812	18175	17993	181
0, Cells Witn No Antibody	87	99	99	8459	9544	9544	9182	627
0, Cells With FITC Only	118	119	109	11555	11672	10555	11261	614
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298

4 Hours	Corresponding MESF Valuess						Mean MESF	SD Of Mean MESF
0	171	166	161	19698	18732	17812	18748	943
0.001	167	169	170	18921	19306	19501	19243	295
0.01	166	168	158	18732	19113	17283	18376	965
0.1	153	161	158	16435	17812	17283	17177	695
1	161	165	155	17812	18544	16769	17708	892
0, Cells Witn No Antibody	87	99	99	8459	9544	9544	9182	627
0, Cells With FITC Only	118	119	109	11555	11672	10555	11261	614
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298

8 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	168	157	162	19113	17110	17993	18072	1004
0.001	169	163	159	19306	18175	17458	18313	932
0.01	164	159	158	18358	17458	17283	17700	577
0.1	164	149	156	18358	15786	16938	17028	1288
1	164	148	155	18358	15628	16769	16918	1371
0, Cells Witn No Antibody	109	112	105	10555	10878	10138	10524	371
0, Cells With FITC Only	127	120	126	12651	11790	12524	12322	465
0, Cells With MHC Class I Antibody	393	373	366	183957	150419	140187	158188	22896

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	242	211	218	40248	29462	31612	33774	5709
0.001	211	212	210	29462	29760	29167	29463	297
0.01	201	213	210	26641	30061	29167	28623	1774
0.1	237	226	234	38273	34262	37135	36557	2067
1	218	162	165	31612	17993	18544	22716	7709
0, Cells Witn No Antibody	77	91	99	7649	8806	9544	8666	955
0, Cells With FITC Only	94	96	104	9076	9260	10037	9458	510
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	251	243	227	44064	40655	34609	39776	4788
0.001	225	201	208	33919	26641	28585	29715	3768
0.01	201	199	207	26641	26110	28299	27017	1142
0.1	235	206	254	37510	28016	45415	36980	8711
1	227	211	230	34609	29462	35670	33247	3321
0, Cells Witn No Antibody	77	91	99	7649	8806	9544	8666	955
0, Cells With FITC Only	94	96	104	9076	9260	10037	9458	510
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

Appendix Table 5.2.6b The Expression Of Alpha-4 By Du145 When Incubated With Varying Concentrations of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 24 hours. Cells were seeded in 24-well TCGPs and cultured until 90-100% confluent. 200μl of the appropriate GM-CSF-supplimented ECLM was added to the wells in triplicate. Median levels of fluorescence were converted to MESF values as described in 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; SD, standard deviation; TCGP, tissue culture grade plate.)

GM-CSF Concentration Of ECLM (ng/ml)

2 Hours

	Median Level Of Fluorescence			Corresponding MESF Vaues			Mean MESF	SD Of Mean MESF
0	111	117	115	10769	11440	11212	11140	341
0.001	113	111	112	10988	10769	10878	10879	109
0.01	113	114	116	10988	11099	11325	11138	172
0.1	117	116	113	11440	11325	10988	11251	235
1	115	114	117	11212	11099	11440	11250	173
0, Cells With No Antibody	113	110	106	10988	10662	10241	10630	375
0, Cells With FITC Only	112	116	116	10878	11325	11325	11176	258
0, Cells With MHC Class I Antibody	182	155	153	22004	16769	16435	18403	3124

4 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	117	112	121	11440	10878	11910	11409	516
0.001	117	118	119	11440	11555	11672	11556	116
0.01	120	118	119	11790	11555	11672	11673	117
0.1	118	119	119	11555	11672	11672	11633	67
1	117	118	120	11440	11555	11790	11595	179
0, Cells With No Antibody	112	114	113	10878	11099	10988	10989	111
0, Cells With FITC Only	117	119	118	11440	11672	11555	11556	116
0, Cells With MHC Class I Antibody	164	157	163	18358	17110	18175	17881	674

8 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	113	116	122	10988	11325	12030	11448	532
0.001	123	119	123	12152	11672	12152	11992	277
0.01	127	127	125	12651	12651	12399	12567	146
0.1	127	123	125	12651	12152	12399	12400	250
1	127	117	126	12651	11440	12524	12205	666
0, Cells With No Antibody	111	109	109	10769	10555	10555	10626	124
0, Cells With FITC Only	117	119	117	11440	11672	11440	11517	134
0, Cells With MHC Class I Antibody	183	165	171	22227	18544	19698	20156	1884

12 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	146	144	151	15317	15011	16107	15478	565
0.001	139	144	146	14275	15011	15317	14868	536
0.01	145	144	139	15163	15011	14275	14817	475
0.1	157	146	143	17110	15317	14861	15762	1189
1	143	141	150	14861	14565	15946	15124	727
0, Cells With No Antibody	109	115	110	10555	11212	10662	10809	353
0, Cells With FITC Only	127	124	124	12651	12275	12275	12400	217
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

24 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	141	141	141	14565	14565	14565	14565	0
0.001	134	144	134	13574	15011	13574	14053	830
0.01	136	134	139	13850	13574	14275	13900	353
0.1	141	138	130	14565	14132	13039	13912	787
1	130	13	131	13039	4017	13171	10075	5247
0, Cells With No Antibody	123	123	126	12152	12152	12524	12276	215
0, Cells With FITC Only	110	109	116	10662	10555	11325	10847	417
0, Cells With MHC Class I Antibody	193	164	166	24580	18358	18732	20557	3489

Appendix Table 5.2.7a The Expression Of Alpha-L By Prostatic Adenocarcinoma Cells, PC3, When Incubated With Varying Concentrations Of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) For 2, 4, 8, 12, and 24 Hours.

GM-CSF Concentration (ng/ml)

2 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	120	122	119	11790	12030	11672	11831	182
0.001	114	112	116	11099	10878	11325	11101	223
0.01	115	111	111	11212	10769	10769	10917	255
0.1	113	110	112	10988	10662	10878	10843	166
1	110	112	115	10662	10878	11212	10917	277
0, Cells With No Antibody	107	94	111	10344	9076	10769	10063	881
0, Cells With FITC Only	88	84	107	8544	8207	10344	9032	1149
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298

4 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	112	120	119	10878	11790	11672	11447	496
0.001	114	106	89	11099	10241	8631	9990	1253
0.01	114	108	107	11099	10449	10344	10631	409
0.1	111	105	108	10769	10138	10449	10452	316
1	115	119	114	11212	11672	11099	11328	304
0, Cells With No Antibody	107	94	111	10344	9076	10769	10063	881
0, Cells With FITC Only	107	102	107	10344	9837	10344	10175	293
0, Cells With MHC Class I Antibody	396	386	420	189595	171444	241392	200810	36298

8 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	113	114	122	10988	11099	10662	10916	228
0.001	111	113	105	10769	10988	10138	10632	441
0.01	110	102	108	10662	9837	10449	10316	428
0.1	110	103	95	10662	9936	9168	9922	747
1	110	114	113	10662	11099	10988	10916	228
0, Cells With No Antibody	107	94	111	10344	9076	10769	10063	881
0, Cells With FITC Only	94	97	73	9076	9354	7347	8592	1087
0, Cells With MHC Class I Antibody	393	373	366	183957	150419	140187	158188	22896

12 Hours

	Median Level Of Fluorescene			Corresponding MESF Vaules			Mean MESF	SD Of Mean MESF
0	139	131	134	14275	13171	13574	13673	559
0.001	129	138	124	12908	14132	12275	13105	944
0.01	125	128	119	12399	12779	11672	12283	562
0.1	129	123	121	12908	12152	11910	12323	521
1	144	132	130	15011	13304	13039	13785	1071
0, Cells With No Antibody	107	94	111	10344	9076	10769	10063	881
0, Cells With FITC Only	140	128	130	14419	12779	13039	13412	882
0, Cells With MHC Class I Antibody	356	383	359	126345	166345	130651	141254	21816

24 Hours

	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
0	146	144	148	15317	15011	15628	15319	308
0.001	126	147	142	12524	15472	14712	14236	1530
0.01	131	135	136	13171	13712	13850	13577	359
0.1	137	133	136	13990	13438	13850	13760	287
1	136	135	135	13850	13712	13712	13758	80
0, Cells With No Antibody	107	94	111	10344	9076	10769	10063	881
0, Cells With FITC Only	130	134	129	13039	13574	12908	13174	353
0, Cells With MHC Class I Antibody	356	383	359	126766	166345	130651	141254	21816

Appendix Table 5.2.7b The Expression Of Alpha-L By Du145 When Incubated With Varying Concentrations of Granulocyte, Monocyte-Colony Stimulating Factor (GM-CSF) for 2,4,8,12, and 24 hours. Cells were seeded in 24-well TCGPs and cultured until 90-100% confluent. 200μl of GM-CSF-supplemented ECLM was added to the wells in triplicate. Median levels of flourescence were converted to MESF as described in 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	362	362	356	108308	108308	102064	106227	3605
ECLM	155	351	350	13961	96179	96179	68773	47469
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	293	277	294	54712	46699	55265	52225	4794
ECLM	347	262	382	93366	40257	132016	88546	46069
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Vaules			Mean MESF	SD Of Mean MESF
HUVEC-CM	346	319	338	81442	62585	75328	73119	9621
ECLM	319	312	325	62585	58455	66357	62466	3953
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	400	347	370	157759	93366	117232	122786	32554
ECLM	317	350	394	69381	96179	148664	104741	40329
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

Appendix Table 5.3.1 a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of CD44 By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	219	216	205	26303	25534	22900	24912	1785
ECLM	249	227	220	35396	28471	26565	30144	4647
ECLM, cells with no antibody	139	ND	ND	11134	NA	NA	11134	NA
ECLM, cells with FITC-conjugated antibody only	162	ND	ND	14028	NA	NA	14028	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	255	264	ND	37562	41061	NA	39312	2474
ECLM	271	252	258	44007	36403	38694	39701	3901
ECLM, cells with no antibody	139	ND	ND	11134	NA	NA	11134	NA
ECLM, cells with FITC-conjugated antibody only	162	ND	ND	14028	NA	NA	14028	NA

Appendix Table 5.3.1 b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of CD44 By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Vaules			Mean MESF	SD Of Mean MESF
HUVEC-CM	424	431	440	174290	186606	203728	188208	14784
ECLM	422	428	402	170923	181224	140629	164259	21102
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	292	242	228	54173	33027	28754	38651	13611
ECLM	286	255	223	51050	37562	27366	38659	11880
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	358	354	352	84735	81446	79850	82010	2491
ECLM	444	419	414	198479	154974	147491	166981	27533
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	395	291	ND	150452	53640	NA	102046	68456
ECLM	301	248	268	59220	35048	42720	45663	12352
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

Appendix Table 5.3.1 c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of CD44 By The Lung Adenocarcinoma Cell Line, A549. Cells were seeded in 24-well TCGPs and cultured to 90-100% confluent. 200µl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4 (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein endothelial cell-conditioned medium; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	362	362	356	108308	108308	102064	106227	3605
ECLM	155	351	350	13961	96179	96179	68773	47469
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	293	277	294	54712	46699	55265	52225	4794
ECLM	347	262	382	93366	40257	132016	88546	46069
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	346	319	338	81442	62585	75328	73119	9621
ECLM	319	312	325	62585	58455	66357	62466	3953
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	400	347	370	157759	93366	117232	122786	32554
ECLM	317	350	394	69381	96179	148664	104741	40329
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

Appendix Table 5.3.2a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of VCAM-1 By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	154	156	146	12945	13208	11945	12699	666
ECLM	159	162	163	13612	14028	14170	13937	290
ECLM, cells with no antibody	139	ND	ND	11134	NA	NA	11134	NA
ECLM, cells with FITC-conjugated antibody only	162	ND	ND	14028	NA	NA	14028	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	135	149	152	10696	12311	12688	11898	1058
ECLM	172	162	160	15511	14028	13749	14429	947
ECLM, cells with no antibody	139	ND	ND	11134	NA	NA	11134	NA
ECLM, cells with FITC-conjugated antibody only	162	ND	ND	14028	NA	NA	14028	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	214	188	146	25033	19354	12771	19053	6137
ECLM	94	93	247	4634	7557	34703	15631	16581
ECLM, cells with no antibody	219	ND	ND	26363	NA	NA	26363	NA
ECLM, cells with FITC-conjugated antibody only	229	ND	ND	29040	NA	NA	29040	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	198	144	174	21367	12521	16850	16913	4423
ECLM	218	238	239	26044	31745	32061	29950	3386
ECLM, cells with no antibody	209	ND	ND	26303	NA	NA	26303	NA
ECLM, cells with FITC-conjugated antibody only	229	ND	ND	29040	NA	NA	29040	NA

Appendix Table 5.3.2b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of VCAM-1 By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	255	158	157	37562	14382	14240	22061	13424
ECLM	152	158	153	13553	14382	13688	13874	445
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	166	163	160	15567	15112	14670	15116	449
ECLM	159	166	164	14525	15567	15262	15118	536
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	136	137	146	11568	11683	12771	12007	664
ECLM	155	149	157	13961	13156	14240	13786	563
ECLM, cells with no antibody	123	ND	ND	10171	NA	NA	10171	NA
ECLM, cells with FITC-conjugated antibody only	155	ND	ND	13961	NA	NA	13961	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	144	145	145	12521	12646	12645	12604	72
ECLM	151	147	155	13420	12982	13961	13454	490
ECLM, cells with no antibody	123	ND	ND	10171	NA	NA	10171	NA
ECLM, cells with FITC-conjugated antibody only	155	ND	ND	13961	NA	NA	13961	NA

Appendix Table 5.3.2c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of VCAM-1 By The Lung Adenocarcinoma Cell Line, A549.
Cells were seeded in 24-well TCGPs and left to become 90-100% confluent. 200µl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein endothelial cell-conditioned medium; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	362	362	356	108308	108308	102064	106227	3605
ECLM	155	351	350	13961	96179	96179	68773	47469
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	293	277	294	54712	46699	55265	52225	4794
ECLM	347	262	382	93366	40257	132016	88546	46069
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	346	319	338	81442	62585	75328	73119	9621
ECLM	319	312	325	62585	58455	66357	62466	3953
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	400	347	370	157759	93366	117232	122786	32554
ECLM	317	350	394	69381	96179	148664	104741	40329
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

Appendix Table 5.3.3a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-4 By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	145	135	148	11274	10191	11621	11029	746
ECLM	157	156	155	12727	12599	12472	12599	128
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	145	149	146	11274	11739	11389	11467	242
ECLM	162	155	160	13386	12472	13118	12992	470
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	181	255	273	18058	37562	44887	33502	13868
ECLM	239	167	161	32061	15722	14815	20866	9706
ECLM, cells with no antibody	219	ND	ND	26303	NA	NA	26303	NA
ECLM, cells with FITC-conjugated antibody only	229	ND	ND	29040	NA	NA	29040	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	174	208	210	16850	23590	24062	21501	4035
ECLM	246	253	244	34361	36826	33687	34958	1652
ECLM, cells with no antibody	219	ND	ND	26303	NA	NA	26303	NA
ECLM, cells with FITC-conjugated antibody only	229	ND	ND	29040	NA	NA	29040	NA

Appendix Table 5.3.3b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-4 By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	157	168	136	14240	15878	11538	13885	2192
ECLM	157	155	161	14240	13961	14815	14339	435
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	165	161	163	15414	14815	15112	15114	300
ECLM	163	162	257	15112	14963	38313	22796	13438
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	152	149	153	15414	14815	15112	15114	300
ECLM	152	157	162	13553	14240	14963	14252	705
ECLM, cells with no antibody	123	ND	ND	10171	NA	NA	10171	NA
ECLM, cells with FITC-conjugated antibody only	155	ND	ND	13961	NA	NA	13961	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	156	150	151	14100	13287	13419	13602	436
ECLM	154	156	146	13824	14100	12771	13565	701
ECLM, cells with no antibody	125	ND	ND	10171	NA	NA	10171	NA
ECLM, cells with FITC-conjugated antibody only	155	ND	ND	13961	NA	NA	13961	NA

Appendix Table 5.3.3c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-4 By The Lung Adenocarcinoma Cell Line, A549.

Cells were seeded in 24-well TCGPs and left to become 90-100% confluent. 200µl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein endothelial cell-conditioned medium; MESF, molecular equivalent to soluble fluorochrome, NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

Appendix Table 5.3.4 a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-5 By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	362	362	356	108308	108308	102064	106227	3605
ECLM	155	351	350	13961	96179	96179	68773	47469
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

4 Hours	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	293	277	294	54712	46699	55265	52225	4794
ECLM	347	262	382	93366	40257	132016	88546	46069
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	346	319	338	81442	62585	75328	73119	9621
ECLM	319	312	325	62585	58455	66357	62466	3953
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Vales			Mean MESF	SD Of Mean MESF
HUVEC-CM	400	347	370	157759	93366	117232	122786	32554
ECLM	317	350	394	69381	96179	148664	104741	40329
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

Appendix Table 5.3.4b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-5 By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	421	426	423	183029	192508	186764	187434	4775
ECLM	420	431	429	181190	202478	197429	193699	11123
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	422	430	424	184887	200443	158659	181330	21118
ECLM	424	419	426	188659	179370	192508	186846	6754
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	386	387	381	111793	112905	166393	130364	31207
ECLM	415	413	417	148958	146039	151936	148978	2949
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	391	395	385	117465	122208	110692	116788	5788
ECLM	4296	423	427	171096	161233	197743	176691	18887
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

Appendix Table 5.3.4c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-5 By The Lung Adenocarcinoma Cell Line, A549.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	181	163	248	18058	15112	35048	22739	10761
ECLM	101	257	253	8181	38313	36826	27773	16984
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	253	241	250	36826	32702	25749	31759	5598
ECLM	168	254	246	15878	37192	34361	29144	11575
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	285	290	297	44920	47165	50498	47528	2807
ECLM	272	274	266	39570	40350	37321	39080	1573
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	290	299	296	47165	51493	50008	49555	2199
ECLM	269	266	264	38429	37321	36600	37450	921
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

Cells were seeded in 24-well TCGPs and left to become 90-100% confluent. 200µl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein endothelial cell-conditioned medium; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	181	190	191	16288	17783	17957	17343	917
ECLM	215	198	200	22694	19226	19605	20508	1902
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA
4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	181	194	192	16288	18490	18133	17637	1182
ECLM	202	198	195	19991	19226	18672	19296	663
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA
12 Hours	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	127	132	158	9619	10100	13015	10911	1838
ECLM	144	140	143	11354	10919	11134	11136	217
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA
24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	123	139	124	9251	10813	9341	9802	877
ECLM	135	133	136	10399	10199	10501	10367	154
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA

Appendix Table 5.3.5a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-L By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	153	152	148	12806	12688	12188	12564	332
ECLM	155	161	159	13076	13888	13612	13525	413
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA
4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	152	152	152	12688	12688	12688	126688	0
ECLM	162	161	159	14028	13888	13612	13843	212
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

Appendix Table 5.3.5b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-L By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	151	156	142	12156	12764	12017	12018	823
ECLM	137	151	156	10601	12156	12764	11841	1113
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
4 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	159	161	172	13143	13401	14919	13821	960
ECLM	170	173	180	14631	15066	16130	15276	771
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
12 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	127	132	129	9619	10100	9808	9842	242
ECLM	137	141	142	10604	11026	11134	10922	280
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
24 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	117	119	118	8725	8897	8810	8811	86
ECLM	127	133	136	9619	10199	10501	10106	448
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

Appendix Table 5.3.5c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Alpha-L By The Lung Adenocarcinoma Cell Line, A549.
Cells were seeded in 24-well TCGPs and left to become 90-100% confluent. 200µl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein enothelial cell-conditioned medium; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	552	545	557	710113	662582	746137	706277	41909
ECLM	521	533	512	522495	588384	477967	529615	55552
ECLM, cells with no antibody	141	ND	ND	12154	NA	NA	12154	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	512	520	512	477967	517349	477967	491094	22737
ECLM	547	552	543	674828	710113	649596	678179	30397
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA
12 Hours	**Median Level Of Fluorescene**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	453	457	469	266564	277329	312301	285398	23912
ECLM	458	462	463	280087	291397	294296	288593	7508
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	539	532	333	624831	582589	81285	429568	302361
ECLM	530	533	534	571171	588384	594236	584597	11990
ECLM, cells with no antibody	141	ND	ND	12155	NA	NA	12155	NA
ECLM, cells with FITC-conjugated antibody only	159	ND	ND	14525	NA	NA	14525	NA

Appendix Table 5.3.6a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Beta-1 By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	335	320	320	76799	66065	66005	69623	6215
ECLM	335	332	180	76799	74508	16054	55787	34429
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	529	523	529	565546	532940	565546	554677	18825
ECLM	435	430	548	223066	571171	682550	492262	239690
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	536	546	540	606115	669172	630592	635293	31790
ECLM	513	537	509	482721	612144	463984	519616	80677
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

Appendix Table 5.3.6b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Beta-1 By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	499	478	509	362232	295139	399345	352238	52817
ECLM	494	491	505	344989	335040	384064	354698	25914
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13887	NA	NA	13887	NA
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	453	449	424	266564	256217	200056	240946	35787
ECLM	468	452	459	309225	263989	282872	285362	22721
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
12 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	488	496	491	325378	351785	335040	337401	13361
ECLM	474	461	462	283845	250041	252492	262126	18849
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	477	472	ND	338033	321712	NA	329873	11541
ECLM	462	453	442	291397	266564	239067	265676	26176
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

Appendix Table 5.3.6c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of Beta-1 By The Lung Adenocarcinoma Cell Line, A549.
Cells were seeded in 24-well TCGPs and left to become 90-100% confluent. 200µl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4. (ECLM, established cell line medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein endothelial cell-conditioned medium; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	395	393	401	150142	147200	159328	152223	6326
ECLM	397	395	394	153144	150142	148664	150650	2283
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA
4 Hours	**Median Level Of Fluorescene**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	395	397	400	150142	153144	157759	153682	3837
ECLM	391	387	396	144315	138713	151636	144888	6481
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA
12 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	406	402	405	167411	160913	165762	164695	3378
ECLM	407	415	417	169076	183007	186666	179583	9281
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA
24 Hours	**Median Level Of Fluorescence**			**Correspoding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	380	397	375	129428	153144	123179	135250	15808
ECLM	378	392	389	126891	145750	141486	138042	9890
ECLM, cells with no antibody	134	ND	ND	11341	NA	NA	11341	NA
ECLM, cells with FITC-conjugated antibody only	183	ND	ND	18419	NA	NA	18419	NA

Appendix Table 5.3.7a. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of ICAM-1 By The Prostatic Adenocarcinoma Cell Line, PC3.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	570	554	566	824096	701145	791472	772238	63692
ECLM	547	551	550	653294	680223	673388	668968	13998
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	556	568	572	631610	710041	738292	693315	55273
ECLM	579	578	544	790463	782790	561843	711699	129836
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA
12 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	533	539	541	504681	535099	545640	528473	21268
ECLM	577	559	558	775192	650366	644053	689870	73958
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	536	536	542	493346	493346	523530	503407	17427
ECLM	584	594	594	793355	875891	875891	848379	47652
ECLM, cells with no antibody	120	ND	ND	8759	NA	NA	8759	NA
ECLM, cells with FITC-conjugated antibody only	148	ND	ND	11621	NA	NA	11621	NA

Appendix Table 5.3.7b. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of ICAM-1 By The Prostatic Adenocarcinoma Cell Line, Du145.

