The Mechanisms of Endothelial Progenitor Cell Homing and Differentiation

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Abstract

Mechanisms of Endothelial Progenitor Cell Homing and Differentiation

By

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Endothelial progenitor cells (EPC) are found circulating in adults and preferentially incorporate into growing vessels at sites of active angiogenesis. The mechanisms for their homing to angiogenic vessels, factors that regulate their differentiation into an endothelial-like phenotype and extent to which these cells become endothelial are not known. Understanding these mechanisms would be valuable for therapeutically directing vascularisation, especially where normal compensatory angiogenic response is lacking or impaired. This project seeks to ex vivo expand EPC cultures as defined in the literature, then aims to define the mechanisms of progenitor cell homing and factors controlling differentiation.

In this study, we have examined the possibility that ex vivo expanded EPC migration and adhesion could be regulated by Angiopoietin -1 (Ang1) and -2 (Ang2), a soluble ligand expressed by endothelial cells at sites of vessel remodelling and angiogenesis

and Vascular Endothelial Growth Factor (VEGF). We show VEGF and Ang1 stimulate EPC migration and show for the first time the same effect is seen with Ang2. This was specific for EPC as the ligand failed to affect endothelial cell migration. Ang2 stimulated EPC migration was inhibited by soluble Tie2 ectodomain. Furthermore, these ligands were found to stimulated adhesion between EPC and endothelial monolayers using cell:cell adhesion assays. The Angiopoietin stimulated increase EPC:endothelial cell interaction was again inhibited by the soluble Tie2 ectodomain. The roles of these soluble mediators and adhesion in signalling differentiation were defined by examining EPC marker expression. These results showed a phenotypic overlap between EPC and monocytes and allowed definition of a monocyte sub-population with endothelial characteristics.

The work in this thesis demonstrates for the first time factors that regulate the homing of EPCs and defines a sub-population of monocytic cells which demonstrate a phenotypic overlap with EPCs which may in turn have an important role in angiogenesis.

The work in this thesis is based on my own independent work

Kathryn Gill

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I would like to thank The Royal College of Surgeons of Edinburgh Robertson Trust Research Fellowship who funded my salary.

Dedication

I dedicate this work to Duncan Gallimore whose love, support and help has never faltered.

Finally, I would also like to dedicate this work to my parents Tom and Eileen to whom I am indebted for their constant encouragement and understanding over the years.

Publications, Presentations and Awards arising from this Work

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Department of Surgery, University of Leicester

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Abstract

FACTORS CONTROLLING EPC MIGRATION AND INCORPORATION INTO SITES OF ANGIOGENESIS

K Gill, N Brindle, PRF Bell

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Abbreviations

Ang 1	Angiopoietin 1
Ang 2	Angiopoietin 2
BAEC	Bovine Aortic Endothelial Cells
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CHO cells	Chinese Hamster Ovary cells
DMEM	Dubecclos Modified Eagles Medium
DTT	Dithiothreitol
ECL	Enhances Chemiluminescence
EDTA	EthyleneDiamine Tetra Acetic acid
EGF	Epidermal Growth Factor
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
HRP	Horse Radish Peroxidase
HUVE	Human Umbilical Vein Endothelial cells
IL-1	Interleukin 1
Ig	Immunoglobin
MHE	Mouse Heart Endothelial cells
PBS	Phosphate Buffered Saline
PAGE	PolyAcrylamide Gel Electrophoresis
PDGF	Platelet Derived Growth Factor

PMA	Phorbol 12-Myristate 13-Acetate
SB	Sample Buffer
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
Tie domains	Tyrosine kinase with Ig and EGF homology
VEGF	Vascular Endothelial Growth Factor
WB	Western Blotting

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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF VASCULAR DEVELOPMENT AND REMODELLING

The embryonic vascular system forms through a combination of two distinct mechanisms, namely vasculogenesis and angiogenesis. Vasculogenesis refers to the earliest stages of vascular development during which, so called angioblasts, undergo differentiation, expansion and coalescence to form a network of primitive tubules and subsequent blood vessels *de novo* which in turn give rise to the heart and first primitive vascular plexus in the embryo and its surrounding yolk sac membranes (13). This is a critical paradigm for embryonic development. In contrast angiogenesis is the process whereby new vessels arise by migration and proliferation of endothelial cells from pre-existing vessels (3, 15).

Although these two processes are distinct in most respects recent evidence suggests they share a number of regulatory mechanisms. Also common to both is the process of remodelling. This is a poorly understood event which gives rise to changes in luminal diameter and vessel wall thickness and involves sprouting and penetration of vessels into previously avascular regions and differential recruitment of associate supporting cells such as smooth muscle cells, pericytes and fibroblasts to suit the microenvironment. (5, 9, 15-17)

1.1.1 Vasculogenesis

In the human embryo, tissues which are vascularised by this process are thought to arise from mesodermal origin e.g. lung, pancreas, spleen, heart tubule and dorsal aorta

(5, 18, 19). Conversely, organs of ectodermal / mesenchymal origin are vascularised by an angiogenic mechanism.

The cardiovascular system is the first organ to develop in the embryo. During the process known as gastrulation, the human embryo epiblast cells invaginate through the primitive streak. Mesodermal cells are subsequently activated and migrate through these extra-embryonic membranes. The first evidence of blood vessel formation can be detected in the pre-liver intra-embryonic site, the so-called splanchnopleural area, of the yolk sac as early as day 17 (20). Progenitor cells of blood and endothelium, the so-called bipotential 'haemangioblasts' differentiate from the migrating mesoderm in this area under the influence of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF). These cells then aggregate to form 'blood islands' next to the endoderm. Each 'blood island' then segregates into a core of embryonic haematopoietic stem cells (HSC), which is surrounded by flattened angioblasts (12, 21, 22). The angioblast is defined as a cell type that has the ability to differentiate into an endothelial cell but has not yet acquired all the characteristic markers. The haematopoietic stem cell has the ability to form the cellular constituents of blood. These cells differentiate concomitantly and in close association (Figure 1).

These blood islands subsequently fuse, with the angioblast forming lumina, ultimately giving rise to the primordial vascular network (Figure 2). The differentiating angioblasts subsequently recruit pericytes and smooth muscle cells to ensheath them. This network extends by sprouting new capillaries, to give rise to an elongated, highly branched vascular plexus by the end of the third week. After the onset of the circulation, the plexus is remodelled many times, until a mature system consisting of vessels of different diameter and function are formed (23).

On day 18 inducing substances from the endoderm of the embryo, namely FGF and VEGF, induce some cells within the mesoderm to differentiate as solitary angioblasts without concomitant haematopoietic stem cell differentiation. These angioblasts then migrate over long distances and fuse with other angioblasts and capillaries to form 'angiocysts' which in turn coalesce into cords which themselves form angioblastic plexuses and vessels in situ (24).

These processes establish the initial configuration of the circulatory system in the embryo. Apart from the mesoderm inducing signals, the controlling mechanisms remain unknown.

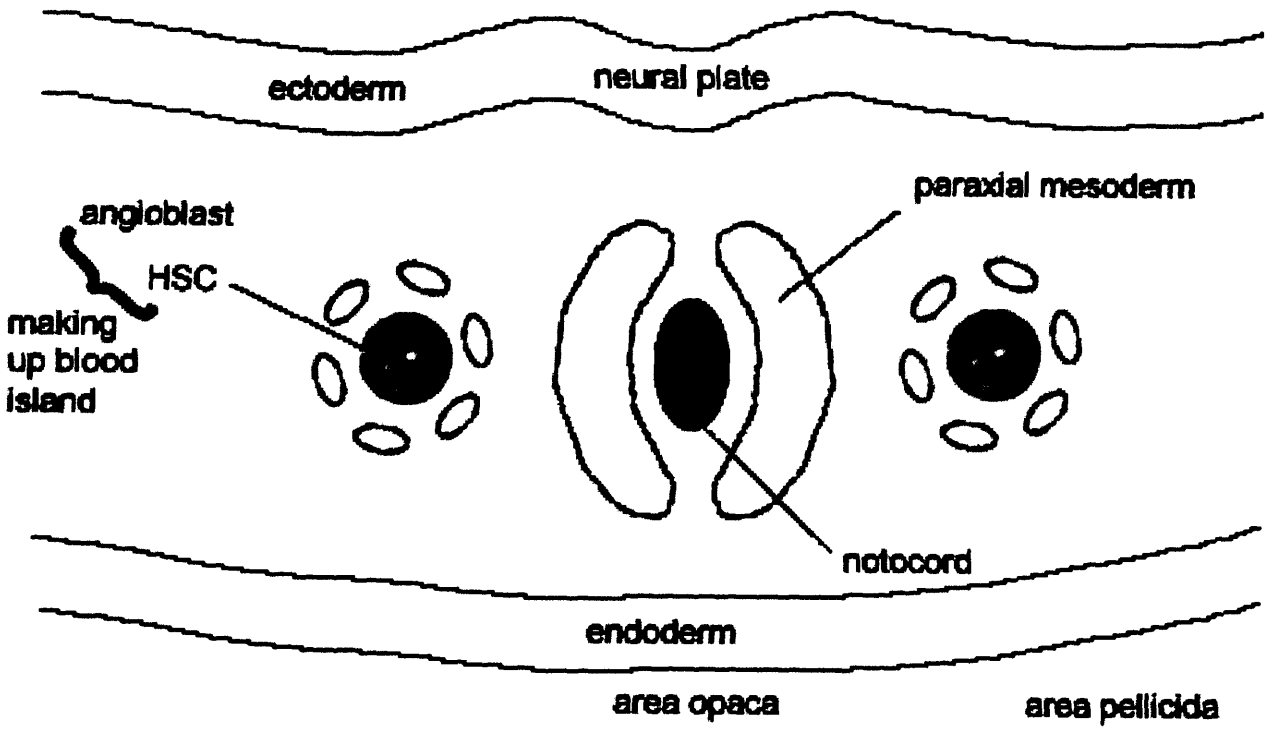


Figure 1.1 Schematic representation of blood island formation within the paraaxial mesoderm.

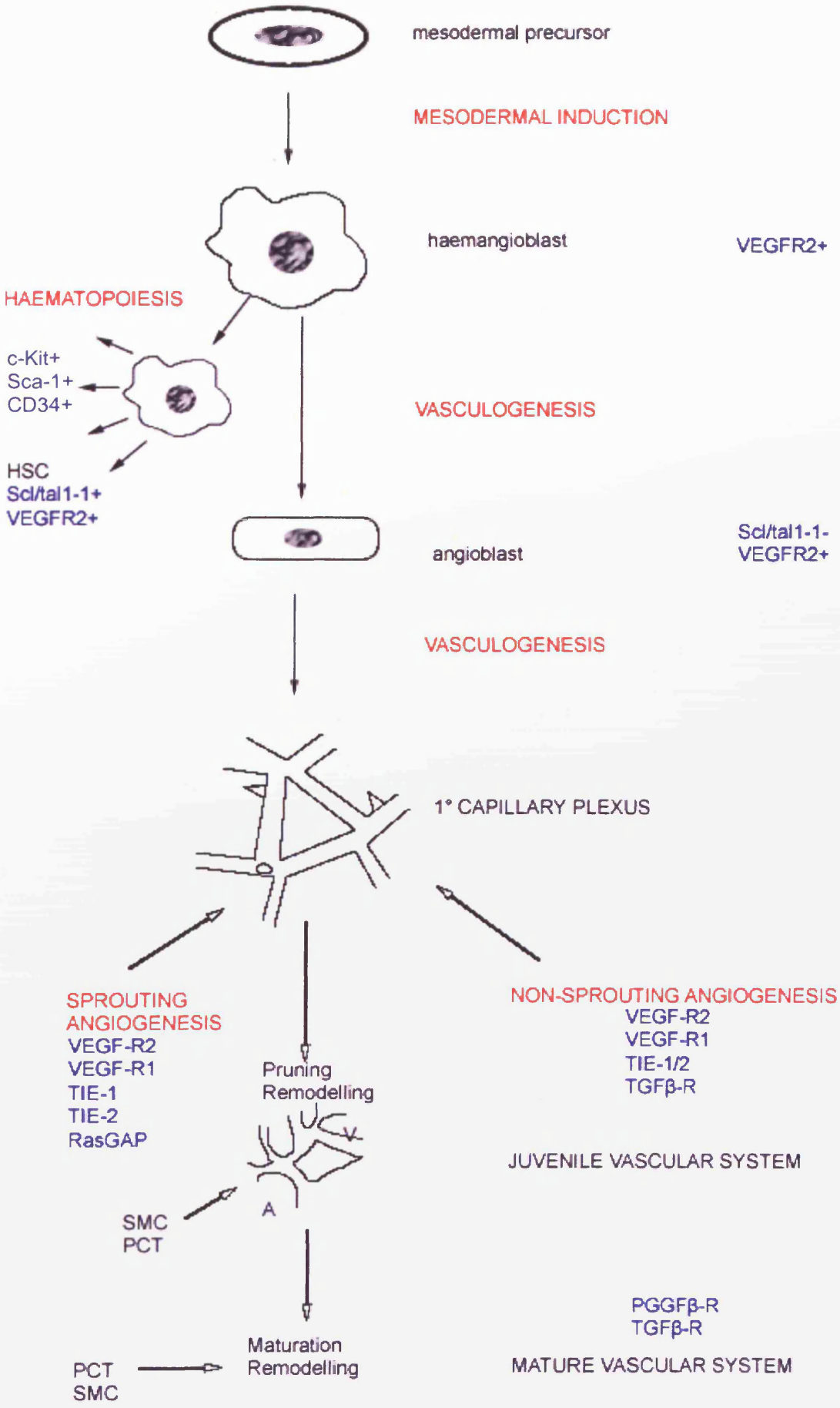

mesodermal precursor
MESODERMAL INDUCTION
haemangioblast
VEGFR2+
HAEMATOPOIESIS
c-Kit+
Sca-1+
CD34+
VASCULOGENESIS
HSC
Scl/tal1-1+
VEGFR2+
angioblast
Scl/tal1-1-
VEGFR2+
VASCULOGENESIS
1° CAPILLARY PLEXUS
SPROUTING
ANGIOGENESIS
VEGF-R2
VEGF-R1
TIE-1
TIE-2
RasGAP
Pruning
Remodelling
NON-SPROUTING ANGIOGENESIS
VEGF-R2
VEGF-R1
TIE-1/2
TGFβ-R
JUVENILE VASCULAR SYSTEM
A
SMC
PCT
PGGFβ-R
TGFβ-R
Maturation
Remodelling
PCT
SMC
MATURE VASCULAR SYSTEM

Figure 1.2. The processes (red labels), molecules (blue labels) and the appearances (black labels) involved in vascular development.

Intermediate steps between haemangioblast differentiation and primary capillary plexus formation remain unknown and, therefore, omitted. A: Arteriole. V:Venule, SMC: Smooth Muscle Cell, PCT: Pericytes, HSC: Haematopoietic Stem Cell. Adapted from Risau, W Mechanisms of Angiogenesis (13).

1.1.2 Angiogenesis

In contrast to vasculogenesis, angiogenesis is the process is the process of blood vessel formation resulting solely from the proliferation, migration and remodelling of endothelial cells from pre-existing blood vessels (25). Angiogenesis occurs throughout the growth of the foetus in utero especially during organogenesis and results in the development of mature blood vessels. Postnatal angiogenesis occurs during wound healing and stages of the female reproductive cycle and has a role in pathological states such as diabetes, psoriasis, rheumatoid arthritis, haemangiomas and the growth and metastases of tumours (26).

The process of angiogenesis is divided into four stages, namely i) initiation phase, characterised by increased permeability of the vessel ii) progression phase whereby proteolytic enzymes are produced which degrade the extracellular matrix to allow endothelial cell migration and proliferation iii) differentiation to form new vessels and iv) the stabilisation and maturation phase with recruitment of pericyte/mesenchymal cells to the vessel wall. Two mechanisms of angiogenesis, sprouting and non-sprouting, are known to exist. Sprouting angiogenesis involves the activation of endothelial cells by angiogenic stimuli leading to the progression phase. A cord of endothelial cells results which ultimately remodel into a new vessel (27). Non-sprouting (intussusceptive) angiogenesis occurs by the splitting of pre-existing vessels by ingrowing columns of connective tissue (27). Eventually this invasion of the lumen in the pre-existing capillary divides the vessel into two.

It has been suggested that vasculogenesis and angiogenesis occur in parallel in the vascularisation of tissues and organs such as the heart and yolk sac (13). However, sprouting angiogenesis appears to predominate in the brain whereas vasculogenesis predominates in the lung where there is a rich preponderance of angioblasts (13).A

period of vessel maturation then follows both processes which involves a combination of both mechanical forces and growth factor inhibition and activation (15). This latter phase will be discussed in the following sections.

1.1.3. Molecular regulation of vasculogenesis and angiogenesis

Since Folkmann's hypothesis that the growth of tumour vasculature requires a combination of angiogenic growth factors and receptor activation in the early 1970's, the understanding of the molecular mechanisms regulating and controlling vasculogenesis and angiogenesis have increased dramatically. It is through a fine balance of activators and inhibitors that the microvascular endothelium is kept in a quiescent state during the entire life span of a normal adult but yet allows rapid proliferation in some physiological and pathological states.

A critical class of molecules regulating vasculogenesis and angiogenesis are the endothelial receptor tyrosine kinases and their ligands. The principle endothelial receptor tyrosine kinase and ligands regulating vessel formation are summarised in Table 1.

The inter-relationships between these factors are not completely understood. Briefly, the receptor tyrosine kinases have a glycosylated extracellular ligand binding domain, single hydrophobic transmembrane region and a cytoplasmic (intracellular) domain that contains a tyrosine kinase catalytic domain. They are classified according to their extracellular structural characteristics (28, 29). Ligand binding results in the receptor assembling into dimers, which enables the cytoplasmic domains to trans-phosphorylate each other on multiple tyrosine residues (28, 29). This is called autophosphorylation. Once activated intracellular signalling proteins bind, usually via their Src homology regions 2 and 3 (SH2 & SH3) to the activated receptor, which

include GTPase activating protein (GAP), phospholypase C-gamma (PLK) and phosphotidylinositol 3' kinase (PI3 kinase) (13).

Growth Factor	Embryonic lethality (time in days)	Stage of vessel development	Receptor
VEGFA (=$VEGF_{121,165,189,206}$)	E10.5-11.5	vasculogenesis	Flt-1(VEGFR1) Flk-1(VEGFR2/KDR
VEGF B	E8.5-9.5		?
VEGF C	E8.5-9.5		VEGFR2&3(Flt4)
VEGF D	E10.5-12		VEGFR2&3(Flt4)
Placental GF			VEGFR 1
Angiopoietin 1	E10.5	angiogenesis	Tie 2(Tek)
Angiopoietin 2	E12.5-P1	maturity	Tie2(Tek)
?			Tie 1
FGF			FGFR
TGFα			EGFR

Table 1: Key receptor tyrosine kinase and their respective ligands involved in vessel formation.

Knockouts animals from the VEGFR/VEGF and Tie/Ang families have exhibited a variety of embryonic defects in vascular development.

It was thought, initially VEGFR 1, 2, 3 and the Tie family were endothelial cell specific. Recent observations, however, have shown VEGFR2, Tie 1 and Tie 2 are also expressed on haematopoietic stem cells (30). Studies in which genes encoding

the TIE 2 receptor and the Angiopoietins were inactivated resulted in embryonic lethality in mice due an absence of remodelling and sprouting of blood vessels (31-33), In similar experiments using gene knockouts of VEGF and its receptors, VEGFR1 and 2, showed VEGF expression in the visceral endoderm is an essential pre-requisite for the normal expansion and organisation of endothelial and haematopoietic lineages in the development and differentiation of the cardiovascular system (13, 21, 23, 34, 35) These observations demonstrate VEGF and the Angiopoietins are amongst the most important regulators of vasculogenesis and angiogenesis.

1.1.3.i. Vascular endothelial growth factor receptors and ligands

The first angiogenic receptor expressed in mesodermal cells is VEGFR2 (called KDR in human, Flk-1 in mouse). Flk-1 deficient mice die in utero at E8.5-9.5 with absent dorsal aorta and blood island formation in the yolk sac and other small vessels, indicating an absolute requirement for Flk-1 in growth/establishment of endothelial cell lineage (21, 34). Later during differentiation the receptor becomes restricted to endothelial and haematopoietic stem cells consistent with its ligand VEGF being a specific endothelial cell growth and permeability factor.

VEGFR1 (Flt-1) deficient mice also display embryonic lethality at E 8.5-9.5 but in comparison to the Flk-1- phenotype, the defect lies more in the organisation of the endothelial cells into tube structures and blood islands, and hence primitive vascular network, than the differentiation of the cells.

The ligand VEGFA is a 45 kDa glycoprotein coded for on human chromosome 6p21.3 (36). Four isomers exist due to exon splicing, which correspond to 121,165,189,206 amino acids in size. 189 and 209 are bound to the extracellular membrane. 165 is the predominant isoform secreted by endothelial cells and smooth muscle cells and pericytes (35). It is a key regulator of vasculogenesis and angiogenesis and has been shown to be a major initiator of neovascularisation during embryonic development (37).

VEGF is secreted in the endoderm of the embryo. In the absence of endoderm no endothelial cells are formed suggesting a paracrine relation ship exists between VEGF secreted in the endoderm and mesodermal cells expressing VEGFR2. In the absence of VEGF, there is down regulation of the receptor, resulting in a lack of differentiation of the angioblast from all mesodermal cells expressing VEGFR2. Mice lacking the VEGF gene die in utero with aberrant blood vessel formation in the yolk

sac and embryo (38). As well as demonstrating defects in angioblast differentiation mice lacking the receptor and its ligand also show defects in the haematopoietic cell lineage providing evidence for a bipotential precursor (39). These observations indicate the VEGF receptor and ligand are necessary for vasculogenesis.

1.1.3.ii The Tie receptor family and ligands

Tie 1 and Tie 2 (in mouse called Tek) are receptor tyrosine kinases that are expressed predominantly on endothelial cells and some haematopoietic cell lines.

Tie 1 is a 135 kDa protein. It is detected in the mouse embryo at E8.0 in mesenchymal angioblasts and blood islands of the yolk sac. The receptor continues to be expressed throughout embryogenesis. Tie 1 null embryos die from internal/subcutaneous haemorrhages and oedema at E713.5, suggesting the receptor is important for endothelial cell differentiation and vessel integrity (15) .

In the adult Tie 1 is upregulated during neovascularisation and down-regulated in quiescent adult tissue (31).

Tie 2 is a 145 kDa glycosylated protein and is first detected in the mouse embryo vasculature of the yolk sac at E7.5. In mouse studies using a transgenic approach, a dominant - negative Tie 2 was expressed under the control of a Tie 2 promoter. This resulted in homozygous mutant death by E9.5 with malformations of their vascular network. Hearts failed to possess organised trabeculae and the dorsal aorta was ruptured and disorganised with a lack of mesenchymal cell support.

From this it was implied the Tie 2 receptor is important in mediating endothelial cell motility and essential for the interaction between endothelial cells and the

extracellular matrix and support cells. The receptor also has a critical role in vasculogenesis promoting network growth (32).

The Angiopoietins are a family of ligands for the Tie 2 receptor. Angiopoietin 1 is first expressed between E9 and 11 around the heart myocardium. Later in development it is found widely throughout the embryo often associated with the mesenchyme adjacent to developing vessels (25, 33)
Angiopoietin 1 is an activating ligand and promotes tyrosine phosphorylation of the Tie2 receptor. Angiopoietin 1 deficient mice display a phenotype similar to Tie 2-/- mice (15, 33) in that the primary capillary plexus fails to fully remodel with reduced interaction and adherence with surrounding support cells and matrix is seen. It has recently been demonstrated the support cells produce Angiopoietin 1 and this acts on the endothelial cells expressing Tie 2, in a paracrine manner (40).
Angiopoietin 1 is constitutively expressed in the adult were it appears to have a role in stabilising mature vessels.

The cloning of Angiopoietin 2 was first described after the discovery of Angiopoietin 1.Angiopoietin 2 displays a 60% homology in amino acid sequence with Angiopoietin 1(41). Overexpression of Angiopoietin 2 in mice results in a lethal phenotype with poor vessel integrity and heart underdevelopment.
There is increased expression of Angiopoietin 2 at sites of vascular remodelling e.g. ovary, placenta and uterus (41) and allows the cells to revert to a more plastic state. In the presence of VGEF the cells can mount a robust angiogenic response similar to that of early embryonic vessels prior to maturation (41). However, in the absence of VEGF, Angiopoietin 2 destabilisation results in frank vessel regression.

Initially it was thought Angiopoietin 2 could bind the Tek receptor but not activate it, therefore a natural antagonist to Angiopoietin 1 (41).However, in studies were Tek was ectopically expressed in non endothelial cells (NIH 3T3 fibroblasts) Angiopoietin 2 was shown to induce phosphorylation. Furthermore high concentration of Angiopoietin 2 (42) was found to stimulate Tie 2 autophosphorylation. Furthermore Angiopoietin 2 has recently been shown to activate the receptor in murine brain endothelial cells via the c Fyn and c Fyc pathway (43). This suggests that Angiopoietin 2 acts not only as originally thought as a natural antagonist to Angiopoietin 1 but may have a direct, stimulatory action on angiogenesis in mature tissue. Isner's group (44) using cornea micropocket assay demonstrated Angiopoietin 2 had a modulatory effect on VEGF induced neovascularisation whereby circumferential extent and length of vessel induced by VEGF and Angiopoietin 2 were significantly increased compared to control ,VEGF /Angiopoietin 1 and VEGF alone. Furthermore, histological examination revealed Angiopoietin 2 and VEGF induced migration of endothelial cells at the tip of capillaries. This confirmed Angiopoietin 2 acts on VEGF-induced neovascularisation to promote the vascular destabilisation and sprouting required to initiate neovascularisation.

1.2 POST-NATAL VASCULARISATION

The processes of vasculogenesis and angiogenesis are not only essential for foetal vascular development but play integral roles in post-natal vascularisation in many physiological and pathological conditions (25, 26, 45).

The vascular endothelium represents a dynamic border between circulating blood and the surrounding tissues. The endothelial monolayer acts as a non-adhesive surface for platelets and leucocytes and has important regulatory factors e.g. NO and prostaglandin production (45, 46). Hence, the ability to regenerate the vascular endothelium in the adult is important for example in the formation of granulation tissue in wound healing and in many physiological conditions such as infantile growth and the ovarian cycle.

There are a number of clinical situations in which it would be desirable to promote vascularisation and augment collateral vessel growth such as occlusive atherosclerotic disease of the coronary arteries or the lower limb vessels leading to myocardial ischaemia and peripheral vascular disease respectively.

Conversely, there are a number of clinical situations in which it would be desirable to inhibit neovascularisation, for example in chronic inflammatory processes such as rheumatoid disease, psoriasis, diabetic retinopathy and in tumour growth or growth of vascular malformations(47).

1.3 ENDOTHELIAL PROGENITOR CELLS

Circulating endothelial circulating cells were first described in the 1960s when impervious Dacron grafts were placed in animal models and their surface found to be coated by a monolayer of endothelial cells (48, 49). It was initially thought these cells were terminally differentiated mature endothelial cells, however subsequently it has been shown mature endothelial cells have relatively poor proliferative potential and it was, therefore, speculated endothelial repair and regeneration may require other cell types possibly found within the circulation (50) .

Asahara's pioneering work in 1997, and subsequent accumulating evidence over the past few years, has overturned the belief that the development of angioblasts and their differentiation into mature endothelial cells occurs exclusively in the embryo. (51-53) It has been suggested that the peripheral blood of adults contains a unique subtype of circulating bone-marrow derived cells with properties reflecting those of embryonic angioblasts , which have the potential to proliferate and differentiate into mature endothelial cells and, so, be involved in post natal vasculogenesis (50) as shown in Figure 3.

These cells have been termed endothelial progenitor cells (EPC).

1.3.1 Evidence for circulating endothelial progenitor cells

As discussed earlier, during embryonic life it is thought endothelial cells and haematopoietic stem cells derive from a common precursor the putative haemangioblast (21). Haematopoietic stem cells have been shown to be present in circulating blood in adults, in quantities sufficient to permit harvesting and readministration for autologous bone marrow transplantation (2). It was thought therefore, given their common ancestry, endothelial progenitor cells could also be present in the circulation. Asahara et al published the first description of putative angioblasts isolated from the mononuclear cell fraction of blood (51, 52)and showed these cells differentiated *in vitro* into endothelial cells. These, angioblasts arose from purified CD34+ fraction of adult peripheral blood. Not only did these cells develop an endothelial phenotype but they also incorporated into new vessels at sites of tissue ischaemia. Following Asahara's innovative work, Rafii et al presented evidence for the existence of circulating bone marrow-derived haemangioblasts in the adult (54). As in Asahara's work this study confirmed the differentiation of a subset of CD34+ cells into an endothelial-like cell which expressed mature endothelial cell marker proteins such as Von Willibrand Factor (vWF) and functionally incorporated Dil-Ac-LDL. Furthermore, it was demonstrated these bone-marrow derived genetically tagged cells, when transplanted into a dog model, covered implanted Dacron grafts. Following this work successive groups have repeated this EPC isolation and gone on to demonstrate these circulating cells have the ability to participate in physiological and pathological neovascularisation in foci of active angiogenesis (2, 3, 52, 55, 56). Furthermore, subsequent studies have consolidated these findings, showing that *ex vivo* expanded EPC from the peripheral adult circulation when transplanted into

animal models of limb ischaemia and myocardial infarction, successfully augment neovascularisation resulting in physiological recovery documented by limb salvage and improvement in myocardial function (4, 57). See table 2.

These seminal studies, therefore, suggested the existence of circulating adult angioblasts, subsequently named EPCs.

SOURCE OF CELLS	SURFACE MARKER PROTEIN	NEOVASCULARISATION MODEL	PAPER
Freshly Isolated			
CD34+	Flk1+,CD45+	Mouse incorporation CD31+ capillaries	Asahara 99
	Tie 2+, Dil-AC-LDL incorporation	Hind limb ischaemia detected (not quantified)	Schatterman 2001
	CD34+ only	MI model	Jackson A 2001
	KDR,AC133+	Neointima on cardiac pacemaker colonised	Peichev 2000
	Adherence, Di-AC-LDL incorporation	Located on implanted prosthetic graft	Shi 98
	CD117,VEGFR2, Tie2, AC133	MI – new vessels	Kocher 2001
BM – MNC	Sca – 1+, Lin-ckit+	MI – incorporation at infarcted sites	Orlic 2001
Ex-vivo Expanded			
Early	CD45-, KDR+	Hind limb ischaemia	Murohawa 2000
Late	AC133+ KDR/Tie2/vWf@ day 14 outgrowth	Late outgrowth + AC133 cells injected in mouse tumour model. 2x size of tumour with both groups	Gehling M 2000
	Endothelial cell growth medium. (7 days)		Kalka C 2002
	Adhesion outgrowth (7 days)	Hind limb ischaemia	
		Hind limb injection outgrowth cells	Kawamoto A 2001
Clinical Studies			
TACT-Trial BM Cells and monocytes	CD34+,Dil-aC-LDL+,lectin+	Intermuscular injection improved blood flow in peripheral vascular disease	
TOPCARE-AMI PB-MNC and BM Cells	PB-MNC: VEGFR2+,CD31+, Dil-AC-LDL,vWf+, BMC;CD34+/CD133+,CD34+/VEGFR2	Intracoronary infusion in patients with MI resulted in increased coronary flow.	

Table2. Experimental and recent clinical studies on EPC isolation and neovascularisation

1.3.2. Isolation and culture of EPCs

As discussed in section *1.4.1*, it has been demonstrated EPCs can be isolated from bone marrow, adult peripheral blood, foetal liver and umbilical cord blood (44, 50, 51, 58-60).

In initial studies putative progenitor cells were isolated from adult human blood using cell surface markers that distinguish a subset of circulating cells with an endothelial phenotype (50, 51). The classical isolation methods described include the use of coated magnetic microbeads coated with anti-CD34 and anti-KDR antibodies or adherence culture of total peripheral blood mononuclear cells (PBMNC). Following isolation the cells are cultured in endothelial specific growth medium to facilitate the growth of endothelial-like cells as it is thought the incubation with specific endothelial growth factors, specific adhesion substrates (e.g. Fibronectin) and extracellular matrix influences the proliferation and differentiation of bone marrow-derived EPCs. After initial adhesion EPCs form, within 3-4 weeks, endothelial monolayers (44, 51, 52, 61). Observations demonstrated these isolated cells have the ability to form capillary tubes in basement matrix containing gels and incorporate acetylated LDL and bind endothelial-specific lectin when co-cultured with CD34- cells (43, 59, 62). It has also been shown that EPCs lose CD34 expression and start to express CD31, Vascular Endothelial Cadherin (VE Cadherin) and vWF, but the timing of marker protein expression has been poorly described (51, 52, 63, 64).

As the surface protein CD34, is not exclusively expressed on haematopoietic stem cells, later studies utilised the more immature stem cell marker, CD133 (formerly AC133) to isolate cells. This antigen is expressed on haematopoietic stem cells and is absent on mature endothelial cells and monocytic cells and has no known biological activity (65). Selected CD133+ cells subsequently differentiated into endothelial-like

cells *in vitro* and demonstrated the same functional outgrowth seen in CD34+ selected cells. It was, therefore, suggested CD133+KDR+ cells were more likely to reflect the true endothelial progenitor population (63, 64)as discussed in section 1.6.1.

Although all of these studies are consistent with the hypothesis that cells with endothelial characteristics, or EPCs, can be isolated from the circulating adult peripheral mononuclear cell population and be cultivated in medium favouring endothelial differentiation and can contribute to neovascularisation, the exact nature of these cells and their origin remain unclear despite refined isolation procedures and immunochemical reagents. In the adult peripheral blood mononuclear fraction, the possible sources of these cells are either 1) mature endothelial cells sloughed from vessel walls, 2) endothelial precursor cells in the circulation, 3) early lineage haematopoietic stem cells/EPCs from the bone marrow or monocytic cells which may have the ability to transdifferentiate to endothelial cells under cultivation selection pressure.

Two major obstacles in the isolation and characterisation of EPCs is the lack of specific markers to differentiate between mature vessel wall endothelial cells and circulating EPCs and the lack of specific functional markers to discriminate between haematopoietic and endothelial progenitors. However there are lines of evidence that EPCs make up the majority of these isolated cells. Firstly, Asahara (51) showed freshly isolated CD34+ cells display a paucity of endothelial markers compared to those plated for 7 days (50), secondly Isner et al (7) has recently shown that in contrast to EPCs, heterologously transplanted differentiated endothelial cells do not incorporate into foci of neovascularisation (9) thirdly, terminally differentiated endothelial cells in peripheral bloods identified using the endothelial P1H12 antibody (53) ranges between 2to 3 /millilitre of blood , whereas the population of circulating

EPCs in normal individuals ranges between 0.5 to 1x103 per millilitre of blood and fourthly, Lin Y et al, using bone marrow transplant recipients (recipients marrow 100% donor genotype determined by restriction fragment length polymorphism +/- cytogenetic analysis) who had received gender mismatched transplants 5-20 months earlier, showed that endothelial outgrowth from cultured PBMNC, after 9 days in culture, was predominantly of the recipient genotype. In comparison however, after one month, endothelial outgrowth was mainly of the donor genotype. Although the recipient genotype was predominantly higher in number (95%) in peripheral blood, endothelial outgrowth expanded ~ 20 fold whereas the donor genotype expanded 1000 fold (53).Together these data suggests that circulating endothelial cells originating from the vessel wall, or from remaining reticular endothelial tissue e.g. the spleen, have limited growth capacity whereas late outgrowth of endothelial cells are mainly from marrow derived cells which demonstrate a more delayed outgrowth but a greater proliferative rate. It was concluded that late outgrowth cells derived from the bone marrow were true circulating EPCs. Furthermore, Shi et al using a canine bone marrow transplantation model, in which marrow cells from the donor and recipient were genetically distinct, implanted an impervious Dacron graft into a mouse model. On removal of the graft 12 weeks later, scattered islands of endothelial cells were found in the absence of any evidence of transmural angiogenesis. Using PCR these cells were shown to be of donor origin (51, 66) and displayed endothelial characteristics. It was, therefore, surmised these cells were bone marrow derived circulating endothelial cells.

Thus, even in the absence of specific EPC marker proteins, there is enough evidence to conclude the adult putative endothelial precursor may reflect a phenotype of bone marrow derived embryonic angioblast. Like embryonic angioblasts these cells have

migrational properties and display the capacity to circulate, proliferate and differentiate into mature endothelial cells and can, ultimately, contribute to vascular repair(44).

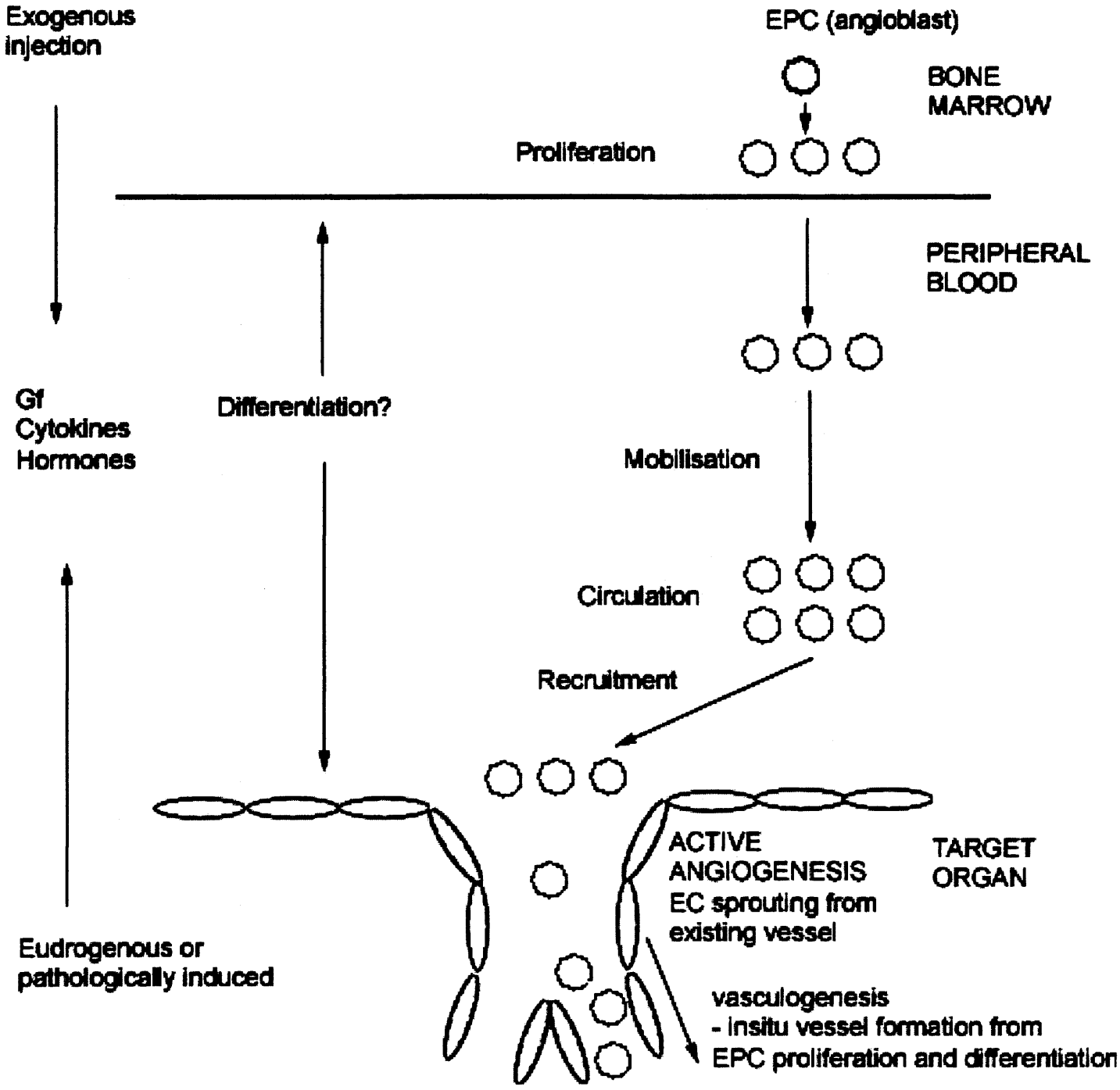


Figure 1.3. Schematic diagram to show the role of endothelial progenitor cells (EPCs) in vasculogenesis and angiogenesis.

Adapted from Sata ,M et al Inflammation, Angiogenesis and Endothelial Progenitor Cells (67)

1.3.3 EPCs in post-natal vascularisation

Asahara, using a bone marrow transplantation model and the Tie 2-ß-lac Z transgenic mice, was the first to demonstrate tissue ischaemia mobilises EPC from bone marrow to peripheral blood and that these mobilised EPC home specifically to sites of neovascularisation and differentiate into mature endothelial cells (50). In this study, transgenic mice expressing β galactoside under the transcription regulation of endothelial cell specific promoters (Flk1/LZ or Tie 2/LZ) were used as bone marrow transplant donors. Tumour cells were implanted subcutaneously after bone marrow transplantation and samples harvested. These disclosed abundant Flk1/LZ and Tie2/LZ fusion transcripts and stained positively for X-gal at one week. Hind limb ischaemia was then induced and lacZ positive EPCs were seen to be incorporated at sites of angiogenesis with the cells being deduced to arise from the transplanted marrow. In a similar experiment Musohara et al (39) induced unilateral hind limb ischaemia in nude rats and on day 3 post-surgery injected *ex vivo* expanded EPCs into the ischaemic thigh muscle. Frozen tissue sections demonstrated incorporation of labelled cells into capillaries within the ischaemic tissue (60). Furthermore, laser Doppler imaging and histological capillary density analysis showed that local transplantation of EPCs quantitatively augmented new vessel formation and blood flow *in vivo*. Kalka et al demonstrated transplantation of *ex vivo* expanded EPCs for therapeutic neovascularisation (57) showed transplantation of adult peripheral blood derived EPCs significantly augmented ischaemia induced neovascularisation in the hind limb and promoted limb salvage in nude mice . These studies provided novel

evidence that exogenously administered EPCs augment impaired neovascularisation in animal models of critical limb ischaemia. Additionally, not only did heterologous cell transplantation improve neovascularisation and blood flow recovery, but it improved biological activity, (i.e. reduced limb necrosis and auto-amputation) by 50% compared to mice receiving differentiated endothelial cells or media from which harvested cells were expanded *ex vivo* prior to transplantation. A similar strategy applied to a myocardial model of ischaemia in the nude rat, demonstrated transplanted human EPCs incorporated into the ischaemic myocardium, enhanced left ventricular function and inhibited fibrosis (3). More recently Kocher et al attempted intravenous infusion of freshly isolated (not cultured) CD34+ mononuclear cells into nude rats with myocardial ischaemia resulting in preservation of left ventricular function and a decrease in cardiomyocyte apoptosis (4). Similarly, CAM-1/Tie2 expressing cells in the bone marrow were shown to contribute to the regeneration of ischaemic cardiac muscle and vascular endothelium (68). These findings that circulating EPCs may home to sites of tissue ischaemia and differentiate into functional endothelial cells *in situ* is consistent with the notion of " postnatal vasculogenesis" (15, 21, 23) a critical paradigm for establishment of the primordial vascular network in the embryo. These data collectively support the hypothesis that EPCs exist within the circulation and target various vascular sites, contributing to vascular repair.

1.4 ENDOTHELIAL PROGENITOR CELL HOMING

Although a number of reports indicate a potential therapeutic role for EPC in ischaemic disease, it is clear that EPC need to home, i.e. recruitment and incorporation of EPCs, to foci of neovascularisation. To date, the homing mechanisms and the differentiation pathway of EPCs remains poorly understood.

EPC homing requires a co-ordinated sequence of signalling events including mobilisation from bone marrow, chemoattraction/ adhesion, local transmigration and tissue invasion and differentiation (69).

1.4.1 Factors controlling endothelial progenitor cell mobilisation and recruitment

There has been much work recently attempting to determine the regulatory role of growth factors in controlling vasculogenesis and angiogenesis. Given the recent work on VEGF as the initial determinant of haemangioblast differentiation into endothelial progenitor cells/angioblasts and haematopoietic stem cells (2, 18, 23, 70)in foetal development, the majority of work has focused on its role in modulating EPC kinetics for postnatal neovascularisation.

The first study of EPC mobilisation was performed by Takahashi T et al (2)who showed cytokine induced EPC mobilisation from bone marrow using a mouse model exogenously administered with granulocyte-macrophage colony stimulating factor (GM-CSF) which has a known stimulatory effect on haematopoietic stem cells, myeloid lineage and endothelial cells (3). Similarly a trial administering GM-CSF to patients with severe coronary artery disease resulted in overall improvement in myocardial perfusion (71). Several studies have shown that VEGF promotes

mobilisation of EPCs and their incorporation into sites of angiogenesis (10, 19, 50, 72, 73). As discussed in section 1.1.3 VEGF promotes angiogenesis and vasculogenesis by inducing proliferation, differentiation and chemotaxis of endothelial cells. It was, therefore, subsequently hypothesised VEGF may have a similar mechanism to induce EPC proliferation and modulate adhesion molecule expression within the bone–marrow to induce mobilisation and migration. Asahara et al observed an increase in circulating EPCs following recombinant human VEGF administration to immunodeficient nude mice (72). VEGF was found to induce mobilisation of bone marrow derived EPC which resulted in an increased EPC numbers generated *in vitro* and augmented corneal vascularisation *in vivo* (44, 72). Physiologically, ischaemia is believed to be a predominant signal to induce EPC mobilisation from bone marrow, with ischaemia up-regulating VEGF (44, 74). This was confirmed by Kalka (10) who also demonstrated an increase in circulating EPC in mice with critical hind limb ischaemia who had received intramuscular gene transfer of naked plasmid DNA encoding human VEGF. Later work by Iwaguro et al (11) showed VEGF gene transfer augmented EPC proliferative activity, adhesion and incorporation into quiescent endothelial cell monolayers *in vitro*, suggesting modulation of adhesion molecule expression by VEGF gene transfer may promote the homing of EPCs to sites of ischaemia and thereby providing potential insights into the mechanisms responsible for the *in vivo* outcomes seen (11). More recent studies, using gene knockout mice, have shown this VEGF induced mobilisation of EPCs from the bone marrow operates via a MMP-9 dependent mechanism, presumably with the latter cleaving adhesive bonds on bone-marrow stromal cells which interact with the integrins on HSCs (75).

Further work by Rafii et al (73) injected adenoviral vector VEGF and Angiopoietin-1 into SCID mice which resulted in remodelling of the bone marrow vasculature and concomitant splenomegaly suggesting activation of VEGF/KDR and Angiopoietin 1/Tek signalling pathways are critical for mobilisation and recruitment of haematopoietic stem cells and endothelial progenitor cells. This work was confirmed by Suda et al (30)who found synergistic effects of both angiopoietins and VEGF on the proliferation and differentiation of Tek expressing cells in the bone marrow. Despite this the roles of the angiopoietin/Tie system on the regulatory function of postnatal vasculogenesis and the downstream signalling pathways have remained unknown and comparative results in human studies are lacking.

A number of studies have described influence of pathological conditions on the number of circulating EPCs. See Table 3.

CONDITION/FACTOR	NUMBER OF EPC IN CIRCULATION
PATHOLOGICAL	
Cardiovascular risk factors(1)	↓
Limb ischaemia(2)	↑
Acute MI(3-5)	↑
Vascular trauma(6, 7)	↑
DRUGS	
HMG-CoA reductase inhibitors(8)	↑
GROWTH FACTORS	
VEGF(9-11)	↑
GM-CSF	↑
OESTROGEN(12)	↑
ERYTHROPOIETIN (EPO)(14)	↑

Table3: Physiological and pathological factors and drugs affecting EPC mobilisation

HMG-CoA reductase inhibitor (statins), used in the prevention of atherosclerosis, provided the first insight into the pharmacological modulation of systemic EPC levels. It has been shown the statins increase the number and functional activity of circulating EPCs *in vitro*, in mouse models and in human patients with coronary artery disease (8, 17, 76). It has been suggested this effect is due to an increase in proliferation, mobilisation and prevention of apoptosis of EPCs (77). It is known VEGF, erythropoietin and oestrogen augment the down-stream signalling PI3

Kinase/AKT –pathway in differentiating endothelial cells and it has recently been shown this pathway plays a role in statin induced increase in EPC levels (77).

To date, however, no clear definition exists of signals that influence the mobilisation, recruitment and differentiation of EPCs in postnatal vasculo- or angiogenesis.

1.4.2 Factors controlling endothelial progenitor cell adhesion and differentiation

To support homing to and incorporation of released EPCs into sites of neovascularisation it is necessary for circulating EPC to adhere to angiogenic foci. The integrins are known to mediate adhesion of HSCs to extra-cellular matrix proteins and mature endothelial cells (78) with the β2 integrin (CD18) being found predominantly on HSC (79). CD18 has been implicated in the homing of EPCs to areas of active angiogenesis, by mediating adhesion and transmigration (80). Moreover, after human EPCs were exposed to a HMG-CoA reductase inhibitor, simvastatin, it was shown the expression of α5β1 and αV β5 subunits were upregulated (81) with the EPCs displaying increased incorporation into the endothelium of balloon damaged vessels following angioplasty. These results support the suggestion that homing to foci of neovascularisation is not only determined by the mobilisation and incorporation of EPCs but also on their adhesiveness which may be influenced by maturation.

These data further suggests the integrin family may play a role in the homing of EPCs. However, their expression on EPCs and exact role remain unknown.

From the published data available it is hypothesised that following homing cells could either be incorporated into sprouting vessels as in the classical paradigm of angiogenesis, coalesce into vessels as in the process of vasculogenesis or be involved

in the modulation of angiogenesis , cellular apoptosis and remodelling of ischaemic tissue (3, 4, 82) as in the myocardial tunnel model described by Molodovan et al (83, 84). In this model monocytes penetrate the extracellular matrix of ischaemic myocardium and induce vasculogenesis within the tunnels formed, by recruiting EPCs and/or transdifferentiation op these monocytes into EPCs.

The start of differentiation of EPCs may be the migration from the bone marrow to the systemic circulation. Alternatively, after homing this differentiation process may be initiated further or completed.

Overall, the maturation of EPCs to a functional endothelial cell is likely to be a pre-requisite to their integration within a vessel. As discussed in section *1.2.3.1* VEGF plays a critical role in stimulating embryonic angioblast differentiation (41) and has been like-wise shown induce differentiation of *ex vivo* expanded CD34+,CD133+ and PBMN adult progenitors (17, 39, 57) and it is likely this growth factor has a similar role *in vivo.*

1.5 PHENOTYPIC VERSATILITY OF EPCS.

1.5.1 Specific endothelial progenitor cell markers

As discussed in section 1.3, despite the plethora of work related to the isolation of progenitor cell, cell surface marker proteins unique to EPCs remain poorly defined. Antibodies to human CD34 identifies both haematopoietic stem cells and EPCs and monoclonal antibodies to the extracellular domain of KDR distinguishes EPCs from lineage-committed haematopoietic stem cells (7).Until recently there has been no marker exclusive to EPCs vs. endothelial cells (58, 85). AC133, an early haematopoietic stem cell marker is a 120KDa transmembrane polypeptide which has been used to differentiate endothelial progenitor cells from terminally differentiated endothelial cells (61, 65). Studies showed that CD34+ VEGFR+ cells also expressed the haematopoietic stem cell marker AC133. Mature endothelial cells do not express AC133. When AC133+VEGFR+ cells from bone marrow were isolated and incubated in the presence of VEGF and FGF they differentiate into a AC133-VEGFR+AcLDL+ colony suggesting AC133 on CD34+ cells could identify a circulating endothelial progenitor cell population (63, 64), with the loss of CD133 reflecting the transformation of circulating EPC into mature endothelial cells. It remains unclear as to when CD133 is lost during differentiation. Circulating EPC have been reported to express a variety of endothelial lineage markers in varying intensity including PECAM-1 (CD31), CD146, VE-Cadherin, vWF, endothelial NO synthase and, upon stimulation, E-Selectin (44, 59). See Table 4.

SOURCE	ISOLATION TECHNIQUE	EPC MARKERS
BM	CD133 BEADS (64)	CD133+ CD146- CD31- vWf – VE Cad-
PB	CD34 BEADS (51) PBMC ADHESION (3, 57, 86)	CD34+ CD133+/- CD31+ vWf + KDR+
UCB	CD34 BEADS (51)	CD133+ CD34+ CD31+ vWf + KDR+

Table 4: Characteristics of EPCs isolated from bone marrow, umbilical cord blood and peripheral blood.

This implies that EPCs may change their phenotype after mobilisation and whilst in the circulation

1.6 CLINICAL APPLICATION OF ENDOTHELIAL PROGENITOR CELLS

With the identification of EPCs as important cells in adult neovascularisation there has been much interest in determining their potential role in 'therapeutic angiogenesis'.

There are three broad areas in which EPCs could be utilised for therapeutic gain - ischaemic disease, tissue engineering and bioartificial grafts and antitumourogenesis. The possibility for infusion of an autologous bone marrow mononuclear cell suspension containing EPCs has been explored. As reviewed above, in animal model studies, there has been a significant improvement in the function of ischaemic limbs and myocardium following human autologous ex vivo expanded EPC transplantation. Indeed the results were successful enough to induce limb salvage and improvement in left ventricular function in animal models (80-82).

As these initial results looked promising, human pilot studies have been initiated. These involved transplant action of autologous *ex vivo* expanded bone marrow derived EPCs. Studies in patients with severe peripheral vascular disease, after 4 weeks of intramuscular injection (gastrocnemius) there was significant improvement in the ankle brachial pressure index and exercise tolerance, with this improvement in function being sustained for 24 weeks (87).

The TOPCARE trial has established the feasibility of EPC transplantation in patients with ischaemic heart disease. In this study 20 patients received progenitor cell infusion into the diseased coronary artery. At 4 months there was a significant

increase in the left ventricular ejection fraction, wall motion at infarction sites and decrease in end systolic left ventricular volumes (5, 88).

EPCs could be harvested and used in the coating of artificial biografts to create a functionally active prosthesis, within the field of tissue engineering (1, 62, 89). A further application may be in the coating of vascular grafts with EPCs thereby creating functionally active vessels prosthesis, which would help avoid the problems of prosthetic graft occlusion (98).

It has been suggested that the ability of EPCs to localise to areas of angiogenesis could be exploited with cells acting as vectors to deliver angiogenic factors to ischaemic tissue. A recent study in primates showed CD34+cell mediated gene delivery into foci of angiogenesis is a feasible strategy. Furthermore, EPCs could be used as cellular vehicles for delivery of toxin genes to disseminate into areas of tumour angiogenesis, in the process of cancer gene therapy (90).

EPCs obviously participate in the revascularisation of ischaemic tissue. Additional experiments are needed to determine the mechanisms that influence EPC mobilisation, homing and differentiation *in vitro* and *vivo* in order to optimise their uses clinically.

1.7 AIMS OF STUDY.

Postnatal neovascularisation has been considered synonymous with proliferation and migration of existing terminally differentiated endothelial cells within the parent vessel i.e. angiogenesis. Recent findings of circulating EPC and their incorporation

into sites of active neovascularisation and the possibility they may differentiate into endothelial cells *in situ* i.e. vasculogenesis calls into question these concepts and suggests new blood vessel formation in the adult may involve embryonic mechanisms.

However, there are a number of unresolved questions involving EPCs in post-natal vasculogenesis, particularly what factors control homing of these cells to sites of vessel formation and repair. Apart from allowing us a greater understanding of the overall process of postnatal neovascularisation such information would potentially allow us to optimise protocols for manipulation of these cells in pro- and anti-angiogenic treatment. As discussed, it is well described in the literature, the Angiopoietins and VEGF are known critical regulators in angiogenesis and are present at high concentrations at these sites.

The hypothesis of this thesis is ' the Angiopoietins and VEGF influence the migration and adhesion of endothelial progenitor cells to and at the sites of angiogenesis and vasculogenesis.'

The aims of this work, therefore, are to define the roles of the Angiopoietins and VEGF in the control migration of EPC and their adhesion to endothelial cells. In addition the mechanisms by which these factors control EPC migration and adhesion will be sought.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. GENERAL REAGENTS

All cell culture media, antibiotics and other reagents were obtained from Gibco-BRL (Paisley,UK) with the exception of FCS, gelatine, MEM and IMDM which were from Sigma.

All tissue culture plastic was from Nunc Products (supplied by Gibco-BRL).

All chemical reagents were obtained from Sigma Aldridge unless otherwise stated.

2.1.2. ANTIBODIES

The cell antibodies used are given in Appendix 1.

2.1.3. SOLUTIONS

All solutions were prepared with doubled distilled de-ionised water. All solutions were pH adjusted using a Whatman PHA 230pH meter and NaOH or HCl.

Phosphate Buffered Saline (PBS)

140Nm NaCl, 2.7mM KCl, 10mM NaPHO4, 1.8Mm kh2po4, pH7.3.

Tris Buffered Saline (TRIS)

25mM Tris, 144mM Na Cl, pH7.4.

Tris-EDTA

10Mm Tris-base, 40mM NaCl, pH7.4.

Protein Transfer Buffer

25mM Tris-HCl Ph8.3, 0.15M glycine, 20% methanol.

Protein Electrophoresis Buffer

250Mm glycine, 25mM Tris-base,0.1% SDS.

Protein Loading Buffer

50mM Tris- HCl pH6.8, 2%SDS, 10% glycerol, 0.1% bromophenol blue, 100mM DTT (latter added immediately prior to use).

2.1.4. GROWTH FACTORS

Human Recombinant Vascular Endothelial Growth Factor (VEGF) was obtained from Peprotech EC Ltd, London.

Stem Cell Growth Factor (SCGF) was obtained from Peprotech EC Ltd, London.

Angiopoietin 2 was obtained from Sigma-Aldrich Corporation, UK.

Angiopoietin-1* was initially supplied by AstraZeneca, UK. It was obtained by collecting the supernatant media from cultured sub-confluent MF58 cells stably expressing human Angiopoietin-1* with a C terminal IgG sequence. In initial experiments, this was received direct from the company and stored at 4^0 C until used. It was not possible to accurately gauge the concentration of the Angiopoietin-1* (Ang-1*) during the initial experiments. However a supply of cells was obtained, and

maintained by G Williams, Department of Surgery, University of Leicester. These cells were incubated in α Modified MEM media supplemented with 10% foetal calf serum, 1% Penicillin and 1%Streptomycin and 1% L-Glutamine. They were then cultured at 37^0 C, 5% CO_2. Media was collected after 48 hours of incubation and the supernatant collected. This ensured standardisation of production of Ang-1*, providing information as to the level of confluence in each culture and allowed for evaluation of concentration of Ang-1* to be determined for each batch, by dot blot analysis.

Where Ang-1* was used in conditioned media, media conditioned by MF58 cells not expressing Ang-1 was used as a control. As indicated in the results chapter, control media contained either 0% or 10% FCS.

Angiopoietin-1 was kindly provided by Ben Dunmore, Department of Surgery, University of Leicester. The cDNA for Angiopoietin-1 was obtained from ATCC, amplified with PCR and the DNA extracted from an agarose gel by Dr.Marie Marron, Department of Surgery, University of Leicester. DNA was then cloned into a pcDNA 3.1 myc/His vector (Invitrogen) using the TA cloning kit (Invitrogen). The DNA was prepared using the Qiagen mini-prep kit and transfected into CHO cells using Superfect (Qiagen). Clones were made using G418 selection. Conditioned media was obtained after 72 hours. Concentration was determined by dot analysis using the α poly Histadine antibody (Sigma). The concentration was estimated at 200 ng/ml.

Purified Angiopoietin-1* was obtained from AstraZeneca, UK. It was dissolved in a solution of PBS, and used at a concentration of concentration of 200ng/ml in

experiments. This allowed for direct analysis of the effect of Angiopoietin-1, without the possible confounding effects of using complete media.

2.2. METHODS

2.2.1. CELL CULTURE

2.2.1i PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMNC)

Ethical approval for the protocol was obtained from the regional Ethics Committee.

Cord blood was collected using a sterile 21G needle into a sterile tube containing anticoagulant. (1:10 solution 0.016M Na^+ Citrate) This was then diluted 1:1 with PBS. Alternatively, Buffy coat was obtained from the regional blood transfusion service .This was anticoagulated with buffered sodium citrate and diluted 1:2 with PBS.

The solutions were then carefully layered over lymphoprep (Robbins Scientific) in ratio 2:1 and centrifuged at 2000rpm for 30min at room temperature (no brake).

Cells at the interface were then carefully removed to prevent any lymphoprep contamination and diluted into PBMC wash buffer (total volume 20ml).

The isolated mononuclear cells were then pelleted by centrifuging at 1500rpm for 10 minutes, resuspended in PBMC wash buffer and washed twice further. The total number of viable cells were then counted using Trypan Blue exclusion.

2.2.1ii ISOLATION AND IN VITRO CULTURE OF HUMAN EPC

Ethical approval for the protocol was obtained from the regional Ethics Committee.

Buffy coat was obtained from the regional blood transfusion service having been anticoagulated with buffered sodium citrate. The anticoagulated buffy coat was diluted 1:2 with PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4,

Ph7.3). Mononuclear cells were isolated using Lymphoprep (Robbins Scientific) density gradient centrifugation. These cells were then washed twice at 300*g* for 7 minutes using PBMC wash medium consisting of MEM supplemented with 0.5M EDTA and 20% FCS.

Mononuclear cells from 150ml buffy coat were resuspended in Medium 199(Invitrogen Life Technologies) supplemented with 20% FCS, 1% penicillin/streptomycin and heparin (100μg/ml) without further enrichment procedures and plated onto either a 6 well plate or T25 (Nunclon) coated with 2% gelatin (Becton Dickinson) and/or fibronectin (0.1mg/ml) at a density of 1 x 10^6 cells/ml. The medium was then supplemented with endothelial cell growth supplement at 0.5mg/ml(Sigma - Aldrich).

The plates were then incubated at 37°C in a humidified environment with 5%CO_2. At 24 hours, unattached cells were removed by washing with medium. For further outgrowth cells were washed every 24 hours.

2.2.1iii HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

HUVE cells were obtained by G Williams Department of Surgery, University of Leicester. Briefly, HUVE cells were isolated from umbilical cord blood by washing the cord with PBS containing penicillin (100U/ml) and streptomycin (0.1mg/ml).A sterile blunt needle was then inserted into the umbilical vein and clamped into place. The vein was then flushed through with PBS to remove any debris and the cord clamped at one end and then distended with collagenase solution (DMEM containing 10mM HEPES buffer pH7.4 and 0.5mg/ml collagenase-Clotridiopeptidase A from Clostridium histolyticum type 2). The cord was then incubated for 15 minutes at 37^0C. Following this the collagenase solution was drained from the cord into a

universal container and the vein flushed with PBS to ensure all cells were retrieved. The cells were then pelleted by spinning for 7 minutes at 300g and the pellet resuspended in complete medium 199 (Gibco, Paisley, Glasgow) supplemented with 2mM glutamine, 500U/ml penicillin, 50μg/ml streptomycin, 20% batch-tested FCS and 0.5μg/ml endothelial growth cell supplement (ECGS). The cell suspension was then placed in a T25 tissue culture flask (Nunc) coated with 2% gelatin. When the cells reached 100% confluence they were routinely passaged at 1:3. For passage cells were washed twice with PBS and incubated with 2ml trypsin/EDTA solution (0.05% trypsin and 0.02% EDTA diluted in PBS), for two minutes or until the cells lifted of the flask with gentle agitation. Complete medium was then added to inactivate the trypsin. The cells were then spun at 400g for 6 minutes to remove the trypsin and resuspended in the desired volume of medium. Cells were used for no longer than 3 passages following isolation.

2.2.1iv BOVINE AORTIC ENDOTHELIAL CELLS (BAEC)

BAEC'S were isolated by Tim Hatch Department of Surgery, University of Leicester from freshly harvested bovine aorta. Cells were routinely cultured in DMEM supplemented with 1% glutamine, 1% streptomycin, 1% penicillin and 10% FCS. Cells were passaged when 100% confluent at a ratio of 1:3. Cells were below passage 6 in all experiments.

2.2.1v.CHINESE HAMSTER OVARY CELLS (CHO)

CHO cell line were purchased from ATCC (American Type Cell Culture) and cultured in α- Minimal Essential Medium Eagle (α- MEM) supplemented with 10%

Foetal Calf Serum and 1% streptomycin, 1% penicillin and 1% L-Glutamine. Cells were passaged at 100% confluence in a ratio of 1:10.

2.2.1vi MOUSE HEART ENDOTHELIAL CELLS (MHE)

MHE'S were a kind donation from Professor Robert Auerbach(University of Wincontin,USA) and routinely cultured in Dulbeccos Modified Eagle Medium(DMEM;Gibco) with supplemented with 10% FCS , 1% penicillin, 1% streptomycin and 1% L-Glutamine. Cells were passaged 1:3 at confluence.

2.2.1.vii.CD 133+ STEM CELLS

CD133+ cell line was obtained from Anthrogenesis,USA. Cells were initially seeded at a density of $1X10^5$ cells /200µl until reaching confluence then passaged 1:2 into 24 well culture dishes. Cells were cultured in Iscoves Modified Dulbeccos Medium (IMDM, Sigma) supplemented with 10% foetal bovine serum (FBS: Sigma) , 10% Horse serum (Sigma) ,10-6 mol/L hydrocortisone (Sigma), 100ng/ml Stem Cell Growth Factor (SCGF) and 50ng/ml Vascular Endothelial Growth Factor (VEGF). Additional feeding was performed depending on cell proliferation.

2.2.1viii CD34+ CELL SELECTION FROM PERIPHERAL BLOOD MONONUCLEAR CELLS

The Dynal CD34 Progenitor Cell Selection System was used. The bCD34 kit contains Dynabeads® CD34 and DETACHaBEAD® CD34. The beads are coated with a mouse Ig G2 (clone 561) which is specific for the class III epitope on the CD34 antigen.

The CD34 antigen is a 105-120 kD heavily glycosylated transmembrane protein expressed mainly on human haematopoietic progenitor and endothelial cells. DETACHaBEAD® CD34 is designed for the detachment of Dynabeads® CD34 and is affinity purified polyclonal antibody against the Fab portion of monoclonal 561. The volume of beads needed was calculated ($4x10^7$ beads/ml of MNC) and resuspended in supernatant. They were then prewashed by placing on MPC™ for 1 minute and the supernatant removed. The beads were then resuspended in 1ml of cold (1-4°c) isolation buffer and the wash repeated. Round bottomed tubes were used throughout.

The cord blood sample was then diluted 1:4 with cold isolation buffer (Per 100ml buffer:

70ml ddH_2O, 10ML 10X PBS, 10ML 20% BSA, 0.6g Tri-sodium citrate, 2ml PS) and the mononuclear cells separated using density gradient centrifugation as previously described. The final pellet of mononuclear cells were then resuspended in isolation buffer at a concentration of $4x10^7 - 4x10^8$cells and maintained at +4°C. Cells were then added to the beads and the cell-bead mixture vortexed for 2-3 seconds to mix and incubated with the beads for 30 minutes at 4^0C with gentle tilt rotation at 10-20rpm.

Following this the cell–bead complexes were vortexed for 2-3 seconds to resuspend and then placed in the Dynal MPC™ magnetic device for 2 minutes to separate bead –rosetted cells from non-targeted cells. The supernatant-containing non-rosetted cells were aspirated whilst still exposed to the magnet. This step was repeated three times. After final wash rosettes were resuspended in 100µl of isolation buffer/$4x10^7$ beads used, to a volume of at least 100µl. To detach the beads from the purified cells 100µl of DETACHaBEAD® was added to the final wash suspension at a concentration of

1:1 of the original concentration of beads used to a volume of at least 100µl and vortexed for 2-3 seconds to mix. This mixture was then incubated at 37^0C for 15 minutes with gentle tilt rotation. Following this 2 ml isolation buffer was added and vortexed for 2-3 seconds. The tube was then placed on the Dynal MPC™ to allow the beads to accumulate on the wall for 2 minutes. Released cells were then transferred into a new tube. These steps were repeated three times pooling released cells into a single tube. The final suspension of CD34+ cells were placed on the Dynal MPC ™ for 2 minutes to remove any residual beads and the supernatant containing cells transferred into fresh tube. The CD34+ were then washed twice in 10ml of isolation buffer and spun at 400g and the pellet resuspended as appropriate.

2.2.2 CELL ASSAYS

2.2.2i ADHESION ASSAYS

An adhesion assay was developed to examine the factors controlling the incorporation of LOC into angiogenic areas by examining the factors that control the interaction of LOC and terminally differentiated endothelial cells. This consisted of a 100% confluent monolayer of endothelial cells prepared on the bottom of a 96 well plate. Fluorescently labelled quiescent LOC in suspension were treated with a variety of cytokines before being aliquoted in equal volumes onto the layer of endothelial cells. Cells were then incubated at 37^0C for the required time. The non-adherent cells were removed by serial washings with serum free RPMI and the number of adherent cells quantified using a fluorescent plate reader.

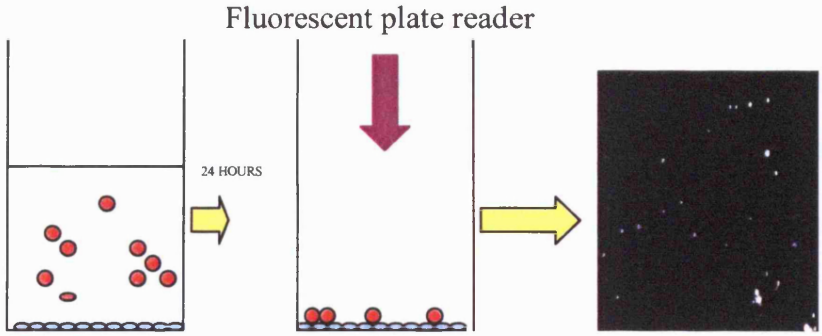


Figure 2.1 Schematic representation of cell adhesion assay.

Human Umbilical Vein Endothelial Cells or Mouse Heart Endothelial Cells at passage 1-3, were dissociated by washing with PBS and then incubated for 12 hours in serum free medium at 37^0C. Cells were then dissociated by washing with PBS followed by 1mM EDTA and incubated with 0.025% trypsin /0.5M EDTA at 37^0C for 3 minutes, ensuring all cells had dissociated, Medium 199 (GIBCO-BRL Life Technologies) with 5% FCS was added to inhibit trypsin. Cells were then centrifuged for 7 minutes at 300g and resuspended in an appropriate volume of serum free medium. Trypan Blue exclusion was then used on a 10µl sample of cells to determine viability and cell count using a haemocytometer.

Cells were then seeded onto a 96 well tissue culture plates (Nunc) at 100,000 cells/ well. Cells were grown for 24 hours, to 100% confluence, quiesced for 12 hours in 100µl serum free medium AT 370C, 5% CO_2 and washed with medium 199.

Late outgrowth cells, having been serum starved for 12 hours, were labelled with 5µg/ml calcein-AM* (Molecular Probes) in PBS at room temperature for 15 minutes, passaged as described previously. Trypan Blue exclusion was used to determine cell

viability and ensure single cell suspension. Cells were then treated with either control medium, Angiopoietin1 (400ng/ml), Angiopoietin 2 (200ng/ml unless stated), VEGF (10ng/ml unless stated) or combination for 15 minutes at 37^0C followed by washing and resuspending in 2.4mls of DMEM, before being divided into appropriate aliquots for dispensing into each well and incubated at 37^0C for 5, 15, 30 and 60 minutes or 24 hours.

After this time nonadherent cells were removed by inverting the plate and tapping the base to remove residual cells and media then washing three times with 200µl RPMI. In order to fix the cells 200µl 0f 4% paraformaldehyde was added. The numbers of adherent cells were then quantified by measuring fluorescence using a Cytofluor plate reader set at 25 reads per well, excitation 485/20, emission 530/25, 85 gain, having confirmed under the microscope that the 0 time point cells had all been rinsed off.

In other experiments both cell types, or the monolayer of terminally differentiated endothelial cells in the 96 well plate, were treated with growth factors as described. In experiments to examine adhesion to the extracellular matrix protein, fibronectin and vitronectin, 96 well tissue culture plates were coated overnight at 4^0C with 1µg/ml of the protein. Prior to use, wells were washed with PBS and blocked by incubation with 2% BSA in PBS at 37^0C for one hour. Experiments then proceeded as above.

Green fluorescent calcein AM is a compound that is converted to a fluorophore only upon uptake and metabolism by living cells and is therefore well retained in live cells and is relatively insensate to PH in the physiological range. It is cell permanent and determines cell viability. In live cells the non-fluorescent calcein AM is converted to a green fluorescent calcein after aectoxymthyl ester hydrolysis by intracellular esterases and is then retained in the cytoplasm. It exhibits emission spectra 494 and 517nm.