2 Hours	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
HUVEC-CM	131	124	122	10002	9341	9161	9501	443
ECLM	133	131	132	10199	10002	10102	10100	99
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
4 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	130	133	126	9904	10199	9525	9876	337
ECLM	135	127	130	10399	9619	9904	9974	395
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
12 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	187	198	210	17270	19226	21614	19370	2175
ECLM	194	184	197	18490	16772	19040	18101	1183
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA
24 Hours	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
HUVEC-CM	195	200	196	18672	19605	18855	19044	494
ECLM	195	200	196	18672	19605	18855	19044	494
ECLM, cells with no antibody	139	ND	ND	11917	NA	NA	11917	NA
ECLM, cells with FITC-conjugated antibody only	150	ND	ND	13287	NA	NA	13287	NA

Appendix Table 5.3.7 c. HUVEC-Conditioned Medium Has No Effect On The Cell Surface Expression Of ICAM-1 By The Lung Adenocarcinoma Cell Line, A549.
Cells were seeded in 24-well TCGPs and left to become 90-100% confluent. 200μl of either HUVEC- conditioned medium (HUVEC-CM) or Established Cell Line Medium (ECLM) was added, in triplicate, to cells. Cells were further cultured for 2, 4, 12, or 24 hours. Median Levels of Fluorescence were converted to MESF values as in Chapter 2.4.2.4 (ECLM, established cell lined medium; FITC, fluorescein isothiocyanate; HUVEC-CM, human umbilical vein endothelial cell-conditioned medium; MESF, mlecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	561	542	520	527	517	558
E-selectin	192	196	201	196	188	185
No Antibody	169	157	161	148	148	154
Secondary FITC-conjugated Antibody	203	193	186	191	192	168

4 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	588	545	560	544	559	544
E-selectin	197	184	204	202	182	184
No Antibody	166	166	157	163	152	161
Secondary FITC-conjugated Antibody	187	185	555	177	171	176

8 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	412	412	408	393	414	400
E-selectin	159	151	156	149	152	151
No Antibody	121	110	121	114	116	117
Secondary FITC-conjugated Antibody	159	135	148	140	138	141

24 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	403	413	395	420	417	422
E-selectin	184	155	163	165	148	151
No Antibody	121	111	113	106	103	106
Secondary FITC-conjugated Antibody	161	142	153	163	150	143

48 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	403	419	418	413	417	406
E-selectin	134	139	138	133	131	135
No Antibody	108	96	101	87	85	104
Secondary FITC-conjugated Antibody	128	116	122	113	105	119

2 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	2879175	2299634	1772709	1925745	1710903	2778792
E-selectin	33609	38383	40722	38383	34918	33700
No Antibody	27889	24195	25370	21755	21755	23355
Secondary FITC-conjugated Antibody	41697	37045	34101	36179	36609	27561

4 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	2278792	232707	2845317	2354688	2811858	2354688
E-selectin	38840	33304	42193	41207	32525	33304
No Antibody	26917	26917	24198	25978	22809	5371
Secondary FITC-conjugated Antibody	34507	33700	2681909	30657	28557	30297

8 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	494090	494090	471256	394636	505918	428704
E-selectin	24778	22540	23914	22013	22809	22540
No Antibody	15807	13787	15807	14551	14899	15076
Secondary FITC-conjugated Antibody	24778	18654	21755	119790	19328	20026

24 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	444191	499969	404084	543131	521494	556134
E-selectin	33304	23633	25978	26600	21755	22540
No Antibody	15807	14043	14380	13237	12775	13237
Secondary FITC-conjugated Antibody	25371	20264	23080	25978	22275	20505

48 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	444191	536744	530432	499969	524194	460237
E-selectin	18434	19558	19328	18218	17792	18654
No Antibody	13553	11760	12476	10572	10325	12927
Secondary FITC-conjugated Antibody	17171	14899	15995	14380	13081	15438

Appendix Table 5.4.1 PC3-Conditioned Medium Does Not Activate Endothelial Cells. HUVECs were seeded in 24-well TCGPs and cultured until 70-100% confluent. 200µl of the appropriate medium was added to the wells. Cells were cultured for a further 2, 4, 8, 24, or 48 hours. Surface expression of E-selectin and PECAM-1 were detected by FACScan analysis. Median levels of fluoresence were converted to MESF values as decribed in Chapter 2.4.2.4. (ECL-CM, established cell line-conditioned medium; ECLM, established cell line medium; ECM, endothelial cell medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; PECAM-1, platelet endothelial cell adhesion molecule; TCGP, tissue culture grade plate.)

2 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	424	424	418	387	413	392
E-selectin	138	132	138	138	127	133
No Antibody	96	95	97	93	97	93
Secondary FITC-conjugated Antibody	126	114	124	124	130	114

4 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	442	430	434	430	426	420
E-selectin	126	116	128	116	125	113
No Antibody	92	92	91	90	91	88
Secondary FITC-conjugated Antibody	115	122	188	115	110	114

12 Hours	Medium Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	419	432	136	442	431	447
E-selectin	147	126	10	172	159	146
No Antibody	101	96	121	145	136	127
Secondary FITC-conjugated Antibody	114	157	237	196	186	143

24 Hours	Median Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	445	449	435	442	452	447
E-selectin	148	164	178	180	166	151
No Antibody	135	113	141	128	126	121
Secondary FITC-conjugated Antibody	190	190	180	154	162	146

48 Hours	Medium Level Of Fluorescence					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	404	400	420	429	423	427
E-selectin	140	134	138	133	130	145
No Antibody	96	96	107	107	104	95
Secondary FITC-conjugated Antibody	115	112	117	127	118	110

2 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	569448	569448	530432	367597	499969	359995
E-selectin	19328	18003	19328	19328	16969	18218
No Antibody	11760	11622	11900	11350	11900	11350
Secondary FITC-conjugated Antibody	16770	14551	16378	16378	17582	14551

4 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	704573	611333	640954	611333	583082	543131
E-selectin	16770	14899	17171	14899	16573	14380
No Antibody	11217	11217	11085	10954	11085	10698
Secondary FITC-conjugated Antibody	14724	15995	15256	14724	13878	14551

12 Hours	Corresponding MESF Values					
	100% ECM	100% ECLM	25% ECL-CM	50% ECl-CM	75% ECL-CM	100% ECL-CM
PECAM-1	730025	765397	648581	704573	793047	747502
E-selectin	34507	26287	31022	31765	26918	22540
No Antibody	18654	14380	20026	17171	16770	15807
Secondary FITC-conjugated Antibody	35753	26600	31765	23355	25673	21246

24 Hours	Corresponding MESF Levels					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	543131	618607	590019	5762224	569448	597040
E-selectin	26600	23355	26287	23355	24778	21499
No Antibody	1451	12625	14210	14380	13394	12775
Secondary FITC-conjugated Antibody	25978	22275	26287	23355	20749	21499

48 Hours	Corresponding MESF Levels					
	100% ECM	100% ECLM	25% ECL-CM	50% ECL-CM	75% ECL-CM	100% ECL-CM
PECAM-1	449477	428704	543131	604144	562751	590019
E-selectin	19790	18434	19328	18218	17582	20996
No Antibody	11760	11760	13394	12477	12927	11622
Secondary FITC-conjugated Antibody	14724	14210	15076	16969	15256	138783

Appendix Table 5.4.2 Du145-Conditioned Medium Does Not Activate Endothelial Cells. HUVECs were seeded in 24-well TCGPs and cultured until 70-100% confluent. 200µl of the appropriate medium was added to the wells. Cells were cultured for a further 2, 4, 12, 24, or 48 hours. Surface expression of E-selectin and PECAM-1 were detected by FACScan analysis. Median levels of fluoresence were converted to MESF values as decribed in Chapter 2.4.2.4. (ECL-CM, established cell line-conditioned medium; ECLM, established cell line medium; ECM, endothelial cell medium; FITC, fluorescein isothiocyanate; MESF, molecular equivalent of soluble fluorochrome; PECAM-1, platelet endothelial cell adhesion molecule; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	99	108	109	9544	10449	10555	10183	555
Unattached Du145	VCAM-1	103	86	90	9936	8374	8718	9009	821
Unmanipulated Du145	VCAM-1	108	115	119	10449	11212	11672	11111	618
Manipulated HUVECs	VCAM-1	117	116	123	11440	11325	12152	11639	448
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	391	431	418	180291	269650	236582	228841	45180
Unattached Du145	MHC Class I	451	455	426	329772	343318	256417	309836	46755
Unmanipulated Du145	MHC Class I	428	420	409	261631	241392	216096	239706	22814
Manipulated HUVECs	MHC Class I	108	144	142	10449	15011	14712	13391	2552
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		123	127	126	12152	12651	12524	12442	259
Unmanipulated Du145 Cells And FITC		130	131	124	13039	13171	12275	12828	484
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	107	108	102	10344	10449	9837	10210	327
Unattached Du145	VCAM-1	92	82	96	8895	8043	9260	8733	624
Unmanipulated Du145	VCAM-1	130	120	122	13039	11790	12030	12286	662
Manipulated HUVECs	VCAM-1	136	138	129	13850	14132	12908	13630	641
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	422	412	414	246300	222720	227248	232089	12514
Unattached Du145	MHC Class I	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated Du145	MHC Class I	408	407	404	213932	211790	205491	210404	4388
Manipulated HUVECs	MHC Class I	146	154	151	15317	16601	16107	16008	648
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		123	127	126	12152	12651	12524	12442	259
Unmanipulated Du145 Cells And FITC		130	131	124	13039	13171	12275	12828	484
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	130	131	123	13039	13171	12152	12787	554
Unattached Du145	VCAM-1	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated Du145	VCAM-1	129	132	127	12908	13304	12651	12954	329
Manipulated HUVECs	VCAM-1	136	140	140	13850	14419	14419	14230	328
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	433	428	424	275133	261631	251308	262690	11948
Unattached Du145	MHC Class I	426	429	442	256417	264277	301216	273970	23921
Unmanipulated Du145	MHC Class I	393	383	382	183957	166345	164679	171660	10682
Manipulated HUVECs	MHC Class I	153	151	151	16435	16107	16107	16216	189
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		123	127	126	12152	12651	12524	12442	259
Unmanipulated Du145 Cells And FITC		130	131	124	13039	13171	12275	12828	484
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

Appendix Table 5.5.1a The Expression Of VCAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26* Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and VCAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; VCAM, vascular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	119	117	114	11672	11440	11099	11404	288
Unattached Du145	VCAM-1	132	129	131	13304	12908	13171	13127	201
Unmanipulated Du145	VCAM-1	122	122	118	12030	12030	11555	11872	274
Manipulated HUVECs	VCAM-1	139	137	101	14275	13990	9738	12668	2541
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	384	386	376	168027	171444	155030	164834	8661
Unattached Du145	MHC Class I	444	457	438	307340	350298	289331	315657	31323
Unmanipulated Du145	MHC Class I	356	363	351	126766	136018	120545	127776	7786
Manipulated HUVECs	MHC Class I	177	184	175	20924	22452	20508	21295	1024
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		78	80	76	7726	7883	7572	7727	156
Unmanipulated Du145 Cells And FITC		123	123	115	12152	12152	11212	11838	543
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	133	129	132	13438	12908	13304	13217	276
Unattached Du145	VCAM-1	133	127	130	13438	12651	13039	13043	394
Unmanipulated Du145	VCAM-1	118	123	123	11555	12152	12152	11953	344
Manipulated HUVECs	VCAM-1	165	141	148	18544	14565	15628	16246	2060
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	394	402	387	185817	201396	173178	186797	14135
Unattached Du145	MHC Class I	465	ND	ND	379667	ND	ND	379667	NA
Unmanipulated Du145	MHC Class I	347	343	301	115789	111220	72881	99963	23565
Manipulated HUVECs	MHC Class I	166	173	171	18732	20099	19698	19510	703
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		78	80	76	7726	7883	7572	7727	156
Unmanipulated Du145 Cells And FITC		123	123	115	12152	12152	11212	11838	543
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	129	127	129	12908	12651	12908	12822	149
Unattached Du145	VCAM-1	138	137	140	14132	13990	14419	14180	219
Unmanipulated Du145	VCAM-1	121	122	121	11910	12030	11910	11950	70
Manipulated HUVECs	VCAM-1	121	123	120	11910	12152	11790	11951	184
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	359	368	358	130651	143037	129343	134344	7557
Unattached Du145	MHC Class I	345	351	298	113481	120545	70714	101580	26963
Unmanipulated Du145	MHC Class I	324	325	324	91863	92792	91863	92173	536
Manipulated HUVECs	MHC Class I	149	150	148	15786	15946	15628	15787	159
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		78	80	76	7726	7883	7572	7727	156
Unmanipulated Du145 Cells And FITC		123	123	115	12152	12152	11212	11838	543
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

Appendix Table 5.5.1b The Expression Of VCAM-1 By HUVECs And Prostatic Adenocarcinoma Cells Of TheDu145 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26* Du145 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. VCAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; VCAM, vascular adhesion molecule; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	113	111	110	10988	10769	10662	10806	167
Unattached Du145	VCAM-1	114	124	113	11099	12275	10988	11454	713
Unmanipulated Du145	VCAM-1	122	122	118	12030	12030	11555	11872	274
Manipulated HUVECs	VCAM-1	102	94	95	9837	9076	9168	9360	415
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	385	368	382	169727	143037	164679	159148	14178
Unattached Du145	MHC Class I	388	388	371	174929	174929	147422	165760	15882
Unmanipulated Du145	MHC Class I	356	363	351	126766	136018	120545	127776	7786
Manipulated HUVECs	MHC Class I	169	175	168	19306	20508	19113	19642	756
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		78	80	76	7726	7883	7572	7727	156
Unmanipulated Du145 Cells And FITC		123	123	115	12152	12152	11212	11838	543
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	122	122	125	12030	12030	12399	12153	213
Unattached Du145	VCAM-1	133	130	114	13438	13039	11099	12526	1251
Unmanipulated Du145	VCAM-1	118	123	123	11555	12152	12152	11953	344
Manipulated HUVECs	VCAM-1	98	97	96	9449	9354	9260	9354	94
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	377	363	364	156598	136018	137394	143336	11505
Unattached Du145	MHC Class I	432	407	409	272378	211790	216096	233421	33806
Unmanipulated Du145	MHC Class I	347	343	301	115789	111220	72881	99963	23565
Manipulated HUVECs	MHC Class I	164	175	208	18358	20508	28585	22484	5392
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		78	80	76	7726	7883	7572	7727	156
Unmanipulated Du145 Cells And FITC		123	123	115	12152	12152	11212	11838	543
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	VCAM-1	122	123	124	12030	12152	12275	12152	122
Unattached Du145	VCAM-1	116	115	120	11325	11212	11790	11442	307
Unmanipulated Du145	VCAM-1	121	122	121	11910	12030	11910	11950	70
Manipulated HUVECs	VCAM-1	117	90	123	11440	8718	12152	10770	1812
Unmanipulated HUVECs	VCAM-1	173	146	189	20099	15317	23610	19675	4163
Attached Du145 (to HUVECs)	MHC Class I	368	355	362	143037	125496	134656	134397	8773
Unattached Du145	MHC Class I	370	386	368	145946	171444	143037	153476	15629
Unmanipulated Du145	MHC Class I	324	325	324	91863	92792	91863	92173	536
Manipulated HUVECs	MHC Class I	183	165	177	22227	18544	20924	20565	1867
Unmanipulated HUVECs	MHC Class I	309	185	195	78992	22679	25080	42250	31842
Unmanipulated Du145 Cells Only		78	80	76	7726	7883	7572	7727	156
Unmanipulated Du145 Cells And FITC		123	123	115	12152	12152	11212	11838	543
Unmanipulated HUVECs Only		122	119	109	12030	11672	10555	11419	770
Unmanipulated HUVECs And FITC		154	155	155	16601	16769	16769	16713	97

Appendix Table 5.5.1c The Expression Of VCAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and VCAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell;VCAM, vascular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	102	100	103	9837	9641	9936	9805	150
Unattached Du145	Alpha L	108	94	90	10449	9076	8718	9414	914
Unmanipulated Du145	Alpha L	104	97	94	10037	9354	9076	9489	494
Manipulated HUVECs	Alpha L	106	88	100	10241	8544	9641	9475	860
Unmanipulated HUVECs	Alpha L	101	87	89	9738	8459	8631	8942	695
Attached Du145 (to HUVECs)	MHC Class I	449	456	462	323201	346790	368376	346122	22595
Unattached Du145	MHC Class I	437	436	440	286434	283566	295214	288405	6069
Unmanipulated Du145	MHC Class I	447	420	421	316761	241392	243834	267329	42826
Manipulated HUVECs	MHC Class I	155	160	168	16769	17634	19113	17838	1185
Unmanipulated HUVECs	MHC Class I	187	170	162	23140	19501	17993	20211	2646
Unmanipulated Du145 Cells Only		85	68	68	8290	6986	6986	7421	753
Unmanipulated Du145 Cells And FITC		83	75	71	8125	7496	7201	7607	472
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	94	79	106	9076	7804	10241	9040	1219
Unattached Du145	Alpha L	93	ND	ND	8985	ND	ND	8985	NA
Unmanipulated Du145	Alpha L	94	91	ND	9076	8806	NA	8941	191
Manipulated HUVECs	Alpha L	115	68	108	11212	6986	10449	9549	2252
Unmanipulated HUVECs	Alpha L	101	87	89	9738	8459	8631	8942	695
Attached Du145 (to HUVECs)	MHC Class I	456	454	ND	346790	339880	ND	343335	4886
Unattached Du145	MHC Class I	445	ND	ND	310449	ND	ND	310449	NA
Unmanipulated Du145	MHC Class I	417	ND	ND	234213	ND	ND	234213	NA
Manipulated HUVECs	MHC Class I	169	174	166	19306	20302	18732	19447	795
Unmanipulated HUVECs	MHC Class I	187	170	162	23140	19501	17993	20211	2646
Unmanipulated Du145 Cells Only		85	68	68	8290	6986	6986	7421	753
Unmanipulated Du145 Cells And FITC		83	75	71	8125	7496	7201	7607	472
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	104	99	84	10037	9544	8207	9263	947
Unattached Du145	Alpha L	88	90	89	8544	8718	8631	8631	87
Unmanipulated Du145	Alpha L	91	99	98	8806	9544	9449	9266	402
Manipulated HUVECs	Alpha L	101	81	104	9738	7963	10037	9246	1121
Unmanipulated HUVECs	Alpha L	101	87	89	9738	8459	8631	8942	695
Attached Du145 (to HUVECs)	MHC Class I	483	479	466	455066	437111	383507	425228	37230
Unattached Du145	MHC Class I	440	446	441	295214	313589	298200	302334	9861
Unmanipulated Du145	MHC Class I	423	441	425	248792	298200	253850	266947	27184
Manipulated HUVECs	MHC Class I	181	176	159	21784	20715	17458	19985	2254
Unmanipulated HUVECs	MHC Class I	187	170	162	23140	19501	17993	20211	2646
Unmanipulated Du145 Cells Only		85	68	68	8290	6986	6986	7421	753
Unmanipulated Du145 Cells And FITC		83	75	71	8125	7496	7201	7607	472
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