LOC were counted, washed with PBS and resuspended in adhesion medium. Cells were incubated with 5μg/ml calcein-AM at room temperature for 15 minutes, washed with adhesion medium and resuspended in serum free medium 199 and the number of calcein labelled cells measured using spectrophotometry.

2.2.2ii MIGRATION ASSAYS

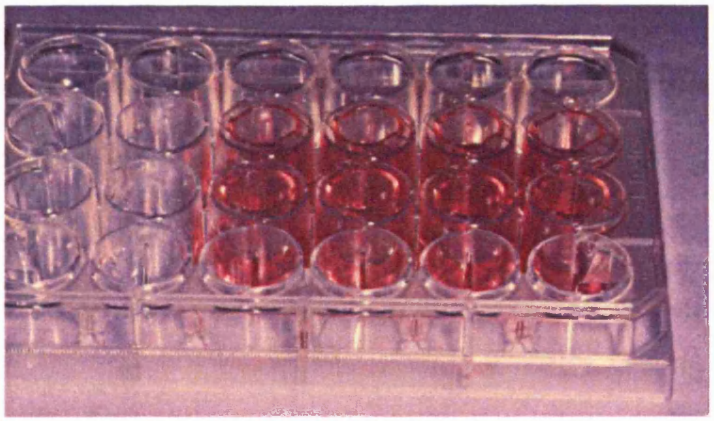
A migration system was developed to determine the factors controlling the 'homing' of LOC to terminally differentiated endothelial cells. Figure 2.1 illustrates the 24 well Boyden Chamber that were used in these experiments to examine the chemoattractant properties of various cytokines and the ability of LOC compared to terminally differentiated endothelial cells to migrate in response to these chemoattractants. Either HUVE cells, MHE cells or late outgrowth cells were grown in the exact same conditions were used in all experiments.

Cells were washed extensively with PBS and then incubated for 12 hours in serum free medium at 370C. Cells were then dissociated by washing with PBS (followed by 1mM EDTA in the case of the late outgrowth cells) and incubated with 0.025% trypsin /0.5M EDTA at 37^0C for 3 minutes, ensuring all cells had dissociated, Medium 199 (GIBCO-BRL Life Technologies) with 5% FCS was added to inhibit trypsin. Cells were then centrifuged for 7 minutes at 300g and resuspended in an appropriate volume of serum free medium.

Trypan Blue exclusion was then used on a 10μl sample of cells to determine viability and cell count using a haemocytometer. 750μ/ of serum free Medium 199 containing 0.25%BSA was placed in the lower chamber of a Transwell tissue culture wells (Falcon, Becton Dickinson, NJ,USA) with either Angiopoietin 1 (400ng/ml), Angiopoietin 2 (200ng/ml unless otherwise stated), VEGF (10ng/ml unless otherwise stated) or in combination.

$1X10^5$ late outgrowth cells were added in 250µl of the same medium to the upper insert (Falcon, Becton Dickinson, NJ, USA) which has a 8µm pore size membrane separating the upper and lower chamber through which cells can pass. Cells were incubated at 370C and allowed to migrate for 6 hours. The membrane was then fixed for at least 2 hours in 70% ethanol at -250C. The membrane was wiped with a cotton bud on the upper side of the membrane and then removed from the insert using a size 11 blade. Harris' Haematoxylin and 0.5% Eosin for 45 seconds each were used to stain the membrane which was then mounted onto glass slides with glycerol. The numbers of migrating cells on the underside of each membrane were counted at 400X magnification in 10 high power fields.

A



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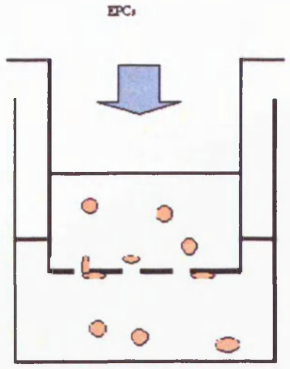

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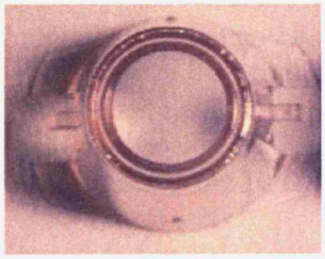


D

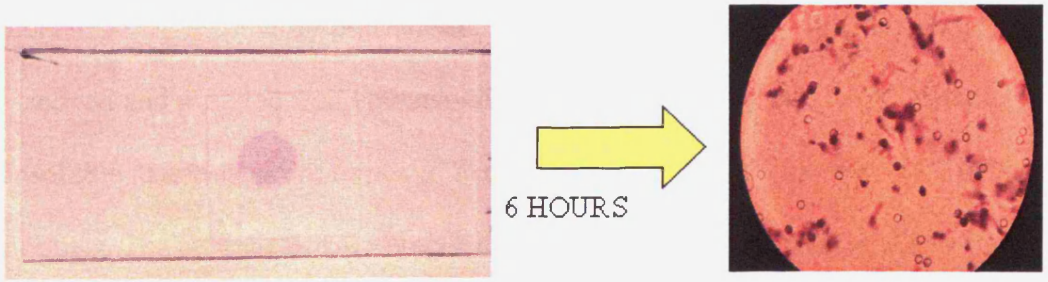


Figure 2.2 Endothelial Progenitor Cell Migration Assay

(A) Migration was measured using a modified Boyden chamber with cell culture inserts containing 8uM pores (Becton Dickinson, Cowley, UK) To the lower chamber was added either, 750ul migration medium containing known concentration of cytokine under investigation. To the upper chamber was added either $0.75x10^5$ LOC or TDEC **(B)** and allowed to migrate, via chemotaxis, to the cytokine under investigation, through 8Um pores to the lower chamber **(C)**. The cell culture insert was fixed in 70% ethanol at -20^0C for 1 hour and then the lower part of the membrane was stained with haematoxylin and eosin before mounting on slides **(D)**. The number of migratory cells was counted at 40X magnification. Only cells with complete nuclei were counted and an average taken from 10 fields of view.

2.2.2iii PROTEIN ANALYSIS

2.2.2iii.a Immunoprecipitation.

To measure expression and phosphorylation, proteins were precipitated using certain antibodies, fractionated dependant upon size and then visualised using immunodetection and enhanced chemiluminescence.

Three week LOC were serum starved for 12 hours and treated with 0.1Mm orthovanadate for 20 minutes. Following this, cells were either stimulated with growth factor or left unstimulated in serum free medium. Cells were washed in 1xPBS and lysed at 40C in 1ml of cold lysis buffer (50Mm Tris Ph7.4 containing 50Mm NaCl, 1% Triton X100, 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM EGTA, 1Mm aminoethylbenzenesulphonic acid). The flasks were scraped, the removed and pipetted into eppendorf tubes and vortexed at 40C for 1 minute. Insoluble materials were removed from the lysate by centrifugation at 12 000g for 10 minutes. The supernatant was incubated for 2 hours or overnight at 40C with

0.4□g/ml of appropriate antibody. The antibody – protein complex was recovered with the addition of 50□l of Protein G- agarose (50mg/ml)(Sigma) in lysis buffer and incubated for a further 2 hours at 4^0C. The immunoprecipitate was collected by centrifuging at 12 000g for 30 seconds and the supernatant discarded or stored at -800C. The immunoprecipitate was washed 3 times with wash buffer (lysis buffer and 5□l of Triton X -100ml to give a final concentration of 0.1% v/v.) Proteins were solubilised in 50ml of 2x sample buffer (125mM Tris pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, 10 Mm EDTA) containing 100mM DTT and the protein eluted by boiling for 5 minutes. Samples were centrifuged for 5 minutes at 12 000g and the supernatant removed and then separated by SDS-polyacrylamide gel electrophoresis.

2.2.2iii.b.Determination of protein concentration

Protein concentrations in cell lysates were determined using the Micro BCA Protein assay reagent kit. (Peirce,USA). The assay utilises the ability of bichinchonic acid (BCA) to bond with Cu+. This assay incorporates the biuet reaction (protein reacting with Cu2+ in an alkaline medium to produce Cu+) and the interaction of two BCA molecules to one cuprous ion (Cu+) in water soluble and leads to a colour change which exhibits a strong absorbance at 562nm.

Individual samples were assayed as per manufacturer's protocol with BSA being used as the standard in this assay.

2.2.2iii c Separation of proteins by sds-polyacrylamide gel electrophoresis. (SDS-PAGE)

Protein samples were routinely resolved using SDS-PAGE and mini Porotean11 cell (Bio-Rad) electrophoresis units. Separation of proteins was performed under denaturing conditions. The proteins were resolved on 10% polyacrylamide gels. The gels contained acrylamide (6.7ml of 30% stock 37.5:1 acrylamide to bisacrylamide, Protogel National Diagnostics, 200□l of 10% SDS, 3.7ml of 2M Tris-HCl pH8.8). Polymerisation was catalysed by the addition of TEMED (1μl/ml gel solution) and 10% ammonium persulphate (10μl/ml gel solution). The solution was mixed and poured between two glass plates then overlaid with water to prevent oxygenation. Following polymerisation the water was removed using filter paper and the stacking gel was cast on top and comb inserted. This was allowed to polymerise. Stacking gel contained 5% acrylamide (30% stock 37.5:1 acrylamide to bisacrylamide, 0.125M Tris –HCl pH6.8, 0.1% SDS, 10μl/ml TEMED and 10μl/ml 10% ammonium persulphate). Protein samples were boiled for five minutes in 1 X reducing sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA, 100mM dithioreitol (DTT) and centrifuged at 1300rpm for 5 minutes. Samples were then loaded onto the gel using a Hamilton syringe and proteins separated by electrophoresis at 200V in running buffer (200mM glycine, 125mM Tris-base, 0.1% SDS). Prestained molecular weight markers (Novex) were electrophoresed on each gel and electrophoresis stopped when the appropriate separation was achieved as judged by the position of the molecular markers.

2.2.2iiid Western Blotting

Following electrophoresis, then proteins were transferred onto nitrocellulose membrane (Hybond ECL, Amersham) using electroblotting. The gel was positioned cathode side of a membrane and sandwiched between Whatmann Filter Paper pre-

soaked in transfer buffer (192Nm glycine, 25 mM Tris bas3, 20% methanol). The 'sandwich' was then inserted into a mini-Trans blot (Biorad) containing 1x transfer buffer and run at a constant current of 0.12 A over 16-18 hours.

Blots were either used immediately or wrapped in Saran wrap and stored at 4°C.

2.2.2iiie Immunoblotting and enhanced chemiluminescence (ecl)

Immunoreacative proteins were visualised using horse-radish peroxidase (HRP) conjugated antibodies and a chemiluminescent detection system. Following electroblotting the free protein binding sites on the membrane were blocked with either 5% BSA in 0.1% Triton X -100 in TBS (0.1%TBS-T) for all antibodies except anti-Tek antibody (R&D Systems) whereby 5% milk was used. Next primary antibodies were diluted to the appropriate concentration in blocking buffer and incubated with the membrane at room temperature for one hour. The membranes were then washed in 0.1% TBS-T three times for 5 minutes. A HRP- conjugated antibody, specific to the primary, was diluted to the appropriate concentration in blocking buffer and incubated with the membrane at room temperature for 1 hour. The membrane was then washed 3 times in 0.1% TBS-T to remove any excess, unbound antibody. Immunoreactive bands were detected using the ECL chemiluminescent detection system. (Amersham Pharmacia Biotech UK Ltd, Bucks, UK) .Developing solution (10 ml of 0.1M Tris -HCl, 22µl, 90 Mm P- Coumaric acid in DMSO,50µl OF 250Mm Lumminol, 5-amino-2,3-dihydro-1,4-pthalazinedione in DMSO, 3µl H_2O_2) was added to the membrane immediately and incubated for 1 minute. Excess developing solution was then drained and the membrane was wrapped in Saran wrap and placed in a cassette and light emission determined using Kodak film (Sigma – Aldridge, Poole, Dorset, UK).

2.2.2iiif Stripping and reprobing nitrocellulose membranes

Repeat reprobing of membranes with different antibodies meant stripping them of previously attached antibodies.

This was achieved by submerging the membrane in stripping buffer (100mM □-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.5) for 45 minutes at 650C using gentile agitation. Membranes were then washed three times in 0.1%TBS-T at room temperature.

Membranes were then blocked in appropriate blocking buffer and re-probed as described above.

2.2.2iiig.Assessment of early and late outgrowth cells protein expression

At set time periods cells were washed in PBS and lysed with 0.75% sodium dodecyl sulphate (SDS).Cell lysates were then scraped using a cell scraper, placed into eppendorfs and sonicated . An aliquot was removed to allow calculation of the protein content of each sample using the BCA protein assay as described. To the whole cell lysate an equal volume of 2X SB-DTT was added, vortexed and then boiled for 5 minutes. Equal amounts of protein were then separated by 10% SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane and probed with either anti-Tie, anti-Tek, anti-KDR and anti- CD40 antibodies. Immunoreactive proteins were visualised using a specific conjugated secondary antibody and chemiluminescent detection system as described in section 2.2.2iiie.

2.2.2iv Immunocytochemistry.

Cells were grown on sterile autoclaved glass coverslips until reaching desired confluency. Medium was removed and cells washed in PBS. Following this cells were

fixed for 10-15 minutes at room temperature in 4% paraformadlehyde. In certain cases cells were then washed in TBS before permeabilising in TBS containing 0.5% TX-100 for 10 minutes. Following this cells were then washed three times in abdil (2% BSA in TBS), before blocking in the same solution for 10minutes at room temperature. Alternatively, cells were grown in 6 well tissue culture dishes (Nunc) until desired confluency. Cells were then trypsinised as described previously and resuspended to 1X105cells/200μL of serum free Medium 199. Using a cytospin machine (Shandon Cytospin-2, Southern Products, Runcorn, UK) 200μL of the cell suspension was placed on each slide at 1000rpm for 3 minutes. The slides were then allowed to air dry at room temperature for 30 minutes before being fixed in 70% acetone for 20 minutes at room temperature and blocked in abdil for one hour. Following migration experiments, the porous membranes (BD Falcon™ Cell Culture Inserts, Franklin Lakes, NJ) were excised from the inserts, washed and fixed with 70% ethanol for 60 minutes at -20°C then blocked in abdil for one hour. The cells were then treated as described below depending on the particular antigen being examined. In all experiments HUVE cells were used as positive controls. All antibodies were monoclonal unless otherwise stated.

2.2.2.iva Detection of von Willibrand Factor

Cells were fixed as described above. Following blocking in abdil the cells were washed three times in TBS-0.15 TX-100 and incubated at room temperature in 200μL of a 1 in 20 dilution in abdil of normal rabbit serum (DAKO) and then washed once. They were then incubated for one hour with 200μL of a 1 in a 50 dilution in abdil of anti-vW for, in the case of controls, normal rabbit serum in a humidified chamber. This was followed by three further washes and a final incubation with a 1 in 100

dilution of FITC conjugated goat anti-rabbit IgG (DAKO). Following three final washes excess water was removed by dabbing the edges with tissue and the coverslips were mounted in 220Mm diazobicylclo-octane (DABCO), dissolved in 90% glycerol,10% PBS,pH8.6. Coverslips were fixed in place using clear nail varnish and viewed under the fluorescent microscope. Photos were taken using a 400ASA film on an Olympus OM4 camera.

2.2.2ivb Detection of CD31 antigen.

An identical procedure to the above using normal mouse serum, TRITC conjugated rabbit anti mouse IgG at a 1 in 30 dilution in abdil, anti CD31 antibody at a 1 in 50 dilution in abdil.

2.2.2iv c Detection of the CD14 antigen

Identical procedure to the above using normal mouse serum, Cy3 conjugated rabbit anti-mouse at a 1 in 50 dilution in abdil, anti CD14 antibody at a 1 in 100 dilution in abdil

2.2.2iv d Detection of the TEK antigen

As the above procedure using normal goat serum, FITC labelled conjugated mouse anti-goat at a 1 in 50 dilution in abdil, anti TEK antibody at a 1 in 100 dilution in abil

2.2.2iv e Detection of the TIE antigen

As the above procedure using normal mouse serum, Cy3 labelled conjugated rabbit anti-goat at a 1 in 50 dilution in abdil, anti-TIE antibody at a 1 in 100 dilution in abdil

2.2.2iv f Detection of CD133 antigen

For identification of CD133 cells either in suspension or grown on coverslips were blocked for 1 hour using abdil then washed three times and incubated at 37°C with normal mouse serum the washed a further three times. Cells were then incubated at 4°C for one hour with PE conjugated anti- CD133 antibody. HUVE cells were used as controls. Cells on coverslips were then mounted as described above. Cells in suspension were pipetted onto slides and allowed to air-dry prior to fixation.

2.2.2v Immunostaining using chromogenic substrate dab.

Cells were immunostained for the following proteins: CD14, VE Cadherin (CD144), VEGFR2 (KDR), CD68, CD34 and the TEK receptor. Following blocking with abdil cells were incubated with the appropriate serum for one hour at room temperature in a humidified chamber. After these cells were incubated with the primary antibody at 370C for one hour (table 1) Cells were then washed three times in abdil prior to blocking with an appropriate secondary antibody (table 1) for 30 minutes at room temperature. Cells were then incubated with an appropriate streptavidin-biotin conjugated antibody for 30 minutes. Cells were then washed once more in TBS before adding DAB substrate buffer as per manufacturer's recommendation, 1 drop in 1ML (DAKO).Controls included the replacement of primary antibody with normal rabbit or mouse serum. Counter staining was performed by adding 50μL of Harris' Haematoxylin (Sigma) to each and leaving for 5 minutes. Each slide was then immersed and rinsed in distilled water. After drying slides were mounted using DABCO and glass coverslips. Slides were visualized using an Olympus BHS microscope.

2.2.2vi. ENDOTHELIAL CELL:CD14+ CELL CULTURE

A co-culture was developed to determine the interaction between CD14+ cells and HUVEC. This consisted of HUVEC grown to 100% confluence to ensure a uniform and reproducible area in 6 well plates as described in section 2.2.1iii, then quiesced in either 0% or 0.5% serum for 12 hours.

CD14+ cells were isolated as described in section 2.2.1vii, washed three times and resuspended in 3ml M199 with either 0% or 0.5% FCS and divided into the necessary aliquots for dispensing. Cell viability was determined using Trypan Blue exclusion and counted using a haemocytometer.

CD14+ cells were added to the confluent lawn of HUVEC and incubated for either 12 or 24 hours at 37°C, 5% CO_2.

At appropriate times the experiment was stopped. Any non-adherent cells were removed by washing the plate three times with PBS. Adherent cells were then fixed using 1ml of 4% paraformaldehyde.

2.2.2vii DAPI staining for cellular apoptosis

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical investigations. When DAPI binds to DNA, its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction, but there is also evidence that DAPI binds to the minor groove, stabilized by hydrogen bonds between DAPI and acceptor groups of AT, AU and IC base pairs. The aim of these experiments (section 3.9) was to determine whether the non-viable, non-adherent cells had undergone necrosis or apoptosis. DAPI identifies nuclear fragmentation and condensation a characteristic feature of apoptosis and was therefore

used in these experiments the primary aim to determine cell non-viability and a suggestion that this was due to apoptosis. However, it would be desirable to confirm the data with other established methods of determining cellular apoptosis such as in situ labelling of DNA strands breaks (TUNEL) , Annexin V binding in combination with PI or measurement of cytochrome C release using, for example , caspase activation assays (91).

Following fixation the cellular co-culture was washed with PBS and DAPI (Sigma-Aldrich, UK) (1ug/ml) at a dilution of 1:1000 in PBS was added at room temperature. After 12 minutes DAPI was removed by washing with PBS. The morphology of cells was determined using fluorescent microscopy (Olympus series) at excitation wavelength 350Nm. Apoptotic nuclei were identified by total fragmentation of the morphology of the nuclear bodies. The percentage of apoptotic nuclei was determined

2.2.2viii FACS analysis

PBMC were harvested by density gradient centrifugation as described in section 2.2.1i. The Required number of cells were resuspended in blocking solution of 2% BSA/FCS in TBS (abdil) 100µl for one hour and washed. Cells were then incubated on ice for 20 minutes followed by one hour with 100µl of a 1:50 dilution in abdil of TEK, CD14 or CD34 monoclonal antibody. This was followed by a further two washes then a further incubation on ice in the dark with a 100µl of a 1:100 dilution in abdil of either anti-goat FITC (fluorosceine isothiocyanite) conjugated to secondary antibody for TEK or anti-mouse Cy3 conjugated to secondary antibody for CD14/34 for 15 minutes. Following a further two washes cells were resuspended in 1 ml PBS for FACS.

2.2.2.ix The matrigel model for in vitro angiogenesis

A variety of in vitro models have been established that may mimic certain cellar and biochemical events during the angiogenic cascade. Some of these models include the aortic ring model, the matrigel tube-forming assay, the fibrin and collagen gel-cord forming assays.

The matrigel and fibrin gel models were used to assess the ability of endothelial progenitor cells to incorporate into in vitro angiogenesis and form tube structures de novo.

Matrigel is a basement membrane matrix extracted from the mouse sarcoma, a tumour rich in the extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparin sulphate proteoglycans, entactin and nidogen. Matrigel is effective for the attachment and differentiation of both normal and transformed anchorage dependant cell types including endothelial cells and has been shown to provide the necessary substrate for angiogenesis. Matrigel was obtained from Becton-Dickinson. The gel was stores at -20°C and thawed at 2-8°C overnight on ice. Using pre-cooled pipettes the matrigel was mixed to homogenicity then 200ul added to cooled 24 well cell culture plates. The plates were then incubated at 37°C for 30 minutes. A homogenous mixture of calcein-AC labelled LOC (as described in section 2.2.2i) and HUVEC were then added to matrigel in specific culture conditions and incubated for various time points as described in the results chapter.

2.2.2.x Statistical evaluation.

Data are presented as means and standard error of the means. Statistical analysis (using Graphpad Prism software) was performed using one-way ANOVA unless

stated otherwise.

APPENDIX 1

ANTIBODIES

Anti-Tie 2 (C-20) and Anti- Tie 2 (C-18) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd, Wiltshire, UK)

Anti- human Tie 2 goat polyclonal was obtained from R & D Systems. Abingdon, Oxfordshire.

Anti- Phosphotyrosine 9PY200 mouse monoclonal antibody was obtained from Transduction Laboratories (Affinity Research Products Ltd, Exeter).

Secondary antibodies anti-mouse HRP, anti-rabbit HRP and anti- goat HRP was obtained from AmershamPharmacia Biotech, Litloe Chalfont, UK.

Anti- goat IgG, anti-mouse IgG, and anti- rabbit IgG, anti-vWf, anti CD31 were obtained from DAKO Ely Cambridgeshire.

Anti-CD133 antibody was obtained from Miltenyi Biotech, Bisley, Surrey.

Anti-CD 14, anti-CD34, anti-CD 144 (VE-Cadherin) and anti-KDR (VEGFR2) were obtained from Peprotech, Peprotech EC Ltd, London UK.

Anti proliferating cell nuclear antigen was obtained from Sigma-Aldrich Corporation, Dorset, UK.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF ENDOTHELIAL PROGENITOR CELLS

3.1 INTRODUCTION

Since Asahara published the first description of isolation of putative EPCs for angiogenesis (51), numerous groups have refined isolation techniques and demonstrated uptake in areas of angiogenesis *in vivo* (3, 5, 56, 57, 76, 92).

Most of these studies characterised EPCs by their expression of antigens CD34, CD133 and KDR (Flk-1) and used classical isolation techniques from umbilical cord and adult peripheral blood, namely, adhesion or magnetic microbead selection. The latter exploiting the surface antigen CD34 and/or AC133 (63, 64). However, as evidence has mounted for these cell types it has become increasingly evident that the EPCs reported comprise of a heterogeneous population with the expression of various antigenic markers and growth characteristcs (93, 94).

A key observation in defining endothelial progenitor cells was reported by Lin et al who, utilizing patients who had received sex mismatched bone marrow transplants, demonstrated by fluorescence *in situ* hybridisation evidence of a two cell populations with early and late outgrowth from cultures of mononuclear cells, with the latter thought to represent the true bone marrow derived EPC population. These late outgrowth cells demonstrated functional neovascularisation in experimental studies (53). This subset of EPCs has been termed Late Outgrowth Cells (LOC). Since this study in 2000, several groups have isolated LOC from adult peripheral blood mononuclear cells which, in contrast to short term culture of mononuclear cells, have been shown to uniformly express endothelial and reduced haematopoietic cell surface antigens (94-96). A hallmark of stem and progenitor cells is their ability to undergo extensive proliferation and give rise to a functional progeny. In these studies, LOC

demonstrate extensive proliferative potential consistent with a progenitor cell, although LOC and early outgrowth cells had comparable *in vivo* angiogenesis.

The aim of the experiments described in this chapter was to isolate endothelial progenitor cells using the prevalent methodologists reported in the literature at the time of the study. Furthermore, the studies sought to define the morphological and phenotypic characteristics of the isolated cells.

3.2 ISOLATION OF PUTATIVE ENDOTHELIAL PROGENITOR CELLS

3.2.1. EPC isolation

Following Asahara's original paper and subsequent publications (3, 5, 56, 57, 76, 92), on the isolation of putative endothelial progenitor cells from adult peripheral blood and umbilical cord blood mononuclear cells, bone marrow and foetal liver, there are now two established techniques for the isolation of these cells, namely adhesion culture and immunomagnetic bead separation. The latter exploits either the haematopoietic /endothelial stem cell markers CD34 and CD133 (63, 64).

In initial experiments, mononuclear cells were harvested from healthy adults, via anonymous pooled buffy coat from the National Blood Service, and umbilical cord blood from the immediate postpartum placenta of normal term infants as described in the 'Materials and Methods' chapter. Briefly, mononuclear cells were isolated from buffy coat and cord blood using lymphoprep density gradient centrifugation. The mononuclear cell layer was collected from the interface, gently washed three times, and cultured. On average 250ml of buffy coat, giving approximately $5x10^5$ mononuclear cells could be obtained from the NBS every 2 days compared to 20 ml of cord blood containing approximately $1x10^3$ mononuclear cells after density gradient separation. The yield of viable cells, using trypan blue exclusion, was 98% in both groups. Twenty four hours following initial plating cells were washed to remove non- and loosely-adherent cells as described in the literature (3, 50, 52)

Early lineage haematopoietic stem cells and endothelial cells share the cell surface antigen CD34 (97-99), which was exploited to isolate putative EPCs using immunomagnetic bead separation. Magnetic beads coated with the CD34 antibody (Dynal, USA) were incubated with the mononuclear cell fraction of adult peripheral blood and umbilical cord blood as described in the method chapter. $1x10^4$ cells could be isolated from 50ml of peripheral blood or approximately 2% of the whole PBMC population (counted using trypan blue exclusion). Using these established techniques and cell culture conditions described in Asahara's original work, cells were plated at a density of $1x10^3$ cells/mm^2 onto fibronectin coated tissue culture plastic.

In initial experiments, CD34+ isolated cells were labelled with the fluorescent vital dye PKH26 and cultured as described by Asahara (49). When CD34+ isolated cells were cultured alone limited initial adherence was seen, but at 24 hours, cells had detached. When co-cultured with CD34- cells in the same ratio as the original MNC population (ratio 1:50, CD34+:CD34-), the majority of fluorescent CD34+ cells were seen to attach. This suggests the culture of CD34+ cells requires the presence of CD34- cells. Over the next 24 hours clusters formed from the CD34+ cells from which spouting cells developed. After three days the cells adherent to the fibronectin matrix but separate from the clusters became detached and were removed in subsequent cell washings.

As described above following initial plating a number of cells were seen to attach and within 24 hours had begin to form clusters Figure 3.1A. These clusters were made up of spherical cells centrally and sprouting spindle shaped cells peripherally (Figure 3.1

B & C). It has been suggested that such clusters, being made up of CD34+ cells , may correspond to the blood-island described in embryonic vasculogenesis (23, 51, 100). Over the following three weeks clusters join by outgrowth of sprouting cells, with cells became more rounded in appearance(Figure 3.1D), until at 21+/-5 days cobblestone monolayers had developed which had the characteristic endothelial appearance (Figure 3.1E). The same phenomenon was noted both for MNC isolated from buffy coat and umbilical cord blood. Cords of cells were observed as early as 4 days in culture, but more commonly from day 7-10. There was great variability in the appearance of these aligned cells, from multiple thin strands of single row cells to complex cords of two or more cells (Figure 3.1 F). Cord organisation continued to increase in complexity up to three weeks when they gradually became disorganised and disappeared or became incorporated into the developing monolayers. This phenomenon has been described by others and is thought to resemble the first stages of vasculogenesis (51, 101). Importantly, when CD34- cells were grown under the same conditions none of the above phenomena were noted, with either no initial adherence or cells becoming detached from the matrix within 3 days of plating. These cells were non-viable when tested using trypan blue exclusion.

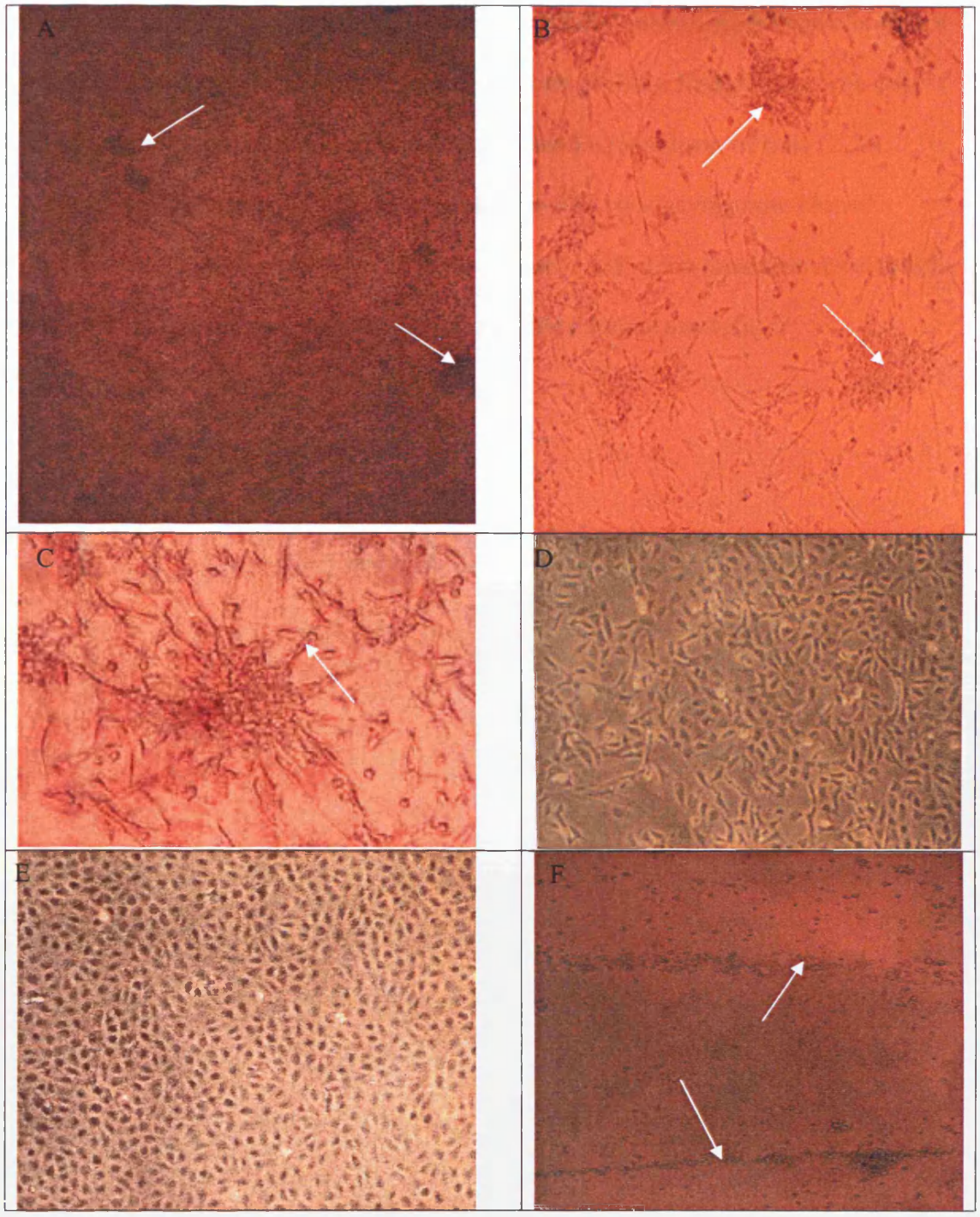


Figure 3.1. Representative pictomicrographs of human adult peripheral blood mononuclear cell cultures.

Mononuclear cells were isolated by density grade centrifugation and cultured under endothelial growth conditions **A**. Early cell clusters at 24 hours viewed at 10X magnification (white arrows) **B**. Cell clusters at 72 hours at 40X magnification

(white arrows) **C**. Cell cluster seen at 300X magnification with sprouting cells seen at the periphery of the cluster (white arrows) **D**. Same culture 10 days later with cells becoming more rounded and clusters fusing to form a monolayer of cells (200X magnification) **E**. Same culture again at 24 days with cells having now formed a typical endothelial monolayer cobblestone appearance F. Line formation developed in 60+/-5% of cultures seen here at 10X magnification (white arrows).

3.2.2 Determination of the optimum culture conditions for EPC growth.

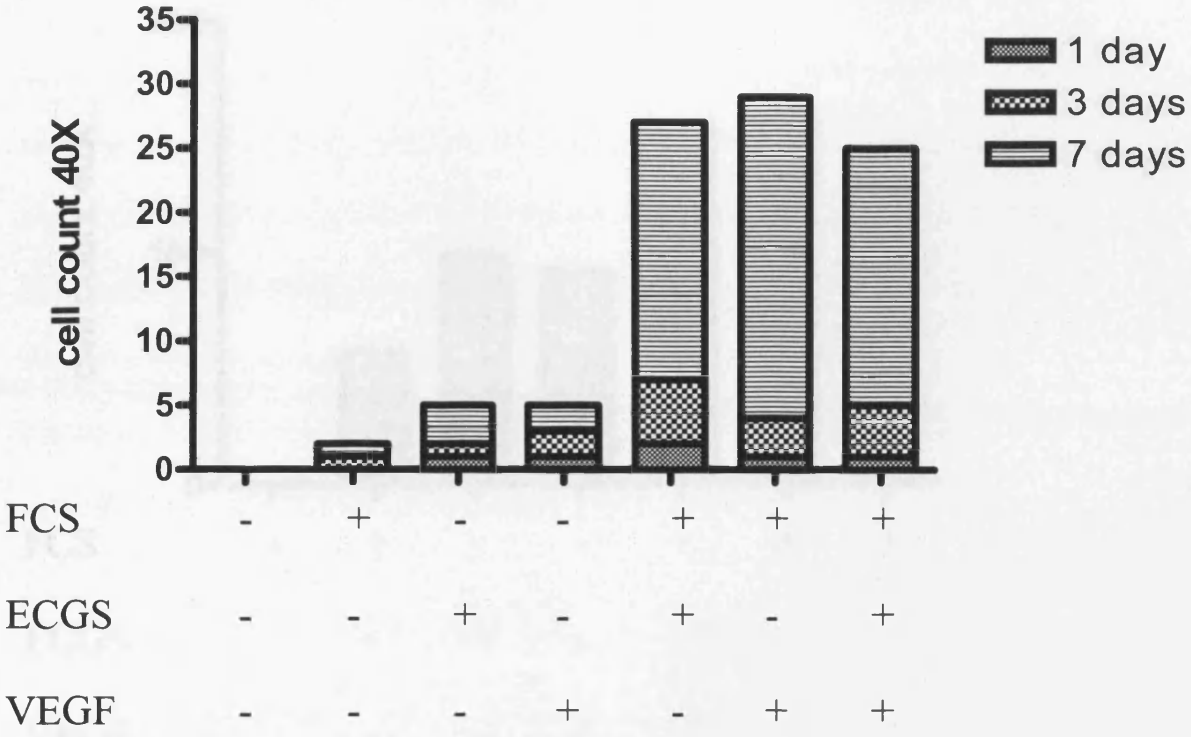
Following harvesting, MNC were plated on fibronectin and cultured in Medium 199 supplemented with 20% foetal calf serum (FCS) with or without ECGS (0.5μg/ml) in the presence or absence of VEGF (10ng/ml) at a density of $1x10^3$ cells/cm^2. The concentration of growth factors and number of cells for plating were the same as those described by Asahara and subsequent papers (51, 52, 57, 63, 64, 72). At 24 hours, non-adherent cells were removed by gentle washing and medium changed every 4 days as described by Asahara (51) .