Appendix Table 5.5.2a The Expression Of αL By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26+ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and αL surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	89	87	87	8631	8459	8459	8516	99
Unattached Du145	Alpha L	103	114	111	9936	11099	10769	10602	599
Unmanipulated Du145	Alpha L	98	89	90	9449	8631	8718	8932	449
Manipulated HUVECs	Alpha L	84	81	85	8207	7963	8290	8153	170
Unmanipulated HUVECs	Alpha L	110	98	94	10662	9449	9076	9729	829
Attached Du145 (to HUVECs)	MHC Class I	366	347	342	140187	115789	110106	122027	15981
Unattached Du145	MHC Class I	347	343	344	115789	111220	112345	113118	2380
Unmanipulated Du145	MHC Class I	346	338	343	114629	105762	111220	110537	4473
Manipulated HUVECs	MHC Class I	161	141	146	17812	14565	15317	15898	1700
Unmanipulated HUVECs	MHC Class I	159	153	143	17458	16435	14861	16251	1308
Unmanipulated Du145 Cells Only		54	33	30	6068	4912	4766	5249	713
Unmanipulated Du145 Cells And FITC		57	85	54	6254	8290	6068	6871	1233
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	100	90	86	9641	8718	8374	8911	655
Unattached Du145	Alpha L	109	111	109	10555	10769	10555	10626	124
Unmanipulated Du145	Alpha L	98	92	90	9449	8895	8718	9021	381
Manipulated HUVECs	Alpha L	98	88	90	9449	8544	8718	8904	480
Unmanipulated HUVECs	Alpha L	110	98	94	10662	9449	9076	9729	829
Attached Du145 (to HUVECs)	MHC Class I	337	327	356	104703	94679	126766	108716	16416
Unattached Du145	MHC Class I	337	323	355	104703	90943	125496	107048	17395
Unmanipulated Du145	MHC Class I	339	301	298	106832	72881	70714	83476	20256
Manipulated HUVECs	MHC Class I	134	127	130	13574	12651	13039	13088	464
Unmanipulated HUVECs	MHC Class I	159	152	143	17458	16270	14861	16196	1300
Unmanipulated Du145 Cells Only		54	33	30	6068	4912	4766	5249	713
Unmanipulated Du145 Cells And FITC		57	85	54	6254	8290	6068	6871	1233
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	101	96	96	9738	9260	9260	9420	276
Unattached Du145	Alpha L	105	98	99	10138	9449	9544	9710	374
Unmanipulated Du145	Alpha L	102	92	88	9837	8895	8544	9092	668
Manipulated HUVECs	Alpha L	105	103	94	10138	9936	9076	9717	564
Unmanipulated HUVECs	Alpha L	110	98	94	10662	9449	9076	9729	829
Attached Du145 (to HUVECs)	MHC Class I	411	388	388	220489	174929	174929	190116	26304
Unattached Du145	MHC Class I	348	349	340	116960	118143	107912	114338	5596
Unmanipulated Du145	MHC Class I	316	310	305	84757	79791	75875	80141	4451
Manipulated HUVECs	MHC Class I	171	164	161	19698	18358	17812	18623	970
Unmanipulated HUVECs	MHC Class I	159	152	143	17458	16270	14861	16196	1300
Unmanipulated Du145 Cells Only		54	33	30	6068	4912	4766	5249	713
Unmanipulated Du145 Cells And FITC		57	85	54	6254	8290	6068	6871	1233
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

Appendix Table 5.5.2b The Expression Of αL By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26+ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and αL surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; MHC, major histocompatability complex; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	81	91	92	7963	8806	8895	8555	514
Unattached Du145	Alpha L	102	91	88	9837	8806	8544	9062	683
Unmanipulated Du145	Alpha L	98	89	90	9449	8631	8718	8932	449
Manipulated HUVECs	Alpha L	80	85	89	7883	8290	8631	8268	374
Unmanipulated HUVECs	Alpha L	110	98	94	10662	9449	9076	9729	829
Attached Du145 (to HUVECs)	MHC Class I	372	368	356	148913	143037	126766	139572	11473
Unattached Du145	MHC Class I	371	361	374	147422	133308	151940	144223	9719
Unmanipulated Du145	MHC Class I	346	338	343	114629	105762	111220	110537	4473
Manipulated HUVECs	MHC Class I	154	148	144	16601	15628	15011	15747	801
Unmanipulated HUVECs	MHC Class I	159	153	143	17458	16435	14861	16251	1308
Unmanipulated Du145 Cells Only		54	33	30	6068	4912	4766	5249	713
Unmanipulated Du145 Cells And FITC		57	85	54	6254	8290	6068	6871	1233
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	103	95	94	9936	9168	9076	9393	472
Unattached Du145	Alpha L	100	97	99	9641	9354	9544	9513	146
Unmanipulated Du145	Alpha L	98	92	90	9449	8895	8718	9021	381
Manipulated HUVECs	Alpha L	104	96	94	10037	9260	9076	9458	510
Unmanipulated HUVECs	Alpha L	110	98	94	10662	9449	9076	9729	829
Attached Du145 (to HUVECs)	MHC Class I	360	350	356	131973	119338	126766	126025	6350
Unattached Du145	MHC Class I	370	382	368	145946	164679	143037	151221	11746
Unmanipulated Du145	MHC Class I	339	301	298	106832	72881	70714	83476	20256
Manipulated HUVECs	MHC Class I	145	140	140	15163	14419	14419	14667	430
Unmanipulated HUVECs	MHC Class I	159	152	143	17458	16270	14861	16196	1300
Unmanipulated Du145 Cells Only		54	33	30	6068	4912	4766	5249	713
Unmanipulated Du145 Cells And FITC		57	85	54	6254	8290	6068	6871	1233
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha L	105	91	93	10138	8806	8985	9310	723
Unattached Du145	Alpha L	99	91	96	9544	8806	9260	9204	372
Unmanipulated Du145	Alpha L	102	92	88	9837	8895	8544	9092	668
Manipulated HUVECs	Alpha L	104	96	97	10037	9260	9354	9550	424
Unmanipulated HUVECs	Alpha L	110	98	94	10662	9449	9076	9729	829
Attached Du145 (to HUVECs)	MHC Class I	351	369	354	120545	144484	124240	129756	12888
Unattached Du145	MHC Class I	371	371	361	147422	147422	133308	142717	8149
Unmanipulated Du145	MHC Class I	316	310	305	84757	79791	75875	80141	4451
Manipulated HUVECs	MHC Class I	152	150	147	16270	15946	15472	15896	402
Unmanipulated HUVECs	MHC Class I	159	152	143	17458	16270	14861	16196	1300
Unmanipulated Du14 Cells Only		54	33	30	6068	4912	4766	5249	713
Unmanipulated Du145 Cells And FITC		57	85	54	6254	8290	6068	6871	1233
Unmanipulated HUVECs Only		65	44	19	6779	5487	4267	5511	1256
Unmanipulated HUVECs And FITC		246	94	231	41902	9076	36030	29003	17505

Appendix Table 5.5.2c The Expression Of αL By HUVECs And Prostatic Adenocarcinoma Cells Of TheDu145 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26.

PKH26* Du145 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. αL surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; SD standard deviation; TCGP, tissue culture grade plate.

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	ICAM-1	588	620	597	1309163	1806569	1433275	1516335	258897
Unattached Du145	ICAM-1	605	632	619	1553441	2038461	1788479	1793460	242548
Unmanipulated Du145	ICAM-1	607	600	601	1585025	1477207	1492149	1518127	58415
Manipulated HUVECs	ICAM-1	97	99	101	9354	9544	9738	9546	192
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	451	437	445	329772	286434	310449	308885	21711
Unattached Du145	MHC Class I	459	473	435	357420	411499	280726	349882	65711
Unmanipulated Du145	MHC Class I	423	421	425	248792	243834	253850	248825	5008
Manipulated HUVECs	MHC Class I	148	145	150	15628	15163	15946	15579	394
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145 Cells Only		128	134	129	12779	13574	12908	13087	427
Unmanipulated Du145 Cells And FITC		125	128	132	12399	12779	13304	12827	454
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	ICAM-1	627	594	581	1938426	1390649	1220110	1516395	375304
Unattached Du145	ICAM-1	619	644	620	1788479	2300119	1806569	1965056	290314
Unmanipulated Du145	ICAM-1	603	609	611	1522486	1617251	1650132	1596623	66276
Manipulated HUVECs	ICAM-1	95	103	98	9168	9936	9449	9518	389
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	412	445	413	222720	310449	224972	252714	50013
Unattached Du145	MHC Class I	429	439	431	264277	292258	269650	275395	14849
Unmanipulated Du145	MHC Class I	419	407	402	238975	211790	201396	217387	19405
Manipulated HUVECs	MHC Class I	139	152	136	14275	16270	13850	14798	1292
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du14 Cells Only		128	134	129	12779	13574	12908	13087	427
Unmanipulated Du145 Cells And FITC		125	128	132	12399	12779	13304	12827	454
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Coresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	ICAM-1	572	554	584	1114456	929804	1257509	1100590	164292
Unattached Du145	ICAM-1	632	604	608	2038461	1537886	1601057	1725801	272607
Unmanipulated Du145	ICAM-1	569	559	573	1081312	977788	1125729	1061610	75913
Manipulated HUVECs	ICAM-1	99	97	102	9544	9354	9837	9578	243
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	395	399	418	187697	195407	236582	206562	26283
Unattached Du145	MHC Class I	417	433	428	234213	275133	261631	256992	20850
Unmanipulated Du145	MHC Class I	389	393	ND	176699	183957	ND	180328	5132
Manipulated HUVECs	MHC Class I	137	134	119	13990	13574	11672	13079	1236
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145 Cells Only		128	134	129	12779	13574	12908	13087	427
Unmanipulated Du145 Cells And FITC		125	128	132	12399	12779	13304	12827	454
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

Appendix Table 5.5.3a The Expression Of ICAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26+ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and ICAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	ICAM-1	573	588	576	1125729	1309163	1160234	1198375	97484
Unattached Du145	ICAM-1	605	612	595	1553441	1666822	1404715	1541659	131450
Unmanipulated Du145	ICAM-1	549	543	547	884175	832366	866556	861032	26343
Manipulated HUVECs	ICAM-1	128	134	128	12779	13574	12779	13044	459
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	376	366	370	155030	140187	145946	147054	7483
Unattached Du145	MHC Class I	423	435	409	248792	280726	216096	248538	32316
Unmanipulated Du145	MHC Class I	342	349	346	110106	118143	114629	114293	4029
Manipulated HUVECs	MHC Class I	144	136	141	15011	13850	14565	14476	586
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145 Cells Only		47	40	33	5655	5271	4912	5280	372
Unmanipulated Du145 Cells And FITC		88	78	82	8544	7726	8043	8105	412
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	ICAM-1	559	563	567	977788	1017952	1059766	1018502	40992
Unattached Du145	ICAM-1	593	598	597	1376724	1447772	1433275	1419257	37541
Unmanipulated Du145	ICAM-1	538	534	524	791518	760288	687498	746435	53376
Manipulated HUVECs	ICAM-1	138	138	141	14132	14132	14565	14276	250
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	343	338	354	111220	105762	124240	113741	9493
Unattached Du145	MHC Class I	406	403	402	209669	203433	201396	204833	4310
Unmanipulated Du145	MHC Class I	330	317	289	97581	85614	64590	82595	16701
Manipulated HUVECs	MHC Class I	132	134	140	13304	13574	14419	13766	582
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145 Cells Only		47	40	33	5655	5271	4912	5280	372
Unmanipulated Du145 Cells And FITC		88	78	82	8544	7726	8043	8105	412
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	ICAM-1	570	573	564	1092249	1125729	1028248	1082075	49530
Unattached Du145	ICAM-1	590	593	556	1335780	1376724	948708	1220404	236184
Unmanipulated Du145	ICAM-1	474	484	477	415661	459669	428401	434577	22645
Manipulated HUVECs	ICAM-1	132	132	139	13304	13304	14275	13627	561
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	328	311	310	95636	80598	79791	85342	8925
Unattached Du145	MHC Class I	376	379	ND	155030	159782	ND	157406	3360
Unmanipulated Du145	MHC Class I	266	276	242	51244	56669	40248	49387	8367
Manipulated HUVECs	MHC Class I	112	106	108	10878	10241	10241	10453	368
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145 Cells Only		47	40	33	5655	5271	4912	5280	372
Unmanipulated Du145 Cells And FITC		88	78	82	8544	7726	8043	8105	412
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

Appendix Table 5.5.3b The Expression Of ICAM-1 By HUVECs And Prostatic Adenocarcinoma Cells Of TheDu145 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26+ Du145 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. ICAM-1 surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular cell adhesion molecule; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD standard deviation; TCGP, tissue culture grade plate.)

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
EXPERIMENT 1									
Attached Du145 (to HUVECs)	ICAM-1	562	575	566	1007759	1148617	1049154	1068510	72396
Unattached Du145	ICAM-1	596	591	593	1418923	1349291	1376724	1381646	35076
Unmanipulated Du145	ICAM-1	549	543	547	884175	832366	866556	861032	26343
Manipulated HUVECs	ICAM-1	114	122	122	11099	12030	12030	11720	537
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	379	356	342	159782	126766	110106	132218	25282
Unattached Du145	MHC Class I	383	400	373	166345	197383	150419	171382	23884
Unmanipulated Du145	MHC Class I	342	349	346	110106	118143	114629	114293	4029
Manipulated HUVECs	MHC Class I	152	137	144	16270	13990	15011	15091	1142
Unmanipulated Du145Cells	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145Cells Only		47	40	33	5655	5271	4912	5280	372
Unmanipulated Du145 Cells And FITC		88	78	82	8544	7726	8043	8105	412
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755
Cell Type	**Marker**	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
EXPERIMENT 2									
Attached Du145 (to HUVECs)	ICAM-1	568	558	560	1070485	967997	987678	1008720	54388
Unattached Du145	ICAM-1	580	575	575	1207893	1148617	1148617	1168375	34223
Unmanipulated Du145	ICAM-1	538	534	524	791518	760288	687498	746435	53376
Manipulated HUVECs	ICAM-1	124	119	121	12275	11672	11910	11952	303
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	344	341	337	112345	109004	104703	108684	3831
Unattached Du145	MHC Class I	355	340	351	125496	107912	120545	117984	9067
Unmanipulated Du145	MHC Class I	330	317	287	97581	85614	63303	82166	17397
Manipulated HUVECs	MHC Class I	148	140	146	15628	14419	15317	15121	628
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145 Cells Only		47	40	33	5655	5271	4912	5280	372
Unmanipulated Du145 Cells And FITC		88	78	82	8544	7726	8043	8105	412
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755
Cell Type	**Marker**	**Median Level Of Fluorescence**			**Corresponding MESF Values**			**Mean MESF**	**SD Of Mean MESF**
EXPERIMENT 3									
Attached Du145 (to HUVECs)	ICAM-1	564	557	562	1028248	958304	1007759	998104	35958
Unattached Du145	ICAM-1	563	564	570	1017952	1028248	1092249	1046150	40254
Unmanipulated Du145	ICAM-1	474	484	477	415661	459669	428401	434577	22645
Manipulated HUVECs	ICAM-1	120	122	123	11790	12030	12152	11991	184
Unmanipulated HUVECs	ICAM-1	104	100	106	10037	9641	10241	9973	305
Attached Du145 (to HUVECs)	MHC Class I	343	344	351	111220	112345	120545	114703	5090
Unattached Du145	MHC Class I	344	332	321	112345	99565	89131	100347	11627
Unmanipulated Du145	MHC Class I	266	276	242	51244	56669	40248	49387	8367
Manipulated HUVECs	MHC Class I	137	130	138	13990	13039	14132	13720	595
Unmanipulated HUVECs	MHC Class I	459	462	468	357420	368376	391305	372367	17291
Unmanipulated Du145Cells Only		47	40	33	5655	5271	4912	5280	372
Unmanipulated Du145 Cells And FITC		88	78	82	8544	7726	8043	8105	412
Unmanipulated HUVECs Only		50	28	29	5829	4671	4718	5073	655
Unmanipulated HUVECs And FITC		63	49	72	6644	5770	7273	6562	755

Appendix Table 5.5.3c The Expression Of ICAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and ICAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	147	142	141	15472	14712	14565	14916	486
Unattached Du145	Alpha 4	143	145	133	14861	15163	13438	14488	921
Unmanipulated Du145	Alpha 4	137	135	139	13990	13712	14275	13992	282
Manipulated HUVECs	Alpha 4	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated HUVECs	Alpha 4	166	162	ND	18732	17993	ND	18362	523
Attached Du145 (to HUVECs)	MHC Class I	387	370	379	173178	145946	159782	159635	13617
Unattached Du145	MHC Class I	374	381	383	151940	163030	166345	160439	7544
Unmanipulated Du145	MHC Class I	362	353	366	134656	122996	140187	132613	8776
Manipulated HUVECs	MHC Class I	205	194	198	27735	24829	25849	26138	1475
Unmanipulated HUVECs	MHC Class I	193	ND	ND	24580	NA	NA	24580	NA
Unmanipulated Du145 Cells Only		89	88	86	8631	8544	8374	8516	131
Unmanipulated Du145 Cells And FITC		304	309	310	75115	78992	79791	77966	2501
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	140	137	138	14419	13990	14132	14180	219
Unattached Du145	Alpha 4	135	139	140	13712	14275	14419	14135	374
Unmanipulated Du145	Alpha 4	123	125	126	12152	12399	12524	12358	190
Manipulated HUVECs	Alpha 4	180	185	187	21566	22679	23140	22462	809
Unmanipulated HUVECs	Alpha 4	166	162	ND	18732	17993	ND	18362	523
Attached Du145 (to HUVECs)	MHC Class I	421	408	394	243834	213932	185817	214528	29013
Unattached Du145	MHC Class I	403	393	397	203433	183957	191513	192968	9819
Unmanipulated Du145	MHC Class I	365	369	372	138783	144484	148913	144060	5078
Manipulated HUVECs	MHC Class I	209	197	197	28875	25590	25590	26685	1896
Unmanipulated HUVECs	MHC Class I	193	ND	ND	24580	NA	NA	24580	NA
Unmanipulated Du145 Cells Only		89	88	86	8631	8544	8374	8516	131
Unmanipulated Du145 Cells And FITC		304	309	310	75115	78992	79791	77966	2501
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	140	145	148	14419	15163	15628	15070	610
Unattached Du145	Alpha 4	150	155	156	15946	16769	16938	16551	531
Unmanipulated Du145	Alpha 4	149	143	149	15786	14861	15786	15478	534
Manipulated HUVECs	Alpha 4	170	178	180	19501	21136	21566	20734	1089
Unmanipulated HUVECs	Alpha 4	166	162	ND	18732	17993	ND	18362	523
Attached Du145 (to HUVECs)	MHC Class I	424	421	433	251308	243834	275133	256758	16346
Unattached Du145	MHC Class I	429	433	428	264277	275133	261631	267013	7155
Unmanipulated Du145	MHC Class I	404	397	413	205491	191513	224972	207326	16805
Manipulated HUVECs	MHC Class I	192	195	199	24334	25080	26110	25175	892
Unmanipulated HUVECs	MHC Class I	193	ND	ND	24580	NA	NA	24580	NA
Unmanipulated Du145 Cells Only		89	88	86	8631	8544	8374	8516	131
Unmanipulated Du145 Cells And FITC		304	309	310	75115	78992	79791	77966	2501
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