Under all culture conditions MNCs formed clusters with limited numbers of attached cells separate from the clusters (figure 3.1A and B). Cells making up the clusters were ovoid, fusiform or elongated in morphology. At 24 hours, sprouting cells appeared around the periphery of the clusters and became spindle –shaped and grew out over the next 72 hours (figure 3.1 C). From 5 days onwards in parallel to the sprouting cells from the clusters, the separate attached cells were also noted to form linear cord-like structures (figure 3.1 E).

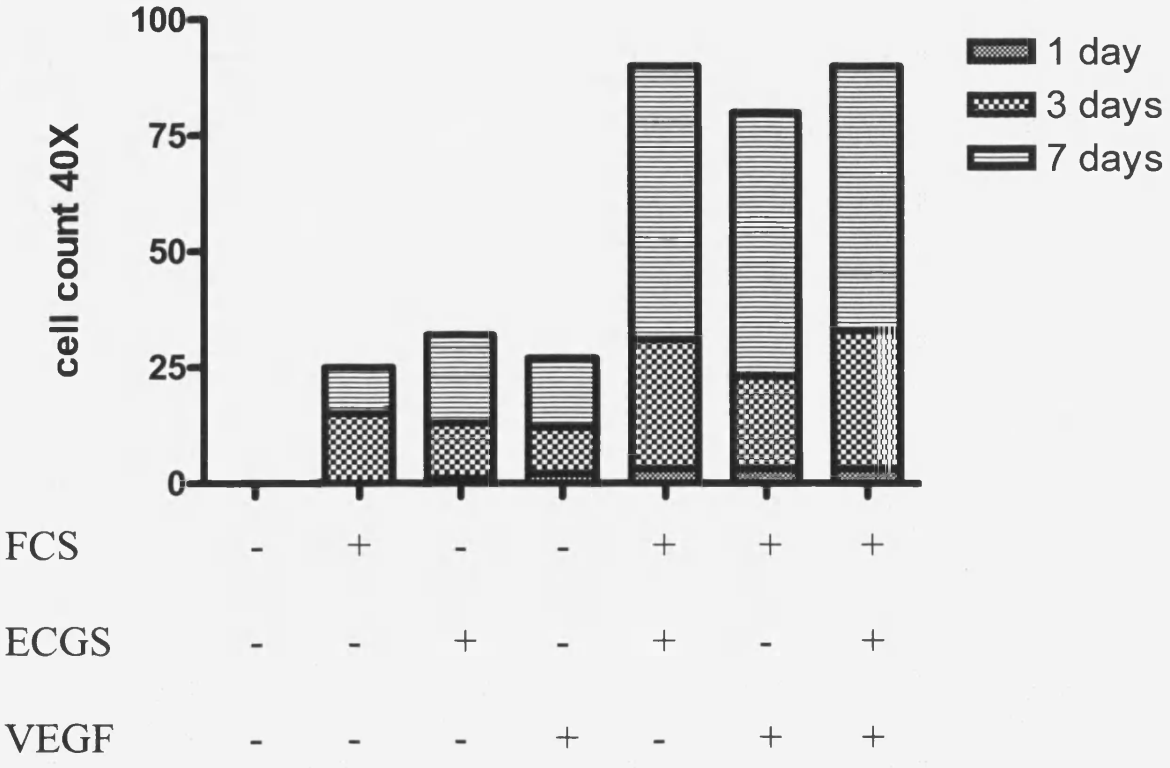
The growth of sprouting clustered and non-clustered cells were determined by cell counting at 1, 3 and 7 days. Five randomly selected high power fields (40X) were counted from cells grown in the presence of ECG, FGF and VEGF. As shown in figure 3.2 there is an increase in cell numbers in clusters, attached and sprouting cells in the presence of FCS and ECGS or FCS and VEGF ($P<0.0001$ Two Way ANOVA,) but no additive effect of all three in combination ($P>0.05$ Two Way ANOVA)for 7 day outgrowth cells. Equivalent trends were noted in umbilical cord blood samples suggesting that cord blood endothelial cell are derived from the same progenitor as adult blood endothelial cells. At the beginning of this project the first publications on

isolation of putative EPCs used only FCS and ECGS in the growth medium and, therefore, these cytokines were used in future experiments unless described otherwise. At 24 days, cells had formed a confluent monolayer with a cobblestone appearance. Clusters of cells appeared primarily between 5 and 15 days of culture. Spindle shaped/sprouting cells developed from these clusters increasingly from day 3 onwards until a cobblestone monolayer was formed from about day 24+/-5 days (figure 3.1 D and E).

A


35
30
25
20
15
10
5
0
cell count 40X
1 day
3 days
7 days
FCS - + - - + + +
ECGS - - + - + - +
VEGF - - - + - + +

B


100
75
50
25
0
cell count 40X
1 day
3 days
7 days
FCS - + - - + + +
ECGS - - + - + - +
VEGF - - - + - + +

C

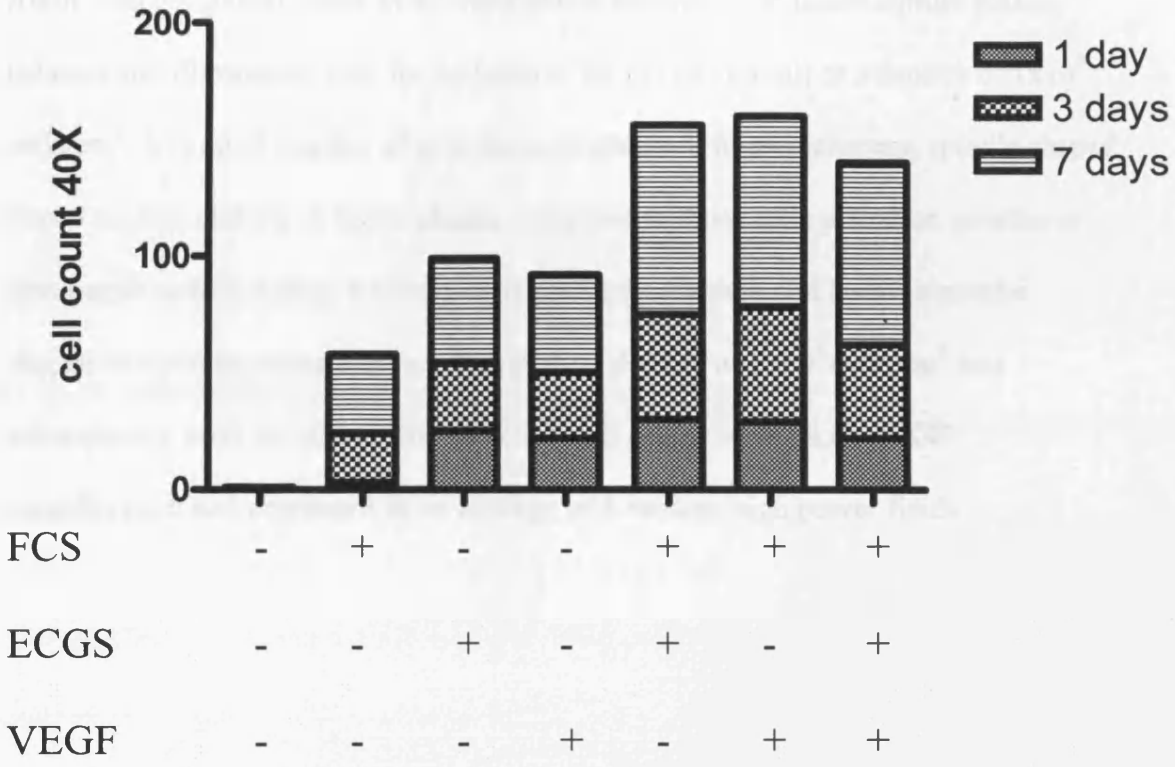


Figure 3.2. Adult peripheral blood and umbilical cord blood mononuclear cell samples grown in various media formulations.

Cells were grown as described in section 3.2.2 under conditions indicated. The number of cells in clusters (A), sprouting cells (B) and non clustered/ attached (C) were counted from 5 random high power fields at 1, 3 and 7 days under different culture conditions.. Results are presented as mean for 10 experiments.

3.2.3. To examine the effects of growth substrate

Adult total peripheral blood MNC were plated separately on tissue culture plastic, gelatine and fibronectin with the addition of ECGS (0.5μg/ml) at a density of $1x10^3$ cells/cm^2. A limited number of cells became attached, formed clusters, spindle shaped over 7 days in culture on tissue plastic compared to those cells plated on gelatine or fibronectin coated dishes, whereby cells promptly attached and became spindle shaped in equal numbers Figure 3.3. A plating density of $1x10^3$ cells/cm^2 was subsequently used for all experiments (51).Cell counts were taken at X40 magnification and expressed as an average of 5 random high power fields.

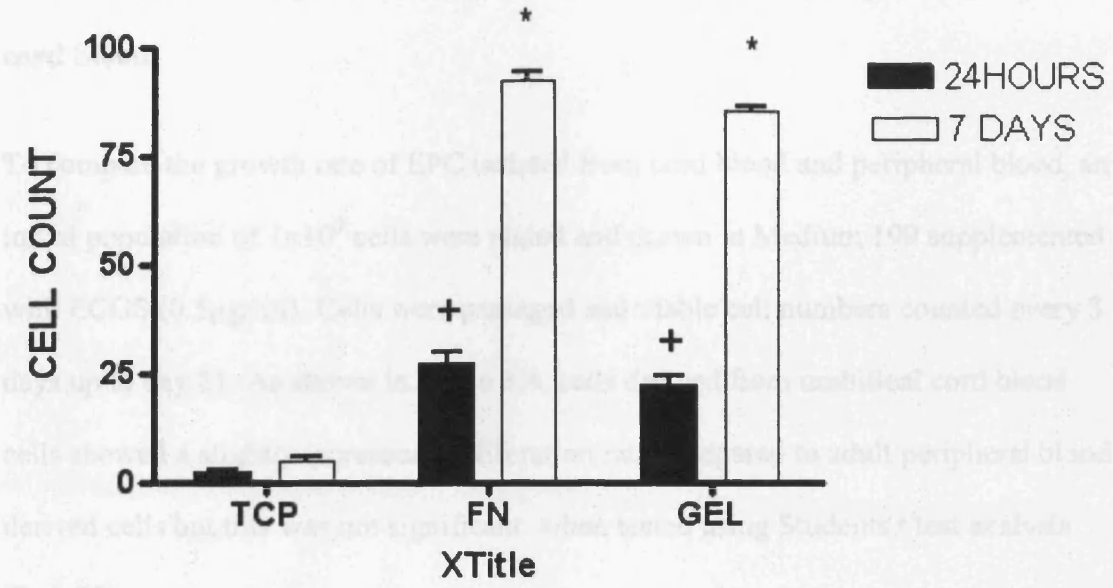


Figure 3.3. Effects of substrate on attached cell number.

The number of attached cells (5 randomly selected high power fields) 24 hours and 7 days after culture on tissue culture plastic (TCP), fibronectin (F) and gelatine (GEL). Results are presented as mean +/- SEM for 20 independent experiments + $P<0.0001$ in comparison with TCP * $P<0.0001$ for comparison between 24 hours and 7 days.

3.2.4 Comparison of growth kinetics of EPC isolated from peripheral blood and cord blood.

To compare the growth rate of EPC isolated from cord blood and peripheral blood, an initial population of $1x10^3$ cells were plated and grown in Medium 199 supplemented with ECGS (0.5μg/ml). Cells were passaged and viable cell numbers counted every 3 days up to day 21. As shown in figure 3.4, cells derived from umbilical cord blood cells showed a slightly increased proliferation rate compared to adult peripheral blood derived cells but this was not significant when tested using Students t test analysis (P=0.05).

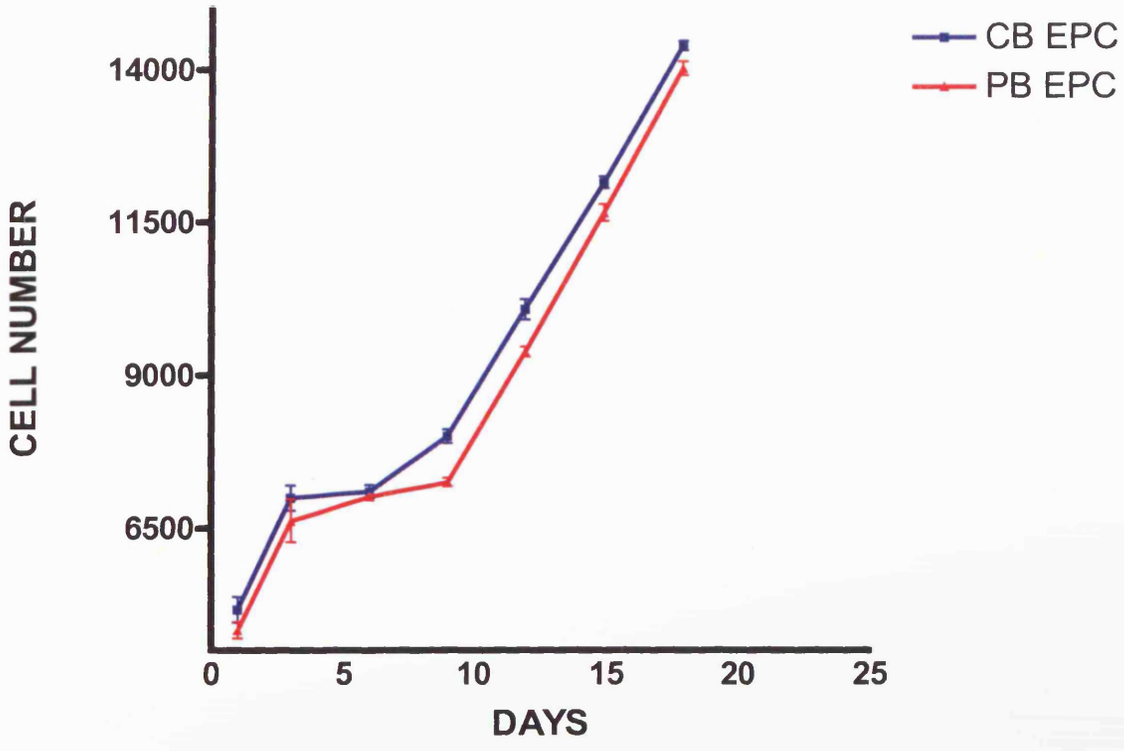


Figure 3.4. Determination of the growth kinetics of plated cells

Umbilical cord blood MNC were plated in parallel and under the same conditions as Adult peripheral blood MNC. Cells were trypsinised and viable cells counted using trypan blue exclusion on consecutive days for up to 21 days. Cell numbers are shown as means +/- SEM for 5 experiments.

3.2.5 To confirm CD34 cells contribute to cluster formation and subsequent development of spindle shaped cells.

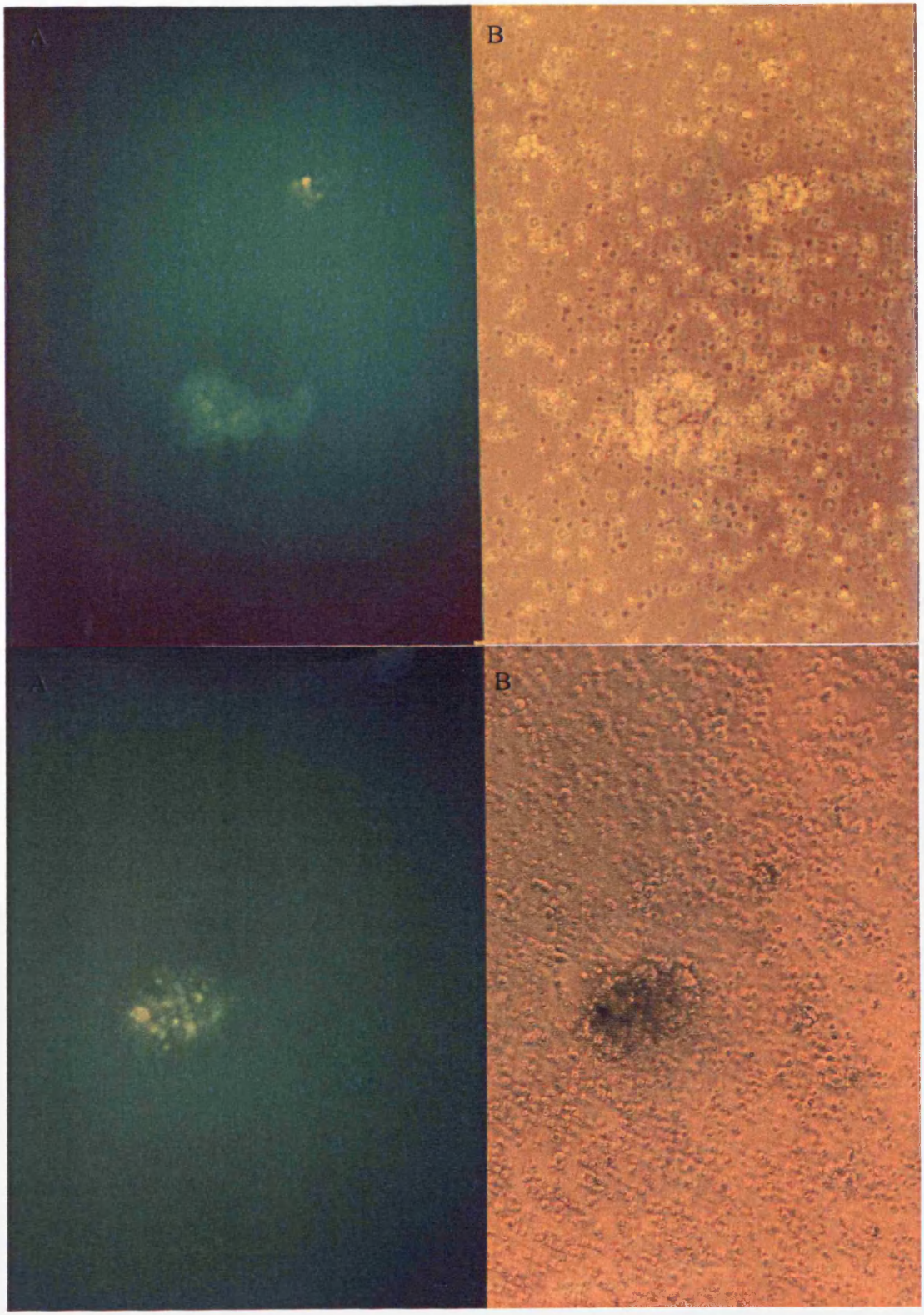
During embryonic development, the primitive vascular network develops from the blood islands which form in the primitive mesoderm in a process known as vasculogenesis(23).These blood islands consist of angioblasts at the periphery and HSCs at the centre. It was therefore suggested, the morphological characteristics and cellular organisation of cultured EPCs might resemble the structure of blood islands i.e. that the cell cluster was the post-natal representative of the blood island (50, 51) .

To investigate this Asahara et al exploited the antigen CD34, shared by haematopoietic stem cells and angioblasts during vasculogenesis. This antigen was used to separate these cells and their progeny, putative angioblasts, from the myeloid population of peripheral blood mononuclear cells (51). CD34 is expressed by all HSC/EPC but lost by haematopoietic cells as they differentiate (97, 98)

Cells were isolated from both CB and PB MNC by means of magnetic cell separation. This consists of incubating the whole MNC population with magnetic beads coated with CD34 (Dynal) as described in the materials and methods. To determine if the CD34 cells contributed to the cell clusters, or putative blood island, isolated cells were labelled with the green fluorescent vital dye PHK67(Sigma) and co-plated with the separated and unlabelled CD34- cells at a density of $1X10^3$ cells onto gelatin. As can be seen in figure 3.5 A and B, these fluorescent trace experiments show that CD34+ cells give rise to the cell clusters or putative blood islands.

In a second set of experiments, CD34+and CD34- cells were plated separately onto tissue culture plastic, fibronectin and gelatin in numbers representative of those prior to isolation. As shown in figure 3.5 C and D, when CD34+ cells, alone, were plated

limited numbers of cell clusters formed with sporadic attached cells. When CD34- cells were plated in the same manner, most cells did not attach. This is in marked contrast to co-culture of CD34+ and CD34 – with a much increased proliferation of attached cell, cluster formation and sprouting cells suggesting that the development of sprouting cells requires interactions between CD34+ and CD34- cells.


A
B
A
B

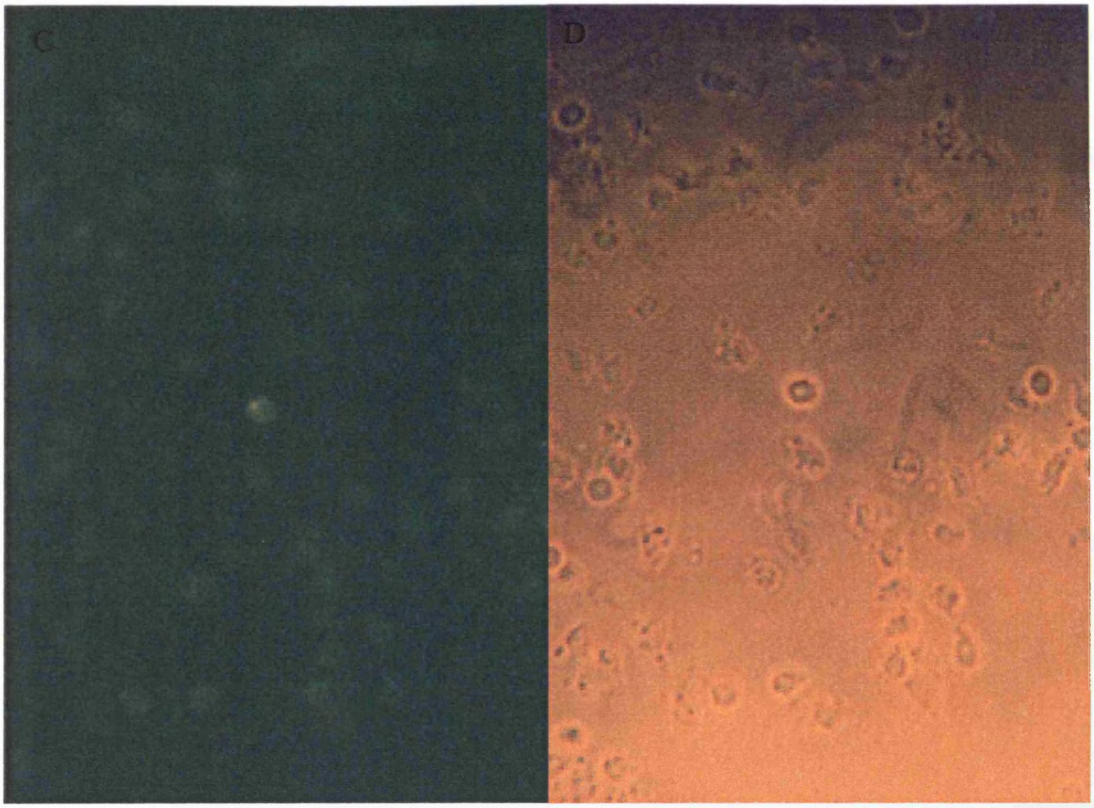


Figure 3.5. CD 34+ cells contribute to cell cluster formation.

A and B. Representative photomicrographs of isolated CD34+ cells were labelled with the fluorescent dye PHK67 and replated with unlabelled CD34- cells and cultured for 24 hours Fluorescent cells were seen to contribute to cell clusters (40X magnification). **B** is the phase image of **A**. **C and D**.CD34+cells do not form cell clusters when isolated by magnetic bead separation and plated in isolation with **D** being the phase image of **C**.

In summary, in all subsequent sections of this thesis EPC were obtained by isolation technique and cultured under the following conditions. I will be referring to freshly isolated cells as peripheral blood mononuclear cells (PBMNC), 7 day outgrowth cells as endothelial progenitor cells (EPC) as described by Asahara (51) and 21 day outgrowth cells as LOC as described by Lin et al (53).

3.3 CHARACTERISATION OF OUTGROWTH CELLS.

As described in section 3.2, cultured cells were established under the same conditions described by Asahara and others and were shown to exhibit the same growth characteristics and morphology as those described for EPC by others (7, 50-53, 57, 59-61, 63-65, 70, 72) In this section the putative EPC were characterised for expression of endothelial markers.

3.3.1 Immunophenotyping and western blotting of outgrowth cells

Freshly isolated PBMC and cells cultured, under the standard conditions described in section 3.2 for EPC, for 1, 3 and 5 days and then assayed for expression of the endothelial and monocyte markers using immunocytochemistry. Cells were probed with the monoclonal antibodies against VEGFR2 and VE Cadherin (CD144), markers of endothelial cell lineage (13, 21, 35, 102-105) as well as CD68 a marker of monocytes lineage (105). Representative photomicrographs of immunostained cells are shown in figure 3.5. In 10 independent preparations 69.2+/- 2% expressed CD68 when freshly isolated (figure 3.5 A and C) which reduced to 16+/- 2.7% (mean +/- SEM) after 3 weeks. VEGFR2 was expressed in 4 +/- 1.6% of the PBMC population which rapidly increased to 55.2+/-3.3% after 7 days in culture then to 70.8+/-3.7% and 85+/- 3.6% after 2 and 3 weeks ($P < 0.001$) culture respectively (figure 3.5 A and B). VE Cadherin was not expressed initially or at 7 days , however , after 14 days

in culture 12.4+/-1.5% of cells stained positively which increased to 25.4+/- 2 % ($P < 0.05$) after 3 weeks in culture (figure 3.5 A and D).

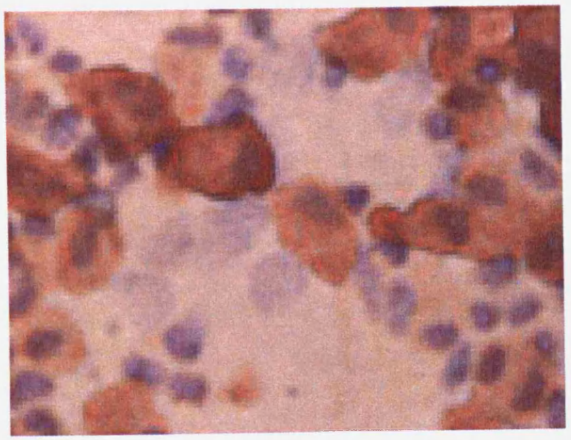
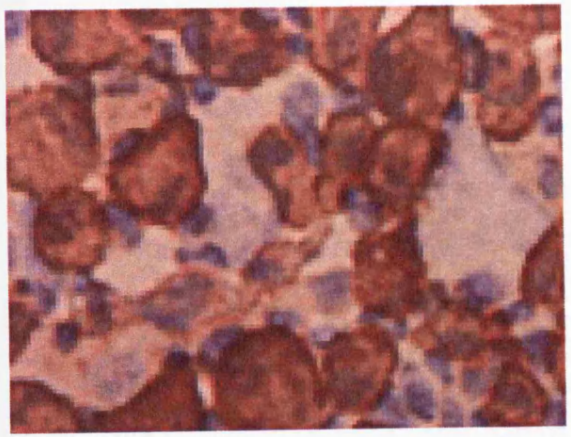
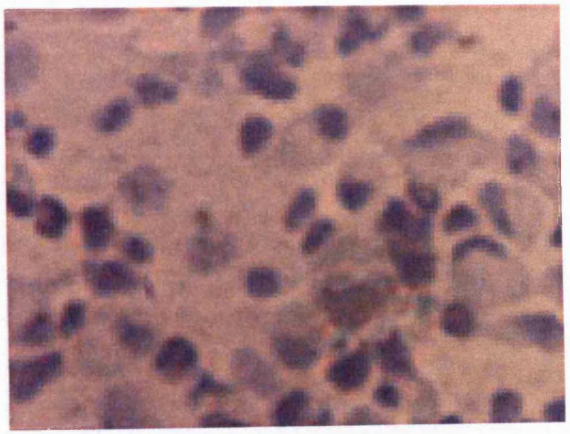
Outgrowth of cells (day)	7	21	Control
VEGFR2			

Figure 3.5A

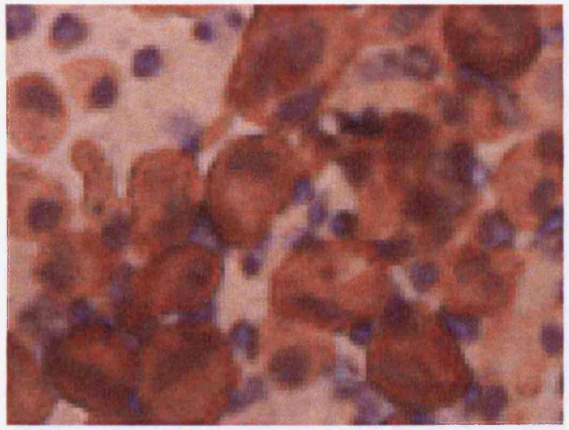
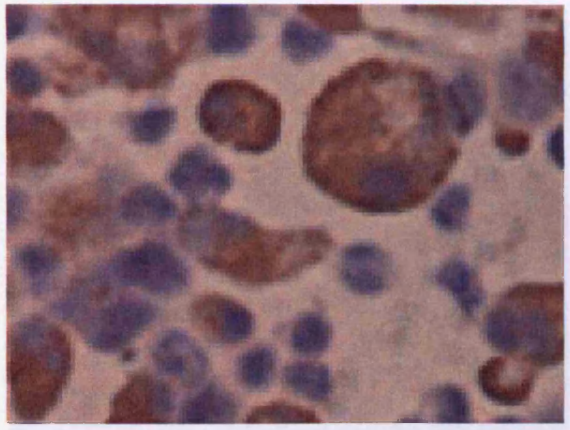
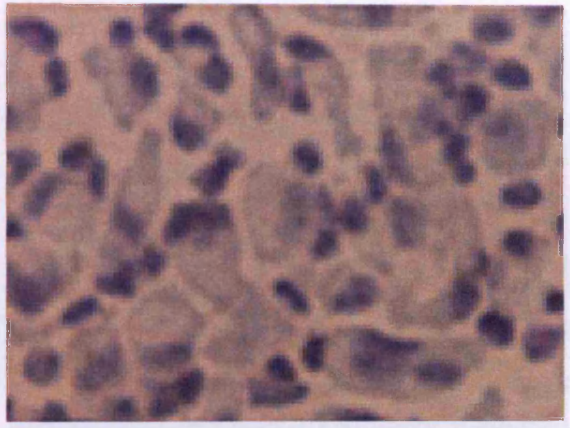
Outgrowth of cells (day)	7	21	Control
CD68			

Figure 3.5B

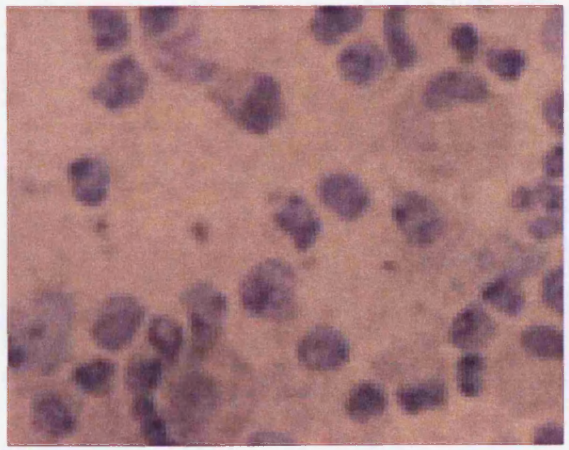
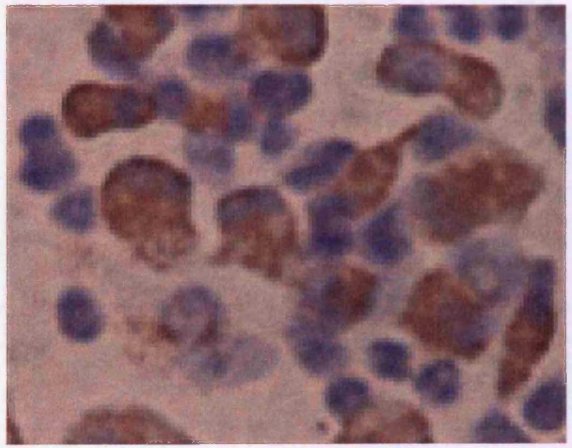
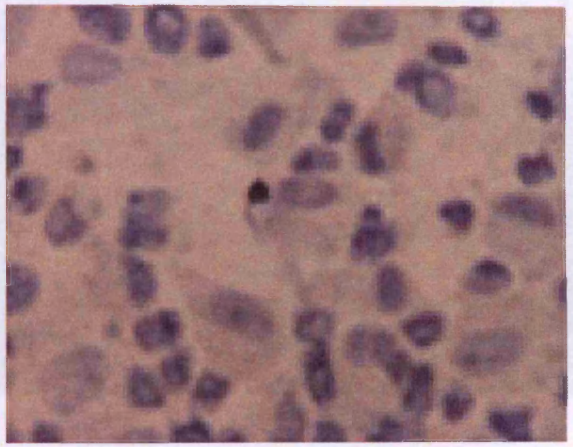
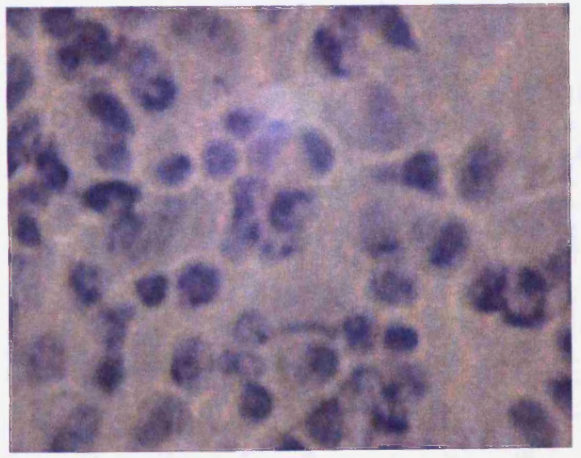
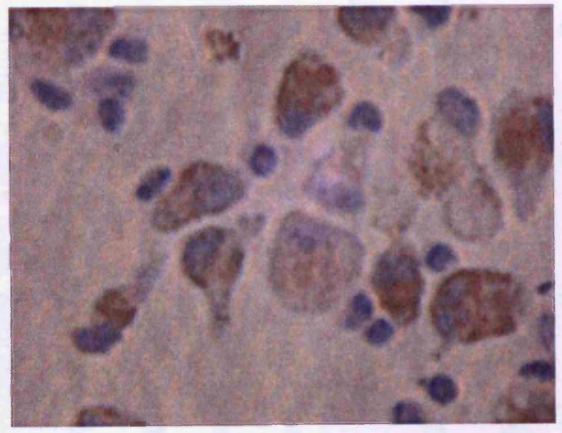
Outgrowth of cells (day)	7	21	Control
VE Cadherin			

Figure 3.5C

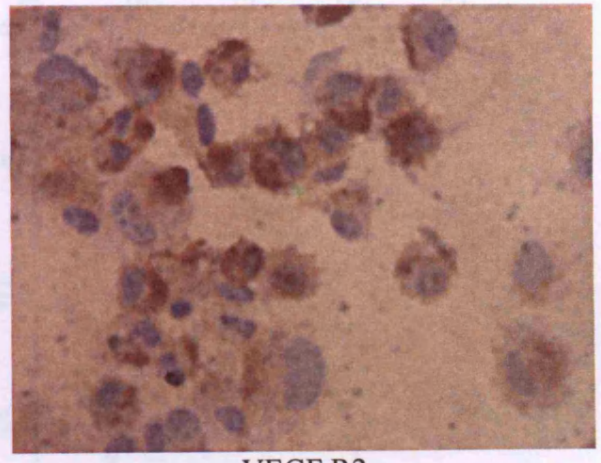
HUVEC



CD68



VE Cadherin



VEGF R2

Figure 3.5D

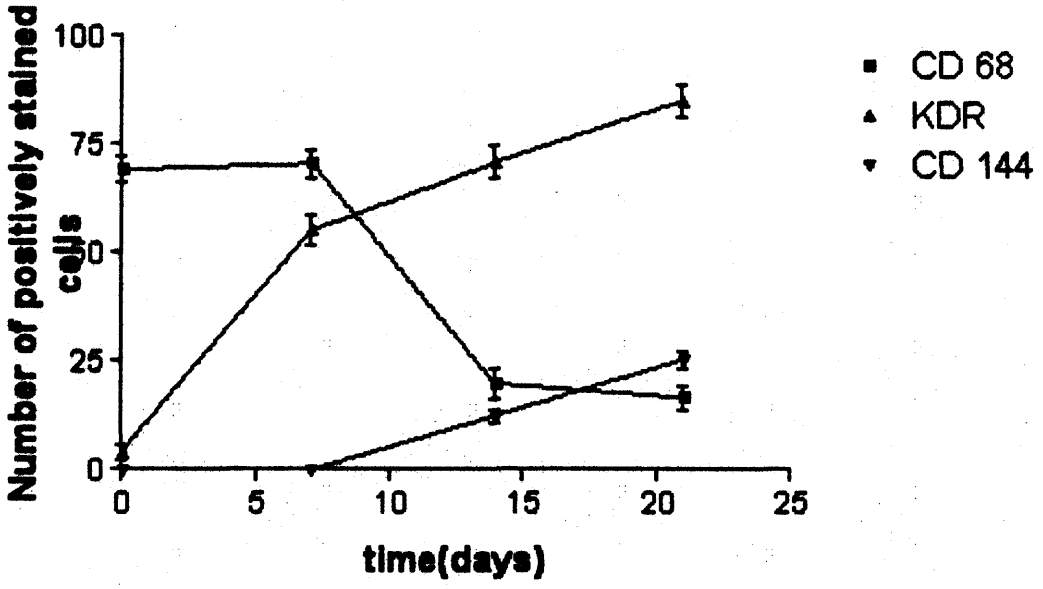


Figure 3.6. Immunophenotyping of outgrowth culture of adult peripheral blood mononuclear cells.

Indirect immunocytochemistry was performed on freshly harvested adult peripheral blood MNC and outgrowth cells at 1, 3 and 5 weeks. Figure 5.6A. Representative photomicrographs of cell staining at 7 and 21 days for VEGFR2. The control column represents cells being stained with secondary antibody alone to exclude any non-specific staining. Figure 3.6B. Representative photomicrographs of cell staining at 7 and 21 days or CD68. FIGURE 3.6C. Representative photomicrographs of cell staining at 7 and 21 days for VE Cadherin. As positive controls HUVEC were used for endothelial cell surface antigens CD68, VEGFR2 and VE Cadherin with representative photomicrographs being shown in Figure 3.6D. A monocyte cell line was used for the CD68 antigen whereby 80% of cells stained positive (results not shown). Figure 3.6.D shows graphical representation of the change in antigen expression on outgrowth cells. Counts of positive stained cells on indirect immunocytochemistry using various monoclonal antibodies were made. Ten random high power fields were selected and the average calculated. The results shown are the

average of three independent experiments. The error bars represent the standard error of the mean.

These data demonstrate a decreased percentage of monocyte marker and increase endothelial markers, VEGFR2 and VE Cad, over the three week culture period. To further characterise the phenotype of the cultured cells.

Western blotting was performed. Freshly isolated PBMC and outgrowth cells at various time points, along with HUVEC were lysed. The amount of protein in each sample assayed using the Bradford Protein Assay and samples standardised accordingly as described in the material and method chapter. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes by electrophoretic transfer. The blot was subsequently probed for various endothelial antigens and the B cell/monocytes marker CD40 (106).As can be seen in figure 3.7, the endothelial cell specific tyrosine kinas receptor Tie 2 was present in small amounts in the initial PBMC sample and expression subsequently increased over the next 5 weeks outgrowth. Interestingly the TIE receptor was absent initially and became apparent at 3 weeks in culture. Western blot confirmed the expression of KDR with immunocytochemistry. The CD40 antigen was initially present in the sample with loss of expression after 1 week.

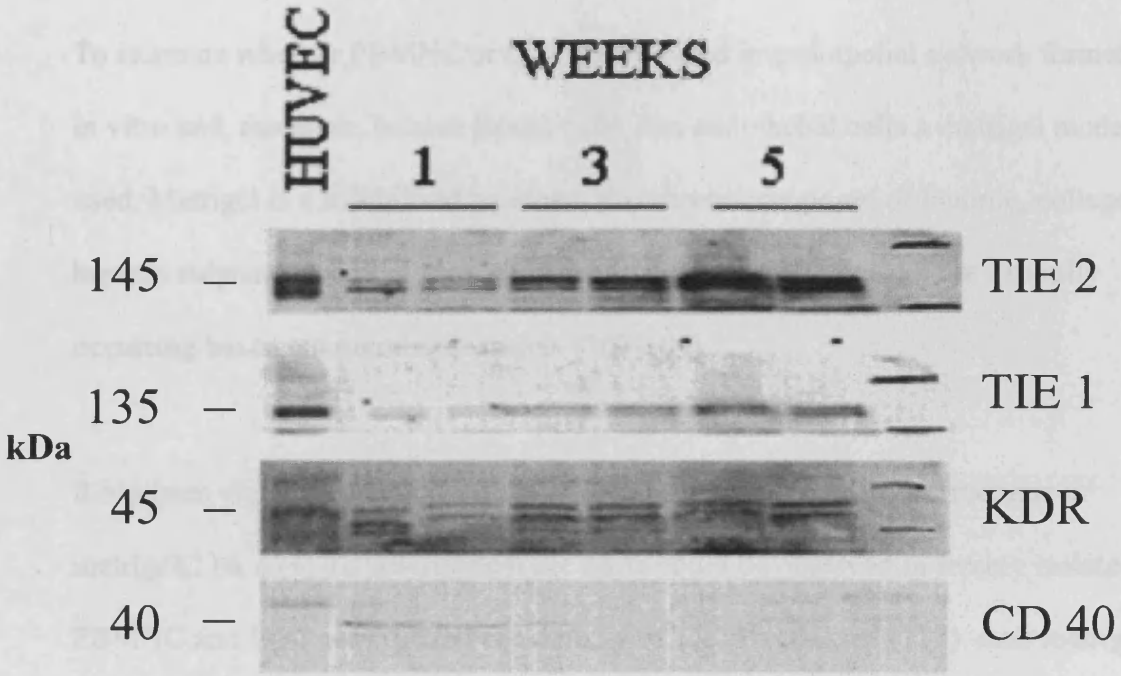


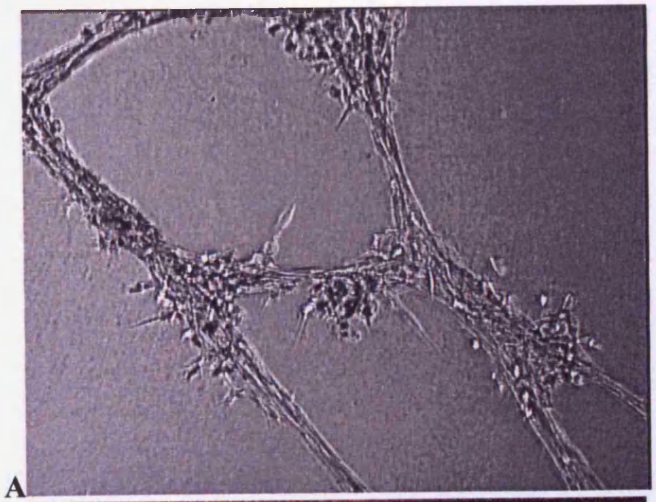
Figure 3.7 Western blot of early and late outgrowth EPCs

Western blotting was performed to characterise the protein expression of PBMNC over a three week outgrowth in angiogenic conditions. Cells were lysed at 1,3 and 5 weeks and the amount of protein standardised using Bradford Protein Assay. Protein on separate samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes and probed for various antigens. Figure 3.6 is representative of 4 preparations.

3.3.2. LOC develop cord-structures in culture

To examine whether PBMNC or LOC participated in endothelial network formation in vitro and, therefore, behave functionally like endothelial cells a matrigel model was used. Matrigel is a solubilised basement membrane composed of laminin, collagen IV, heparin sulphate, proteoglycan, enactin and nidogen and emulates the naturally occurring basement membrane matrix (107-109).

It has been well documented that endothelial cells form cord-like structures in matrigel(110, 111) To determine if the same could be observed in freshly isolated PBMNC and LOC were plated at a density of $1X10^5$ cells/cm^2 (112) onto matrigel in medium 199 supplemented with 20% FCS and ECGS. HUVEC were plated in parallel at the same concentration and under the same conditions. HUVEC formed organised cord structures after 15 hours (figure3.8) as described in the literature (50, 110, 111). PBMC adhered within 2 hours but remained disorganised whereas, LOC became more organised (figure 3.8) with cell clusters seen to connect with each other via cord-structures following 15 hours of culture. These distinct cords were evident in >70% of the samples. These networks are similar to those described by others using LOC.



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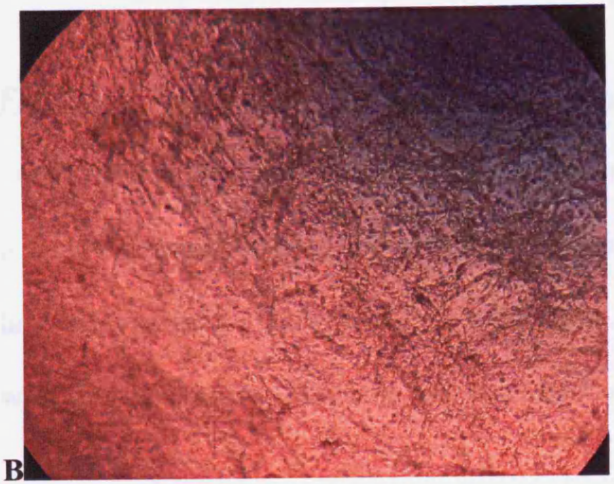

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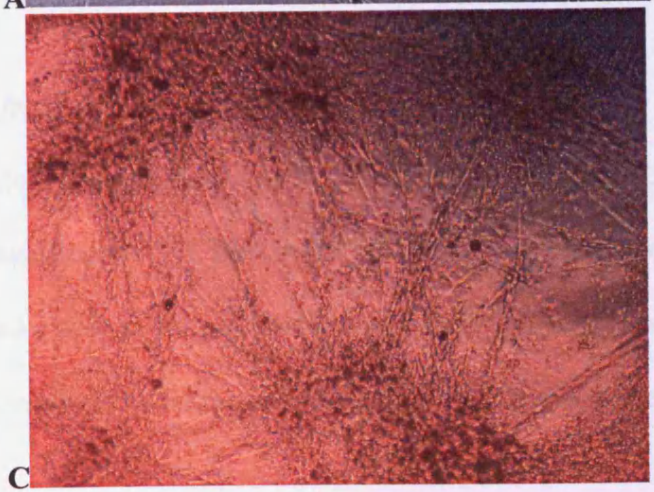

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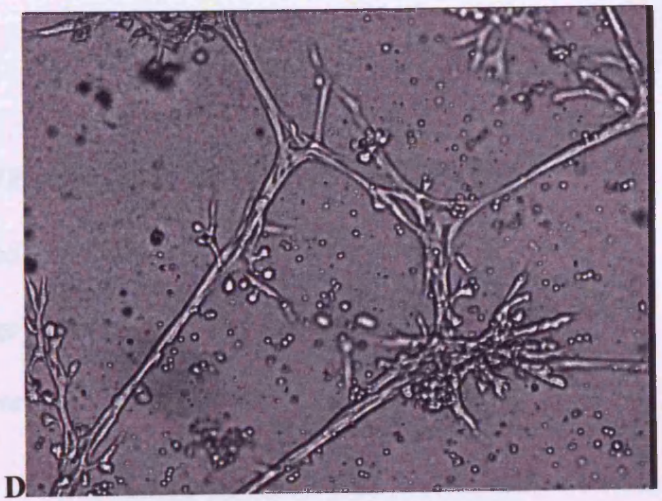

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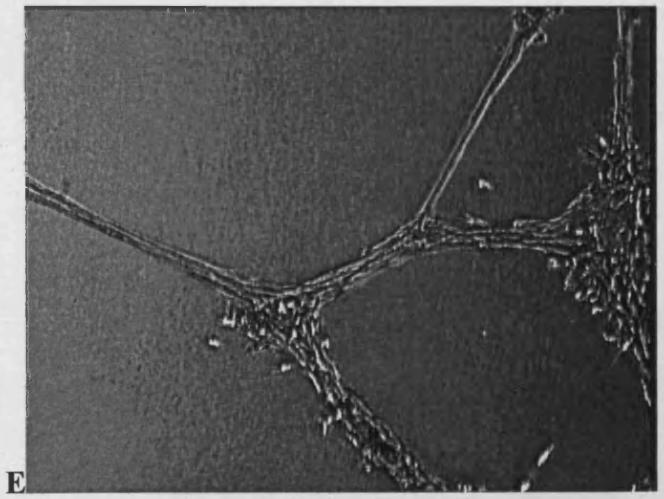


Figure 3.8. Endothelial cells and LOC form cord like structures in a matrigel model.

Freshly isolated PBMNC and LOC were plated onto a matrigel model and cultured in parallel with HUVE cells. At 15 hours the HUVE cells had formed characteristic cord like structures 400X magnification (**A**). In contrast LOC had not yet developed the characteristic cord like structures, however, unlike the HUVEC culture LOC remained viable in culture. LOC culture at day 10 phase image 10X (**B**), 40X (**C**) and 400X (**D**) at which stage characteristic cords had developed connecting the initial cell clusters. (**E**) 400X magnification of LOC at 14 days.

3.3.3. LOC adhere and incorporate into an endothelial ell monolayer

In order to contribute to angiogenesis LOC would have to adhere to and incorporate into an endothelium undergoing active angiogenesis. To investigate this, freshly isolated PBMNC were plated onto sub confluent layers of HUVEC, having previously determined the viability of isolated cells using trypan blue exclusion. After 12 hours in culture it was observed HUVEC had changed morphology and were dissociating from the basement membrane. The same phenomenon was noted when PBMNC were plated with Bovine Aortic Endothelial Cells (BAEC). In contrast, however, when LOC were plated onto sub confluent monolayers of HUVEC and BAEC they incorporated into the layer without any obvious cell death as shown in figure 3.9.

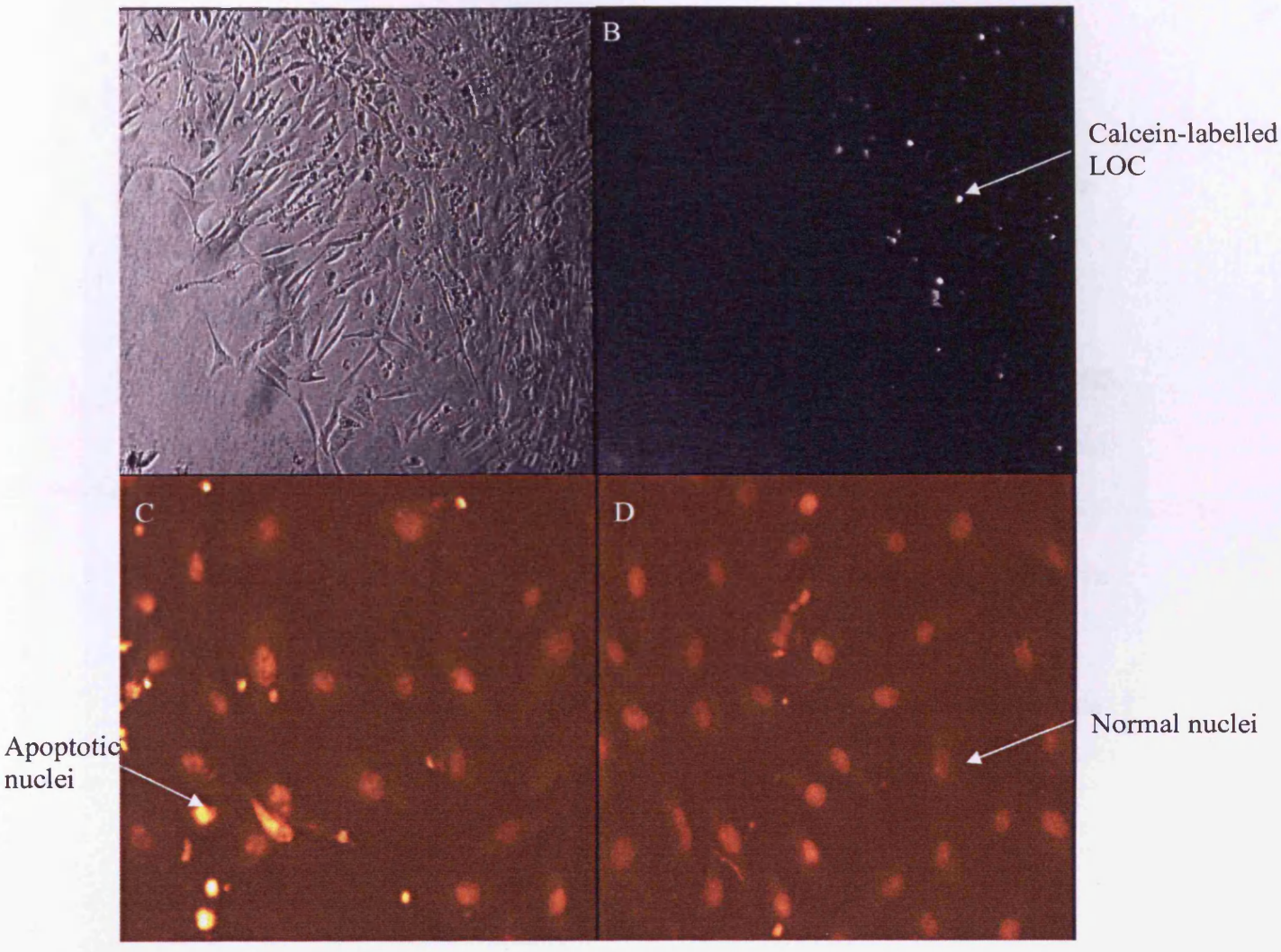


Figure 3.9. LOC adhere to HUVEC

A. Calcein labelled LOC were plated onto a HUVEC monolayer. After 6 hours non-adherent cells were removed by serial washings. LOC were seen to incorporate into the endothelial cell layer in preference to the gelatine/fibronectin substrate **B**. Photomicrographs were taken at 40X magnification. **C** PBMNC and LOC were plated directly onto a 80% subconfluent monolayer of HUVEC after cell viability was confirmed using trypan blue exclusion. Within 12 hours HUVEC when plated with PBMNC were seen to undergo apoptosis as determined by DAPI staining. **D** In comparison co-culture of HUVEC with LOC no HUVEC apoptosis seen. Photomicrographs were taken at 80X magnification.

3.3.4 LOC contributes to endothelial cord formation in culture.

Terminally differentiated endothelial cells have the ability to form cord structures in culture (107-109, 112). To determine whether LOC could contribute to endothelial network formation, calcein labelled LOC were co-plated alone or with unlabelled HUVEC on matrigel in medium 199 supplemented with 20% FCS and 5mg/ml ECGS. As seen in figure 3.9A HUVEC form cords independent of any LOC activity. Within 2 hours, fluorescent labelled LOC were contacting HUVEC and beginning to incorporate into the cords (Figure 3.10 B and C). Further cells were incorporated in a time dependant manner up to 6 hours (Figure 3.10 D-H).

These results suggest that outgrowth cells actively form endothelial like networks de novo but also incorporate into networks when they are co-cultured with mature endothelial cells on matrigel in culture.

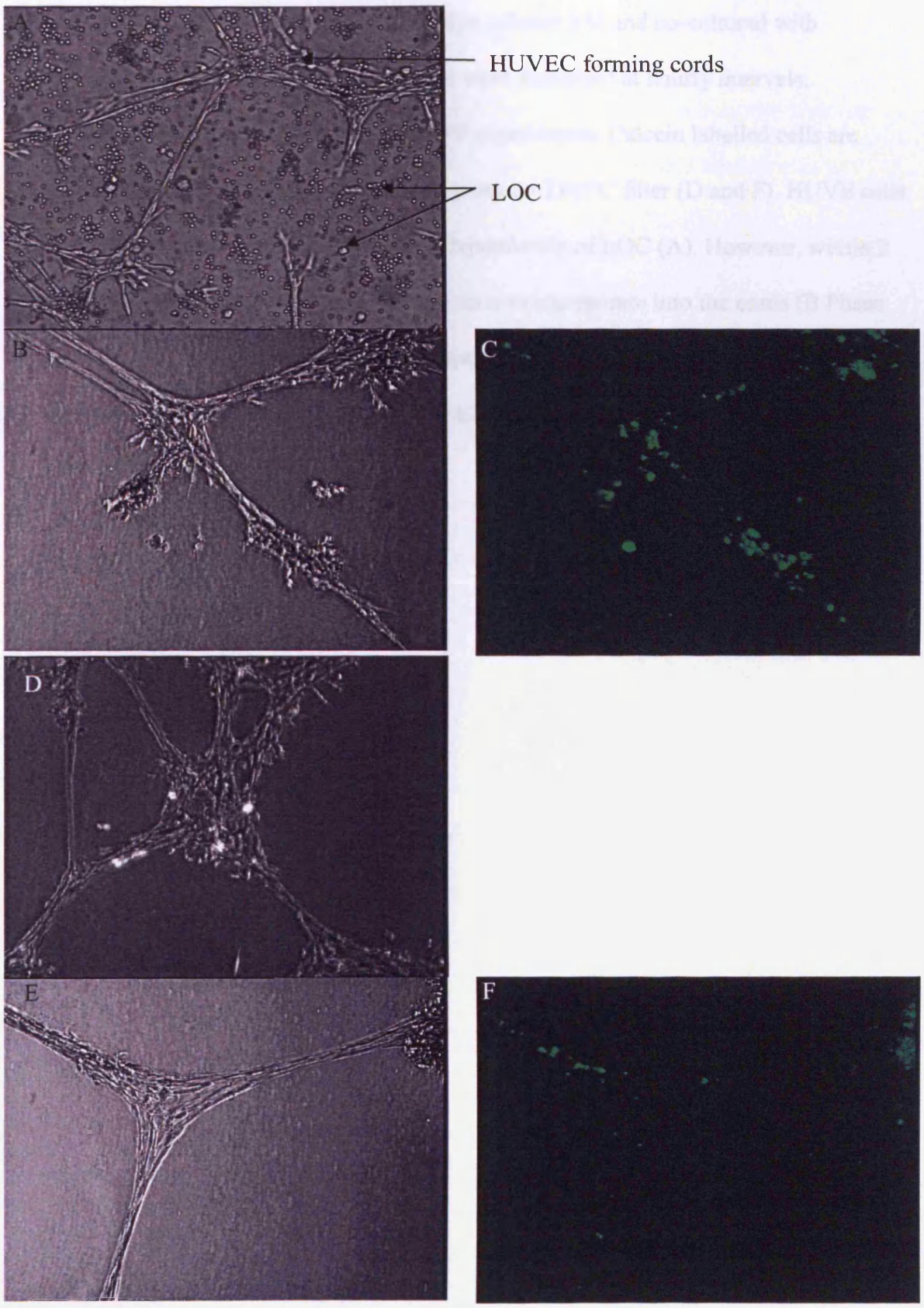


Figure 3.10. LOC incorporate into endothelial cord structures.

LOC were labelled with the fluorescent dye calcein-AM and co-cultured with unlabelled HUVEC on matrigel. Cultures were examined at hourly intervals.. Photomicrographs are representative of 10 experiments. Calcein labelled cells are bright cells on phase images A and D or green on TRITC filter (D and F). HUVE cells were seen to form cord like structures independently of LOC (A). However, within 2 hours of the cords developing LOC were seen to incorporate into the cords (B Phase image at 200X magnification with corresponding TRITC filter image C). LOC incorporation continues to 4 hours (D) until at 6 hours all cells are incorporated into the HUVE cell cords (E and F).

3.4. DISCUSSION

The purpose of this chapter was to reproduce the isolation and culture methods for endothelial progenitor cells available at the time of the study and characterise the phenotype of the cultured cells (7, 50-53, 57, 59-61, 63-65, 70, 72).

Asahara et al (51), provided the first evidence of circulating endothelial progenitor cells in the adult. These cells could contribute to post-natal neovascularisation and suggest the possibility of a vasculogenic–like process in adults. The paradigm of vasculogenesis in the embryo begins with cluster, or blood island, formation in the embryonic mesoderm which consists of haematopoietic stem cells in the centre and angioblasts at the periphery (23, 100, 113). These two cell types are thought to be derived from a common precursor (114) and share antigenic determinants including CD34 and the TIE receptors (15, 55, 115). CD34 is a transmembrane glycoprotein constitutively expressed on endothelial and haematopoietic progenitor cells (116, 117). It has recently been discovered, adult blood stem cells and the bone marrow are in dynamic equilibrium with each other thus allowing migration of cells between the two (54). Using this information Asahara et al (51) exploited the CD34 antigen to identify and isolate a cell population that included endothelial from adult peripheral blood.

Results presented in this chapter confirm that CD34 + cells, derived from the adult peripheral blood and umbilical cord blood, formed cell clusters, endothelial cords and outgrowth cells and displayed the morphological characteristics of endothelial progenitor cells as described by Asahara et al (3, 51, 57, 62, 89, 96, 118). CD34 + cells, isolated by immunoseparation techniques, when cultured alone failed to proliferate. However, when co-plated with CD34 - cells, they proliferated and

exhibited the morphological characteristics described above. Furthermore, it was shown that within this co-culture the CD34+ cells contributed to the cluster formation as described in Asahara's original work. This suggests cross talk between CD34+ and CD34- cells is necessary to promote cell growth, most probably by growth factor or extracellular matrix component being produced by cells that are removed during the isolation procedure.

These results show, there was no difference in characteristics and proliferation between adult peripheral blood mononuclear cells derived from buffy coat and umbilical cord blood cells the former was used in all subsequent chapter investigations due to availability and ease of acquisition.

To date, there still remains no uniform definition of an EPC and therefore, they remain primarily defined by their expression of cell surface antigens (119). Cells with a combination of Flk-1 /KDR/VE Cadherin/CD31/Tie-1 and Tie-2 surface antigens have been interpreted as cells in intermediate stages during differentiation of embryonic stem cell derived endothelial cells (58, 114). Results detailed in this chapter, showed that adherent cells derived from peripheral blood and umbilical cord blood MNC formed colonies containing cells expressing CD34, VEGFR2 and Tie1 and 2 and reduction in the expression of the monocytic lineage markers CD40 and CD68.

In addition the cells expressed the endothelial marker VE Cadherin and formed homogenous monolayers and cord structures in three dimensional matrices. VE Cadherin was expressed only after 14 days in culture when a more homogenous layer of cells were forming (i.e.>70% confluence). This would be consistent with the suggestion of Nishikawa's (120) that the co-expression of VE Cadherin and VEGFR2

denotes the point of divergence of endothelial cells from haematopoietic lineages. In parallel the expression of CD40 and CD68 decreased considerably at this time. Furthermore Tie1 expression was apparent after only three weeks in culture. It is known that VEGFR2 is functionally important during vasculogenesis (21).Consistent with the hypothesis that these cells are progenitor in nature, immunocytochemistry and western blotting revealed relatively high expression of VEGFR in freshly isolated cells and early outgrowth colonies. This progression to an endothelial phenotype with increasing endothelial cell marker expression, strongly suggests an endothelial precursor and that these cultures are not made up of proliferating mature endothelial cells.

It has been shown in functional studies that mature EC can undergo matrix dependant morphogenic changes into capillary structures (107-109, 112). In the present study LOC formed cord like structures in matrigel three weeks after seeding. possibly mimicking formation of a primordial network in the embryo (23, 100).Furthermore, this suggests that LOC possess endothelial- like properties in being able to form networks similar to endothelial cells.

In this study it was seen co-plating early outgrowth cells with mature endothelial cell lead to endothelial cell death, whereas in comparison co-plating with LOC resulted in adherence to and incorporation into mature endothelial cell monolayers and cord structures. This would suggest the development of cells at three weeks have undergone changes that lead to a different functional behaviour or, alternatively, have

arisen from a different cell type. It is noteworthy that Lin et al (53) using gender mismatched bone marrow transplant models, found that when cells were harvested and cultured in the same manner as described in the present study, the early outgrowth cells consisted of recipient genotype whilst the late outgrowth cells consisted of donor genotype the latter displaying a 1000 fold expansion. Based on these results it is logical to speculate that late outgrowth cells have a large ability to proliferate and act as precursor cells in this lineage.

Therefore, the LOC isolated and cultured in the present study are likely to be the same type as that described by Lin et al and these cells are considered as angioblast-like endothelial cells. These findings have recently been confirmed by Gulati et al (95) who explored the lineage relationships between early EPCs and LOC in culture. He concluded that EPCs were derived primarily from a CD14+ sub fraction of PBMNC and late outgrowth cells were derived exclusively from the CD14- fraction. It would be interesting to examine CD14 status of the LOC cells in the present study. This will be addressed in chapter 5. Furthermore, LOC exhibited markedly greater capacity for capillary morphogenesis in vitro and in vivo matrigel models entirely consistent with the findings in this study.

In summary, work detailed in this chapter demonstrates putative endothelial progenitor cells isolated from adult peripheral blood mononuclear cells and, when cultured, are the same as those described in the literature in terms of morphology, phenotype and functional behaviour. Importantly, in combination with the findings of Lin and Hristov (53, 121), the results presented here suggest that that LOC represent the true endothelial progenitor cell that displays marked capillary morphogenesis in vitro, are phenotypically more endothelial and allow co-culture with HUVEC and

functionally contribute to new vessel formation. Hence, three week LOC will be used in all future experiments described in this thesis unless stated otherwise.

CHAPTER 4

INVESTIGATION INTO THE HOMING MECHANISMS OF LATE OUTGROWTH ENDOTHELIAL PROGENITOR CELLS

4.1 INTRODUCTION

In vivo studies have shown late outgrowth endothelial progenitor cells (LOC) incorporate into sites of angiogenesis (3, 53, 57, 62, 94), increasing blood flow at areas of ischaemia (see chapter 1). Although these reports indicate a therapeutic role for LOC in ischaemic disease, it is clear that LOC need to home, i.e. recruitment and incorporation of EPCs, to foci of neovascularisation. At the time of writing, however, there is little understanding as to the mechanisms of LOC incorporation into sites of angiogenesis.

Incorporation of cells into specific sites requires two processes (i) the recruitment of cells from the circulation and (ii) the adherence of cells at the site (69). Both of these processes would be necessary in the homing of LOC to angiogenic vessels. Once 'homed', in order to contribute to new blood cells formation cells would have to differentiate into functional cells with endothelial-like properties as shown in Figure 4.1.

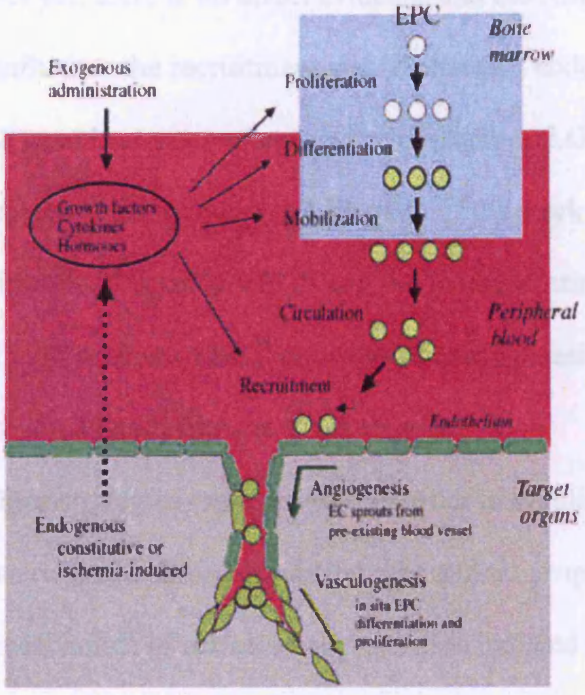


Figure 4.1 Schematic representation of endothelial progenitor cell homing

Incorporation of LOC at sites of angiogenesis involves both the recruitment of cells from bone marrow and hence the circulation and then the adherence of cells at the sites of active angiogenesis. Adapted from Sata,M Inflammation, Angiogenesis and Endothelial Progenitor Cells (67)

Cytokines are present within the circulation and at high concentrations at sites of angiogenesis (9, 13, 25, 41, 47, 72, 115). In particular VEGF and the angiopoietins are cytokines that play important roles in the process of angiogenesis (47) being present at high concentrations in *in vivo* models of myocardial ischaemia, peripheral ischaemia and tumours.(26) These observations suggest that one of the functions of these cytokines may be to act as chemoattractant to LOC and regulate the interaction between recruited LOC and angiogenically active endothelial cells (72).

As yet, there is no direct evidence that the Angiopoietins or VEGF can directly influence the recruitment and adhesion of endothelial progenitor cells. VEGF and Ang 2 would act as agonists via their receptors. LOC have been shown to express these receptors in section 3.3.1.The aim of the work in this chapter is to determine the factors , primarily VEGF and the Anpiopoietins, controlling the recruitment and incorporation of EPC into sites of angiogenesis and examine some of the mechanisms that may contribute to these effects.

Two strategies were adopted in order to do this. Firstly, Boyden chamber experiments were established to study the migrational properties of LOC and the cellular mechanism of action of any cytokine induced migration. Secondly, an adhesion assay was devised to determine whether VEGF and the Angiopoietins could directly influence the adhesion between LOC and endothelial cells, which then provided a platform to investigate the mechanism of this cellular interaction and the factors which augment this adhesion.

The experiments in this chapter are divided into two areas, the former concentrating on the migrational characteristics of LOC, the latter describing the experiments performed to attempt to define adhesional characteristics.

4.2. FACTORS CONTROLLING THE MIGRATION OF LATE OUTGROWTH ENDOTHELIAL PROGENITOR CELLS.

The purpose of this part of the study was to determine whether VEGF or the angiopoietins affect the migratory properties of endothelial progenitor cells.

A modified Boyden chamber was used to measure migration toward chemoattractants. Figure 4.2 illustrates the 24 well Boyden chamber used during these experiments. LOC were added to the top chamber and allowed to migrate through 8 uM pores to the lower chamber. The chemoattractant properties of VEGF and the Angiopoietins were tested by adding to the lower chamber. LOC present on the membrane were counted at termination of the experiments at 6 hours as shown in figure 4.3.

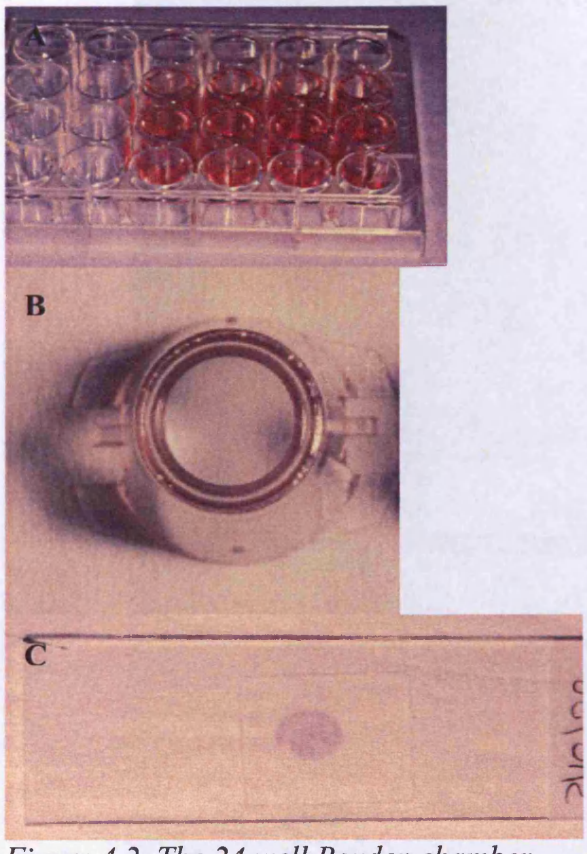


Figure 4.2. The 24 well Boyden chamber

Migration was measured using a modified 24 well Boyden chamber with cell culture inserts containing 8μM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added either, 750μl migration medium, 750μl migration medium containing 10ng/ml VEGF, 400ng/ml Ang 1 or 200ng/ml Ang 2. To the upper chamber was added 2x 10^5 LOC or endothelial cells in 250μl of migration medium. B. The cell culture insert at the base of the upper chamber was fixed in 70% ethanol at -20°C for 1 hour then carefully removed and the lower part of the membrane was stained using haematoxylin and eosin before mounting on slides. C. Migratory LOC where counted at 40X magnification.