Appendix Table 5.5.4a The Expression Of α4 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26* Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α4 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	163	165	ND	18175	18544	NA	18359	261
Unattached Du145	Alpha 4	169	176	ND	19306	20715	NA	20010	996
Unmanipulated Du145	Alpha 4	154	152	163	16601	16270	18175	17015	1018
Manipulated HUVECs	Alpha 4	184	174	ND	22452	20302	NA	21377	1520
Unmanipulated HUVECs	Alpha 4	164	161	ND	18358	17812	NA	18085	386
Attached Du145 (to HUVECs)	MHC Class I	414	420	411	227248	241392	220489	229710	10667
Unattached Du145	MHC Class I	390	408	ND	178486	213932	ND	196209	25064
Unmanipulated Du145	MHC Class I	377	378	376	156598	158182	155030	156603	1576
Manipulated HUVECs	MHC Class I	216	210	201	30982	29167	26641	28930	2180
Unmanipulated HUVECs	MHC Class I	213	158	ND	30061	17283	NA	23672	9035
Unmanipulated Du145 Cells Only		115	115	119	11212	11212	11672	11365	266
Unmanipulated Du145 Cells And FITC		141	138	137	14565	14132	13990	14229	299
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	165	180	171	18544	21566	19698	19936	1525
Unattached Du145	Alpha 4	171	ND	ND	19698	NA	NA	19698	NA
Unmanipulated Du145	Alpha 4	156	156	163	16938	16938	18175	17350	714
Manipulated HUVECs	Alpha 4	181	191	197	21784	24090	25590	23821	1917
Unmanipulated HUVECs	Alpha 4	213	158	ND	30061	17283	ND	23672	9035
Attached Du145 (to HUVECs)	MHC Class I	399	399	417	195407	195407	234213	208342	22405
Unattached Du145	MHC Class I	391	ND	ND	180291	NA	NA	180291	NA
Unmanipulated Du145	MHC Class I	389	378	394	176699	158182	185817	173566	14082
Manipulated HUVECs	MHC Class I	181	191	197	21784	24090	25590	23821	1917
Unmanipulated HUVECs	MHC Class I	213	158	ND	30061	NA	NA	30061	NA
Unmanipulated Du145 Cells Only		115	115	119	11212	11212	11672	11365	266
Unmanipulated Du145 Cells And FITC		141	138	137	14565	14132	13990	14229	299
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	153	153	ND	16435	16435	NA	16435	0
Unattached Du145	Alpha 4	159	169	ND	17458	19306	NA	18382	1307
Unmanipulated Du145	Alpha 4	380	401	395	161398	199380	187697	182825	19454
Manipulated HUVECs	Alpha 4	158	156	ND	17283	16938	NA	17111	244
Unmanipulated HUVECs	Alpha 4	164	161	ND	18358	17812	NA	18085	386
Attached Du145 (to HUVECs)	MHC Class I	401	385	412	199380	169727	222720	197275	26559
Unattached Du145	MHC Class I	375	ND	ND	153477	NA	NA	153477	NA
Unmanipulated Du145	MHC Class I	380	401	395	161398	199380	187697	182825	19454
Manipulated HUVECs	MHC Class I	183	182	193	22227	22004	24580	22937	1427
Unmanipulated HUVECs	MHC Class I	114	ND	ND	11099	NA	NA	11099	NA
Unmanipulated Du145 Cells Only		115	115	119	11212	11212	11672	11365	266
Unmanipulated Du145 Cells And FITC		141	138	137	14565	14132	13990	14229	299
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

Appendix Table 5.5.4b The Expression Of α4 By HUVECs And Prostatic Adenocarcinoma Cells Of TheDu145 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26* Du145 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. α4 surface expresseion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	161	161	163	17812	17812	18175	17933	209
Unattached Du145	Alpha 4	160	156	152	17634	16938	16270	16947	682
Unmanipulated Du145	Alpha 4	154	152	163	16601	16270	18175	17015	1018
Manipulated HUVECs	Alpha 4	185	189	191	22679	23610	24090	23460	718
Unmanipulated HUVECs	Alpha 4	164	161	ND	18358	17812	NA	18085	386
Attached Du145 (to HUVECs)	MHC Class I	394	378	378	185817	158182	158182	167394	15956
Unattached Du145	MHC Class I	293	298	311	67244	70714	80598	72852	6929
Unmanipulated Du145	MHC Class I	377	378	376	156598	158182	155030	156603	1576
Manipulated HUVECs	MHC Class I	235	229	230	37510	35313	35670	36164	1179
Unmanipulated HUVECs	MHC Class I	213	158	ND	30061	17283	NA	23672	9035
Unmanipulated Du145 Cells Only		115	115	119	11212	11212	11672	11365	266
Unmanipulated Du145 Cells And FITC		141	138	137	14565	14132	13990	14229	299
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	157	155	154	17110	16769	16601	16826	259
Unattached Du145	Alpha 4	139	145	160	14275	15163	17634	15691	1741
Unmanipulated Du145	Alpha 4	156	156	163	16938	16938	18175	17350	714
Manipulated HUVECs	Alpha 4	179	166	168	21350	18732	19113	19731	1415
Unmanipulated HUVECs	Alpha 4	164	161	ND	18358	17812	NA	18085	386
Attached Du145 (to HUVECs)	MHC Class I	374	389	368	151940	176699	143037	157226	17442
Unattached Du145	MHC Class I	317	288	ND	85614	63944	NA	74779	15323
Unmanipulated Du145	MHC Class I	389	378	394	176699	158182	185817	173566	14082
Manipulated HUVECs	MHC Class I	257	251	260	46807	44064	48241	46371	2123
Unmanipulated HUVECs	MHC Class I	213	158	ND	30061	17283	NA	23672	9035
Unmanipulated Du145 Cells Only		115	115	119	11212	11212	11672	11365	266
Unmanipulated Du145 Cells And FITC		141	138	137	14565	14132	13990	14229	299
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 4	161	167	157	17812	18921	17110	17948	913
Unattached Du145	Alpha 4	187	174	168	23140	20302	19113	20852	2069
Unmanipulated Du145	Alpha 4	163	167	164	18175	18921	18358	18485	389
Manipulated HUVECs	Alpha 4	168	180	173	19113	21566	20099	20259	1234
Unmanipulated HUVECs	Alpha 4	213	158	ND	30061	17283	NA	23672	9035
Attached Du145 (to HUVECs)	MHC Class I	368	369	383	143037	144484	166345	151289	13059
Unattached Du145	MHC Class I	316	304	308	84757	75115	78201	79358	4924
Unmanipulated Du145	MHC Class I	380	401	395	161398	199380	187697	182825	19454
Manipulated HUVECs	MHC Class I	213	210	216	30061	29167	30982	30070	908
Unmanipulated HUVECs	MHC Class I	213	158	ND	30061	17283	NA	23672	NA
Unmanipulated Du145 Cells Only		115	115	119	11212	11212	11672	11365	266
Unmanipulated Du145 Cells And FITC		141	138	137	14565	14132	13990	14229	299
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

Appendix Table 5.5.4c The Expression Of α4 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α4 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 5	352	348	348	121764	116960	116960	118561	2774
Unattached Du145	Alpha 5	330	332	ND	97581	99565	NA	98573	1403
Unmanipulated Du145	Alpha 5	351	328	339	120545	95636	106832	107671	12475
Manipulated HUVECs	Alpha 5	373	336	354	150419	103655	124240	126104	23438
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	427	406	427	259011	209669	259011	242564	28488
Unattached Du145	MHC Class I	413	406	ND	224972	209669	NA	217321	10821
Unmanipulated Du145	MHC Class I	393	372	365	183957	148913	138783	157218	23704
Manipulated HUVECs	MHC Class I	149	132	143	15786	13304	14861	14650	1255
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		84	82	71	8207	8043	7201	7817	540
Unmanipulated Du145 Cells And FITC		88	96	82	8544	9260	8043	8616	612
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 5	339	325	329	106832	92792	96604	98743	7260
Unattached Du145	Alpha 5	324	318	ND	91863	86480	NA	89172	3806
Unmanipulated Du145	Alpha 5	345	335	333	113481	102617	100572	105557	6939
Manipulated HUVECs	Alpha 5	391	336	357	180291	103655	128048	137331	39153
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	417	410	406	234213	218282	209669	220721	12453
Unattached Du145	MHC Class I	411	387	406	220489	173178	209669	201112	24789
Unmanipulated Du145	MHC Class I	407	352	361	211790	121764	133308	155620	48985
Manipulated HUVECs	MHC Class I	144	134	129	15011	13574	12908	13831	1075
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		84	82	71	8207	8043	7201	7817	540
Unmanipulated Du145 Cells And FITC		88	96	82	8544	9260	8043	8616	612
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 5	348	335	338	116960	102617	105762	108446	7539
Unattached Du145	Alpha 5	354	323	335	124240	90943	102617	105933	16894
Unmanipulated Du145	Alpha 5	375	349	323	153477	118143	90943	122854	31355
Manipulated HUVECs	Alpha 5	375	337	332	153477	104703	99565	119248	29754
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	399	386	401	195407	171444	199380	188743	15113
Unattached Du145	MHC Class I	393	382	388	183957	164679	174929	174522	9645
Unmanipulated Du145	MHC Class I	387	390	376	173178	178486	155030	168898	12300
Manipulated HUVECs	MHC Class I	138	124	133	14132	12275	13438	13282	938
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		84	82	71	8207	8043	7201	7817	540
Unmanipulated Du145 Cells And FITC		88	96	82	8544	9260	8043	8616	612
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

Appendix Table 5.5.5a The Expression Of α5 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α5 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 5	378	353	339	158182	122996	106832	129336	26256
Unattached Du145	Alpha 5	382	369	362	164679	144484	134656	147940	15307
Unmanipulated Du145	Alpha 5	378	360	340	158182	131973	107912	132689	25142
Manipulated HUVECs	Alpha 5	289	280	273	64590	58997	54984	59524	4825
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	370	401	388	145946	199380	174929	173418	26749
Unattached Du145	MHC Class I	393	387	386	183957	173178	171444	176193	6780
Unmanipulated Du145	MHC Class I	378	360	340	158182	131973	107912	132689	25142
Manipulated HUVECs	MHC Class I	110	167	164	10662	18921	18358	15980	4615
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		83	74	81	8125	7421	7963	7836	368
Unmanipulated Du145 Cells And FITC		97	90	90	9354	8718	8718	8930	367
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 5	ND	ND	ND	NA	NA	NA	NA	NA
Unattached Du145	Alpha 5	365	341	339	138783	109004	106832	118206	17853
Unmanipulated Du145	Alpha 5	403	398	400	203433	193450	197383	198089	5029
Manipulated HUVECs	Alpha 5	338	304	308	105762	75115	78201	86359	16874
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	408	371	389	213932	147422	176699	179351	33334
Unattached Du145	MHC Class I	398	394	383	193450	185817	166345	181871	13977
Unmanipulated Du145	MHC Class I	403	398	400	203433	193450	197383	198089	5029
Manipulated HUVECs	MHC Class I	187	26	158	23140	4578	17283	15000	9489
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		83	74	81	8125	7421	7963	7836	368
Unmanipulated Du145 Cells And FITC		97	90	90	9354	8718	8718	8930	367
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Value			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	Alpha 5	379	384	386	159782	168027	171444	166418	5995
Unattached Du145	Alpha 5	361	348	365	133308	116960	138783	129684	11354
Unmanipulated Du145	Alpha 5	373	339	356	150419	106832	126766	128006	21820
Manipulated HUVECs	Alpha 5	292	267	261	66570	51762	48729	55687	9546
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	397	376	381	191513	155030	163030	169858	19176
Unattached Du145	MHC Class I	394	392	388	185817	182115	174929	180954	5536
Unmanipulated Du145	MHC Class I	330	304	308	97581	75115	78201	83632	12178
Manipulated HUVECs	MHC Class I	187	170	174	23140	19501	20302	20981	1912
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		83	74	81	8125	7421	7963	7836	368
Unmanipulated Du145 Cells And FITC		97	90	90	9354	8718	8718	8930	367
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

Appendix Table 5.5.5b The Expression Of α5 By HUVECs And Prostatic Adenocarcinoma Cells Of TheDu145 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ Du145 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. α5 surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD standard deviation; TCGP, tissue culture grade plate.

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached Du145 (to HUVECs)	Alpha 5	394	381	404	185817	163030	205491	184780	21249
Unattached Du145	Alpha 5	381	377	367	163030	156598	141605	153744	10994
Unmanipulated Du145	Alpha 5	386	364	407	171444	137394	211790	173542	37242
Manipulated HUVECs	Alpha 5	334	300	334	101589	72151	101589	91777	16996
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	374	365	343	151940	138783	111220	133981	20781
Unattached Du145	MHC Class I	393	386	372	183957	171444	148913	168104	17759
Unmanipulated Du145	MHC Class I	378	360	340	158182	131973	107912	132689	25142
Manipulated HUVECs	MHC Class I	195	197	193	25080	25590	24580	25083	505
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		83	74	81	8125	7421	7963	7836	368
Unmanipulated Du145 Cells And FITC		97	90	90	9354	8718	8718	8930	367
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached Du145 (to HUVECs)	Alpha 5	381	385	379	163030	169727	159782	164180	5071
Unattached Du145	Alpha 5	372	368	367	148913	143037	141605	144518	3872
Unmanipulated Du145	Alpha 5	368	356	349	143037	126766	118143	129315	12642
Manipulated HUVECs	Alpha 5	429	402	373	264277	201396	150419	205364	57033
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	371	386	361	147422	171444	133308	150724	19281
Unattached Du145	MHC Class I	387	370	379	173178	145946	159782	159635	13617
Unmanipulated Du145	MHC Class I	403	398	400	203433	193450	197383	198089	5029
Manipulated HUVECs	MHC Class I	154	158	143	16601	17283	14861	16248	1249
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		83	74	81	8125	7421	7963	7836	368
Unmanipulated Du145 Cells And FITC		97	90	90	9354	8718	8718	8930	367
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached Du145 (to HUVECs)	Alpha 5	406	379	380	209669	159782	161398	176949	28348
Unattached Du145	Alpha 5	364	365	375	137394	138783	153477	143218	8912
Unmanipulated Du145	Alpha 5	373	339	356	150419	106832	126766	128006	21820
Manipulated HUVECs	Alpha 5	198	191	202	25849	24090	26910	25616	1424
Unmanipulated HUVECs	Alpha 5	359	350	348	130651	119338	116960	122316	7316
Attached Du145 (to HUVECs)	MHC Class I	347	355	361	115789	125496	133308	124864	8777
Unattached Du145	MHC Class I	352	345	347	121764	113481	115789	117011	4275
Unmanipulated Du145	MHC Class I	330	304	308	97581	75115	78201	83632	12178
Manipulated HUVECs	MHC Class I	198	191	202	25849	24090	26910	25616	1424
Unmanipulated HUVECs	MHC Class I	206	187	184	28016	23140	22452	24536	3033
Unmanipulated Du145 Cells Only		83	74	81	8125	7421	7963	7836	368
Unmanipulated Du145 Cells And FITC		97	90	90	9354	8718	8718	8930	367
Unmanipulated HUVECs Only		92	85	92	8895	8290	8895	8693	349
Unmanipulated HUVECs And FITC		137	141	142	13990	14565	14712	14423	381

Appendix Table 5.5.5c The Expression Of α5 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α5 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	246	239	235	41902	39051	37510	39488	2228
Unattached Du145	CD44	254	270	273	45415	53349	54984	51249	5119
Unmanipulated Du145	CD44	268	274	275	52286	55540	56102	54643	2060
Manipulated HUVECs	CD44	270	268	267	53349	52286	51762	52466	808
Unmanipulated HUVECs	CD44	304	350	ND	75115	119338	ND	97227	31270
Attached Du145 (to HUVECs)	MHC Class I	434	421	424	277915	243834	251308	257686	17914
Unattached Du145	MHC Class I	432	437	454	272378	286434	339880	299564	35615
Unmanipulated Du145	MHC Class I	409	420	432	216096	241392	272378	243289	28189
Manipulated HUVECs	MHC Class I	172	176	172	19898	20715	19898	20170	472
Unmanipulated HUVECs	MHC Class I	340	318	ND	107912	86480	ND	97196	15155
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du14 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	251	248	239	44064	42753	39051	41956	2600
Unattached Du145	CD44	252	249	253	44510	43186	44960	44218	922
Unmanipulated Du145	CD44	266	271	264	51244	53888	50223	51785	1892
Manipulated HUVECs	CD44	287	282	265	63303	60197	50731	58077	6549
Unmanipulated HUVECs	CD44	304	350	ND	75115	119338	ND	97226	31270
Attached Du145 (to HUVECs)	MHC Class I	433	436	434	275133	283566	277915	278871	4297
Unattached Du145	MHC Class I	439	439	447	292258	292258	316761	300425	14147
Unmanipulated Du145	MHC Class I	423	406	413	248792	209669	224972	227811	19715
Manipulated HUVECs	MHC Class I	165	173	168	18544	20099	19113	19252	787
Unmanipulated HUVECs	MHC Class I	340	318	ND	107912	86480	ND	97196	15155
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	233	237	233	36763	38273	36763	37266	872
Unattached Du145	CD44	251	263	261	44064	49720	48729	47504	3020
Unmanipulated Du145	CD44	262	252	261	49222	44510	48729	47487	2590
Manipulated HUVECs	CD44	273	275	272	54984	56102	54434	55173	850
Unmanipulated HUVECs	CD44	304	350	ND	75115	119338	ND	97226	31270
Attached Du145 (to HUVECs)	MHC Class I	424	417	414	251308	234213	227248	237590	12380
Unattached Du145	MHC Class I	441	441	442	298200	298200	301216	299205	1741
Unmanipulated Du145	MHC Class I	397	380	409	191513	161398	216096	189669	27396
Manipulated HUVECs	MHC Class I	163	167	169	18175	18921	19306	18801	575
Unmanipulated HUVECs	MHC Class I	340	318	ND	107912	86480	ND	97196	15155
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

Appendix Table 5.5.6a The Expression Of CD44 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26+ Du145 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and CD44 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	214	228	219	30365	34959	31932	32418	2335
Unattached Du145	CD44	281	289	280	59594	64590	58997	61061	3071
Unmanipulated Du145	CD44	223	207	227	33243	28299	34609	32050	3320
Manipulated HUVECs	CD44	155	166	153	16769	18732	16435	17312	1241
Unmanipulated HUVECs	CD44	209	215	199	28875	30672	26110	28552	2298
Attached Du145 (to HUVECs)	MHC Class I	ND	ND	ND	NA	NA	NA	NA	NA
Unattached Du145	MHC Class I	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated Du145	MHC Class I	344	367	335	112345	141605	102617	118856	20293
Manipulated HUVECs	MHC Class I	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated HUVECs	MHC Class I	174	187	177	20302	23140	20924	21456	1492
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	226	224	227	34262	33580	34609	34150	524
Unattached Du145	CD44	273	271	275	54984	53888	56102	54992	1107
Unmanipulated Du145	CD44	223	230	225	33243	35670	33919	34277	1252
Manipulated HUVECs	CD44	149	146	162	15786	15317	17993	16365	1429
Unmanipulated HUVECs	CD44	261	256	279	48729	46338	58407	51158	6390
Attached Du145 (to HUVECs)	MHC Class I	334	325	334	101589	92792	101589	98657	5079
Unattached Du145	MHC Class I	411	426	403	220489	256417	203433	226780	27046
Unmanipulated Du145	MHC Class I	328	330	305	95636	97581	75875	89697	12010
Manipulated HUVECs	MHC Class I	168	168	161	19113	19113	17812	18679	751
Unmanipulated HUVECs	MHC Class I	174	187	177	20302	23140	20924	21456	1492
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	205	208	207	27735	28585	28299	28207	433
Unattached Du145	CD44	276	279	279	56669	58407	58407	57828	1003
Unmanipulated Du145	CD44	237	233	219	38273	36763	31932	35656	3312
Manipulated HUVECs	CD44	132	131	127	13304	13171	12651	13042	345
Unmanipulated HUVECs	CD44	261	250	279	48729	43623	58407	50253	7509
Attached Du145 (to HUVECs)	MHC Class I	349	343	349	118143	111220	118143	115835	3997
Unattached Du145	MHC Class I	410	406	401	218282	209669	199380	209110	9463
Unmanipulated Du145	MHC Class I	337	343	329	104703	111220	96604	104176	7322
Manipulated HUVECs	MHC Class I	169	165	165	19306	18544	18544	18798	440
Unmanipulated HUVECs	MHC Class I	174	187	177	20302	23140	20924	21456	1492
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

Appendix Table 5.5.6b The Expression Of CD44 By HUVECs And Prostatic Adenocarcinoma Cells Of TheDu145 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26.