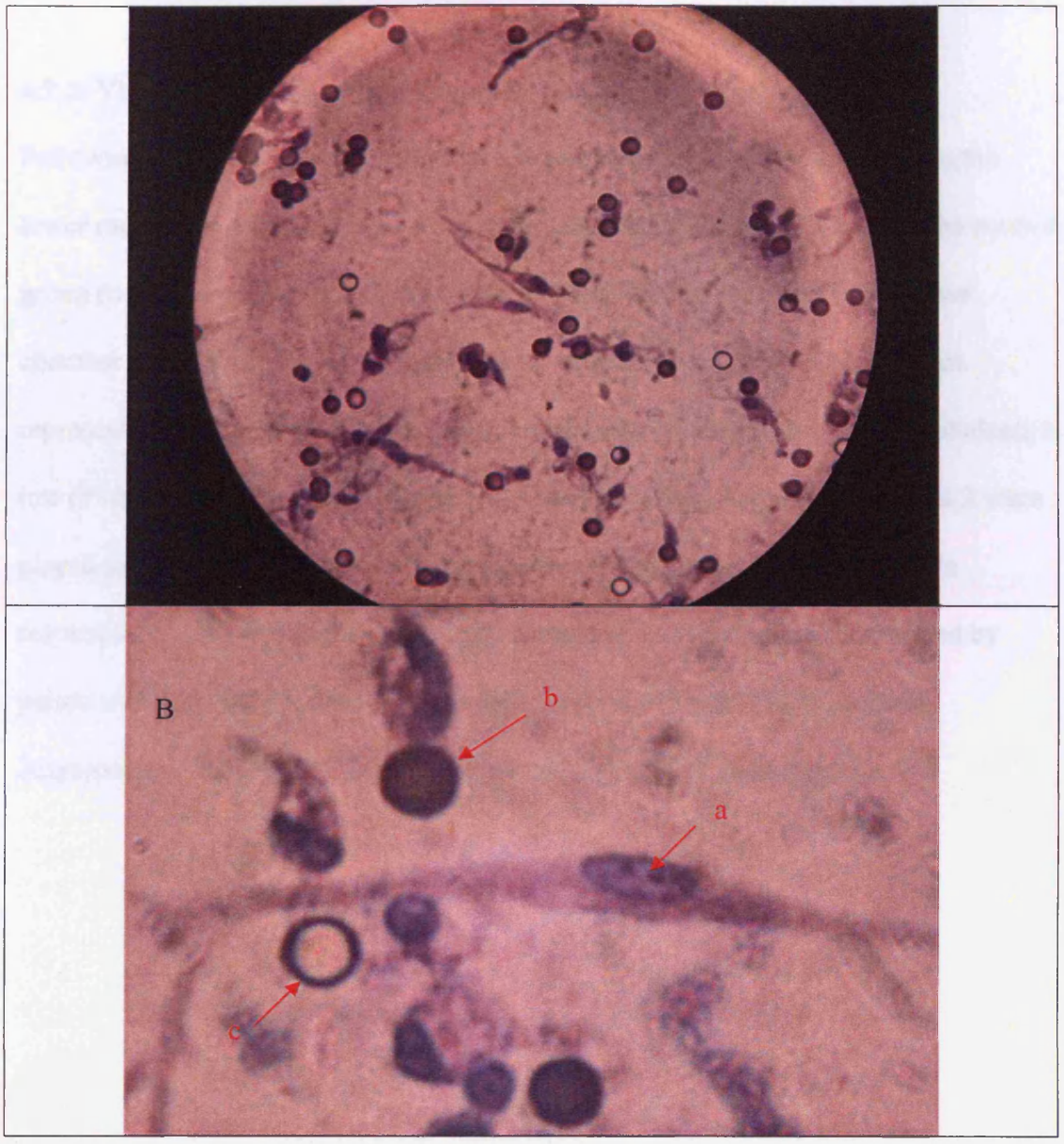


Figure 4.3 Representative view of migrating cells after H & E staining on transwell filter.

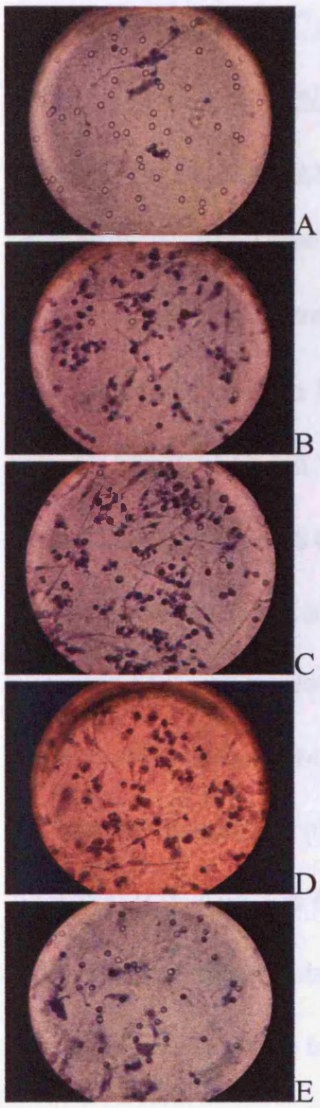
Following fixing, staining and mounting the migrated cells on the undersurface of the transwell filter were counted using an average from ten fields of view.

Photomicrographs of representative filters at 10X magnification (**A**).

Photomicrograph at 100X magnification to show only cells with complete nuclei were included (**B**). Migrated cell with complete nucleus (a). Pore with cell present within (b). Empty pore (c).

4.2.1. VEGF and the Angiopoietins stimulate LOC migration

Following 6 hours of migration through the transwell filter the mean count on the lower membrane, representing the migrated cells, had reached 1.12 cells in the control group (no chemoattractant). In comparison, when VEGF is present in the lower chamber the mean count on the lower membrane had reached 10.15 cells. This represented a highly significant increase in migration determined by paired Students t-test ($P<0.001$, $n=10$). Similar effects were observed when Angiopoietin 1 and 2 were placed in the lower chamber, with increase in migration of 16.3 and 19.8 cells respectively. This was highly significant compared with control as determined by paired t-test ($P<0.0001$, $n=10$). These data demonstrate that VEGF and the Angiopoietins induce LOC migration. The results are shown in figure 4.4.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B
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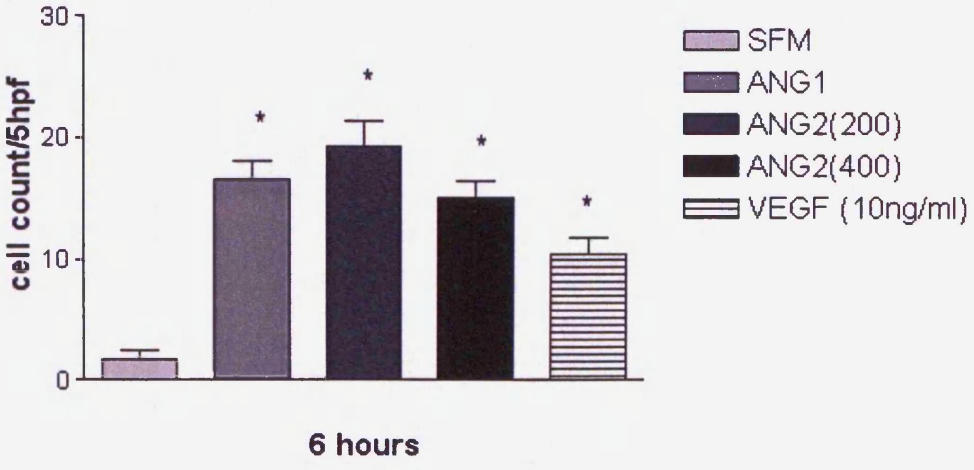

30
20
10
0
cell count/5hpf
*
*
*
*
SFM
ANG1
ANG2(200)
ANG2(400)
VEGF (10ng/ml)
6 hours

Figure 4.4. Shows LOC chemotaxis through uncoated inserts towards Ang 1, Ang 2 at 200ng/ml and 400ng/ml, VEGF AT 10ng/ml and also towards no chemoattractant (original magnification x40).

Migration was measured as described above. To the lower chamber was added either, 750µl migration medium, 750µl migration medium containing 10ng/ml VEGF or 400ng/ml Angiopoietin 1or 200/400ng/ml Angiopoietin 2. To the upper chamber was added $0.75x10^5$ LOC in 500µl of migration medium. After 6 hours, the cell culture insert was fixed in 70% ethanol at -20°C for 1 hour and then the lower part of the membrane was stained using haematoxylin and eosin before mounting on slides. Migrated LOC where counted at x40 magnification only cells that complete nuclei were counted and an average taken from ten fields of view used. A

E show representative photomicrographs for control (A), Ang 1(B), Ang 2 at → 200ng/ml (C), Ang 2 at 400ng/ml (D) and VEGF (E). F shows the results of 10 independent experiments on LOC migration. Data is shown as mean and SEM, *P<0.0001 for agonist treated cells compared with controls.

4.2.2. Time Course of Migration Assay

Initial experiments were conducted to examine the rate of migration of LOC in the presence of Angiopoietin 2 and VEGF using the Boyden Chamber Assay as described. The experiments were performed over a 10 hour time course.

As shown in figure 4.5, Ang 2 and VEGF had a direct effect on the rate of migration of LOC. There was increased rate of migration up to 6 hours in a time dependant manner. The migratory effect induced by Ang 2 and VEGF diminished until after 10 hours there was no detectable difference in migration in Ang 2 and VEGF treated cells and controls. These results show Angiopoietin 2 and VEGF have a direct effect on migration of LOC and this chemoattractant effect is time dependant. Cells in the upper chamber were isolated from the medium, fixed and counted using Trypan Blue Exclusion. At 6 and 10 hours 96% of cells were viable using trypan blue exclusion.

A 6 hour time point was used for all future experiments.

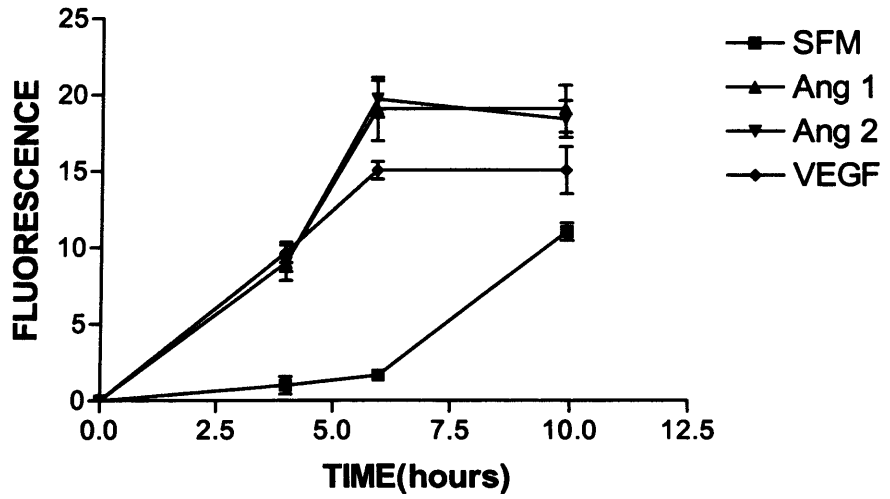


Figure 4.5. Time course showing effect of Ang 2 on migration over time

Migration was measured using a modified 24 well Boyden chamber with cell culture inserts containing 8μM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added 200ng/ml of Ang 2, 100ng/ml Ang 1 and 200ng/ml VEGF.

A time course was carried out with time points at 0, 4, 6 and 10 hours.

To the upper chamber was added $0.75x10^5$ LOC in 500μl of migration medium. The cell culture insert was fixed in 70% ethanol at -20°C for 1 hour and then the lower part of the membrane was stained using haematoxylin and eosin before mounting on slides. Migratory LOC were counted at 40X magnification only cells that complete nuclei were counted and an average taken from ten fields of view used. Data is presented from three independent experiments as mean and SEM.

4.2.3. Dose response curve of Angiopoietin 2 and VEGF on LOC migration.

Having established a migratory response of LOC toward VEGF and the Angiopoietins, concentration dependence was determined for future experiments. Boyden chamber experiments were repeated as described and stopped at 6 hours. It has been described previously (43) that Angiopoietin 2 at a concentration of 800ng/ml can induce endothelial cell survival ,migration and tube like structure formation and therefore, concentrations up to this were studied.

Angiopoietin 2 has a dose dependant effect on LOC migration, showing maximal migratory effect at 200ng/ml. In all future experiments Angiopoietin 2 at 200ng/ml was used.

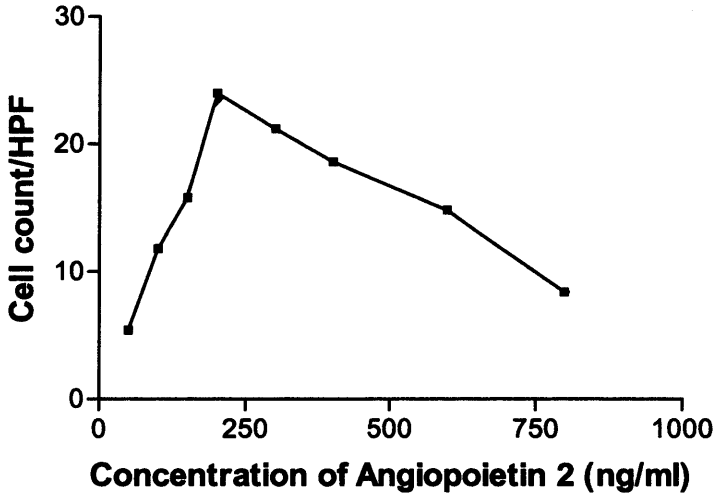


Figure 4.6. Concentration Dependence of Angiopoietin 2 on LOC migration

Migration was measured using a modified 24 well Boyden chamber with cell culture inserts containing 8μM pores. To the lower chamber was added either, 750μl migration medium, 750μl migration medium containing 25,50,100,150 200 or 400ng/ml Ang 2. To the upper chamber was added $0.75x10^5$ LOC in 500μl of migration medium. The cell culture insert was fixed in 70% ethanol at -20°C for 1 hour and then the lower part of the membrane was stained using haematoxylin and eosin before mounting on slides. Migratory LOC where counted at 40X magnification only cells that complete nuclei were counted and an average taken from ten fields of view used. Data is presented from one representative experiment as the mean.

4.2.4 Comparative Effect of Various Cytokines on Terminally Differentiated Endothelial Cell Migration.

As described in Chapter 3 LOC are developing endothelial cell characteristics. In order to determine whether the angiopoietins and VEGF have a positive migratory effect on terminally differentiated endothelial cell migration in the same manner as LOC, HUVE and BAE cells were used in Boyden Chamber experiments in the same manner as described previously. These cells are representative of venous and arterial endothelial cells.

Angiopoietin 1 and VEGF increased the rate of HUVEC migration, similar to that seen with LOC, a 4- and 2-fold increase in migration respectively. Experiments were repeated in the exact same manner with BAE cells with Ang1 and VEGF stimulating migration in a similar manner. Ang 1 and VEGF induced significantly greater migration of HUVEC than control medium (Fig 4.7). These results were significant when mean results were analysed by paired t-tests. In comparison, however, with LOC migration Ang 2 had no effect on the rate of HUVE or BAE cell migration, Figure 4.7. In contrast, Ang 2 did not significantly affect HUVEC migration.

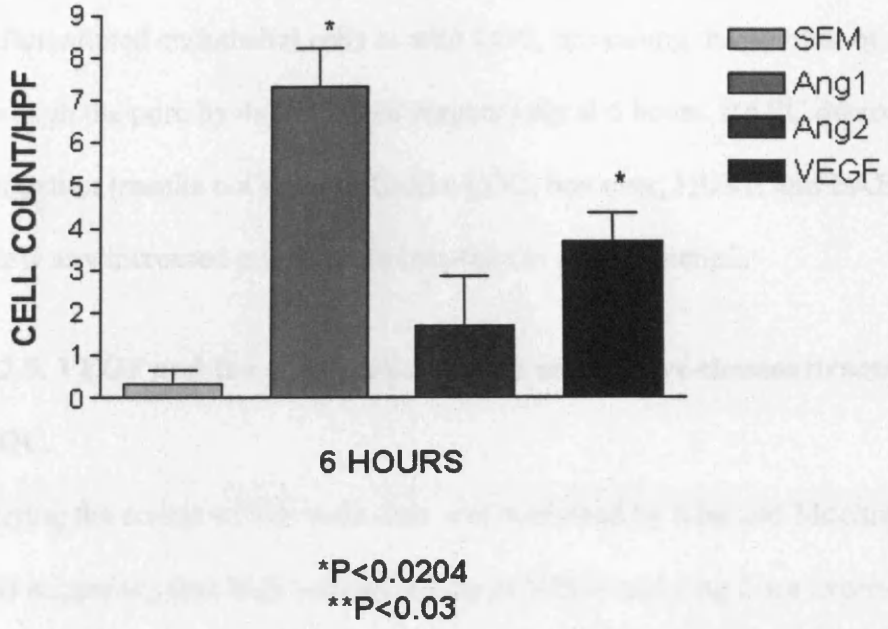


Figure 4.7. HUVEC have a positive migratory response to Angiopoietin1 and VEGF.

HUVEC were grown to confluence, trypsinised and resuspended. Migration was measured using a 24 well modified Boyden Chamber with cell culture inserts containing 8mM pores. To the lower chamber was added either 750µL of migration medium or 750µL of migration medium containing 10ng/ml VEGF, 100ng/ml Ang 1 or 200ng/ml Ang 2. To the upper insert was added $0.75x10^5$ HUVEC in 500µL of migration medium. After 6 hours the cell culture insert was removed, fixed with 70% ethanol at -20^0C for one hour, stained with H&E before mounting on slides. Migratory cells were counted at 40X magnification with an average of 10 high power fields taken. Each experiment was repeated in triplicate and data shown as mean and SEM, * $P<0.001$ for agonist treated cells compared with controls.

These results show Ang 1 and VEGF have a similar migratory effect with terminally differentiated endothelial cells as with LOC, increasing the number of cells migrating through the pore by 4- and 2-fold respectively at 6 hours. BAEC demonstrated similar migration (results not shown). Unlike LOC, however, HUVE and BAE cells did not show any increased migration in response to Angiopoietin2.

4.2.5. VEGF and the Angiopoietins have an additive chemoattractant effect on LOC.

During the course of this work data was published by Kim and Mochizuki et al (42, 43) suggesting that high concentrations of VEGF and Ang 2 are expressed at sites of active angiogenesis. Having established VEGF and the Angiopoietins have chemoattractant properties for LOC independently, experiments were performed to determine if these factors have any synergistic effect on LOC migration.

Boyden Chamber experiments were repeated in the same manner as described previously. VEGF and Ang 1 and/or Ang 2 were added to the lower chambers at concentrations of 100 and 200ng/ml as determined in earlier concentration experiments. As controls, migration medium, Ang 1 and 2 were added independently to the lower chambers.

As found in previous experiments LOC demonstrated an increase in migration in response to Ang 1 and Ang 2. However, when VEGF and Ang 2 were used in combination there was a 55.8 % (range 52.9 - 58.9%) increase in migration of LOC into the lower chamber ($P<0.001$ paired t test) over VEGF and Ang-2 alone. In comparison, VEGF and Ang 1 in combination did not induce LOC migration significantly above controls alone. The results are shown in Figure 4.8.

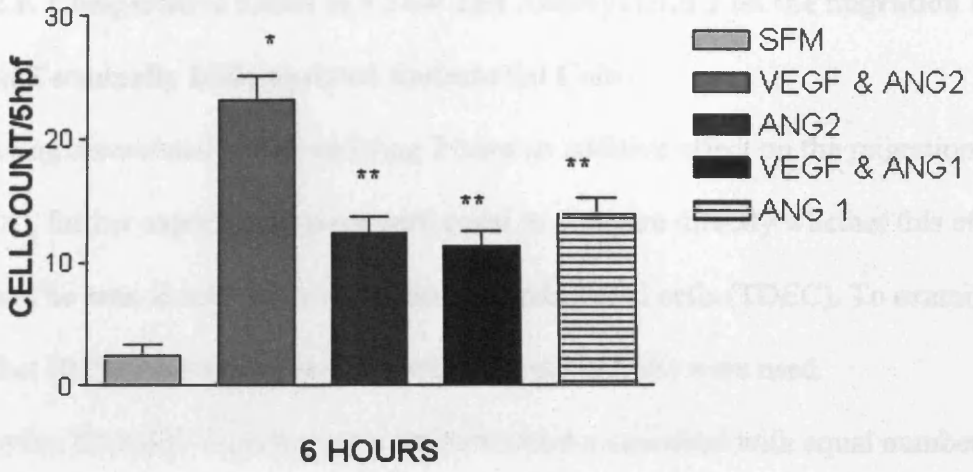


Figure 4.8. VEGF and Angiopoietin 2 have a synergist effect on LOC migration

LOC were grown as described previously. Migration was measured using a 24 well modified Boyden Chamber with cell culture inserts containing 8mM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added either 750µL of migration medium or 750µL of migration medium containing 10ng/ml VEGF with 100ng/ml Ang 1 or 200ng/ml Ang 2 or the Angiopoietins alone. To the upper insert was added $0.75x10^5$ LOC in 500µL of migration medium. After 6 hours the cell culture insert was removed, fixed with 70% ethanol at -20^0C for one hour, stained with H&E before mounting on glass slides. Migratory cells were counted at 40X magnification with an average of 10 high power fields taken. Each experiment was repeated in triplicate. This graph demonstrates an increase in LOC migration in the presence of Ang 1 and 2 as previously demonstrated. When VEGF is added to Ang 2 there is a 52.9% increase of migration which is not seen with VEGF in combination with Ang1. This suggests VEGF and Ang 2 may have a complementary effect on the migration of LOC. Data is presented from three independent experiments as mean and SEM, *P< 0.0001 for agonist treated cells compared with controls.

4.2.6. Comparative Effect of VEGF and Angiopoietin 2 on the migration of LOC and Terminally Differentiated Endothelial Cells.

Having determined VEGF and Ang 2 have an additive effect on the migration of LOC, further experiments were performed to compare directly whether this effect could be seen in terminally differentiated endothelial cells (TDEC). To examine this effect HUVE and mouse heart endothelial cells (MHE) were used.

Boyden Chamber experiments were performed a described with equal number of LOC and HUVE/MHE were added to the upper inserts. Again there was a 5.9 fold increase in the rate of migratory LOC in the presence of VEGF and Angiopoietin 2 over control. This effect was not seen with MHE or HUVEC, both showing only a 2 fold increase in migration in response to VEGF. This effect is shown in figure 4.9.

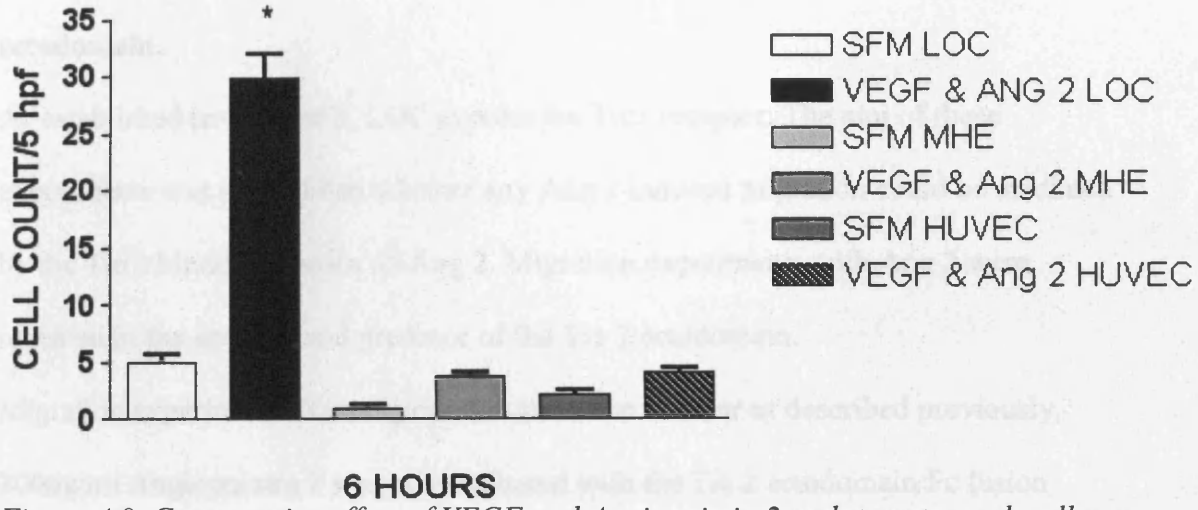


Figure 4.9. Comparative effect of VEGF and Angiopoietin 2 on late outgrowth cell and endothelial cell migration.

LOC and MHE were grown as described previously. Migration was measured using a 24 well modified Boyden Chamber with cell culture inserts containing 8mM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added either 750µL of migration medium or 750µL of migration medium containing 10ng/ml VEGF with 200ng/ml Ang 2 or migration medium alone. To the upper insert was added $0.75x10^5$ LOC in 500µL of migration medium. After 6 hours the cell culture insert was removed, fixed with 70% ethanol at -20^0C for one hour, stained with H&E before mounting on slides. Migratory cells were counted at 40X magnification with an average of 10 high power fields taken. Each experiment was repeated in triplicate. Data is presented as mean and standard deviation of 10 high power fields and is representative of three independent experiments. These results show VEGF and Angiopoietin2 do not have a combined effect on HUVEC migration that is demonstrated with LOC. Data is presented from three independent experiments as mean and SEM, *P<0.0006 compared with migration in the absence of cytokine.

4.2.7. Inhibition of Angiopoietin2 mediated migration of LOC by Tie 2 ectodomain.

As established in Chapter 3, LOC express the Tie2 receptor. The aim of these experiments was to establish whether any Ang 2 induced migration could be mediated by the Tie 2 binding domain on Ang 2. Migration experiments with Ang 2 were repeated in the absence and presence of the Tie 2 ectodomain.

Migration experiments were repeated in the same manner as described previously. 200ng/ml Angiopoietin 2 was pre-incubated with the Tie 2 ectodomain/Fc fusion protein (R&DSystems) at a concentration of 20μg/ml for competitive inhibition. The Tie 1 Fc fusion protein was used at the same concentration for control.

As shown in figure 4.10 inclusion of the Tie 2 ectodomain, but not the Tie 1 ectodomain, inhibited the ability of Angiopoietin2 to stimulate LOC migration. This data demonstrates the soluble Tie2 ectodomain binding of Ang 2 reduces the ability of the ligand to stimulate migration suggesting any Ang 2 induced migration is dependant on the ability of the ectodomain binding site on Ang 2.

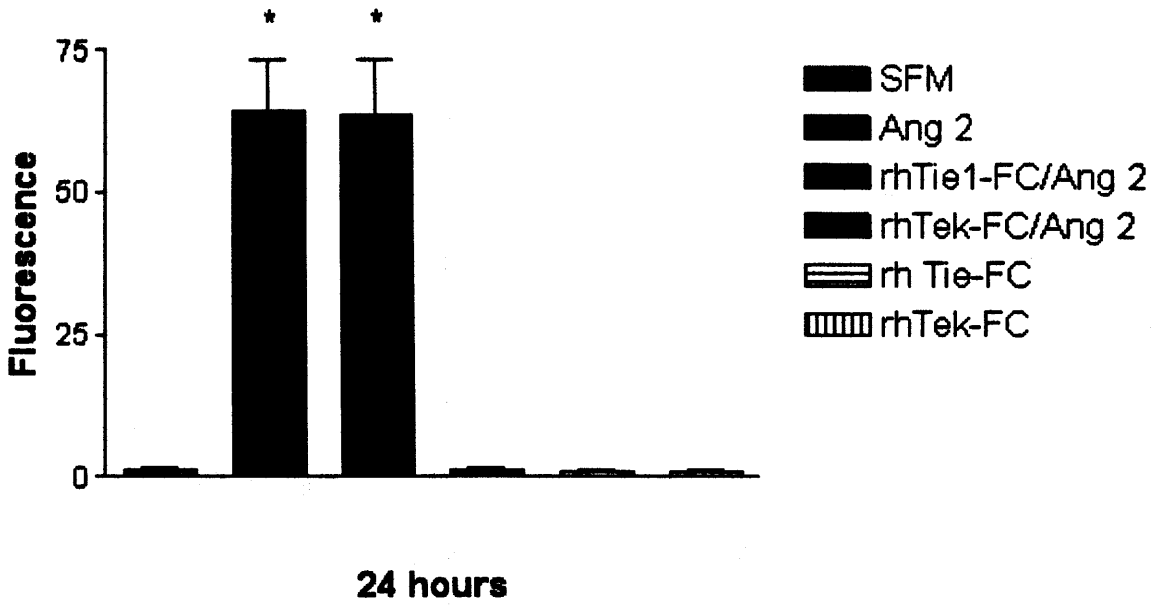


Figure 4.10. Ang 2 induced migration is blocked with the Tie 2 ectodomain

Experiments were performed to examine the effects of competitive inhibition with the Tie 2 ectodomain. LOC were trypsinised and $0.75X10^5$ cells resuspended in 500µl of migration medium and seeded into the upper well of the Boyden Chamber. In the lower chamber 200ng/ml of Ang 2 with either Tie 2/Fc or the Tie 1/Fc(control) ectodomain at a concentration of 20µg/ml were added to the same medium having been pre-incubated at room temperature for 30 minutes. Inserts were removed at 6 hours, washed, stained and 10 high power fields (20X) counted. Similar results were obtained from three independent experiments. Data is presented as mean and standard deviation of 10 high power fields and representative of three independent experiments,*$P<0.01$ .compared with migration in the absence of ligand and fusion proteins using Students *t* test.

4.2.8. Tie 2 activation and downstream signalling via the intracellular protein PI3 Kinase.

At the outset of this project it was known that Ang 2 could inhibit Ang 1 mediated phosphorylation of Tie 2 in endothelial cells (104). The finding that 200ng/ml Ang 2 stimulated migration of LOC suggested the ligand may be activating signalling pathways in LOC.

PI3 Kinase has been shown to be an important in the downstream signalling in Angiopoietin 1 mediated migration of mature endothelial cells (122, 123) and further work by Moduzuki et al 2002 (43) suggested Angiopoietin 2 stimulated chemotaxis in mature endothelial cells at high concentration with this effect being mediated through the PI3 Kinase pathway.

In light of this data, further work was performed to determine whether PI3 Kinase was a candidate signalling molecule downstream of Tie 2 and therefore involved in the Angiopoietin 2 mediated chemotaxis response of LOC. Migration assays were performed, therefore, in the presence and absence of PI3 Kinase inhibitors Wortmannin and LY294002 (42, 103, 122, 124).

In order to examine this effect Boyden Chamber experiments were used as described previously. PI3 Kinase inhibitors, Wortmannin and LY 294002 at concentrations of 100nM and 10μM respectively, were preincubated with LOC for 15 minutes at 37^0C prior to initiation of migration assays. LOC viability was determined pre- and post-experiment by Trypan blue exclusion and found in all experiments to be greater than 98%.

As shown in figure 4.11, Wortmannin and LY294002 caused a marked inhibition of the ability of Ang 2 to stimulate LOC migration. These data suggest PI3 Kinase has a role in Ang 2 stimulated migration of LOC.

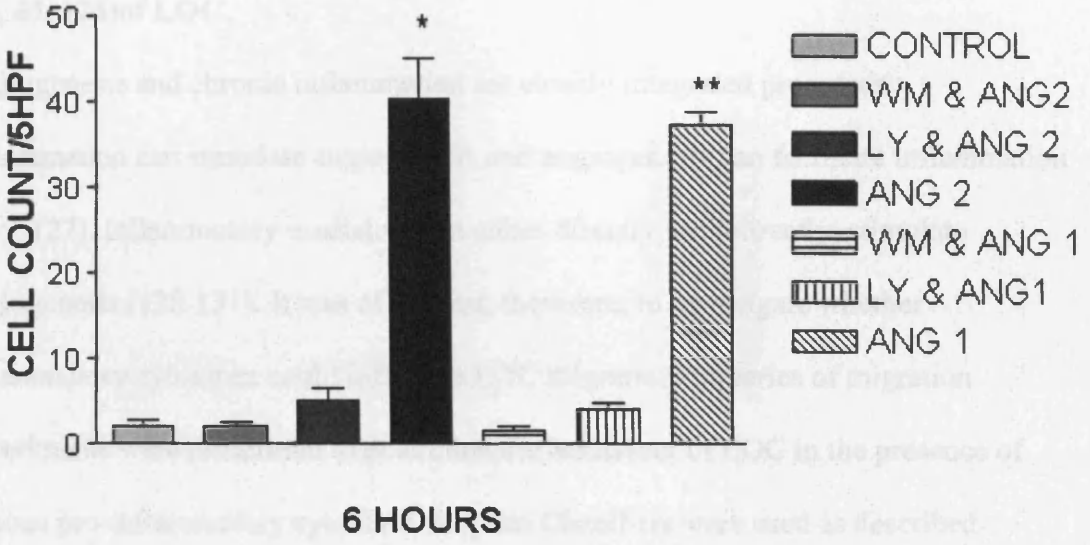


Figure 4.11. Tie 2 activation and downstream signalling via the intracellular protein PI3 Kinase on Angiopoietin stimulated LOC migration.

LOC were trypsinised and having been pre-incubated with the PI3 Kinase inhibitors Wortmannin (100Nm) and LY 294004 (10μM) for 15 minutes at 37^0C, trypsinised and resuspended in migration medium and further incubated for 5 minutes at 37^0C. Ang 1(400ng/ml) and Ang (200ng/ml) in migration medium were added to the lower chamber as described earlier. At 6 hours the cell culture insert was removed and fixed in 70% ethanol at -20^0C for one hour after which the membrane was stained using haematoxylin and eosin before mounting on slides. Migratory cells were counted at 40X magnification. The data is presented as mean and standard deviation of 10 random high power fields from one experiment and is representative of two independent experiments *P<0.001 compared to migration in the absence of antagonist.

4.2.9. Analysis of the effect of various inflammatory cytokines on the migration (25, 45, 125)of LOC.

Angiogenesis and chronic inflammation are closely integrated processes. Inflammation can stimulate angiogenesis and angiogenesis can facilitate inflammation (126, 127). Inflammatory mediators can either directly, or indirectly, stimulate angiogenesis (128-131). It was of interest, therefore, to investigate whether inflammatory cytokines could influence LOC migration. A series of migration experiments were performed to determine the behaviour of LOC in the presence of various pro-inflammatory cytokines. Boyden Chambers were used as described previously. Tumour Necrosis Factor –α (TNF-α) and interleukin-1β (IL-1β) were chosen as representative cytokines for their well documented pro-inflammatory, including migratory, effects on haematopoietic cells (132) (125, 133, 134).
Boyden chamber experiments were performed as described previously. TNF- α or IL-1β were added to the lower chamber. VEGF (10ng/ml) and migratory medium alone were used as positive and negative controls.
These experiments demonstrated no positive migratory response of LOC in the presence of TNF and IL-1 above basal conditions at these concentrations.

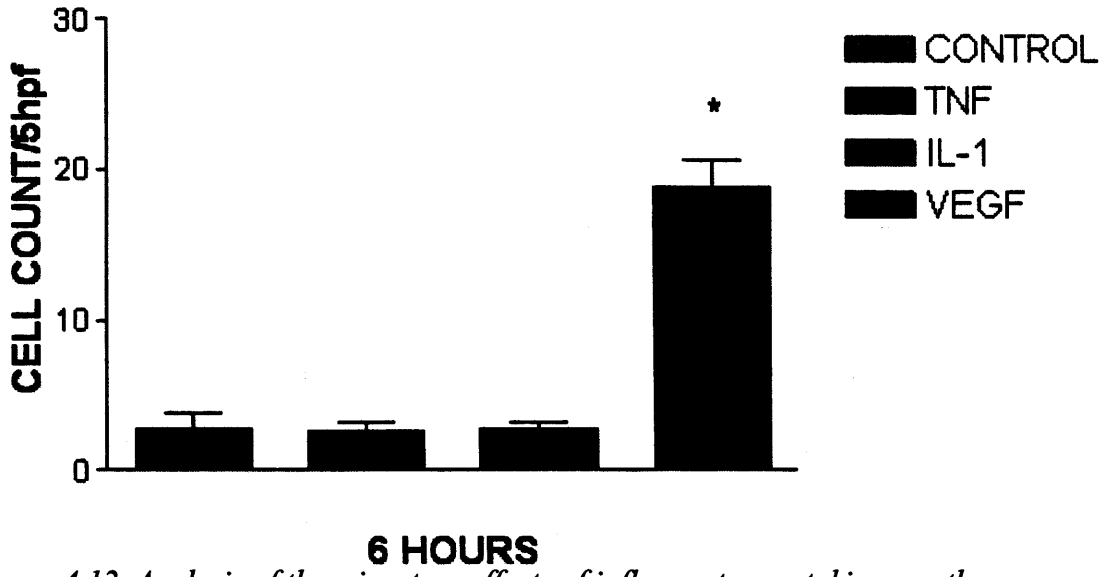


Figure 4.12. Analysis of the migratory effects of inflammatory cytokines on the migration of LOC.