PKH26+ Du145 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. CD44 surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; NA, not applicable; ND, not done; SD standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	217	210	216	31295	29167	30982	30481	1149
Unattached Du145	CD44	222	228	233	32910	34959	36763	34877	1928
Unmanipulated Du145	CD44	223	207	227	33243	28299	34609	32050	3320
Manipulated HUVECs	CD44	201	179	182	26641	21350	22004	23332	2885
Unmanipulated HUVECs	CD44	174	187	177	20302	23140	20924	21456	1492
Attached Du145 (to HUVECs)	MHC Class I	369	356	358	144484	126766	129343	133531	9573
Unattached Du145	MHC Class I	394	386	389	185817	171444	176699	177987	7273
Unmanipulated Du145	MHC Class I	344	367	335	112345	141605	102617	118856	20293
Manipulated HUVECs	MHC Class I	201	179	182	26641	21350	22004	23332	2885
Unmanipulated HUVECs	MHC Class I	174	187	177	20302	23140	20924	21456	1492
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	207	216	215	28299	30982	30672	29984	1468
Unattached Du145	CD44	220	215	110	32255	30672	10662	24529	12036
Unmanipulated Du145	CD44	226	224	227	34262	33580	34609	34150	524
Manipulated HUVECs	CD44	141	144	135	14565	15011	13712	14429	660
Unmanipulated HUVECs	CD44	261	256	279	48729	46338	58407	51158	6390
Attached Du145 (to HUVECs)	MHC Class I	326	329	329	93731	96604	96604	95646	1659
Unattached Du145	MHC Class I	385	390	380	169727	178486	161398	169870	8545
Unmanipulated Du145	MHC Class I	328	330	305	95636	97581	75875	89697	12010
Manipulated HUVECs	MHC Class I	163	159	156	18175	17458	16938	17523	621
Unmanipulated HUVECs	MHC Class I	174	187	177	20302	23140	20924	21456	1492
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values For Medians			Mean MESF	SD Of Mean MESF
Attached Du145 (to HUVECs)	CD44	220	200	213	32255	26374	30061	29563	2972
Unattached Du145	CD44	205	213	209	27735	30061	28875	28890	1163
Unmanipulated Du145	CD44	237	233	219	38273	36763	31932	35656	3312
Manipulated HUVECs	CD44	171	144	158	19698	15011	17283	17331	2344
Unmanipulated HUVECs	CD44	261	250	279	48729	43623	58407	50253	7509
Attached Du145 (to HUVECs)	MHC Class I	323	327	330	90943	94679	97581	94401	3328
Unattached Du145	MHC Class I	395	403	403	187697	203433	203433	198188	9085
Unmanipulated Du145	MHC Class I	337	343	329	104703	111220	96604	104176	7322
Manipulated HUVECs	MHC Class I	159	157	162	17458	17110	17993	17520	445
Unmanipulated HUVECs	MHC Class I	174	187	177	20302	23140	20924	21456	1492
Unmanipulated Du145 Cells Only		92	94	97	8895	9076	9354	9108	231
Unmanipulated Du145 Cells And FITC		145	149	138	15163	15786	14132	15027	836
Unmanipulated HUVECs Only		88	103	ND	8544	9936	NA	9240	984
Unmanipulated HUVECs And FITC		94	108	ND	9076	10449	NA	9763	971

Appendix Table 5.5.6c The Expression Of CD44 By HUVECs And Prostatic Adenocarcinoma Cells From The Du145 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with the fluorescent membrane dye PKH26. PKH26* Du145 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and CD44 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell;MESF, molecular equivalent of soluble fluorochrome; NA, not applicabel; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs	VCAM-1	111	111	111	10769	10769	10769	10769	0
Unattached PC3	VCAM-1	123	116	110	12152	11325	10662	11379	747
Unmanipulated PC3	VCAM-1	80	73	81	7883	7347	7963	7731	335
Manipulated HUVECs	VCAM-1	90	90	91	8718	8718	8806	8747	51
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs	MHC Class I	162	153	151	17993	16435	16107	16845	1007
Unattached PC3	MHC Class I	160	140	146	17634	14419	15317	15790	1659
Unmanipulated PC3	MHC Class I	145	121	121	15163	11910	11910	12994	1879
Manipulated HUVECs	MHC Class I	172	160	159	19898	17634	17458	18330	1361
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		66	48	48	6847	5713	5713	6091	655
Unmanipulated PC3 Cells And FITC		97	78	77	9354	7726	7649	8243	963
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs	VCAM-1	112	130	108	10878	13039	10449	11455	1388
Unattached PC3	VCAM-1	108	114	119	10449	11099	11672	11074	612
Unmanipulated PC3	VCAM-1	97	76	72	9354	7572	7273	8067	1125
Manipulated HUVECs	VCAM-1	75	87	69	7496	8459	7057	7671	717
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs	MHC Class I	162	153	151	17993	16435	16107	16845	1007
Unattached PC3	MHC Class I	160	145	147	17634	15163	15472	16090	1346
Unmanipulated PC3	MHC Class I	131	110	120	13171	10662	11790	11874	1257
Manipulated HUVECs	MHC Class I	129	145	137	12908	15163	13990	14021	1128
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		66	48	48	6847	5713	5713	6091	655
Unmanipulated PC3 Cells And FITC		97	78	77	9354	7726	7649	8243	963
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs	VCAM-1	127	114	112	12651	11099	10878	11543	966
Unattached PC3	VCAM-1	131	122	119	13171	12030	11672	12291	782
Unmanipulated PC3	VCAM-1	111	85	73	10769	8290	7347	8802	1768
Manipulated HUVECs	VCAM-1	98	86	83	9449	8374	8125	8649	704
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs	MHC Class I	168	166	162	19113	18732	17993	18612	569
Unattached PC3	MHC Class I	174	163	152	20302	18175	16270	18249	2017
Unmanipulated PC3	MHC Class I	148	129	119	15628	12908	11672	13403	2024
Manipulated HUVECs	MHC Class I	168	163	161	19113	18175	17812	18367	671
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		66	48	48	6847	5713	5713	6091	655
Unmanipulated PC3 Cells And FITC		97	78	77	9354	7726	7649	8243	963
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

Appendix Table 5.5.7a The Expression Of VCAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and VCAM-1 surface expression was detected by flow cytometry. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; NA, not applicable; ND, not done; VCAM, vascular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Cooresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs	VCAM-1	144	133	137	15011	13438	13990	14147	798
Unattached PC3	VCAM-1	151	146	141	16107	15317	14565	15330	771
Unmanipulated PC3	VCAM-1	137	115	111	13990	11212	10769	11990	1746
Manipulated HUVECs	VCAM-1	82	64	64	8043	6711	6711	7155	769
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs	MHC Class I	224	215	212	33580	30672	29760	31337	1995
Unattached PC3	MHC Class I	215	201	215	30672	26641	30672	29328	2327
Unmanipulated PC3	MHC Class I	214	191	159	30365	24090	17458	23971	6454
Manipulated HUVECs	MHC Class I	135	119	120	13712	11672	11790	12391	1145
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		166	92	93	18732	8895	8985	12204	5653
Unmanipulated PC3 Cells And FITC		128	130	119	12779	13039	11672	12497	726
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs	VCAM-1	140	132	137	14419	13304	13990	13904	563
Unattached PC3	VCAM-1	146	135	136	15317	13712	13850	14293	889
Unmanipulated PC3	VCAM-1	192	157	132	24334	17110	13304	18249	5603
Manipulated HUVECs	VCAM-1	75	56	62	7496	6192	6577	6755	670
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs	MHC Class I	212	194	206	29760	24829	28016	27535	2500
Unattached PC3	MHC Class I	205	212	210	27735	29760	29167	28887	1041
Unmanipulated PC3	MHC Class I	192	157	132	24334	17110	13304	18249	5603
Manipulated HUVECs	MHC Class I	114	80	87	11099	7883	8459	9147	1715
Unmanipulated HUVECs	MHC Class I	434	439		277915	292258	3524	191232	162718
Unmanipulated PC3 Cells Only		166	92	93	18732	8895	8985	12204	5653
Unmanipulated PC3 Cells And FITC		128	130	119	12779	13039	11672	12497	726
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

EXPERIMENT 3

Cell Type	Marker	Medain Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs	VCAM-1	68	ND	ND	6986	ND	ND	6986	NA
Unattached PC3	VCAM-1	145	142	143	15163	14712	14861	14912	230
Unmanipulated PC3	VCAM-1	147	174	140	15472	20302	14419	16731	3137
Manipulated HUVECs	VCAM-1	56	ND	ND	6192	ND	ND	6192	NA
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs	MHC Class I	132	128	134	13304	12779	13574	13219	404
Unattached PC3	MHC Class I	219	212	206	31932	29760	28016	29902	1962
Unmanipulated PC3	MHC Class I	207	182	201	28299	22004	26641	25648	3263
Manipulated HUVECs	MHC Class I	78	71	74	7726	7201	7421	7449	264
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		166	92	93	18732	8895	8985	12204	5653
Unmanipulated PC3 Cells And FITC		128	130	119	12779	13039	11672	12497	726
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

Appendix Table 5.5.7b The Expression Of VCAM-1 By HUVECs And Prostatic Adenocarcinoma Cells Of The PC3 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ PC3 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. VCAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; NA, not applicable; ND, not done; VCAM, vascular cell adhesion molecule; MESF, molecular equivalent to soluble fluorochrome; SD standard deviation; TCGP, tissue culture grade plate.

Appendices

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	VCAM-1	147	145	134	15472	15163	13574	14736	1018
Unattached PC3	VCAM-1	124	123	122	12275	12152	12030	12152	122
Unmanipulated PC3	VCAM-1	137	115	111	13990	11212	10769	11990	1746
Manipulated HUVECs	VCAM-1	76	77	61	7572	7649	6511	7244	636
Unmanipulated HUVECs	VCAM-1	151	146	141	16107	15317	14565	15330	771
Attached PC3 (to HUVECs)	MHC Class I	223	222	224	33243	32910	33580	33244	335
Unattached PC3	MHC Class I	212	207	195	29760	28299	25080	27713	2394
Unmanipulated PC3	MHC Class I	214	191	159	30365	24090	17458	23971	6454
Manipulated HUVECs	MHC Class I	135	119	120	13712	11672	11790	12391	1145
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		166	92	93	18732	8895	8985	12204	5653
Unmanipulated PC3 Cells And FITC		128	130	119	12779	13039	11672	12497	726
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	VCAM-1	142	139	142	14712	14275	14712	14566	253
Unattached PC3	VCAM-1	122	119	114	12030	11672	11099	11601	469
Unmanipulated PC3	VCAM-1	134	108	108	13574	10449	10449	11491	1804
Manipulated HUVECs	VCAM-1	45	34	29	5543	4962	4718	5074	424
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs)	MHC Class I	211	214	236	29462	30365	37890	32572	4627
Unattached PC3	MHC Class I	179	164	163	21350	18358	18175	19294	1783
Unmanipulated PC3	MHC Class I	192	157	132	24334	17110	13304	18249	5603
Manipulated HUVECs	MHC Class I	83	74	77	8125	7421	7649	7732	359
Unmanipulated HUVECs	MHC Class I	434	439	433	277915	292258	275133	281769	9190
Unmanipulated PC3 Cells Only		166	92	93	18732	8895	8985	12204	5653
Unmanipulated PC3 Cells And FITC		128	130	119	12779	13039	11672	12497	726
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	VCAM-1	160	158	158	17634	17283	17283	17400	203
Unattached PC3	VCAM-1	112	114	109	10878	11099	10555	10844	274
Unmanipulated PC3	VCAM-1	147	174	140	15472	20302	14419	16731	3137
Manipulated HUVECs	VCAM-1	57	20	20	6254	4310	4310	4958	1123
Unmanipulated HUVECs	VCAM-1	101	70	86	9738	7128	8374	8414	1305
Attached PC3 (to HUVECs)	MHC Class I	219	216	215	31932	30982	30672	31195	656
Unattached PC3	MHC Class I	160	161	164	17634	17812	18358	17935	377
Unmanipulated PC3	MHC Class I	207	182	201	28299	22004	26641	25648	3263
Manipulated HUVECs	MHC Class I	110	104	102	10662	10037	9837	10178	430
Unmanipulated HUVECs	MHC Class I	434	439	433	292258	275133	275133	280841	9887
Unmanipulated PC3 Cells Only		166	92	93	18732	8895	8985	12204	5653
Unmanipulated PC3 Cells And FITC		128	130	119	12779	13039	11672	12497	726
Unmanipulated HUVECs Only		90	81	ND	8718	7963	NA	8340	534
Unmanipulated HUVECs And FITC		151	141	133	16107	14565	13438	14703	1340

Appendix Table 5.5.7c The Expression Of VCAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26.

PKH26+ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and VCAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; NA, not applicable; ND, not done; VCAM, vascular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
E	Attached PC3 (to HUVECs)	Alpha 4	118	125	112	11555	12399	10878	11611	762
X	Unattached PC3	Alpha 4	122	107	113	12030	10344	10988	11121	851
P	Unmanipulated PC3	Alpha 4	112	86	90	10878	8374	8718	9323	1358
E	Manipulated HUVECs	Alpha 4	128	141	120	12779	14565	11790	13045	1406
R	Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
I	Attached PC3 (to HUVECs)	MHC Class I	206	197	205	28016	25590	27735	27114	1327
M	Unattached PC3	MHC Class I	186	178	187	22908	21136	23140	22395	1096
E	Unmanipulated PC3	MHC Class I	194	167	159	24829	18921	17458	20402	3903
N	Manipulated HUVECs	MHC Class I	196	137	139	25334	13990	14275	17866	6468
T	Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
1	Unmanipulated PC3 Cells Only		69	49	54	7057	5770	6068	6299	674
	Unmanipulated PC3 Cells And FITC		92	88	66	8895	8544	6847	8095	1095
	Unmanipulated HUVECs Only		76	53	56	7572	6007	6192	6590	855
	Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
	Attached PC3 (to HUVECs)	Alpha 4	120	105	101	11790	10138	9738	10556	1088
E	Unattached PC3	Alpha 4	94	92	99	9076	8895	9544	9172	335
X	Unmanipulated PC3	Alpha 4	100	67	81	9641	6916	7963	8173	1374
P	Manipulated HUVECs	Alpha 4	144	132	129	15011	13304	12908	13741	1118
E	Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
R	Attached PC3 (to HUVECs)	MHC Class I	190	186	193	23849	22908	24580	23779	838
I	Unattached PC3	MHC Class I	164	174	185	18358	20302	22679	20446	2164
M	Unmanipulated PC3	MHC Class I	136	128	136	13850	12779	13850	13493	619
E	Manipulated HUVECs	MHC Class I	129	110	112	12908	10662	10878	11483	1239
N	Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
T	Unmanipulated PC3 Cells Only		69	49	54	7057	5770	6068	6299	674
2	Unmanipulated PC3 Cells And FITC		92	88	66	8895	8544	6847	8095	1095
	Unmanipulated HUVECs Only		76	53	56	7572	6007	6192	6590	855
	Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
	Attached PC3 (to HUVECs)	Alpha 4	26	90	85	4578	8718	8290	7195	2277
E	Unattached PC3	Alpha 4	106	107	108	10241	10344	10449	10345	104
X	Unmanipulated PC3	Alpha 4	108	95	80	10449	9168	7883	9167	1283
P	Manipulated HUVECs	Alpha 4	25	91	77	4532	8806	7649	6996	2210
E	Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
R	Attached PC3 (to HUVECs)	MHC Class I	181	183	192	21784	22227	24334	22782	1363
I	Unattached PC3	MHC Class I	180	170	162	21566	19501	17993	19687	1794
M	Unmanipulated PC3	MHC Class I	179	157	144	21350	17110	15011	17824	3229
E	Manipulated HUVECs	MHC Class I	166	142	154	18732	14712	16601	16682	2011
N	Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
T	Unmanipulated PC3 Cells Only		69	49	54	7057	5770	6068	6299	674
3	Unmanipulated PC3 Cells And FITC		92	88	66	8895	8544	6847	8095	1095
	Unmanipulated HUVECs Only		76	53	56	7572	6007	6192	6590	855
	Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

Appendix Table 5.5.8a The Expression Of α4 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26+ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α4 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
E	Cell Type	Marker								
X	Attached PC3 (to HUVECs)	Alpha 4	135	121	124	13712	11910	12275	12632	953
P	Unattached PC3	Alpha 4	135	123	121	13712	12152	11910	12591	978
E	Unmanipulated PC3	Alpha 4	129	110	97	12908	10662	9354	10975	1798
R	Manipulated HUVECs	Alpha 4	129	73	92	12908	7347	8895	9717	2870
I	Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
M	Attached PC3 (to HUVECs)	MHC Class I	220	212	220	32255	29760	32255	31423	1441
E	Unattached PC3	MHC Class I	258	252	240	47280	44510	39446	43745	3972
N	Unmanipulated PC3	MHC Class I	195	176	153	25080	20715	16435	20743	4323
T	Manipulated HUVECs	MHC Class I	144	119	125	15011	11672	12399	13028	1756
1	Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
	Unmanipulated PC3 Cells Only		88	80	76	8544	7883	7572	8000	496
	Unmanipulated PC3 Cells And FITC		125	102	111	12399	9837	10769	11002	1297
	Unmanipulated HUVECs Only		76	63	56	7572	6644	6192	6802	704
	Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

	Cell Type	Marker	Median Level Of Fluoescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
E	Cell Type	Marker								
X	Attached PC3 (to HUVECs)	Alpha 4	126	133	125	12524	13438	12399	12787	567
P	Unattached PC3	Alpha 4	131	124	127	13171	12275	12651	12699	450
E	Unmanipulated PC3	Alpha 4	112	113	55	10878	10988	6130	9332	2774
R	Manipulated HUVECs	Alpha 4	111	123	118	10769	12152	11555	11492	693
I	Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
M	Attached PC3 (to HUVECs)	MHC Class I	206	208	204	28016	28585	27458	28020	564
E	Unattached PC3	MHC Class I	260	240	251	48241	39446	44064	43917	4399
N	Unmanipulated PC3	MHC Class I	204	112	148	27458	10878	15628	17988	8538
T	Manipulated HUVECs	MHC Class I	95	95	95	9168	9168	9168	9168	0
2	Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
	Unmanipulated PC3 Cells Only		88	80	76	8544	7883	7572	8000	496
	Unmanipulated PC3 Cells And FITC		125	102	111	12399	9837	10769	11002	1297
	Unmanipulated HUVECs Only		76	63	56	7572	6644	6192	6802	704
	Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
E	Cell Type	Marker								
X	Attached PC3 (to HUVECs)	Alpha 4	137	119	121	13990	11672	11910	12524	1275
P	Unattached PC3	Alpha 4	123	115	117	12152	11212	11440	11601	490
E	Unmanipulated PC3	Alpha 4	112	98	91	10878	9449	8806	9711	1061
R	Manipulated HUVECs	Alpha 4	94	70	74	9076	7128	7421	7875	1050
I	Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
M	Attached PC3 (to HUVECs)	MHC Class I	220	229	219	32255	35313	31932	33166	1866
E	Unattached PC3	MHC Class I	229	230	234	35313	35670	37135	36039	966
N	Unmanipulated PC3	MHC Class I	179	170	153	21350	19501	16435	19095	2483
T	Manipulated HUVECs	MHC Class I	164	148	153	18358	15628	16435	16807	1403
3	Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
	Unmanipulated PC3 Cells Only		88	80	76	8544	7883	7572	8000	496
	Unmanipulated PC3 Cells And FITC		125	102	111	12399	9837	10769	11002	1297
	Unmanipulated HUVECs Only		76	63	56	7572	6644	6192	6802	704
	Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

Appendix Table 5.5.8b The Expression Of α4 By HUVECs And Prostatic Adenocarcinoma Cells Of The PC3 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26.

PKH26+ PC3 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. α4 surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 24.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs)	Alpha 4	133	124	127	13438	12275	12651	12788	594
Unattached PC3	Alpha 4	136	124	124	13850	12275	12275	12800	910
Unmanipulated PC3	Alpha 4	129	110	97	12908	10662	9354	10975	1798
Manipulated HUVECs	Alpha 4	15	86	68	4098	8374	6986	6486	2181
Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
Attached PC3 (to HUVECs)	MHC Class I	214	228	214	30365	34959	30365	31896	2652
Unattached PC3	MHC Class I	237	225	219	38273	33919	31932	34708	3243
Unmanipulated PC3	MHC Class I	195	176	153	25080	20715	16435	20743	4323
Manipulated HUVECs	MHC Class I	157	144	137	17110	15011	13990	15370	1590
Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
Unmanipulated PC3 Cells Only		88	80	76	8544	7883	7572	8000	496
Unmanipulated PC3 Cells And FITC		125	102	111	12399	9837	10769	11002	1297
Unmanipulated HUVECs Only		76	63	56	7572	6644	6192	6802	704
Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

(EXPERIMENT 1)

Cell Type	Marker	Median level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs)	Alpha 4	118	118	120	11555	11555	11790	11634	136
Unattached PC3	Alpha 4	128	124	123	12779	12275	12152	12402	332
Unmanipulated PC3	Alpha 4	112	113	55	10878	10988	6130	9332	2774
Manipulated HUVECs	Alpha 4	64	61	58	6711	6511	6318	6513	197
Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
Attached PC3 (to HUVECs)	MHC Class I	199	213	210	26110	30061	29167	28446	2072
Unattached PC3	MHC Class I	223	221	221	33243	32581	32581	32802	382
Unmanipulated PC3	MHC Class I	204	112	148	27458	10878	15628	17988	8538
Manipulated HUVECs	MHC Class I	112	100	100	10878	9641	9641	10053	714
Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
Unmanipulated PC3 Cells Only		88	80	76	8544	7883	7572	8000	496
Unmanipulated PC3 Cells And FITC		125	102	111	12399	9837	10769	11002	1297
Unmanipulated HUVECs Only		76	63	56	7572	6644	6192	6802	704
Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

(EXPERIMENT 2)

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached PC3 (to HUVECs)	Alpha 4	124	118	114	12275	11555	11099	11643	592
Unattached PC3	Alpha 4	129	116	117	12908	11325	11440	11891	883
Unmanipulated PC3	Alpha 4	112	98	91	10878	9449	8806	9711	1061
Manipulated HUVECs	Alpha 4	103	91	79	9936	8806	7804	8849	1067
Unmanipulated HUVECs	Alpha 4	105	107	61	10138	10344	6511	8998	2156
Attached PC3 (to HUVECs)	MHC Class I	235	215	236	37510	30672	37890	35357	4062
Unattached PC3	MHC Class I	233	227	229	36763	34609	35313	35561	1098
Unmanipulated PC3	MHC Class I	179	170	153	21350	19501	16435	19095	2483
Manipulated HUVECs	MHC Class I	154	116	141	16601	11325	14565	14164	2661
Unmanipulated HUVECs	MHC Class I	166	206	108	18732	28016	10449	19066	8788
Unmanipulated PC3 Cells Only		88	80	76	8544	7883	7572	8000	496
Unmanipulated PC3 Cells And FITC		125	102	111	12399	9837	10769	11002	1297
Unmanipulated HUVECs Only		76	63	56	7572	6644	6192	6802	704
Unmanipulated HUVECs And FITC		96	86	99	9260	8374	9544	9060	611

(EXPERIMENT 3)

Appendix Table 5.5.8c The Expression Of α4 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26+ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α4 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	174	178	173	20302	21136	20099	20512	550
Unattached PC3	Alpha L	178	186	178	21136	22908	21136	21727	1023
Unmanipulated PC3	Alpha L	170	184	184	19501	22452	22452	21468	1704
Manipulated HUVECs	Alpha L	132	135	132	13304	13712	13304	13440	235
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	278	276	266	57822	56669	51244	55245	3513
Unattached PC3	HLA-ABC	267	266	269	51762	51244	52815	51940	800
Unmanipulated PC3	HLA-ABC	262	242	257	49222	40248	46807	45426	4644
Manipulated HUVECs	HLA-ABC	199	199	184	26110	26110	22452	24891	2112
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		149	151	155	15786	16107	16769	16221	501
Unmanipulated PC3 Cells And FITC		182	189	181	22004	23610	21784	22466	997
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	201	205	205	26641	27735	27735	27371	632
Unattached PC3	Alpha L	209	211	ND	28875	29462	ND	29168	415
Unmanipulated PC3	Alpha L	209	209	201	28875	28875	26641	28130	1290
Manipulated HUVECs	Alpha L	123	128	132	12152	12779	13304	12745	577
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	276	277	277	56669	57243	57243	57052	331
Unattached PC3	HLA-ABC	265	286	284	50731	62669	61421	58274	6562
Unmanipulated PC3	HLA-ABC	281	286	277	59594	62669	57243	59835	2721
Manipulated HUVECs	HLA-ABC	181	186	177	21784	22908	20924	21872	995
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		149	151	155	15786	16107	16769	16221	501
Unmanipulated PC3 Cells And FITC		182	189	181	22004	23610	21784	22466	997
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	211	213	214	29462	30061	30365	29962	459
Unattached PC3	Alpha L	215	219	221	30672	31932	32581	31728	971
Unmanipulated PC3	Alpha L	220	202	204	32255	26910	27458	28874	2940
Manipulated HUVECs	Alpha L	111	112	109	10769	10878	10555	10734	165
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	283	285	286	60806	62042	62669	61839	948
Unattached PC3	HLA-ABC	281	280	277	59594	58997	57243	58611	1222
Unmanipulated PC3	HLA-ABC	282	281	288	60197	59594	63944	61245	2357
Manipulated HUVECs	HLA-ABC	177	180	181	20924	21566	21784	21425	447
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		149	151	155	15786	16107	16769	16221	501
Unmanipulated PC3 Cells And FITC		182	189	181	22004	23610	21784	22466	997
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

Appendix Table 5.5.9a The Expression Of αL By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and αL surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	176	172	180	20715	19898	21566	20726	834
Unattached PC3	Alpha L	187	192	175	23140	24334	20508	22660	1958
Unmanipulated PC3	Alpha L	170	180	183	19501	21566	22227	21098	1422
Manipulated HUVECs	Alpha L	131	125	135	13171	12399	13712	13094	660
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	237	252	256	38273	44510	46338	43040	4228
Unattached PC3	HLA-ABC	268	272	264	52286	54434	50223	52314	2105
Unmanipulated PC3	HLA-ABC	262	242	257	49222	40248	46807	45426	4644
Manipulated HUVECs	HLA-ABC	171	188	196	19698	23374	25334	22802	2861
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		112	113	119	10878	10988	11672	11180	430
Unmanipulated PC3 Cells And FITC		153	156	157	16435	16938	17110	16828	351
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	175	169	172	20508	19306	19898	19904	601
Unattached PC3	Alpha L	184	182	184	22452	22004	22452	22303	258
Unmanipulated PC3	Alpha L	185	169	172	22679	19306	19898	20627	1801
Manipulated HUVECs	Alpha L	119	113	123	11672	10988	12152	11604	585
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	244	243	232	41067	40655	36395	39372	2587
Unattached PC3	HLA-ABC	261	261	260	48729	48729	48241	48567	282
Unmanipulated PC3	HLA-ABC	261	251	260	48729	44064	48241	47011	2564
Manipulated HUVECs	HLA-ABC	159	141	137	17458	14565	13990	15338	1858
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		112	113	119	10878	10988	11672	11180	430
Unmanipulated PC3 Cells And FITC		153	156	157	16435	16938	17110	16828	351
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	167	175	170	18921	20508	19501	19643	803
Unattached PC3	Alpha L	187	186	193	23140	22908	24580	23543	906
Unmanipulated PC3	Alpha L	178	185	187	21136	22679	23140	22318	1049
Manipulated HUVECs	Alpha L	96	99	96	9260	9544	9260	9355	164
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	242	238	248	40248	38660	42753	40554	2064
Unattached PC3	HLA-ABC	262	265	259	49222	50731	47758	49237	1486
Unmanipulated PC3	HLA-ABC	245	261	257	41482	48729	46807	45673	3754
Manipulated HUVECs	HLA-ABC	159	150	170	17458	15946	19501	17635	1784
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		112	113	119	10878	10988	11672	11180	430
Unmanipulated PC3 Cells And FITC		153	156	157	16435	16938	17110	16828	351
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

Appendix Table 5.5.9b The Expression Of αL By HUVECs And Prostatic Adenocarcinoma Cells Of The PC3 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ PC3 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. αL surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; SD standard deviation; TCGP, tissue culture grade plate.)

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	167	159	163	18921	17458	18175	18184	732
Unattached PC3	Alpha L	166	172	167	18732	19898	18921	19183	626
Unmanipulated PC3	Alpha L	170	180	153	19501	21566	16435	19167	2582
Manipulated HUVECs	Alpha L	128	118	124	12779	11555	12275	12203	615
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	263	254	245	49720	45415	41482	45539	4120
Unattached PC3	HLA-ABC	230	248	226	35670	42753	34262	37562	4551
Unmanipulated PC3	HLA-ABC	262	242	257	49222	40248	46807	45426	4644
Manipulated HUVECs	HLA-ABC	254	233	219	45415	36763	31932	38036	6831
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		112	113	119	10878	10988	11672	11180	430
Unmanipulated PC3 Cells And FITC		153	156	157	16435	16938	17110	16828	351
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	168	195	170	19113	25080	19501	21231	3339
Unattached PC3	Alpha L	179	178	177	21350	21136	20924	21137	213
Unmanipulated PC3	Alpha L	185	169	172	22679	19306	19898	20627	1801
Manipulated HUVECs	Alpha L	118	154	111	11555	16601	10769	12975	3164
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	246	241	239	41902	39845	39051	40266	1471
Unattached PC3	HLA-ABC	254	259	256	45415	47758	46338	46504	1181
Unmanipulated PC3	HLA-ABC	261	251	260	48729	44064	48241	47011	2564
Manipulated HUVECs	HLA-ABC	174	172	179	20302	19898	21350	20517	749
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		112	113	119	10878	10988	11672	11180	430
Unmanipulated PC3 Cells And FITC		153	156	157	16435	16938	17110	16828	351
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha L	182	173	167	22004	20099	18921	20341	1556
Unattached PC3	Alpha L	165	162	172	18544	17993	19898	18811	980
Unmanipulated PC3	Alpha L	178	185	187	21136	22679	23140	22318	1049
Manipulated HUVECs	Alpha L	116	109	100	11325	10555	9641	10507	843
Unmanipulated HUVECs	Alpha L	111	115	97	10769	11212	9354	10445	970
Attached PC3 (to HUVECs)	HLA-ABC	245	239	253	41482	39051	44960	41831	2970
Unattached PC3	HLA-ABC	224	220	214	33580	32255	30365	32066	1616
Unmanipulated PC3	HLA-ABC	245	261	257	41482	48729	46807	45673	3754
Manipulated HUVECs	HLA-ABC	164	166	181	18358	18732	21784	19625	1879
Unmanipulated HUVECs	HLA-ABC	473	479	472	411499	437111	407378	418663	16109
Unmanipulated PC3 Cells Only		112	113	119	10878	10988	11672	11180	430
Unmanipulated PC3 Cells And FITC		153	156	157	16435	16938	17110	16828	351
Unmanipulated HUVECs Only		70	71	65	7128	7201	6779	7036	226
Unmanipulated HUVECs And FITC		112	107	105	10878	10344	10138	10454	382

Appendix Table 5.5.9c The Expression Of αL By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and αL surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	ICAM-1	355	356	351	125496	126766	120545	124269	3287
Unattached PC3	ICAM-1	353	347	333	122996	115789	100572	113119	11448
Unmanipulated PC3	ICAM-1	350	331	321	119338	98568	89131	102346	15454
Manipulated HUVECs	ICAM-1	156	152	155	16938	16270	16769	16659	347
Unmanipulated HUVECs	ICAM-1	160	157	163	17634	17110	18175	17639	532
Attached PC3 (to HUVECs)	CD3	166	166	162	18732	18732	17993	18485	427
Unattached PC3	CD3	185	188	205	22679	23374	27735	24596	2741
Unmanipulated PC3	CD3	177	174	173	20924	20302	20099	20442	430
Manipulated HUVECs	CD3	120	188	115	11790	23374	11212	15459	6861
Unmanipulated HUVECs	CD3	128	128	126	12779	12779	12524	12694	147
Unmanipulated PC3 Cells Only		167	167	166	18921	18921	18732	18858	109
Unmanipulated PC3 Cells And FITC		176	177	177	20715	20924	20924	20855	121
Unmanipulated HUVECs Only		104	98	100	10037	9449	9641	9709	300
Unmanipulated HUVECs And FITC		119	128	124	11672	12779	12275	12242	554

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	ICAM-1	360	354	349	131973	124240	118143	124785	6931
Unattached PC3	ICAM-1	344	328	353	112345	95636	122996	110326	13791
Unmanipulated PC3	ICAM-1	330	331	321	97581	98568	89131	95093	5187
Manipulated HUVECs	ICAM-1	158	160	164	17283	17634	18358	17758	549
Unmanipulated HUVECs	ICAM-1	141	154	149	14565	16601	15786	15651	1025
Attached PC3 (to HUVECs)	CD3	167	165	172	18921	18544	19898	19121	699
Unattached PC3	CD3	216	348	193	30982	116960	24580	57507	51587
Unmanipulated PC3	CD3	177	174	173	20924	20302	20099	20442	430
Manipulated HUVECs	CD3	118	116	153	11555	11325	16435	13105	2886
Unmanipulated HUVECs	CD3	126	125	124	12524	12399	12275	12399	125
Unmanipulated PC3 Cells Only		167	167	166	18921	18921	18732	18858	109
Unmanipulated PC3 Cells And FITC		176	177	177	20715	20924	20924	20855	121
Unmanipulated HUVECs Only		104	98	100	10037	9449	NA	9743	416
Unmanipulated HUVECs And FITC		119	128	124	11672	12779	12275	12242	554

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	ICAM-1	333	326	328	100572	93731	95636	96646	3531
Unattached PC3	ICAM-1	319	344	317	87355	112345	85614	95105	14956
Unmanipulated PC3	ICAM-1	330	331	321	97581	98568	89131	95093	5187
Manipulated HUVECs	ICAM-1	141	154	149	14565	16601	15786	15651	1025
Unmanipulated HUVECs	ICAM-1	160	167	166	17634	18921	18732	18429	695
Attached PC3 (to HUVECs)	CD3	174	173	175	20302	20099	20508	20303	204
Unattached PC3	CD3	194	224	209	24829	33580	28875	29094	4380
Unmanipulated PC3	CD3	177	174	173	20924	20302	20099	20442	430
Manipulated HUVECs	CD3	126	125	124	12524	12399	12275	12399	125
Unmanipulated HUVECs	CD3	128	128	126	12779	12779	12524	12694	147
Unmanipulated PC3 Cells Only		167	167	166	18921	18921	18732	18858	109
Unmanipulated PC3 Cells And FITC		176	177	177	20715	20924	20924	20855	121
Unmanipulated HUVECs Only		104	98	100	10037	9449	NA	9743	416
Unmanipulated HUVECs And FITC		119	128	124	11672	12779	12275	12242	554

Appendix Table 5.5.10a The Expression Of ICAM-1 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26+ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and ICAM-1 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular cell adhesion molecule; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	ICAM-1	342	338	336	110106	105762	103655	106508	3290
Unattached PC3	ICAM-1	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated PC3	ICAM-1	326	330	327	93731	97581	94679	95330	2006
Manipulated HUVECs	ICAM-1	141	137	154	14565	13990	16601	15052	1372
Unmanipulated HUVECs	ICAM-1	150	152	155	15946	16270	16769	16328	415
Attached PC3 (to HUVECs)	CD3	181	188	180	21784	23374	21566	22241	987
Unattached PC3	CD3	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated PC3	CD3	172	171	174	19898	19698	20302	19966	308
Manipulated HUVECs	CD3	117	122	119	11440	12030	11672	11714	297
Unmanipulated HUVECs	CD3	132	138	133	13304	14132	13438	13625	444
Unmanipulated PC3 Cells Only		167	167	166	18921	18921	18732	18858	109
Unmanipulated PC3 Cells And FITC		176	177	177	20715	20924	20924	20855	121
Unmanipulated HUVECs Only		104	98	100	10037	9449	NA	9743	416
Unmanipulated HUVECs And FITC		119	128	124	11672	12779	12275	12242	554

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD of Mean MESF
Attached PC3 (to HUVECs)	ICAM-1	334	334	338	101589	101589	105762	102980	2409
Unattached PC3	ICAM-1	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated PC3	ICAM-1	326	330	327	93731	97581	94679	95330	2006
Manipulated HUVECs	ICAM-1	168	159	155	19113	17458	16769	17780	1205
Unmanipulated HUVECs	ICAM-1	150	152	155	15946	16270	16769	16328	415
Attached PC3 (to HUVECs)	CD3	194	188	ND	24829	23374	ND	24101	1029
Unattached PC3	CD3	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated PC3	CD3	172	171	174	19898	19698	20302	19966	308
Manipulated HUVECs	CD3	122	118	ND	12030	11555	ND	11793	336
Unmanipulated HUVECs	CD3	132	138	133	13304	14132	13438	13625	444
Unmanipulated PC3 Cells Only		167	167	166	18921	18921	18732	18858	109
Unmanipulated PC3 Cells And FITC		176	177	177	20715	20924	20924	20855	121
Unmanipulated HUVECs Only		104	98	100	10037	9449	9641	9709	300
Unmanipulated HUVECs And FITC		119	128	124	11672	12779	12275	12242	554

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	ICAM-1	340	337	332	107912	104703	99565	104060	4211
Unattached PC3	ICAM-1	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated PC3	ICAM-1	326	330	327	93731	97581	94679	95330	2006
Manipulated HUVECs	ICAM-1	135	139	141	13712	14275	14565	14184	434
Unmanipulated HUVECs	ICAM-1	150	152	155	15946	16270	16769	16328	415
Attached PC3 (to HUVECs)	CD3	183	186	183	22227	22908	22227	22454	393
Unattached PC3	CD3	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated PC3	CD3	172	171	174	19898	19698	20302	19966	308
Manipulated HUVECs	CD3	114	120	115	11099	11790	11212	11367	371
Unmanipulated HUVECs	CD3	132	138	133	13304	14132	13438	13625	444
Unmanipulated PC3 Cells Only		167	167	166	18921	18921	18732	18858	109
Unmanipulated PC3 Cells And FITC		176	177	177	20715	20924	20924	20855	121
Unmanipulated HUVECs Only		104	98	100	10037	9449	NA	9743	416
Unmanipulated HUVECs And FITC		119	128	124	11672	12779	12275	12242	554

Appendix Table 5.5.10b The Expression Of ICAM-1 By HUVECs And Prostatic Adenocarcinoma Cells Of The PC3 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26+ PC3 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. ICAM-1 surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular cell adhesion molecule; MESF, molecular equivalent to soluble fluorochrome; SD standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha 5	304	302	308	75115	73618	78201	75645	2337
Unattached PC3	Alpha 5	310	314	334	79791	83068	101589	88149	11754
Unmanipulated PC3	Alpha 5	293	297	298	67244	70006	70714	69321	1834
Manipulated HUVECs	Alpha 5	341	359	354	109004	130651	124240	121298	11119
Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
Attached PC3 (to HUVECs)	MHC Class I	267	268	276	51762	52286	56669	53573	2695
Unattached PC3	MHC Class I	262	258	262	49222	47280	49222	48575	1121
Unmanipulated PC3	MHC Class I	243	257	250	40655	46807	43623	43695	3076
Manipulated HUVECs	MHC Class I	222	231	227	32910	36030	34609	34517	1562
Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
Unmanipulated PC3 Cells Only		102	108	106	9837	10449	10241	10176	311
Unmanipulated PC3 Cells And FITC		148	150	145	15628	15946	15163	15579	394
Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha 5	302	306	304	73618	76642	75115	75125	1512
Unattached PC3	Alpha 5	307	295	305	77418	68611	75875	73968	4703
Unmanipulated PC3	Alpha 5	305	304	301	75875	75115	72881	74624	1556
Manipulated HUVECs	Alpha 5	350	356	353	119338	126766	122996	123033	3714
Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
Attached PC3 (to HUVECs)	MHC Class I	274	275	279	55540	56102	58407	56683	1519
Unattached PC3	MHC Class I	271	264	275	53888	50223	56102	53404	2969
Unmanipulated PC3	MHC Class I	267	267	261	51762	51762	48729	50751	1751
Manipulated HUVECs	MHC Class I	214	232	223	30365	36395	33243	33334	3016
Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
Unmanipulated PC3 Cells Only		102	108	106	9837	10449	10241	10176	311
Unmanipulated PC3 Cells And FITC		148	150	145	15628	15946	15163	15579	394
Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha 5	325	301	298	92792	72881	70714	78796	12170
Unattached PC3	Alpha 5	282	299	298	60197	71429	70714	67446	6289
Unmanipulated PC3	Alpha 5	289	287	292	64590	63303	66570	64821	1646
Manipulated HUVECs	Alpha 5	394	386	359	185817	171444	130651	162637	28618
Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
Attached PC3 (to HUVECs)	HLA-ABC	265	260	260	50731	48241	48241	49071	1437
Unattached PC3	MHC Class I	261	267	256	48729	51762	46338	48943	2719
Unmanipulated PC3	MHC Class I	257	256	253	46807	46338	44960	46035	960
Manipulated HUVECs	MHC Class I	211	208	195	29462	28585	25080	27709	2319
Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
Unmanipulated PC3 Cells Only		102	108	106	9837	10449	10241	10176	311
Unmanipulated PC3 Cells And FITC		148	150	145	15628	15946	15163	15579	394
Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

Appendix Table 5.5.11a The Expression Of α5 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26* PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α5 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; sd, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha 5	244	243	246	41067	40655	41902	41208	635
Unattached PC3	Alpha 5	255	262	254	45874	49222	45415	46837	2078
Unmanipulated PC3	Alpha 5	268	276	271	52286	56669	53888	54281	2218
Manipulated HUVECs	Alpha 5	379	384	392	159782	168027	182115	169975	11293
Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
Attached PC3 (to HUVECs)	MHC Class I	221	237	226	32581	38273	34262	35039	2924
Unattached PC3	MHC Class I	249	247	242	43186	42325	40248	41920	1510
Unmanipulated PC3	MHC Class I	217	223	215	31295	33243	30672	31737	1341
Manipulated HUVECs	MHC Class I	159	174	164	17458	20302	18358	18706	1454
Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
Unmanipulated PC3 Cells Only		162	161	155	17993	17812	16769	17525	661
Unmanipulated PC3 Cells And FITC		170	193	184	19501	24580	22452	22178	2551
Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha 5	258	259	257	47280	47758	46807	47282	476
Unattached PC3	Alpha 5	264	261	264	50223	48729	50223	49725	862
Unmanipulated PC3	Alpha 5	275	273	269	56102	54984	52815	54634	1671
Manipulated HUVECs	Alpha 5	379	387	377	159782	173178	156598	163186	8799
Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
Attached PC3 (to HUVECs)	MHC Class I	231	239	235	36030	39051	37510	37531	1511
Unattached PC3	MHC Class I	250	250	270	43623	43623	53349	46865	5615
Unmanipulated PC3	MHC Class I	246	236	255	41902	37890	45874	41888	3992
Manipulated HUVECs	MHC Class I	173	175	177	20099	20508	20924	20510	413
Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
Unmanipulated PC3 Cells Only		162	161	155	17993	17812	16769	17525	661
Unmanipulated PC3 Cells And FITC		170	193	184	19501	24580	22452	22178	2551
Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

EXPERIMENT 3

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	Alpha 5	260	260	260	48241	48241	48241	48241	0
Unattached PC3	Alpha 5	259	262	264	47758	49222	50223	49068	1240
Unmanipulated PC3	Alpha 5	264	261	267	50223	48729	51762	50238	1517
Manipulated HUVECs	Alpha 5	390	383	386	178486	166345	171444	172091	6096
Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
Attached PC3 (to HUVECs)	MHC Class I	227	237	237	34609	38273	38273	37052	2116
Unattached PC3	MHC Class I	242	244	241	40248	41067	39845	40387	622
Unmanipulated PC3	MHC Class I	239	226	230	39051	34262	35670	36328	2461
Manipulated HUVECs	MHC Class I	147	151	157	15472	16107	17110	16229	826
Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
Unmanipulated PC3 Cells Only		162	161	155	17993	17812	16769	17525	661
Unmanipulated PC3 Cells And FITC		170	193	184	19501	24580	22452	22178	2551
Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

Appendix Table 5.5.11b The Expression Of α5 By HUVECs And Prostatic Adenocarcinoma Cells Of The PC3 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26* PC3 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re-cultured for 24 hours. Cells were removed from the plate by trypsinisation. α5 surface expresseion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; SD standard deviation; TCGP, tissue culture grade plate.)