LOC were grown as described previously. Migration was measured using a 24 well modified Boyden Chamber with cell culture inserts containing 8mM pores. To the lower chamber was added either 750μL of migration medium or 750μL of migration medium containing VEGF, IL-1 or TNF. To the upper insert was added $0.75x10^5$ LOC in 500μL of migration medium. After 6 hours the cell culture insert was removed, fixed with 70% ethanol at -20^0C for one hour, stained with H&E before mounting on slides. Migratory cells were counted at 40X magnification with an average of 10 high power fields taken. Each experiment was repeated in triplicate. This graph demonstrates an increase in LOC migration in the presence of VEGF as previously demonstrated. There was no demonstrable increase in migration in the presence of the pro-inflammatory cytokines. Data is presented from three independent experiments as mean and SEM, *$P<0.001$ compared to migration in the absence of growth factor.

4.3. FACTORS CONTROLLING ADHESION BETWEEN ENDOTHELIAL PROGENITOR CELLS AND ENDOTHELIAL CELLS

4.3.1. LOC adhere to mature endothelial cells in a time dependant manner

Having established in Chapter 3, cells adhere to HUVEC on a subconfluent monolayer and incorporate into endothelial cords in matrigel, further experiments were performed to determine if LOC adhered specifically to an endothelial cell monolayer and, if so, determine the rate of adherence and potential regulators. As previously discussed VEGF and the Angiopoietins were investigated. Adhesion assays were performed as described earlier (Section 2.2.2i). Briefly, calcein labelled LOC were plated onto a confluent lawn of either HUVEC under basal conditions, at varying time points cells were washed to remove any non-adherent cells. The number of calcein- labelled LOC adherent to HUVEC were then quantified using a fluorescent plate reader.

The initial experiment was run over one hour time course with 5, 15, 30, and 60 minute time points to determine the rate of basal adhesion. The results of these experiments are shown in Figure 4.13.

Adhesion of LOC to endothelial cells occurs in a time dependant manner, with the maximum adhesion occurring between 15 and 30 minutes. This basal rate of adhesion diminishes until there is no significant increase of adhesion between 30 and 60 minutes. This was reproducible with cells of small vessels Mouse Heart Endothelial

(MHE) and large vessels namely of the aorta Bovine Aortic Endothelial Cells (BAEC).

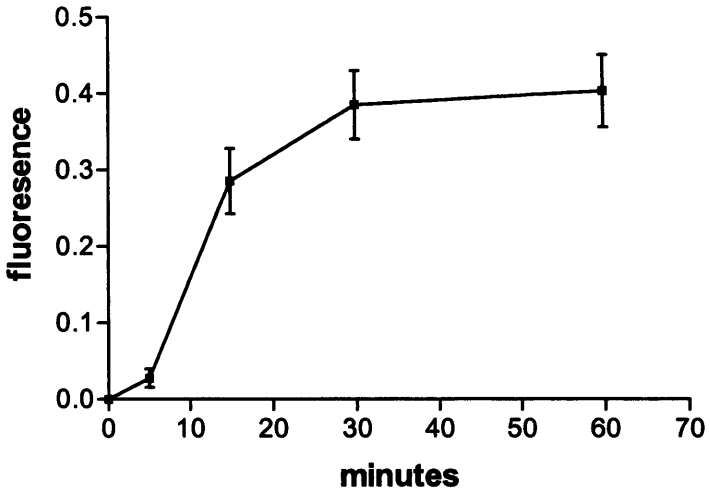


Figure 4.13. Time course showing basal LOC and endothelial cell adhesion over time.

Serum starved LOC were labelled with 5mg/ml Calcein for 15 minutes at room temperature. Following passage cells were resuspended in serum free medium. Cells were then aliquoted, in triplicate, onto confluent lawns of HUVE, MHE or BAE Cells. A time course was carried out, with the experiment being stopped at 5, 15, 30 and 60 minute time points. In order to do these non-adherent cells were removed with serial washings. The number of remaining adherent cells was quantified using a cytoflour fluorescent plate reader. The average of the triplicate readings was calculated. Each experiment, with all three different cell types was performed three times.

This graph shows an example of the increase in basal adhesion between HUVEC and LOC. The greatest rate of adhesion is between the 5 and 30 minute time points with very little increase in the rate between 30 and 60 minutes, therefore, for further experiments the 30 minute time point was used. Date is presented from three

independent experiments as mean and SEM. Similar results were seen with all endothelial cell types namely MHE and BAEC. (Results not shown).

4.3.2. LOC adhere specifically to mature endothelial cells

Having established basal adhesion rates between endothelial cell and LOC, further experiments were performed to determine if this adhesion was specific for endothelial cells. 15 and 30 minute time points were selected on the basis of the time course experiments. Adhesion assays as previously described were employed, this time using monolayers of MHE and CHO (Chinese hamster ovary) cells, the latter not of endothelial origin.

As shown in figure 4.14, there was an increase in adhesion of LOC onto cells of endothelial lineage compared to CHO, with a mean ratio of 1.56 (range 1.49-1.65) and this was shown to be significant when analysed by paired 't' test applied ($p<0.0004$).

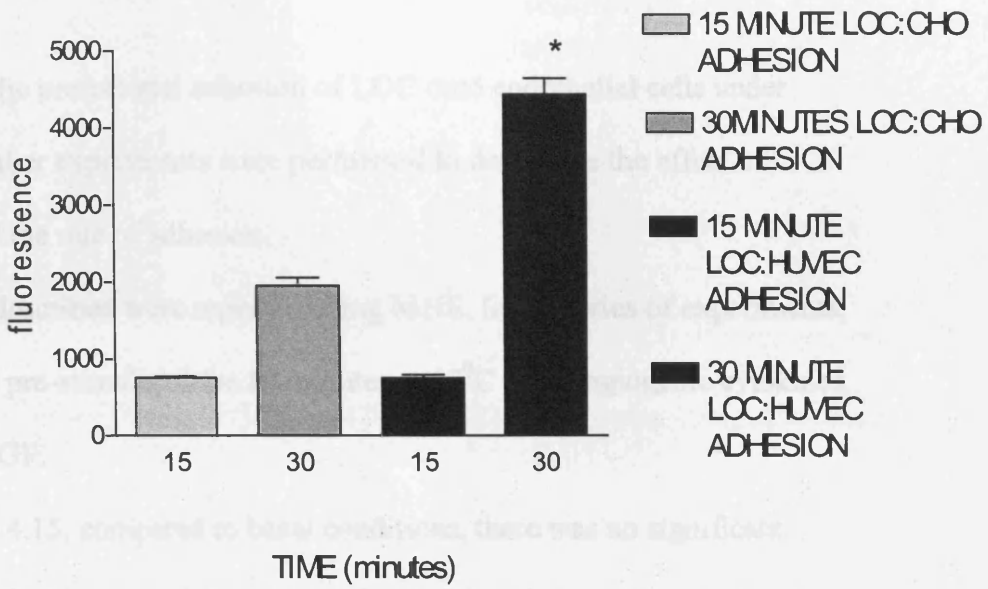


Figure 4.14. LOC preferentially adhere to endothelial cells

HUVEC and CHO were grown to confluence in 96 well plates then serum starved for 12 hours. Quiescent LOC were labelled with Calcein (5μg/ml) for 15 minutes at room temperature then aliquoted in triplicate onto the confluent HUVEC or CHO. After 30 minutes any non-adherent cells were removed using serial washings with RPMI. Cytofluor plate readings were used to quantify the amount of adherence. An average of the readings was calculated. There is a significant increase in adhesion between LOC and MHE compared to that between LOC and CHO at the 30 minute time point. Three experiments showed a mean average increase of 60.75% in LOC adhesion onto MHE compared to CHO. ($P<0.0004$ using Student t-test). Data is shown as mean +/- SEM for three experiments.

4.3.3. Analysis of the effects of various cytokines on the rate of adhesion of LOC onto endothelial cells (MHE, BAEC and HUVEC).

Having determined the preferential adhesion of LOC onto endothelial cells under basal conditions, further experiments were performed to determine the effect of various cytokines on the rate of adhesion.

Adhesion assays as described were repeated using MHE. In the series of experiments, however, LOC were pre-stimulated for 15 minutes at 37^0C with angiogenic cytokines, Ang 1, Ang 2 or VEGF.

As shown in Figure 4.15, compared to basal conditions, there was no significant increase in the rate of adhesion, at 30 minutes when LOC were pre-stimulated with VEGF, Ang 1 or 2 at concentrations of 10, 400 and 200ng/ml respectively. These experiments were repeated at 100ng/ml VEGF and 800ng/ml Ang 2 (43) which, again, had no effect on the rate of adhesion. Experiments were repeated using Ang 1, Ang 2 and VEGF in combination to determine in there was any effect on LOC adhesion onto endothelial cells. As shown in figure 4.16, the cytokines in combination had no effect on the rate of adhesion between LOC and endothelial cells.

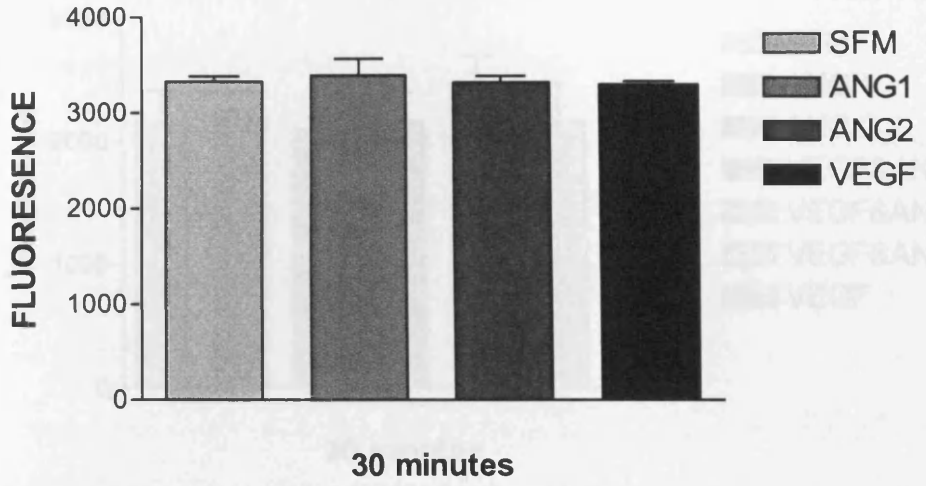


Figure 4.15 Various cytokines have no effect on adhesion between LOC and endothelial cells over 30 minutes.

In separate experiments HUVEC, MHE or BAEC were grown to confluence in 96 well plates and serum starved for 12 hours. Quiescent LOC were labelled with 5μg/ml Calcein for 15 minutes at room temperature. Cells were trypsinised and resuspended in serum free medium containing Ang 1 (400ng/ml), Ang 2 (200ng/ml) and VEGF (100ng/ml) for 15 minutes at 37^0C. Following this cells were aliquoted in triplicate onto the lawn of endothelial cells. After 30 minutes any non-adherent cells were removed by serial washing with RPMI. A cytoflour plate reader was used to quantify the amount of adhesion. An average of the triplicate readings was calculated.
This figure shows representative results of three experiments with data been shown as mean +/- SEM. There was no significant increase in adhesion between LOC and endothelial cells in the presence of VEGF, Ang 1 or 2 compared to serum free medium over 30 minutes.

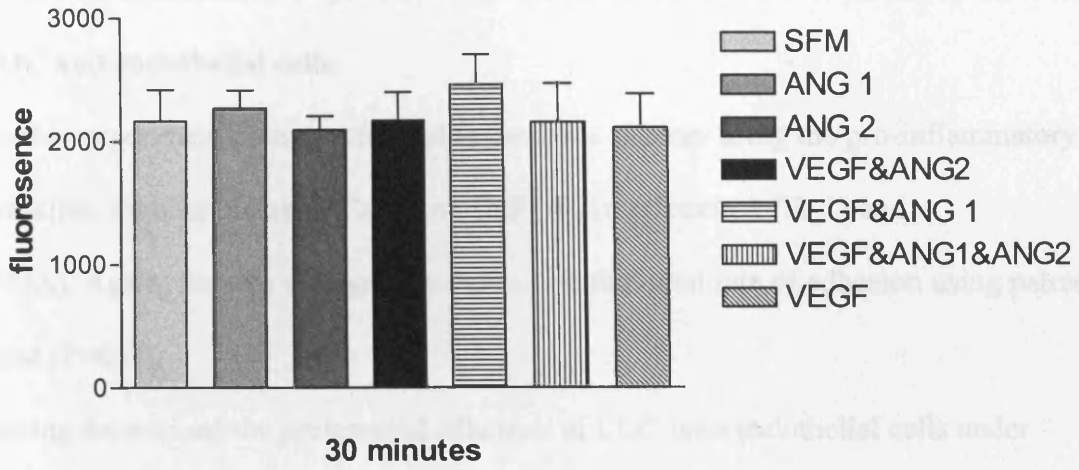


Figure 4.16. Angiopoietins and VEGF have no affect on adherence of LOC onto fibronectin.

Fibronectin was incubated, at a concentration of 10mg/ml in PBS, for 1 hour at 37^0C to coat 96 cell plates. LOC were passaged and labelled with 5mg/ml Calcein for 15 minutes at room temperature. Cells were then resuspended in serum free medium containing Angiopoietin 1(400ng/ml), Angiopoietin 2(200ng/ml), VEGF (100ng/ml) or in combination for 15 minutes at 37^0C. Cells were then aliquoted in triplicate onto the fibronectin coated pates. The experiment was stopped at 30 minutes and any non-adherent cells removed by serial washings with RPMI. The experiment was repeated 3 times and an average of the triplicates calculated.

Figure 4.16 shows a representative graph of the results. There was no statistical difference (using paired t-test) between cells pre-stimulated with various cytokines alone, or in combination, on adhesion of LOC onto fibronectin above control levels.

4.3.4. Pro-inflammatory cytokines have no effect on the rate of adhesion between LOC and endothelial cells.

Further experiments were performed in the same manner using the pro-inflammatory cytokines Tumour Necrosis Factor-α (TNF- α), Interleukin-1 (IL-1) and (PMA). Again, the was no significant effect on the basal rate of adhesion using paired t-test (P=0.02)

Having determined the preferential adhesion of LOC onto endothelial cells under basal conditions, further experiments were performed to determine the effect of various pro-inflammatory cytokines on the rate of adhesion of LOC and endothelial cells.

Adhesion assays as described were repeated using MHE. In the series of experiments, however, LOC were pre-stimulated for 15 minutes at 37^0C with pro-inflammatory cytokines, TNF-α, IL-1β and PMA.

As shown in Figure 4.15, compared to basal conditions, there was no significant increase in the rate of adhesion, at 30 minutes when LOC were pre-stimulated with any of the cytokines used.

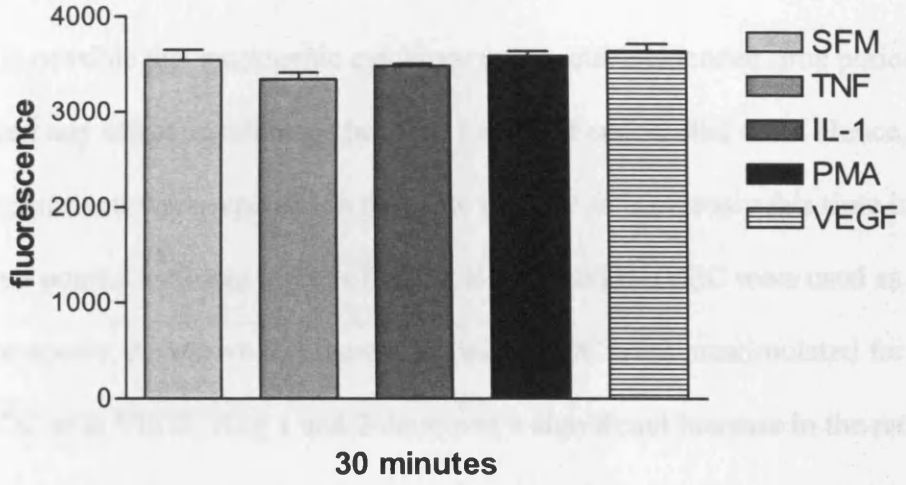


Figure 4.17. Pro-inflammatory cytokines have no effect on adhesion.

HUVEC were grown to confluence on 96 well plates. LOC were labelled with Calcein-AM for 15 minutes at room temperature, passaged and pre-treated with inflammatory cytokines as discussed in the text. Cells were then aliquoted in triplicate onto the quiescent MHE. The experiment was stopped after 24 hours and any non-adherent cells removed with serial washes. The amount of adhesion was quantified using a cytofluor plate reader.

Data is shown as mean +/- SEM for three experiments. This result indicates pro-inflammatory cytokines do not induce any significant increase in adherence between LOC and endothelial cells.

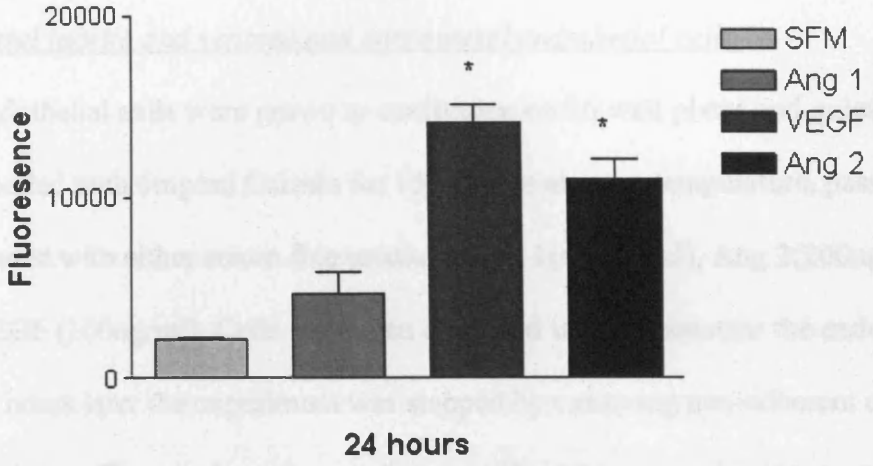
4.3.5. Analysis of VEGF and the Angiopoietins 1 and 2 on the rate of adhesion of LOC onto endothelial cells over 24 hours.

It is possible that angiogenic cytokines may require extended time periods in order to exert any effect on adhesion between LOC and endothelial cells. Hence, adhesion experiments were repeated in the same manner as previously this time using a 24 hour time point. Confluent layers of MHE, BAEC and HUVEC were used as described previously. As shown in Figure 4.18, when LOC were prestimulated for 15 minutes at 37^0C with VEGF, Ang 1 and 2 there was a significant increase in the ratio of adhesion of 2.1,7.3 and 4.5 respectively above basal levels (paired t-test $P<0.001$) at 24 hours. These results show that VEGF, Angiopoietin 1 and Angiopoietin 2 can increase adhesion between LOC and arterial, venous and small vessel endothelial cells. This suggests that, although basal adherence is maximal at 30 minutes, any up-regulation of adhesion molecules, mediated by these cytokines requires longer time periods.

The same experiments were repeated with HUVEC and BAEC replacing MHE to determine if the same effect is seen with large vessel, arterial and venous cell lines. These results are shown in Figure 4.18. Again, there was significance increase of cell adhesion in both series of experiments ($P<0.001$ using paired t-test) when cells were incubated with VEGF and Angiopoietin 2. The experiments were repeated three times with a mean increase adhesion of 6.6 and 6.9 fold for VEGF on HUVEC and BAEC respectively and 5.1 and 4 fold for Angiopoietin 2 on HUVEC and BAEC respectively.

These series of experiments show a VEGF and Angiopoietin 2 induced increase in adhesion of LOC to mature endothelial cells is repeatable with cells of a variety of endothelial origin.

A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Fluoresence
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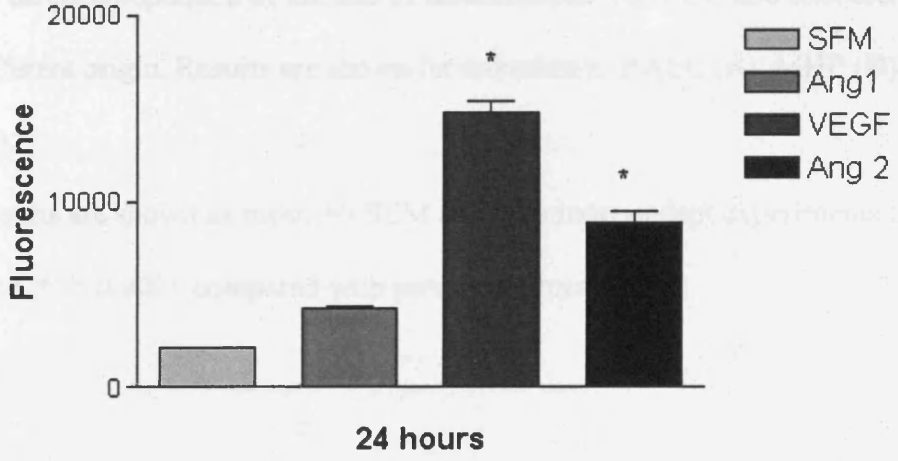
B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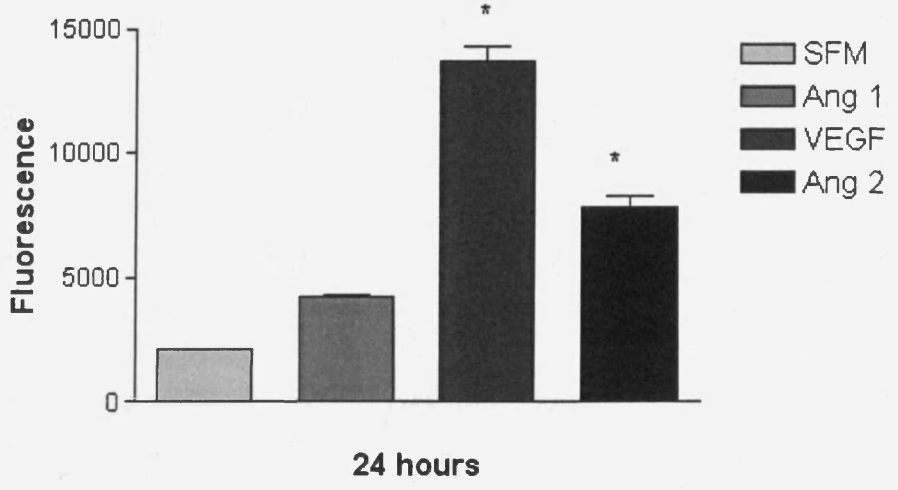
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VEGF
Ang 2
24 hours

Figure 4.18. Ang 2 and VEGF significantly increase adhesion of LOC onto large vessel (aortic and venous) and microvessel endothelial cells.

Endothelial cells were grown to confluence on 96 well plates and quiesced. LOC were labelled with 5mg/ml Calcein for 15 minutes at room temperature, passaged and pre-treated with either serum free medium, Ang 1(400ng/ml), Ang 2(200ng/ml) and VEGF (100ng/ml). Cells were then aliquoted in triplicate onto the endothelial cells. 24 hours later the experiment was stopped by removing non-adherent cells by serial washings. The number of non-adherent cells was measured using a cytofluor plate reader. An average of the triplicate readings was calculated .These experiments allow for direct comparison of the rate of adhesion between LOC and endothelial cells of different origin. Results are shown for adhesion to BAEC (**A**), MHE (**B**) and HUVEC (**C**).

Results are shown as mean +/- SEM for three independent experiments for each cell type .* $P<0.0001$ compared with serum free medium.

4.3.6. Optimum cytokine concentration to induce LOC adhesion onto mature endothelial cells

Having established upregulation of adhesion of LOC onto a variety of endothelial cell lines in the presence of VEGF and Ang 2, the concentration dependence of this effect was determined. A confluent lawn of MHE was used as previously and the experiment stopped at 24 hours. The range of Ang 2 concentrations of 0, 50, 100, 200, 400, 800ng/ml were examined. It has been shown that Ang 2 at supraphysiological levels enhances endothelial cells survival through the phosphotidylinositol 3 kinase/Akt signal transduction pathway, therefore levels above this concentration were studied, namely 1000ng/ml (42).

As shown in Figure4.19, Ang 2 had a dose dependant response, showing maximum adhesion at a concentration of 200ng/ml. At concentrations above this level there was decreased LOC:EC adhesion.

In all future experiments, unless stated otherwise, 10ng/ml VEGF and 200ng/ml of Ang 2 were used.

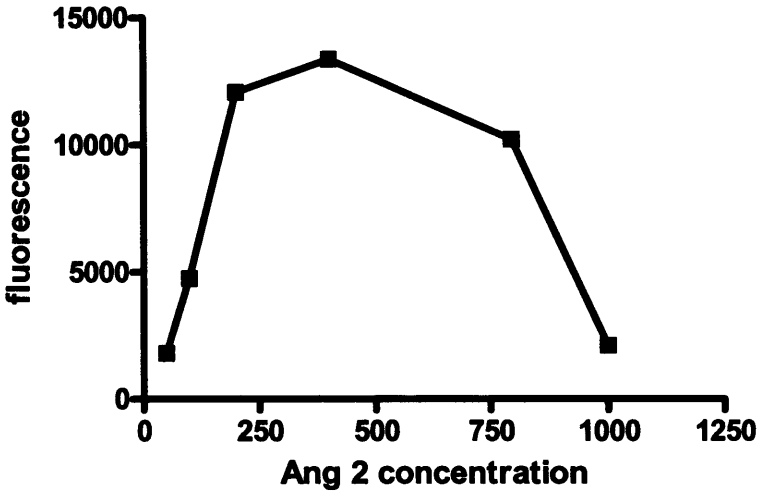


Figure 4.19. Concentration dependence of Angiopoietin 2 effects on LOC adhesion to endothelial cells.

MHE were grown to confluence on 96 well plates and quiesced. Serum starved LOC were labelled with 5mg/ml Calcein for 15 minutes at room temperature, passaged then pre-treated with Angiopoietin 2 at 50,100, 200,400 and 800ng/ml. Cells were aliquoted in triplicate onto the endothelial cells. The experiment was stopped at 24 hours by removing non-adherent cells with serial washings. The number of adherent cells was quantified using a cytofluor fluorescent plate reader. The average of each triplicate was calculated. This graph is representative of two experiments.

4.3.7 Comparative effects of cytokine induced adhesion between mature endothelial cells.

As previously shown in chapter 3 LOC are developing endothelial cell characteristics. In order to determine the extent to which LOC have differentiated, adhesion experiments were performed in the same manner as previously this time assessing whether these cytokines have a similar effect in inducing homotypic terminally differentiated endothelial cell adhesion. The same concentration of cytokines as in the LOC experiments was used. As Figure 4.20 shows, neither VEGF nor the Angiopoietins had any significant effect on adhesion between MHE, at those concentrations and conditions that induce adhesion of LOC onto MHE.
Further experiments were conducted to see if a similar effect could be induced in microvascular terminally differentiated endothelial cells. Identical adhesion experiments were performed with MHE onto MHE and compared directly with LOC adhesion in the presence of VEGF and the Angiopoietins. The results are shown in Figure 4.21.

These results suggest that LOC, although expressing endothelial like characteristics compared to one week cell, in terms of adhesion regulation under these conditions respond differently to the cytokines VEGF and Ang 2.

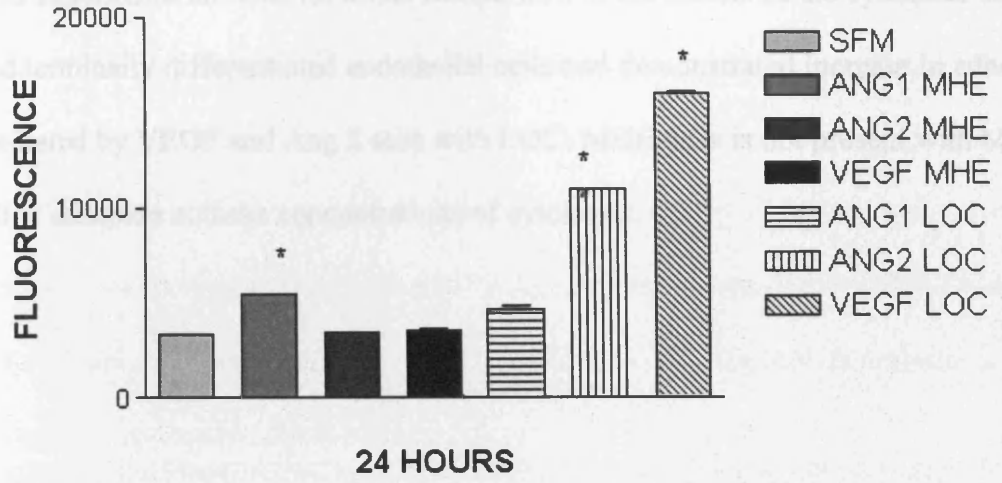


Figure 4.20. Comparative effect of adhesion of LOC and terminally differentiated endothelial cells onto endothelial cells in the presence of various cytokines.

MHE were grown to confluence on 96 well plates. LOC and MHE in equal numbers were labelled with 5mg/ml Calcein, passaged and pre-treated with VEGF and the Angiopoietins for 15 minutes at 37^0C. Cells were then aliquoted in triplicate onto MHE. The experiment was stopped at 24 hours and non-adherent cells removed by serial washings. The number of adherent cells was quantified using a Cytofluor fluorescent plate reader. Data is presented from three independent experiments as the mean and SEM. * $P<0.001$ compared to adhesion in the absence of cytokine.

This experiment allowed for direct comparison of the effects of the cytokines on LOC and terminally differentiated endothelial cells and demonstrated increase in adhesion mediated by VEGF and Ang 2 seen with LOC: MHE cells is not present with MHE: MHE adhesion at these concentrations of cytokines.

4.3.8. Pre-conditioned LOC upregulate adhesion onto mature endothelial cells

Data showing increased adhesion at 24 hours between LOC and endothelial cells suggests upregulation of adhesion molecules. However, it is not known whether the Ang 1, Ang 2 or VEGF is acting on LOC or endothelial cells. To test this each cell type was pre-incubated independently for 15 minutes and washed with PBS to remove any residual medium containing cytokines before continuing with the adhesion experiment as described previously.

As can be seen in Figure 4.21, when MHE are pre-stimulated and washed, there was no difference in adhesion rates between the two cell types.

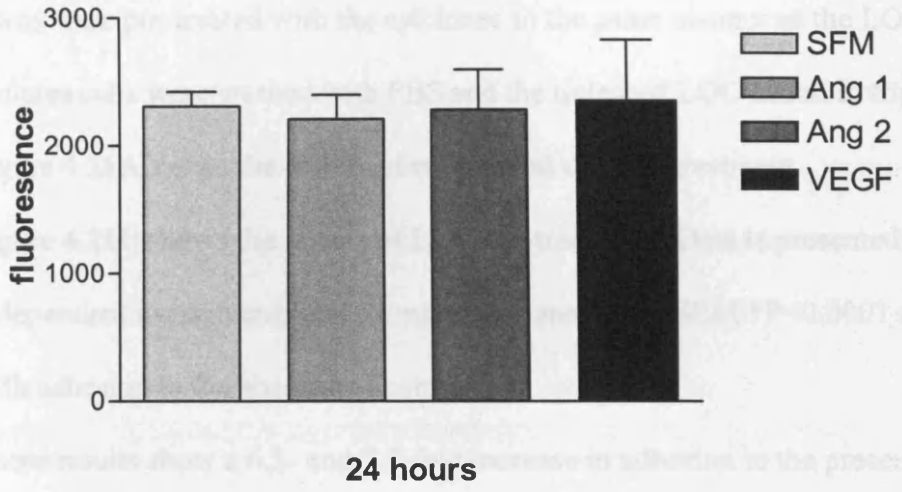
This was in contrast to LOC pre-stimulation. Here there was an increase in adhesion from basal levels of 7.1 for Ang 2 and 7.67 with VEGF. These results are shown in Figure 4.22.

These results show that, in the presence of VEGF and Ang 2, LOC adhesion to endothelial cells increases above basal levels. Neither cytokine demonstrated similar effects with terminally differentiated endothelial cells.

These results also suggest the mechanism of upregulation of adhesion is mediated by LOC as opposed the endothelial cell alone or in combination.

These results also imply there is an immediate (within 15 minutes) upregulation mediated by LOC, which then subsequently requires a further 24 hours for any increase in adhesion to be seen.

A



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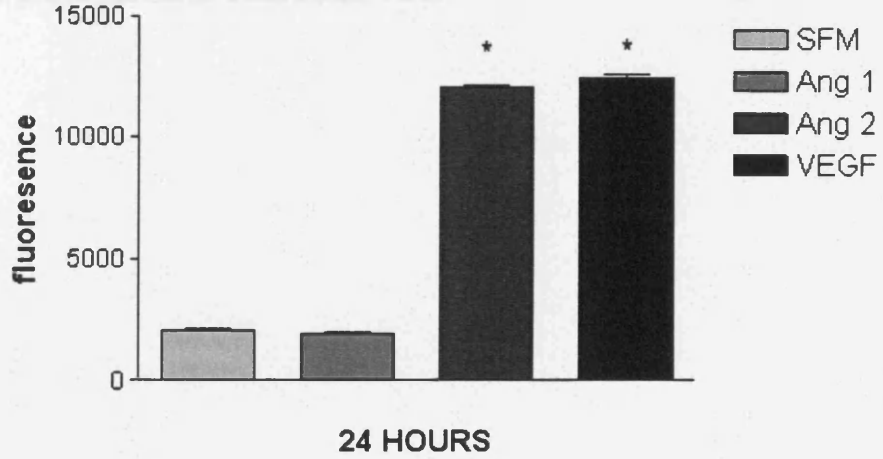


Figure 4.21 Effects of LOC and mature endothelial cell independent pre-conditioning on cell:cell adhesion

MHE and HUVEC were grown to confluence on 96 well plates. LOC were labelled with 5mg/ml calcein for 15 minutes at room temperature and passaged. They were then pre-treated with VEGF, Angiopoietin1 or 2 for 15 minutes at 37^0C for 15 minutes then cytokine removed by serial washings. Cells were then aliquoted equally in triplicate onto MHE or HUVEC. After 24 hours the experiment was stopped by removing adherent cells with serial washes. The amount of adherent cells was

quantified using a fluorescent plate reader. In parallel experiments the endothelial lawns were pre-treated with the cytokines in the same manner as the LOC. After 15 minutes cells were washed with PBS and the quiesced LOC added in triplicate.

Figure 4.21A shows the results of endothelial cell pre-treatment.

Figure 4.21B shows the results of LOC pre-treatment. Data is presented from three independent experiments and shown as the mean and SEM.*P<0.0001 compared with adhesion in the absence of cytokine.

These results show a 6.3- and 7.7-fold increase in adhesion in the presence of Ang 2 and VEGF respectively, only when LOC are pre-treated compared to the terminally differentiated endothelial cells. This suggests any up-regulation in adhesion is via the LOC as opposed to endothelial cells.

4.3.9. Inhibition of Angiopoietin 2 induced adhesion with the Tie-2 ectodomain.

The aim of this series of experiments was to establish whether the increase in adhesion seen between LOC and endothelial cells in the presence of Ang 2 was due to any activation of the Tie 2 receptor, and should if this is the case, be blocked by competitive inhibition with the Tie 2 ectodomain. The rhTie 2 ectodomain-FC chimera at a concentration of 20μg/ml was used for competitive inhibition. rhTie 1-FC chimera at the same concentration was used as control.

As can be seen in Figure 4.21, in the presence of the rhTie 2-FC chimera there was a marked reduction of Angiopoietin 2 induced adhesion to basal levels. In the control group Ang 2 increased adhesion to X 9 above basal levels and the rhTie 1-FC chimera had no effect on this upregulation.

These experiments suggest Tie 2 binding sites on Ang 2 is required for the pro-adhesive effect of this ligand.