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
E	Attached PC3 (to HUVECs	Alpha 5	277	270	260	57243	53349	48241	52944	4514
X	Unattached PC3	Alpha 5	268	271	280	52286	53888	58997	55057	3505
P	Unmanipulated PC3	Alpha 5	268	276	271	52286	56669	53888	54281	2218
E	Manipulated HUVECs	Alpha 5	378	389	377	158182	176699	156598	163826	11176
R	Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
I	Attached PC3 (to HUVECs	MHC Class I	226	216	233	34262	30982	36763	34002	2899
M	Unattached PC3	MHC Class I	228	217	232	34959	31295	36395	34216	2630
E	Unmanipulated PC3	MHC Class I	217	223	215	31295	33243	30672	31737	1341
N	Manipulated HUVECs	MHC Class I	164	156	175	18358	16938	20508	18601	1797
T	Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
1	Unmanipulated PC3 Cells Only		162	161	155	17993	17812	16769	17525	661
	Unmanipulated PC3 Cells And FITC		170	193	184	19501	24580	22452	22178	2551
	Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
	Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
E	Attached PC3 (to HUVECs	Alpha 5	279	270	271	58407	53349	53888	55215	2777
X	Unattached PC3	Alpha 5	285	278	283	62042	57822	60806	60223	2170
P	Unmanipulated PC3	Alpha 5	275	273	269	56102	54984	52815	54634	1671
E	Manipulated HUVECs	Alpha 5	366	325	345	140187	92792	113481	115487	23761
R	Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
I	Attached PC3 (to HUVECs	MHC Class I	233	233	243	36763	36763	40655	38060	2247
M	Unattached PC3	MHC Class I	248	248	237	42753	42753	38273	41260	2587
E	Unmanipulated PC3	MHC Class I	246	236	255	41902	37890	45874	41888	3992
N	Manipulated HUVECs	MHC Class I	171	160	184	19698	17634	22452	19928	2417
T	Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
2	Unmanipulated PC3 Cells Only		162	161	155	17993	17812	16769	17525	661
	Unmanipulated PC3 Cells And FITC		170	193	184	19501	24580	22452	22178	2551
	Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
	Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

	Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
E	Attached PC3 (to HUVECs	Alpha 5	273	269	274	54984	52815	55540	54446	1440
X	Unattached PC3	Alpha 5	278	273	280	57822	54984	58997	57268	2063
P	Unmanipulated PC3	Alpha 5	264	261	267	50223	48729	51762	50238	1517
E	Manipulated HUVECs	Alpha 5	414	397	405	227248	191513	207570	208777	17898
R	Unmanipulated HUVECs	Alpha 5	376	380	354	155030	161398	124240	146889	19872
I	Attached PC3 (to HUVECs	MHC Class I	245	237	241	41482	38273	39845	39867	1605
M	Unattached PC3	MHC Class I	235	235	236	37510	37510	37890	37637	219
E	Unmanipulated PC3	MHC Class I	239	226	230	39051	34262	35670	36328	2461
N	Manipulated HUVECs	MHC Class I	160	151	158	17634	16107	17283	17008	800
T	Unmanipulated HUVECs	MHC Class I	467	472	488	387386	407378	478551	424438	47917
3	Unmanipulated PC3 Cells Only		162	161	155	17993	17812	16769	17525	661
	Unmanipulated PC3 Cells And FITC		170	193	184	19501	24580	22452	22178	2551
	Unmanipulated HUVECs Only		102	102	101	9837	9837	9738	9804	57
	Unmanipulated HUVECs And FITC		132	134	131	13304	13574	13171	13350	206

Appendix Table 5.5.11c The Expression Of α5 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26⁺ PC3 cells were then added to the HUVECs and cell mixtures were incubated for 24 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and α5 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	CD44	278	287	297	57822	63303	70006	63710	6102
Unattached PC3	CD44	324	324	338	91863	91863	105762	96496	8025
Unmanipulated PC3	CD44	350	303	275	119338	74363	56102	83268	32545
Manipulated HUVECs	CD44	240	251	251	39446	44064	44064	42525	2666
Unmanipulated HUVECs	CD44	293	235	261	67244	37510	48729	51161	15015
Attached PC3 (to HUVECs)	CD3	133	133	132	13438	13438	13304	13393	78
Unattached PC3	CD3	126	121	124	12524	11910	12275	12236	309
Unmanipulated PC3	CD3	120	128	127	11790	12779	12651	12407	538
Manipulated HUVECs	CD3	126	129	130	12524	12908	13039	12824	267
Unmanipulated HUVECs	CD3	132	130	123	13304	13039	12152	12831	603
Unmanipulated PC3 Cells Only		129	125	121	12908	12399	11910	12406	499
Unmanipulated PC3 Cells And FITC		125	111	130	12399	10769	13039	12069	1170
Unmanipulated HUVECs Only		134	130	125	13574	13039	12399	13004	588
Unmanipulated HUVECs And FITC		137	206	135	13990	28016	13712	18573	8179

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	CD44	311	308	309	80598	78201	78992	79263	1221
Unattached PC3	CD44	338	320	337	105762	88238	104703	99568	9826
Unmanipulated PC3	CD44	350	303	275	119338	74363	56102	83268	32545
Manipulated HUVECs	CD44	256	257	256	46338	46807	46338	46494	271
Unmanipulated HUVECs	CD44	293	235	261	67244	37510	48729	51161	15015
Attached PC3 (to HUVECs)	CD3	131	127	128	13171	12651	12779	12867	271
Unattached PC3	CD3	120	122	120	11790	12030	11790	11870	138
Unmanipulated PC3	CD3	120	128	127	11790	12779	12651	12407	538
Manipulated HUVECs	CD3	134	131	130	13574	13171	13039	13261	279
Unmanipulated HUVECs	CD3	132	130	123	13304	13039	12152	12831	603
Unmanipulated PC3 Cells Only		129	125	121	12908	12399	11910	12406	499
Unmanipulated PC3 Cells And FITC		125	111	130	12399	10769	13039	12069	1170
Unmanipulated HUVECs Only		134	130	125	13574	13039	12399	13004	588
Unmanipulated HUVECs And FITC		137	206	135	13990	28016	13712	18573	8179

Appendix Table 5.5.12a The Expression Of CD44 By HUVECs And Prostatic Adenocarcinoma Cells From The PC3 Cell Line When Co-cultured For 1 Hour. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26*.PC3 cells were then added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Attached, unattached, and unmanipulated cell populations were separated and CD44 surface expression was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD, standard deviation; TCGP, tissue culture grade plate.)

EXPERIMENT 1

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	CD44	222	244	249	32910	41067	43186	39054	5425
Unattached PC3	CD44	393	349	385	183957	118143	169727	157275	34629
Unmanipulated PC3	CD44	459	453	445	357420	336477	310449	334782	23532
Manipulated HUVECs	CD44	179	180	182	21350	21566	22004	21640	333
Unmanipulated HUVECs	CD44	293	235	261	67244	37510	48729	51161	15015
Attached PC3 (to HUVECs)	CD3	133	138	136	13438	14132	13850	13807	349
Unattached PC3	CD3	123	125	121	12152	12399	11910	12153	245
Unmanipulated PC3	CD3	117	118	122	11440	11555	12030	11675	313
Manipulated HUVECs	CD3	116	118	126	11325	11555	12524	11802	636
Unmanipulated HUVECs	CD3	132	130	123	13304	13039	12152	12831	603
Unmanipulated PC3 Cells Only		112	113	130	10878	10988	13039	11635	1217
Unmanipulated PC3 Cells And FITC		115	114	114	11212	11099	11099	11137	65
Unmanipulated HUVECs Only		134	130	125	13574	13039	12399	13004	588
Unmanipulated HUVECs And FITC		137	206	135	13990	28016	13712	18573	8179

EXPERIMENT 2

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached PC3 (to HUVECs)	CD44	232	239	257	36395	39051	46807	40751	5410
Unattached PC3	CD44	385	366	368	169727	140187	143037	150984	16294
Unmanipulated PC3	CD44	459	453	445	357420	336477	310449	334782	23532
Manipulated HUVECs	CD44	187	189	181	23140	23610	21784	22845	948
Unmanipulated HUVECs	CD44	293	235	261	67244	37510	48729	51161	15015
Attached PC3 (to HUVECs)	CD3	138	138	134	14132	14132	13574	13946	322
Unattached PC3	CD3	123	126	126	12152	12524	12524	12400	215
Unmanipulated PC3	CD3	117	118	122	11440	11555	12030	11675	313
Manipulated HUVECs	CD3	123	120	116	12152	11790	11325	11756	414
Unmanipulated HUVECs	CD3	132	130	123	13304	13039	12152	12831	603
Unmanipulated PC3 Cells Only		112	113	130	10878	10988	13039	11635	1217
Unmanipulated PC3 Cells And FITC		115	114	114	11212	11099	11099	11137	65
Unmanipulated HUVECs Only		134	130	125	13574	13039	12399	13004	588
Unmanipulated HUVECs And FITC		137	206	135	13990	28016	13712	18573	8179

Appendix Table 5.5.12b The Expression Of CD44 By HUVECs And Prostatic Adenocarcinoma Cells Of The PC3 Cell Line When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded into 24-well TCGPs and left to become confluent. Freshly trypsinised PC3 cells were stained with the fluorescent membrane dye PKH26. PKH26* PC3 cells were added to the HUVECs and cell mixtures were incubated for 1 hour under standard tissue culture conditions. Unattached cells were aspirated and attached cells were trypsinised from the TCGP. Cells were washed and re-seeded separately in fresh TCGPs. Cells were then re cultured for 24 hours. Cells were removed from the plate by trypsinisation. CD44 surface expresesion was detected by flow cytometric analysis. Median levels of fluorescence were converted to MESF values as in 2.4.2.4. (FITC, Fluorescin isothiocyanate; HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent to soluble fluorochrome; SD standard deviation; TCGP, tissue culture grade plate.)

Marker	Median Level Of Fluorescene			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
E-selectin	89	95	99	6738	7156	7450	7115	358
CD44	113	103	115	8575	7755	8749	8360	531
ICAM-1	95	93	97	7156	7014	7302	8158	144
VCAM-1	94	86	88	7085	6538	6671	6764	285
Alpha-4	90	94	96	6806	7085	7229	7040	215
Alpha-5	118	110	116	9017	8320	8837	8725	362
Alpha-L	96	101	93	7229	7601	7014	7281	297
Beta-1	97	94	97	7302	7085	7302	7229	125
Cells Only	70	63	59	5567	5189	4985	5247	296
Cells with FITC Conjuagted Antibod	92	90	84	6944	6806	6408	6719	278

Appendix Table 5.5.13 Mouse Anti-human Monoclonal Antibidies Against Cell Adhesion Molecules Do Not Recognise Epitopes On The Surface Of LLC PK1 Cells. LLC PK1 cells were subjected to a standard FACScan analysis as described in Chapter 2.4.2. Median levels of fluorescence were converted to MESF values as described in Chapter 2.4.2.4.(ICAM, intercellular cell adhesion molecule; MESF, molecular equivalent of soluble flourochrome; SD, standard deviation; VCAM, vascular cell adhesion molecule.)

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Cell Type	Marker	Median Level OF Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached Du145	ICAM-1	502	515	537	426948	485610	606840	506466	91742
Unmanipulated Du14	ICAM-1	486	488	493	363555	370933	390040	374843	13669
Attached Du145	CD3	212	214	219	23182	32653	24871	26902	5052
Unmanipulated Du14	CD3	227	235	267	26952	29208	40282	32147	7135
Du145 Cells Only		199	201	197	20344	20757	19939	20347	409

Appendix Table 5.5.14 The Expression Of ICAM-1 By Du145 Cells And LLC PK1 Cells When Co-cultured For 1 Hour And Re-cultured For 24 Hours. LLC PK1 cells were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised Du145 cells were stained with PKH26 and co-cultured with LLC PK1 cells for 1 hour. Unattached Du145 cells removed and attached Du145 cells and HUVECs were trypsinsed and re-cultured for 24 hours in the absence of unattached Du145 cells. Cells were removed from the TCGP by trypsinisation and Du145 cells were analysed for ICAM-1 expression by FACScan. Median levels of fluorescence were converted to MESF values as described in Chapter 2.4.2.4. (HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; TCGP, tissue culture grade plate.)

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Attached A549 (to HUVECs)	CD44	494	564	501	508337	1028248	545440	694008	290054
Unattached A549	CD44	476	473	ND	424112	411499	NA	417805	8919
Unmanipulated A549	CD44	452	451	468	333108	329772	391305	351395	34603
Manipulated HUVECs	CD44	353	387	367	122996	173178	141605	145926	25369
Unmanipulated HUVECs	CD44	376	357	352	155030	128048	121764	134947	17673
Attached A549 (to HUVECs)	CD3	202	196	200	26910	25334	26374	26206	802
Unattached A549	CD3	205	207	209	27735	28299	28875	28303	570
Unmanipulated A549	CD3	208	203	204	28585	27183	27458	27742	743
Manipulated HUVECs	CD3	222	211	230	32910	29462	35670	32681	3110
Unmanipulated HUVECs	CD3	242	227	223	40248	34609	33243	36033	3713

Appendix Table 5.5.15a The Expression Of CD44 By HUVECs And Lung Adenocarcinoma Cells, A549, Following A 1 Hour Co-culture. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised A549 cells were stained with the fluorescent membrane dye PKH26. PKH26+ A549 cells were then co-cultured in direct contact with the confluent monolayers of HUVECs. Cells were cultured for 1 hour under standard tissue culture conditions. Unattached A549 cells were aspirated and attached cells were trypsinised from the TCGP. Attached, unattached and unmanipulated cells were analysed for CD44 cell surface expression by flow cytometry as described in Chapter 2.4.2. Median levels of fluorescence were converted to MESF values as described in Chapter 2.4.2.4. (HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; SD,

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean
Unattached A549	CD44	476	488	488	424112	478551	478551	460404	31430
Unmanipulated A549	CD44	452	451	468	333108	329772	391305	351395	34603
Manipulated HUVECs (now unattached)	CD44	185	185	185	22679	22679	22679	22679	0
Unmanipulated HUVECs	CD44	376	357	352	155030	128048	121764	134947	17673
Unattached A549	CD3	198	200	201	25849	26374	26641	26288	403
Unmanipulated A549	CD3	208	203	204	28585	27183	27458	27742	743
Manipulated HUVECs (now unattached)	CD3	176	189	191	20715	23610	24090	22805	1826
Unmanipulated HUVECs	CD3	245	214	217	41482	30365	31295	34381	6167

Appendix Table 5.5.15b The Expression Of CD44 By HUVECs And Lung Adenocarcinoma Cells, A549, When Co-cultured For 1 Hour And Re-cultured For 24 Hours. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised A549 cells were stained with the fluorescence membrane dye, PKH26. PKH26+ A549 cells were added to the confluent monolayers of HUVECs and incubated for 1 hour. Unattached cells were aspirated from the TCGP and re-cultured for 24 hours. Unattached cells were aspirated from the TCGP and attached cells were trypsinised from the TCGP. Cell populations were examined for CD44 cell surface expression by flow cytometric analysis, as described in Chapter 2.4.2. Median levels of fluorescence were converted to MESF values as described in Chapter 2.4.2.4. (HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached A549 (to HUVECs)	CD44	466	460	459	383507	361035	357420	367321	14134
Unattached A549	CD44	434	451	443	277915	329772	304263	303983	25929
Unmanipulated A549	CD44	420	425	439	241392	253850	292258	262500	26513
Manipulated HUVECs	CD44	327	327	ND	94679	94679	NA	94679	0
Unmanipulated HUVECs	CD44	350	355	351	119338	125496	120545	121793	3264
Attached A549 (to HUVECs)	CD3	220	218	217	32255	31612	31295	31721	489
Unattached A549	CD3	213	213	212	30061	30061	29760	29960	174
Unmanipulated A549	CD3	216	204	205	30982	27458	27735	28725	1960
Manipulated HUVECs	CD3	212	217	215	29760	31295	30672	30576	772
Unmanipulated HUVECs	CD3	231	229	230	36030	35313	35670	35671	359

Appendix Table 5.5.16a The Expression Of CD44 By HUVECs And Lung Adenocarcinoma Cells, A549, After A 1 Hour Co-culture. HUVECs were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised A549 cells were stained with the fluorescent membrane dye, PKH26. PKH26⁺ cells were added to the HUVECs and cells were co-cultured in direct contact with each other for 1 hour. Unattached cells were collected by aspiration and attached cells by trypsinisation from the TCGP. Cell surface expression of CD44 was detected by flow cytometric analysis, as described in Chapter 2.4.2. Median levels of fluorescence were converted to MESF values, as described in Chapter 2.4.2.4. (HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

Cell Type	Marker	Median Level Of Fluorescence			Corresponding MESF Values			Mean MESF	SD Of Mean MESF
Attached A549 (to TCGP)	CD44	457	455	449	350298	343318	323201	338939	14069
Unattached A549	CD44	429	ND	ND	264277	NA	NA	264277	NA
Unmanipulated A549	CD44	420	425	439	241392	253850	292258	262500	26513
Manipulated And Attached HUVECs	CD44	306	ND	ND	76642	NA	NA	76642	NA
Manipulated And Unattached HUVECs	CD44	184	184	184	22452	22452	22452	22452	0
Unmanipulated HUVECs	CD44	350	355	351	119338	125496	120545	121793	3264
Attached A549 (to TCGP)	CD3	219	222	245	31932	32910	41482	35441	5254
Unattached A549	CD3	ND	ND	ND	NA	NA	NA	NA	NA
Unmanipulated A549	CD3	216	217	215	30982	31295	30672	30983	312
Manipulated And Attached HUVECs	CD3	219	222	243	31932	32910	40655	35166	4779
Manipulated And Unattached HUVECs	CD3	176	189	191	20715	23610	24090	22805	1826
Unmanipulated HUVECs	CD3	231	229	230	36030	35313	35670	35671	359

Appendix Table 5.5.16b The Expression Of CD44 By HUVECs And Lung Adenocarcinoma Cells, A549, After A 1 Hour Co-culture And A 24 Hour Re-culture. HUVECS were seeded in 24-well TCGPs and left to become confluent. Freshly trypsinised A549 cells were stained with the fluorscent membrane dye, PKH26. PKH26⁺ cells were added to the confluent monolayers of HUVECS. Cells were co-cultured in direct contact with each other for 1 hour. Unattached cells and attached cells were collected by aspiration and trypsinisation, respectively. The individual cell populations were re-cultured separately for 24 hours. The original unattached cells were now attached and were collected by trypsinsation. The originally attached cells were now unattached and were collected by aspiration. Cell surface expression of CD44 by each population of cells was detected by flow cytometric analysis, as desccribed in Chapter 2.4.2. Median levels of fluorescence were converted to MEFS values as described in Chapter 2.4.2.4. (HUVEC, human umbilical vein endothelial cell; MESF, molecular equivalent of soluble fluorochrome; NA, not applicable; ND, not done; SD, standard deviation; TCGP, tissue culture grade plate.)

CD44

EXPERIMENT 1

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	73	ND	25	ND	95	ND	3	ND
	69	ND	29	ND	95	ND	3	ND
	65	ND	33	ND	94	ND	3	ND
	67	ND	32	ND	95	ND	3	ND
	65	ND	29	ND	94	ND	4	ND
	69	ND	31	ND	95	ND	3	ND
Mean Percentage	68	NA	30	NA	95	NA	3	NA
SD of the Mean	3	NA	3	NA	1	NA	0	NA

EXPERIMENT 2

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	65	ND	32	ND	90	ND	7	ND
	66	ND	31	ND	91	ND	6	ND
	63	ND	34	ND	90	ND	6	ND
	65	ND	33	ND	92	ND	5	ND
	67	ND	31	ND	92	ND	6	ND
	63	ND	34	ND	91	ND	6	ND
Mean Percentage	65	NA	33	NA	91	NA	6	NA
SD of the Mean	2	NA	1	NA	1	NA	1	NA
Mean Of All Experiments	66	NA	31	NA	93	NA	5	NA
SD of the Mean	3	NA	3	NA	2	NA	2	NA

ICAM-1

EXPERIMENT 1

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	40	ND	60	ND	19	ND	79	ND
	34	ND	66	ND	15	ND	83	ND
	27	ND	73	ND	18	ND	80	ND
	32	ND	71	ND	18	ND	80	ND
	29	ND	67	ND	20	ND	77	ND
	27	ND	73	ND	12	ND	85	ND
Mean Percentage	32	NA	68	NA	17	NA	81	NA
SD of the Mean	5	NA	5	NA	3	NA	3	NA

EXPERIMENT 2

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	35	ND	66	ND	22	ND	76	ND
	34	ND	65	ND	17	ND	82	ND
	35	ND	65	ND	16	ND	82	ND
	33	ND	66	ND	19	ND	79	ND
	32	ND	67	ND	11	ND	86	ND
	30	ND	68	ND	13	ND	85	ND
Mean Percentage	33	NA	66	NA	16	NA	82	NA
SD of the Mean	2	NA	1	NA	4	NA	4	NA

EXPERIMENT 3

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	36	ND	16	ND	63	ND	82	ND
	34	ND	14	ND	66	ND	85	ND
	34	ND	13	ND	65	ND	85	ND
	34	ND	13	ND	65	ND	86	ND
	34	ND	12	ND	66	ND	86	ND
	31	ND	9	ND	68	ND	90	ND
Mean Percentage	34	NA	13	NA	66	NA	86	NA
SD of the Mean	2	NA	2	NA	2	NA	3	NA
Mean Of All Experiments	33	NA	49	NA	33	NA	83	NA
SD of the Mean	3	NA	27	NA	24	NA	4	NA

VCAM-1

EXPERIMENT 1

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	48	96	27	12	82	70	9	28
	45	66	25	11	76	66	12	25
	44	83	26	16	73	69	13	20
	45	79	38	14	75	56	9	23
	39	71	37	19	64	50	10	24
	39	70	35	19	64	48	11	26
Mean Percentage	43	78	31	15	72	60	11	24
SD of the Mean	4	11	6	3	7	10	2	3

EXPERIMENT 2

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	47	77	25	11	73	60	9	13
	40	66	29	20	63	58	8	10
	42	61	22	22	65	53	9	15
	54	76	27	11	76	63	7	29
	42	60	33	20	66	56	6	36
	41	62	33	20	65	49	6	43
Mean Percentage	44	67	28	17	68	57	8	24
SD of the Mean	5	8	4	5	5	5	1	14

EXPERIMENT 3

	ATTACHED CELLS				UNATTACHED CELLS			
	PKH26+ PC3 CELLS		PKH26- HUVECs		PKH26+ PC3 CELLS		PKH26- HUVECs	
Percentage Of FACScan Sample	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
	50	75	30	2	79	71	7	7
	39	76	22	6	62	62	10	4
	39	72	20	6	61	72	10	8
	53	78	30	2	82	68	3	13
	44	76	30	3	66	70	4	12
	42	68	27	4	67	65	4	14
Mean Percentage	45	74	27	4	70	68	6	10
SD of the Mean	6	4	4	2	9	4	3	4
Mean Of All Experiments	44	73	29	12	70	61	8	19
SD of the Mean	5	9	5	7	7	8	3	11

Appendix Table 5.5.17 The Distribution Of Prostatic Adenocarcinoma PC3 Cells And Human Umbilical Vein Endothelial Cells (HUVECs) In Attached And Unattached Cell Suspensions Generated By Direct Co-cultures Of Either 1 Or 24 Hours. (Continued overpage.)(NA, not applicable; ND, not done.)

| | | ATTACHED CELLS | | | | UNATTACHED CELLS | | | |
| | | PKH26+ PC3 CELLS | | PKH26- HUVECs | | PKH26+ PC3 CELLS | | PKH26- HUVECs | |
		1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
Alpha 4 — EXPERIMENT 1	Percentage Of FACScan Sample	63	63	17	2	76	67	9	8
		72	71	19	8	60	60	11	6
		61	67	19	9	66	62	8	4
		59	85	15	6	80	75	8	9
		70	78	19	13	67	67	12	15
		56	76	24	11	63	67	17	16
	Mean Percentage	64	73	19	8	69	66	11	10
	SD of the Mean	6	8	3	4	8	5	3	5
EXPERIMENT 2	Percentage Of FACScan Sample	86	75	14	5	60	67	6	8
		45	59	12	5	49	54	7	5
		45	61	11	4	57	54	5	6
		60	73	21	12	61	61	11	18
		50	55	20	13	64	53	5	19
		52	59	18	11	60	50	5	20
	Mean Percentage	56	64	16	8	59	57	7	13
	SD of the Mean	16	8	4	4	5	6	2	7
EXPERIMENT 3	Percentage Of FACScan Sample	72	76	53	11	73	71	13	11
		65	68	25	13	72	62	14	13
		66	69	25	12	70	58	15	15
		79	81	15	12	75	73	15	15
		66	68	19	16	67	64	22	25
		66	74	22	17	67	62	21	27
	Mean Percentage	69	73	27	14	71	65	17	18
	SD of the Mean	6	5	14	2	3	6	4	7
	Mean Of All Experiments	63	70	20	10	66	63	11	13
	SD of the Mean	11	8	9	4	8	7	5	7
Alpha 5 — EXPERIMENT 1	Percentage Of FACScan Sample	71	81	31	11	83	77	19	13
		70	79	33	2	58	79	42	11
		72	75	28	10	60	71	30	18
		64	89	32	8	66	87	26	10
		52	86	35	11	55	85	36	10
		59	88	34	9	53	88	37	9
	Mean Percentage	65	83	32	9	63	81	32	12
	SD of the Mean	8	6	2	3	11	7	8	3
EXPERIMENT 2	Percentage Of FACScan Sample	68	77	31	11	66	74	26	12
		73	80	26	9	77	77	22	11
		72	80	26	9	74	78	25	12
		70	87	24	8	65	84	23	11
		65	87	27	7	79	87	11	9
		67	88	25	7	81	84	11	11
	Mean Percentage	69	83	27	9	74	81	20	11
	SD of the Mean	3	5	2	2	7	5	7	1
EXPERIMENT 3	Percentage Of FACScan Sample	66	86	32	8	84	82	10	9
		70	86	29	7	77	85	14	8
		72	83	25	8	55	78	30	12
		73	90	27	4	79	83	20	8
		72	87	27	3	85	82	13	8
		71	89	28	4	85	82	14	9
	Mean Percentage	71	87	28	6	78	82	17	9
	SD of the Mean	3	2	2	2	12	2	7	2
	Mean Of All Experiments	68	84	29	8	71	81	23	11
	SD of the Mean	5	5	3	3	11	5	10	2
Alpha L — EXPERIMENT 1	Percentage Of FACScan Sample	42	81	55	17	71	75	27	27
		43	81	54	16	70	79	26	22
		47	82	51	16	72	81	26	21
		50	83	52	14	84	81	19	16
		48	83	54	14	49	84	21	15
		46	79	52	16	7	84	26	14
	Mean Percentage	46	82	53	16	59	81	24	19
	SD of the Mean	3	2	2	1	28	3	3	5
EXPERIMENT 2	Percentage of FACScan Sample	58	89	40	7	86	89	13	9
		54	91	43	5	85	91	12	8
		54	87	43	8	ND	89	ND	9
		53	89	45	7	77	85	20	8
		54	89	45	6	85	91	12	5
		52	90	46	6	85	90	12	6
	Mean Percentage	54	89	44	7	84	89	14	8
	SD of the Mean	2	1	2	1	4	2	3	2
EXPERIMENT 3	Percentage Of FACScan Sample	46	95	50	2	82	82	15	9
		45	95	51	2	84	83	13	8
		49	95	45	2	83	87	13	6
		50	93	48	10	83	87	15	13
		47	95	52	10	84	85	13	15
		46	94	52	10	82	84	15	19
	Mean Percentage	47	95	50	6	83	85	14	12
	SD of the Mean	2	1	3	4	1	2	1	5
	Mean Of All Experiments	49	88	49	9	75	85	18	13
	SD of the Mean	4	5	4	5	2.0	4	6	6

Appendix Table 5.5.17 The Distribution Of Prostatic Adenocarcinoma PC3 Cells And Human Umbilical Vein Endothelial Cells (HUVECs) In Attached And Unattached Cell Suspensions Generated By Direct Co-cultures Of Either 1 Or 24 Hours. PC3 cells were stained with the fluorescent membrane stain, PKH26. PKH26+ PC3 cells were incubated in direct contact with confluent monolayers of HUVECs for either 1 or 24 hours. Unattached cells were collected by aspiration and attached cells were collected by trypsinisation. Cell populations were analysed by FACScan. PKH26+ PC3 cells and PKH26- HUVECs could be distinguished using the FL1 detector of the FACScan. The percentage of PKH26+ and PKH26- cells in each FACScan sample was calculated by the FACScan. (NA, not applicable; ND, not done.)

		ATTACHED CELLS				UNATTACHED CELLS			
		PKH26+ Du145 CELLS		PKH26- HUVECs		PKH26+ Du145 CELLS		PKH26- HUVECs	
		1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours	1 Hour	24 Hours
CD44 EXPERIMENT 1	Percentage Of FACScan Sample	70	98	28	3	85	84	13	22
		73	98	25	3	83	81	16	24
		71	98	27	3	85	82	14	23
		73	74	27	24	92	65	14	32
		76	70	24	28	90	48	16	48
		74	73	25	24	94	49	11	47
	Mean Percentage	73	85	26	14	88	68	14	33
	SD of the Mean	2	14	2	12	4	17	2	12
EXPERIMENT 2	Percentage Of FACScan Sample	70	94	31	6	72	77	20	20
		71	95	31	6	66	78	24	20
		68	95	33	6	70	69	22	21
		74	81	23	19	86	42	8	43
		70	80	27	20	89	54	7	33
		71	79	25	20	87	46	8	41
	Mean Percentage	71	87	28	13	78	61	15	30
	SD of the Mean	2	8	4	7	10	16	8	11
		PKH26+ Du145 CELLS		PKH26- HUVECs		PKH26+ Du145 CELLS		PKH26- HUVECs	
	Percentage Of FACScan Sample	70	90	31	9	72	84	20	25
		71	89	29	9	73	88	17	19
		70	89	31	10	77	88	16	19
		75	81	21	20	87	53	5	41
		72	78	23	23	79	49	9	46
		74	89	21	11	44	50	11	46
	Mean Percentage	72	86	26	14	72	69	13	33
	SD of the Mean	2	5	5	6	15	20	6	13
	Mean Of All Experiments	72	86	27	14	80	66	14	32
	SD of the Mean	2	9	4	9	12	17	5	11
ICAM-1 EXPERIMENT 1	Percentage Of FACScan Sample	45	63	54	33	91	27	8	60
		29	65	71	31	95	20	4	66
		43	64	56	32	91	26	1	62
		45	75	53	23	84	26	11	61
		41	67	55	30	90	27	6	64
		40	70	58	25	85	18	7	72
	Mean Percentage	41	67	58	29	89	24	6	64
	SD of the Mean	6	5	7	4	4	4	3	4
EXPERIMENT 2	Percentage Of FACScan Sample	40	70	60	29	94	28	5	68
		51	68	48	31	94	26	5	70
		46	68	52	31	95	24	4	72
		46	79	53	20	92	34	8	66
		49	78	51	21	93	25	6	74
		61	79	38	20	87	27	13	72
	Mean Percentage	49	74	50	25	93	27	7	70
	SD of the Mean	7	6	7	6	3	4	3	3
EXPERIMENT 3	Percentage Of FACScan Sample	30	66	66	29	89	22	9	64
		34	61	60	34	87	21	11	67
		30	54	66	39	91	21	7	65
		60	79	38	15	89	26	10	69
		55	80	42	15	93	21	8	73
		48	81	50	15	88	18	9	77
	Mean Percentage	43	70	54	25	90	22	9	69
	SD of the Mean	13	11	12	11	2	3	1	5
	Mean Of All Experiments	44	70	54	26	90	24	7	68
	SD of the Mean	9	8	9	7	3	4	3	5
VCAM-1 EXPERIMENT 1	Percentage Of FACScan Sample	72	63	24	10	26	59	66	33
		70	88	27	10	17	51	76	37
		71	89	26	10	14	42	80	45
		73	63	23	7	76	50	13	15
		73	85	23	12	76	43	15	47
		67	88	27	8	72	37	12	52
	Mean Percentage	71	79	25	10	47	47	44	38
	SD of the Mean	2	13	2	2	31	8	34	13
EXPERIMENT 2	Percentage of FACScan Sample	76	87	23	10	46	70	50	23
		79	88	20	10	27	40	69	48
		80	89	19	9	32	41	61	34
		74	88	21	6	ND	39	10	48
		73	92	20	3	ND	33	10	44
		71	93	21	1	ND	49	9	29
	Mean Percentage	76	90	21	7	35	45	35	38
	SD of the Mean	4	2	1	4	10	13	28	11
EXPERIMENT 3	Percentage Of FACScan Sample	77	ND	20	7	ND	75	68	39
		73	ND	24	12	ND	72	71	23
		71	ND	26	8	ND	77	77	47
		82	ND	16	7	ND	ND	15	ND
		79	ND	19	11	ND	ND	18	ND
		71	ND	17	8	ND	ND	13	ND
	Mean Percentage	76	NA	20	9	ND	75	44	36
	SD of the Mean	5	NA	4	2	ND	3	31	12
	Mean Of All Experiments	74	84	22	8	43	52	41	38
	SD of the Mean	4	10	3	3	26	15	30	11

Appendix Table 5.5.17 The Distribution Of Prostatic Adenocarcinoma Du145 Cells And Human Umbilical Vein Endothelial Cells (HUVECs) In Attached And Unattached Cell Suspensions Generated By Direct Co-cultures Of Either 1 Or 24 Hours. (Continued overpage.)

Group	Exp	Measure	Attached PKH26+ Du145 1 Hour	Attached PKH26+ Du145 24 Hours	Attached PKH26- HUVECs 1 Hour	Attached PKH26- HUVECs 24 Hours	Unattached PKH26+ Du145 1 Hour	Unattached PKH26+ Du145 24 Hours	Unattached PKH26- HUVECs 1 Hour	Unattached PKH26- HUVECs 24 Hours
Alpha 4	Experiment 1	Percentage Of FACScan Sample	72	91	ND	13	82	42	6	57
			74	91	ND	13	83	44	7	56
			72	92	ND	12	76	29	13	46
			78	73	27	10	91	54	6	44
			74	79	31	12	93	60	4	39
			73	81	31	10	93	55	4	43
		Mean Percentage	74	85	30	12	86	47	7	48
		SD of the Mean	2	8	2	1	7	11	3	7
	Experiment 2	Percentage Of FACScan Sample	70	85	27	14	78	37	11	63
			71	84	26	15	81	54	11	46
			72	84	25	16	80	45	11	56
			82	86	18	14	87	63	16	37
			76	84	23	17	89	47	13	53
			75	85	24	16	84	ND	18	ND
		Mean Percentage	74	85	24	15	83	49	13	51
		SD of the Mean	4	1	3	1	4	10	3	10
	Experiment 3	Percentage Of FACScan Sample	77	89	21	13	70	39	16	62
			80	87	17	16	53	37	12	64
			74	85	23	17	87	43	15	58
			76	88	21	10	74	38	19	55
			76	89	22	10	77	22	17	72
			71	90	26	9	25	12	66	85
		Mean Percentage	76	88	22	13	64	32	24	66
		SD of the Mean	3	2	3	3	22	12	21	11
		Mean Of All Experiments	75	86	24	13	78	42	15	55
		SD of the Mean	3	5	4	3	16	13	14	12
Alpha 5	Experiment 1	Percentage Of FACScan Sample	52	97	45	12	57	55	32	61
			43	88	52	24	64	45	27	67
			44	97	52	10	ND	38	ND	73
			51	80	46	20	74	45	17	55
			36	67	61	33	55	33	37	64
			38	53	59	46	ND	28	ND	71
		Mean Percentage	44	80	53	24	63	41	28	65
		SD of the Mean	7	18	7	14	9	10	9	7
	Experiment 2	Percentage Of FACScan Sample	41	91	60	25	71	45	56	44
			35	77	72	36	64	30	60	61
			39	68	69	40	ND	32	49	58
			41	62	63	32	46	28	37	65
			29	57	68	37	43	28	45	63
			32	49	64	44	45	26	ND	64
		Mean Percentage	36	67	66	36	54	32	49	59
		SD of the Mean	5	15	4	7	13	7	9	8
	Experiment 3	Percentage Of FACScan Sample	52	90	48	7	71	44	25	53
			48	71	51	26	58	36	35	61
			47	68	53	29	59	43	32	54
			65	81	30	23	62	30	32	64
			56	65	39	40	60	31	31	63
			57	70	38	36	54	31	36	63
		Mean Percentage	54	74	43	27	61	36	32	60
		SD of the Mean	7	9	9	12	6	6	4	5
		Mean Of All Experiments	45	74	54	29	59	36	37	61
		SD of the Mean	10	15	12	11	9	8	12	7
Alpha L	Experiment 1	Percentage Of FACScan Sample	47	83	55	29	86	78	15	42
			50	84	51	27	71	79	15	48
			51	87	51	25	84	67	15	58
			42	71	58	28	84	42	16	58
			36	67	65	32	87	60	12	39
			30	67	70	32	92	54	7	46
		Mean Percentage	43	77	58	29	84	63	13	49
		SD of the Mean	8	9	8	3	7	14	3	8
	Experiment 2	Percentage of FACScan Sample	48	80	46	22	86	43	14	21
			42	78	45	19	88	38	12	20
			49	77	46	20	ND	38	ND	25
			40	72	60	22	84	37	11	52
			39	70	61	22	ND	30	ND	55
			49	71	51	21	ND	34	ND	55
		Mean Percentage	45	75	52	21	86	37	12	38
		SD of the Mean	5	4	7	1	2	4	2	18
	Experiment 3	Percentage Of FACScan Sample	37	85	70	18	82	91	12	12
			31	87	61	16	78	88	ND	14
			34	88	59	12	79	88	ND	13
			27	63	71	33	92	69	11	29
			31	72	67	26	91	76	11	23
			28	69	71	28	89	96	13	25
		Mean Percentage	31	77	67	22	85	85	12	19
		SD of the Mean	4	11	5	8	6	10	1	7
		Mean Of All Experiments	40	76	59	24	85	62	13	35
		SD of the Mean	8	8	9	6	6	22	2	17

Appendix Table 5.5.18 The Distribution Of Prostatic Adenocarcinoma Du145 Cells And Human Umbilical Vein Endothelial Cells (HUVECs) In Attached And Unattached Cell Suspensions Generated By Direct Co-cultures Of Either 1 Or 24 Hours. Du145 cells were stained with the fluorescent membrane stain, PKH26. PKH26+ PC3 cells were incubated in direct contact with confluent monolayers of HUVECs for either 1 or 24 hours. Unattached cells were collected by aspiration and attached cells were collected by trypsinisation. Cell populations were analysed by FACScan. PKH26+ Du145 cells and PKH26- HUVECs could be distinguished using the FL1 detector of the FACScan. The percentage of PKH26+ and PKH26- cells in each FACScan sample was calculated by the FACscan.

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