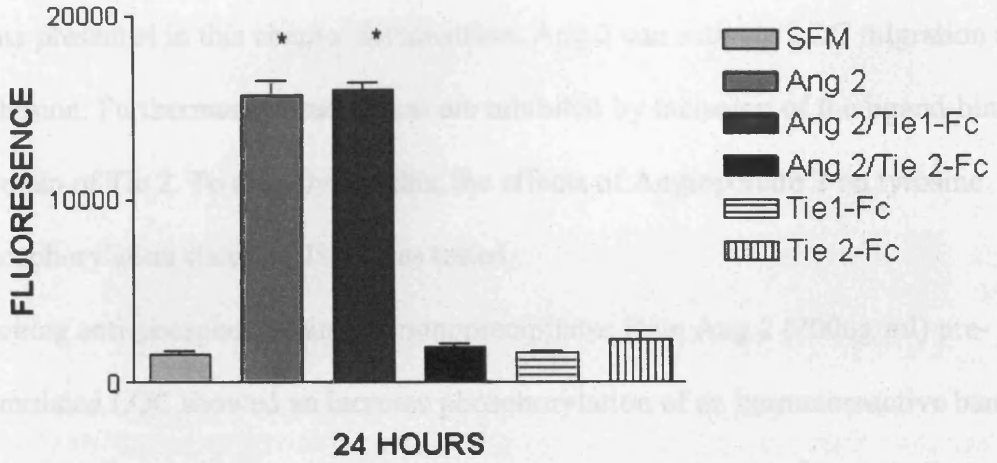


Figure 4.22. Inhibition of Ang 2 induced adhesion with the Tie-2 ectodomain

Experiments were performed to examine the effects of competitive inhibition with the Tie-2 ectodomain. Quiescent LOC were labelled with 5mg/ml Calcein-AM. They were passaged and pre-treated with media containing either Ang 2 or serum free control medium for 15 minutes. Either the Tie-2/FC ectodomain or the Tie-1/FC ectodomain at a concentration of 20μg/ml were also added to the cell suspension. Cells were then added in triplicate to a confluent lawn of quiescent MHE in 96 well plates. After 24 hours any non-adherent cells were removed by serial washings. The number of adherent cells was quantified using a cytofluor fluorescent plate reader. An average of the triplicate readings was calculated and the results shown above. * $P<0.0001$ compared with adhesion in the absence of ligand and fusion protein. Data is presented from three independent experiments as mean and SEM.

Thee experiments show any increase in adhesion induced by Ang 2 can be competitively inhibited with the Tie –2/Fc ectodomain to control levels. The Tie-1/Fc ectodomain had displayed no competitive inhibition implying Tie-2/Fc ectodomain inhibition is due to the Tie-2 portion of the molecule as opposed to the Fc portion.

4.3.10. Angiopoietin 2 stimulates Tie-2 receptor phosphorylation.

Data presented in this chapter demonstrates Ang 2 can activate LOC migration and adhesion. Furthermore, these effects are inhibited by inclusion of the ligand-binding domain of Tie 2. To directly test this, the effects of Angiopoietin 2 on tyrosine phosphorylation status of Tie 2 was tested.

Probing anti-phosphotyrosine immunoprecipitates from Ang 2 (200ng/ml) pre-stimulated LOC showed an increase phosphorylation of an immunoreactive band corresponding to the size of the Tie-2 receptor compared to Angiopoietin 2 pre-conditioned HUVEC. This was repeated five times with marked variability in the level of activation of Tie-2 by Ang 2, but overall this suggests Ang 2 acts an activating ligand for Tie-2 on LOC that may be responsible for inducing migration.

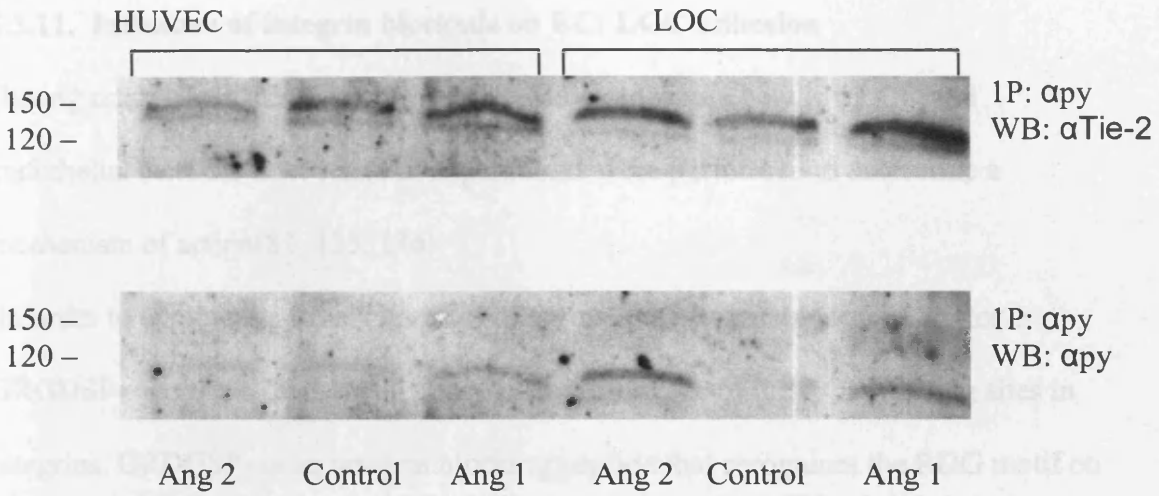


Figure 4.23. Phosphorylation of Tie-2 ectodomain on LOC following Angiopoietin-2 pre-conditioning.

Quiesced HUVEC (H) and LOC (L) were placed in contact with Ang 1 (A1) and Ang 2 (A2) for 10 minutes and phosphorylation of the Tie-2 receptor was compared. Immunoprecipitation (IP) was performed using the anti- phosphotyrosine antibody (α Py). Western blot **A** was probed with anti-Tie-2 antibody (α Tie-2) then stripped and re-probed with anti-phosphotyrosine antibody **B**. C represents control blot probed with secondary antibody alone. These results are representative of two independent experiments.

4.3.11. Influence of integrin blockade on EC: LOC adhesion

Having established VEGF and Ang 2 influences adhesion between LOC and endothelial cells the next series of experiments were performed to determine a mechanism of action(81, 135, 136).

In order to explore the involvement of integrins, the integrin binding inhibitor – GRGDSP- was used. This acts by competitive blockade of the RGD binding sites in integrins. GRDGSP- is an integrin blocking peptide that recognises the RDG motif on the integrin molecule. -GRADGSP- was used as a control peptide. LOC were fluorescently labelled, trypsinised and pre-treated in suspension for 15 minutes in a solution containing either the active peptide, RGD, or the control peptide, RAD, at a concentration of 2.5μg/ml. and either control media or VEGF at a concentration of 10ng/ml. LOC and MHE/HUVEC adhesion experiments were repeated as described previously.

RGD blocking in the absence of VEGF reduced adhesion between LOC and endothelial cells by 1.34 but was not significant using paired t-test ($P=0.19$, $n=3$). To compare any effect of integrin blockade in the presence of VEGF, three groups were used. VEGF alone, RGD peptide +/- VEGF, and RAD peptide +/- VEGF. RGD peptide reduced VEGF induced adhesion by 43% of the value seen in the presence of the RAD peptide ($P<0.018$ using paired 't'-test, $n=3$). These results demonstrate that a component of the effect of VEGF induced adhesion between EC and LOC is RGD related and, therefore, likely that part of the effect of VEGF induced adhesion is integrin mediated. As the increase in adhesion is still seen in the presence of the blocking peptide, it is possible that another mechanism may be involved in the VEGF induced adhesion.

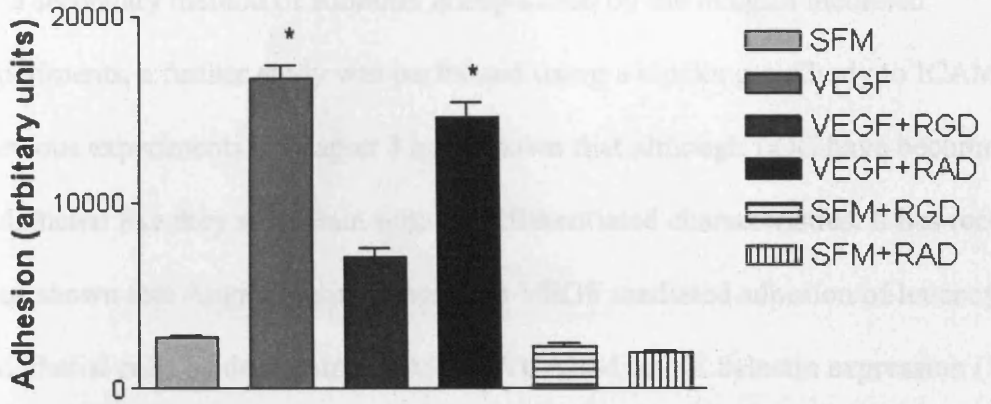


Figure 4.24. Influence of integrin blockade on EC: LOC adhesion

VEGF has been shown to mediate integrin adhesion. These experiments aimed to determine if VEGF induced increase in adhesion between LOC and endothelial cells could be integrin mediated.

MHE were grown to confluence in 96 well plates. Quiescent LOC were labelled with 5mg/ml Calcein-AM, passaged and aliquoted into six groups. Two were control groups, pre-treated with either serum free control medium or VEGF .Two were treated with the active –RGD- peptide (2.5µg/ml) and two treated with the control peptide –RAD- (2.5µg/ml). After 24 hours any non-adherent cells were removed with serial washings. The number of adherent cells was quantified using a cytofluor plate reader. In the presence of the blocking peptide the VEGF induced adhesion is reduced by 51.48 +/- 9% .* $P<0.0001$ compared to adhesion in the absence of ligand and blocking peptide. Data is presented from three independent experiments as mean and SEM of three independent experiments.

4.3.12. Influence of ICAM-1 blockade on EC:LOC adhesion

As a secondary method of adhesion is implicated by the integrin mediated experiments, a further study was performed using a blocking antibody to ICAM-1. Previous experiments in chapter 3 have shown that although LOC have become more endothelial like they still retain some undifferentiated characteristics. It has recently been shown that Angiopoietin 1 decreases VEGF mediated adhesion of leucocytes to endothelial cells by decreasing ICAM-1, VCAM-1 and E Selectin expression (103, 137). Further more, VEGF has been implicated in the expression of ICAM-1mediated adhesion on mature endothelial (136) and inflammatory cells (138) involved in angiogenesis. To determine whether any endothelial or remaining functional haematopoietic or monocyte properties could account for the remaining mechanism of adhesion, blocking peptides to the leukocyte adhesion molecule ICAM-1 (CD18) were used to examine the influence of VEGF induced adhesion of LOC onto mature endothelial cells.

LOC were trypsinised and fluorescently labelled. α CD18 (PEPROTECH) was pre-incubated with LOC for 15 minutes at 37^0C to block any ICAM-1 binding sites and control immunoglobulin peptide, at concentrations of 10μl/ml. Adhesion experiments were then performed as described previously. The results are shown in Figure 4.25. α CD18 antibody alone had no effect on basal adhesion. As previously, VEGF increased adhesion to above control levels. When α CD18 was pre-incubated with LOC in the presence of VEGF there was a reduction of this VEGF mediated adhesion. These results show that a component of VEGF induced adhesion between mature endothelial cells and LOC is mediated by ICAM-1.

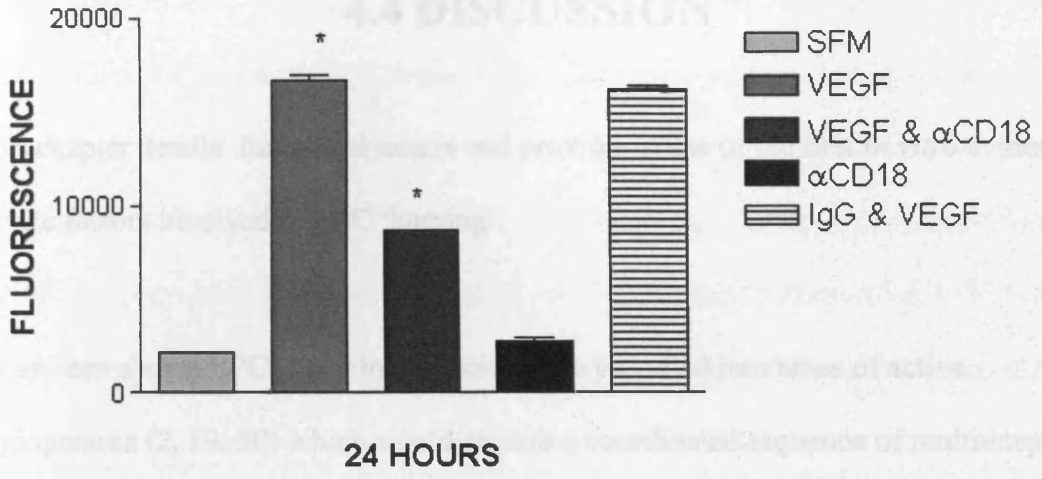


Figure 4.25. Influence of ICAM-1 blockade on EC:LOC adhesion

VEGF has been shown to influence ICAM-1 mediated adhesion. In order to determine potential mechanisms and the extent to which LOC still express functional monocytes adhesion molecules, blocking experiments to the adhesion ICAM-1 were performed. MHE were grown to confluence in 96 well plates and quiesced. LOC were labelled with Calcein-AM, passaged and split into five equal aliquots. Two were control groups in either serum free control medium or VEGF at 100ng/ml. Two groups were treated with an antibody to CD18 in the presence of VEGF or in control vehicle of serum free medium. A further group was treated with VEGF along with mouse IgG immunoglobulin). Results are shown as mean +/- SEM for three independent experiments * $P<0.0001$ compared with control.

4.4 DISCUSSION

This chapter details functional assays and provides some of the first *in vitro* evidence for the factors involved in EPC 'homing'.

It has been shown EPCs have the capacity to be recruited into areas of active angiogenesis (2, 19, 50) which would require a coordinated sequence of multi-step adhesion and signalling events. In this study we have extended these observations by demonstrating VEGF and the Angiopoietins significantly induce late outgrowth EPC (LOC) migration and adhesion. VEGF induced a significant increase in the migration of cells. VEGF has been shown to be effective at mobilising EPCs into the peripheral circulation (19, 72). Furthermore, it has been demonstrated increased circulating VEGF levels is accompanied by increased PBMNCs which comprises a significant number of bone marrow derived EPCs as defined by KDR/AC133 expression (6). Given the role of VEGF in both angiogenesis and vasculogenesis during foetal development and pathological states (21, 139), these findings are consistent with the notion that VEGF modulates EPCs in post-natal vascularisation.

Ang 1 has been shown to induce chemotactic migration of sprouting mature endothelial cells and Ang 2 , whilst not inducing endothelial cell migration, has been suggested to initiate angiogenesis by blocking the effect of Ang 1 (13, 33, 41, 140, 141). The work detailed in this chapter show both Ang 1 and 2 increase the migration of LOC. Additionally, VEGF and Ang 2 act in an additive manner to induce LOC migration. On this basis, it may be hypothesised that EPCs have different roles with different outcomes depending on the combination of angiogenic signals. For example, in the presence of VEGF and Ang 1 EPCs may contribute to vessel maturation whilst

in the presence of Angiopoietin 2 and VEGF they may mount an angiogenic response similar to that of the embryonic angioblasts in initiating neovascularisation prior to vessel maturation (41). This notion is further supported by Asahara et al (142) who, using corneal micropocket assays, demonstrated that Ang 1 had a maturation effect on VEGF induced neovascularisation. In contrast Ang 2 resulted in vascular destabilisation and sprouting, both of which are required to initiate neovascularisation. This would be consistent with previous findings showing that Ang 2 may collaborate with VEGF at the leading edge of vascular sprouts or anastomotic areas by allowing cells to revert to a more plastic state and hence be more responsive to VEGF sprouting signals (143). These reports would be consistent with our findings that EPCs mobilise in response to VEGF and Ang 2, with both in combination having an additional effect. It is possible that these cytokines, present at the tips of sprouting capillaries, are responsible for the homing of EPCs specifically to these areas. Furthermore, histological examination of Asahara's assays revealed isolated migrating endothelial like cells at the leading tip of capillaries and so speculate the isolated, migrating endothelial like cells seen at these sites are, in fact, EPC that may become involved in anastomosis to other capillary tips or sprouting vessels.

As shown, VEGF and Ang 2 regulate the interaction between EPC and mature EC and following adherence, it is possible that differentiation signals may be induce EPC maturation to an endothelial phenotype. Alternatively, Maisonpierre et al have shown previously that EC continue to undergo apoptosis in the presence of Ang 2, with VEGF conferring an anti-apoptotic effect (41). It could, therefore, be hypothesised that EPCs could have an initial apoptotic effect on mature endothelial cells, hence allowing vessels regression before the modulating effects of VEGF could take place

in inducing EPC differentiation into a more mature endothelial phenotype (39) and induce capillary sprouting and anastomosis.

The positive migratory effect of the Angiopoietins seen in this study suggested Tie-2 expression on LOC. Co-administration of soluble Tie 2 ectodomain with Ang 2 resulted in reduced LOC migration to control levels. These findings would imply an agonist effect of Ang 2 on the receptor. Previous groups have not been able to show evidence of Tie 2 activation by Ang 2 on mature endothelial cells (41). Having found in chapter 3 that LOC express the Tie 2 receptor from an early stage, and that Ang 2 is associated with Tie 2 phosphorylation, this work would suggest that in contrast to endothelial cells, LOC may constitute a state that allows signalling from the Tie 2 receptor following Ang 2 binding.

During the course of this work evidence was published showing Ang 2 could induce auto-phosphorylation of the Tie 2 receptor at high concentrations (42). This work was supported by Mochizuki et al who demonstrated Ang 2 stimulated chemotaxis of mature EC via the PI3 Kinase pathway (43). Several studies have indicated that the activation of the PI3 Kinase/AKT pathway may play an important role in statin induced increase in EPC levels (17, 77).The work in this chapter showed the application of PI3 Kinase inhibitors reduced migration of LOC to control levels implying the Ang 2 induced migration of LOC could utilise a similar down-stream signalling pathway.

It is now established the homotypic interaction between EC is important in new blood vessel formation (32), which would suggest that adhesion between EC and EPCs is fundamental to the development of new blood vessels. Furthermore, one of the initial

steps of homing of EPC would involve their adhesion to EC, activated presumably by cytokines, and the subsequent transmigration of cells through the EC monolayer. This study provides the first evidence that VEGF and the Angiopoietins can stimulate EC:EPC adhesion which would be important for localising progenitor cells to angiogenic endothelial cells allowing them to participate in new vessel formation. LOC demonstrated preferential binding to EC with initial constitutive adhesion seen within 30 minutes over control. However, over 24 hours, VEGF, Ang 1 and 2 were seen to induce further adhesion. This effect occurred only after LOC pre-activation suggesting upregulation or modulation of LOC adhesion molecules. It has been demonstrated Ang 1and 2 regulate endothelial cell adhesion within 30 minutes (144), suggesting LOC, although phenotypically and morphologically more endothelial like as discussed in chapter 3, may employ different signalling pathways in regulating cognate adhesion molecules compared to mature EC. Moreover, soon after this work Iwaguo et al (11) showed VEGF gene transfer enhanced adhesion and incorporation of EPCs into a quiescent endothelial cell monolayer over 24 hours, which would support this speculation.

The Integrins are known to mediate the adhesion of various cell types including haematopoietic stem cells and leucocytes to EC (78) (145). Furthermore, VEGF induced EC adhesion is mediated via an integrin pathway (136). β1 integrins are expressed on EC and haematopoietic cells, whereas β2 integrins (CD18 or ICAM-1) are found preferentially on haematopoietic cells (79) which raised the possibility that the increased LOC:EC adhesion induced by VEGF could be mediated by an integrin mechanism. Indeed, inhibition of the integrin with the RGD peptide blocked

VEGF induced EPC migration. However, the incomplete suppression of adhesion suggests other mechanisms may be involved.

ICAM-1 is a member of the immunoglobulin family. It has recently been shown that VEGF can up-regulate ICAM-1 through the PI3 Kinase/AKT pathway resulting in migration of mature endothelial cells (124) and can mediate the adhesion of haematopoietic progenitor cells (80, 146). This study shows the β2 integrins are required for VEGF induced adhesion of LOC to EC. This may represent a secondary mechanism involved in the increased adhesion that is seen in the presence of VEGF. The work detailed here shows heterotypic adhesion between mature endothelial cells and LOC. Furthermore, Ang 2 and VEGF can directly support LOC:EC adhesion, the latter of which is in part mediated by the integrins and ICAM-1. Studies have shown the homing of progenitor cells to different tissues is dependant on distinct adhesion molecules (147). It is conceivable that LOC at different stages in their differentiation pathway may use distinct adhesion molecules or mechanisms for homing to angiogenic sites. Further studies into the exact molecular basis by which VEGF enhances this upregulation of adhesion molecules are warranted. Overall, the recruitment and incorporation of endothelial progenitor cells requires a coordinated sequence of multi-step adhesive and signalling events including adhesion and migration (e.g., by integrins), chemoattraction (e.g., by VEGF and the Angiopoietins), and finally the differentiation to endothelial cells.

As VEGF upregulation coincident with Ang 2 expression at leading tips of sprouting angiogenic capillaries and areas of vessel co-option, suggest it may be plausible these factors are responsible for recruiting EPCs to these areas and subsequently maybe

regulators in the involvement of EPCs in the formation of new vessels or anastomosis of developing vessels with this mechanism being mediated, in part, by the integrins and ICAM-1. Further work is necessary to determine the molecular basis of this effect.

Despite limitations, the findings in this study provide potential insights into the mechanism for EPC uptake in areas of angiogenesis in the in vivo studies detailed in chapter 1.

CHAPTER 5

CD14 EXPRESSING CELLS RESPOND TO ANGIOPOIETIN-2

5.1. INTRODUCTION

Asahara et al reported isolation of EPCs from purified CD34+ populations (51) and subsequently that putative EPCs could be grown from non-purified total peripheral blood mononuclear cell populations under endothelial growth conditions (19, 76, 148). During the course of this work, increasing evidence emerged suggesting EPCs were not a single cell population but a more heterogeneous population including monocytes expressing endothelial cell characteristics (82, 94, 149). Asahara's group subsequently generated similar EPCs from the non-selected human peripheral blood monocytes and after 7 days in culture over 90% of these cells expressed CD14, a monocyte marker, in addition to expressing flk-1, VE-Cadherin and CD31 (150). Among the CD14+ cells that do take on an endothelial phenotype, two distinct phenotypes have been described: late and early outgrowth cells (53, 64). Gulati et al defined this subpopulation further by showing early outgrowth endothelial progenitor cells were derived from CD14+ population whist late outgrowth endothelial progenitor cells were derived from the CD14-population of monocytes, with the latter exhibiting capacity for capillary morphogenesis (95).

It is established the angiogenically active microvasculature can progress down one of two routes; regression or remodelling (41, 140, 151, 152). Ang 2 has been shown to be induced in the endothelium of vessels undergoing active remodelling (41, 140, 153) which led to the suggestion Ang 2 could play a key role in vessel destabilisation. Furthermore, Maisonpierre et al suggested EC would continue to undergo apoptosis in the presence of Ang 2, however, the presence of VEGF is a

critical regulator of this, offering a anti-apoptotic protection at high concentration and pro-apoptotic effects at low concentration (151) .

Given the evidence that CD14+ cells represent the early outgrowth cells as described in chapter 3, and can go on to assume endothelial characteristics, they would be the primary cells recruited from the blood to sites of angiogenesis and interact with the endothelium to undergo angiogenesis. The aim of this study is to test the ability of angiogenic factors VEGF and the Angiopoietins as described in chapter 4, to stimulate migration and adhesion of these cells from whole blood PBMNC and determine a mechanism of interaction of these cells with endothelium..

5.2 RESULTS

5.2.1 Angiopoietin 2 stimulates Peripheral Blood Mononuclear Cell migration.

Having identified the early expression of Tie-2 on putative EPCs in chapter 3 and shown in chapter 4 that Ang 2 is a chemoattractant in inducing LOC migration this phenomenon was utilised to determine if a sub-fraction of cells from the whole peripheral blood mononuclear cell (PBMNC) population could be sub-selected, by Ang 2 induced migration, that would later develop LOC phenotype.

Initial experiments were performed to determine any chemoattractant effect of Angiopoietin 2 on the heterogeneous population of PBMNC. Boyden chamber experiments, as described in chapter 4, were repeated using PBMNC isolated by density gradient centrifugation. 2 X 10^5 PBMNC were resuspended in migration medium and seeded into the upper chamber. Angiopoietin 2 at a concentration of 200ng/ml in migration medium was added to the lower chamber.

The experiment was terminated at 6 hours. The count of migrated cells on the lower chamber had reached a 5.5 (range 5.2-5.7) fold increase over control. Angiopoietin 1 showed no detectable chemoattractant effect over the control at six hours.

These results are shown in figure 5.1.

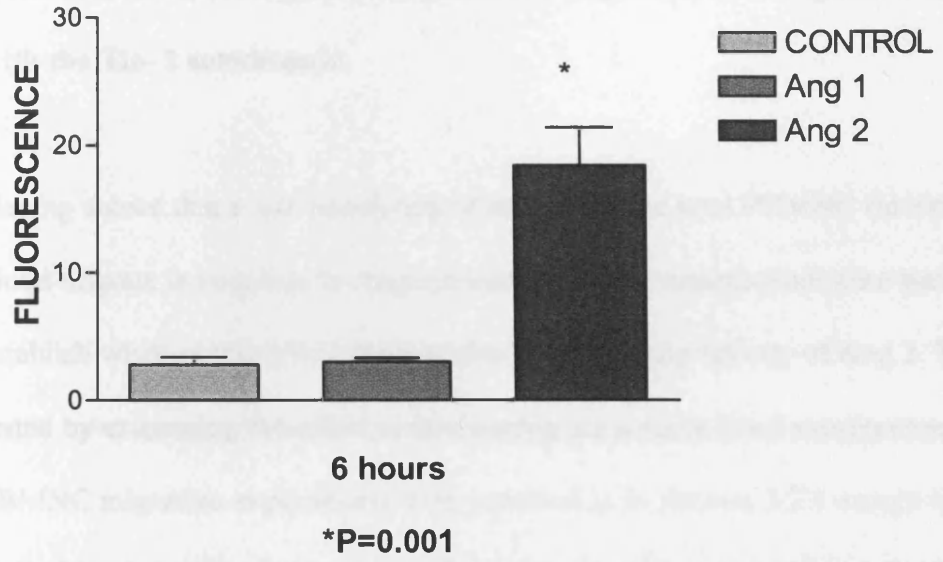


Figure 5.1. Angiopoietin 2 induces migration of peripheral blood mononuclear cells

PBMC were isolated from buffy coat using density gradient centrifugation. Cells were counted and examined for viability using trypan blue exclusion. 200,000 cells were added to the upper well of a modified Boyden chamber with cell culture inserts containing 8μM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added either, 750μl migration medium (control), 750μl migration medium containing 100ng/ml of Angiopoietin 1 or 200ng/ml Angiopoietin 2. After 6 hours, as discussed in chapter 4, the cell culture insert was fixed in 70% ethanol at -20^0C for 1 hour, cells removed from the upper aspect of the membrane. The lower side of the membrane was stained using haematoxylin and eosin before mounting on slides. Migratory cells where counted at 40X magnification. The average of five fields was calculated. Error bars show standard errors of the mean .Data is presented from three independent experiments as mean and SEM, $P<0.0001$ for agonist treated cells compared to controls.

5.2.2. Inhibition of Angiopoietin 2 induced migration by competitive inhibition with the Tie- 2 ectodomain.

Having shown that a sub-population of cells from the total PBMNC fraction of whole blood migrate in response to Angiopoietin 2, further experiments were performed to establish whether this effect required the Tie-2 binding activity of Ang 2. This was tested by examining the effect of introducing the soluble Tie 2 ectodomain. PBMNC migration experiments were repeated as in Section 5.2.1 except that in some wells 20μg/ml of Tie 2 ectodomain/Fc fusion protein was included in the lower chamber. The Tie 1-Fc fusion protein was used as control.

Inclusion of rhTie 2 –Fc chimera resulted in a total loss of Angiopoietin 2 mediated migration. As shown in figure5.2, Angiopoietin 2 increases PBMNC migration by 6 fold in the control group, this effect been blocked by the Tie 2 –Fc chimera resulting in a migration rate similar to basal level. This data confirms that the Tie 2-binding activity of Ang 2 is required for the effect of the ligand on migration.

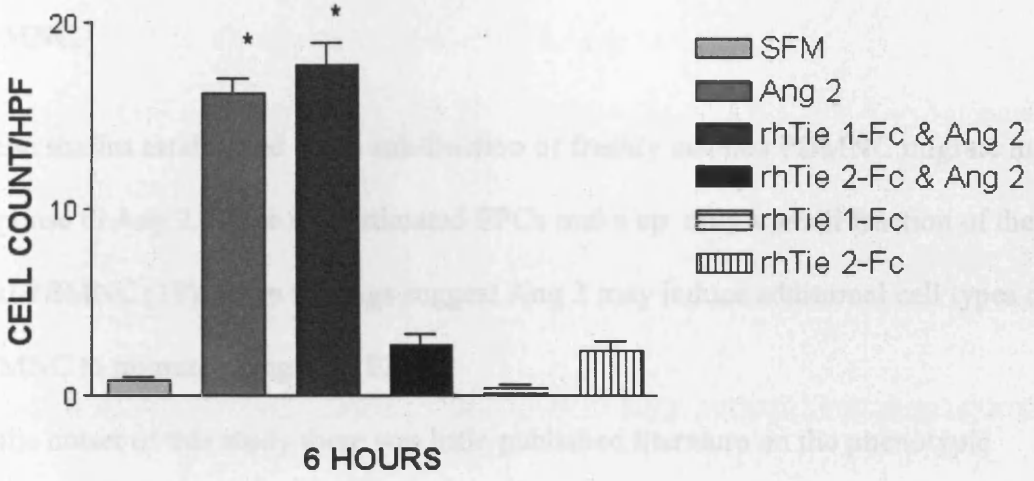


Figure 5.2. Angiopoietin 2 induced migration is blocked with the Tie-2 ectodomain.

Experiments were performed to examine the effects of competitive inhibition with the Tie-2 ectodomain. $2x10^5$ freshly isolated PBMNC were added to the upper well of a modified Boyden chamber. To the lower chambers were added migration media alone or in combination with Angiopoietin 2. In some wells, Ang 2 was added in the presence of 20μg/ml Tie-2/Fc or Tie1/Fc-ectodomain. After 6 hours at 37^0C the membranes were fixed in ethanol for 1 hour at -20^0C. Following this cells were stained with haematoxylin and eosin on the lower end only. Migrated cells were counted at 40X magnification. An average of five high power field counts were calculated and the results shown in figure 5.2.Data is presented from three independent experiments as mean and SEM, *P<0.0001 compared with migration in the absence of ligand and fusion proteins.

5.2.3. Angiopoietin 2 acts as chemoattractant to the CD14+ sub- population of PBMNC.

These studies established that a sub-fraction of freshly isolated PBMNC migrate in response to Ang 2. Since it is estimated EPCs make up only a small fraction of the total PBMNC (19) ,these findings suggest Ang 2 may induce additional cell types of PBMNC to migrate along with EPCs.

At the outset of this study there was little published literature on the phenotypic overlap of endothelial cells, monocytes and angioblasts. During the course of this work, however, evidence was published which led us to consider the idea that a subset of CD14+ monocytes within the PBMC population could be the migrating cell type. Firstly, it has been demonstrated CD14+ cells can develop an endothelial like phenotype in culture (95, 150, 154, 155). Secondly, these cells incorporate into the neovasculature in models of ischaemia and contribute to collateral vessel formation (82, 150).

A series of experiments were conducted to determine the phenotype of the cells on the upper and lower surface of the transwell membrane filter following Ang 2 induced migration. Migration experiments were repeated as in section 5.2.1.

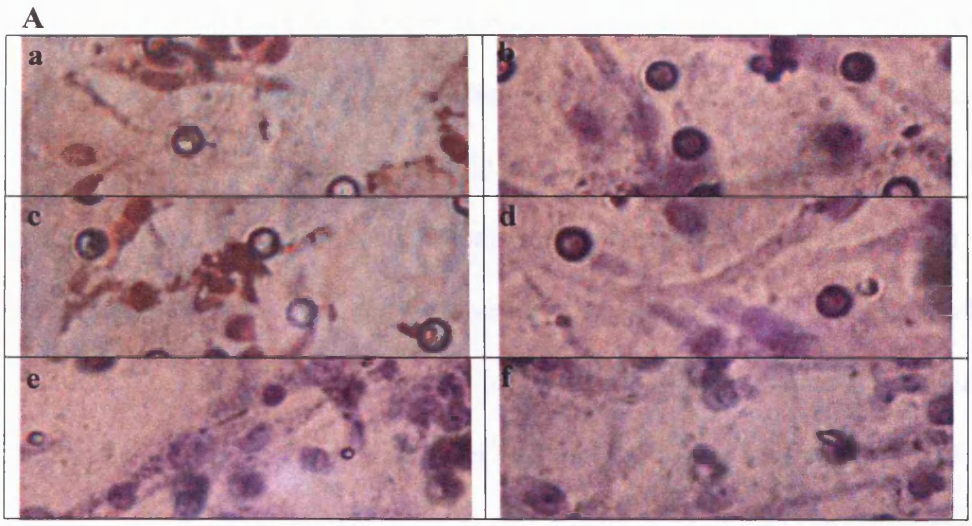
The filters were removed at termination of the experiment, washed and cells on the surface not under investigation removed by careful wiping and washings. The filters were then fixed and immunolabelled with monoclonal antibodies to CD14, CD34 and Tie-2. 32% of cells on the upper and lower membranes stained positively for CD14. Incubation with relevant secondary antibody alone yielded no labelled cells.

These results are shown in figure 5.3.

The ratio of CD34+ cells on the upper surface to the lower surface was also 1:1, however, only 2% of cells stained positively for CD34.

In comparison, immunotyping for Tie 2+ cells revealed a ratio of 1:6.5 on the upper membrane compared to the lower.

These results imply that a subset of CD14+ and Tie 2+ cells migrate in response to Ang 2.



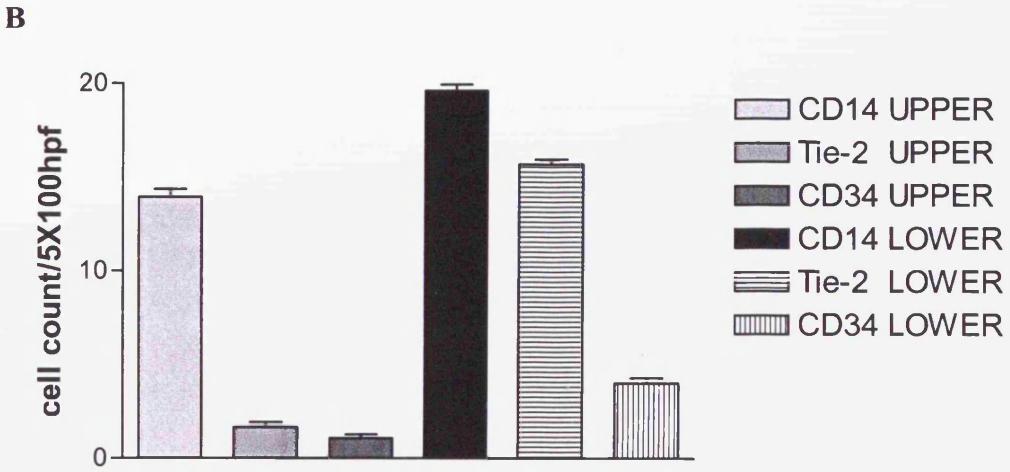


Figure 5.3 Immunocytochemistry of transwell membranes

Experiments were performed to determine the phenotype of the cells present on the upper and lower surface of transwell membranes following Ang 2 induced migration of whole PBMC. 2 X 10^5 cells were added to the upper well of a modified Boyden chamber with cell culture inserts containing 8μM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added either, 750μl migration medium (control),

750µl migration medium alone or containing 200ng/ml Ang 2. After 6 hours, the cell culture insert was fixed in 70% ethanol at -20°C for 1 hour. Following fixation the upper or lower membrane, depending on the side under investigation, was vigorously wiped to remove adherent cells. Membranes were then incubated with the relevant blocking reagent for one hour followed by the relevant monoclonal antibody (as described in Material and Methods) for one hour and corresponding secondary antibody for one hour. DAB staining was then performed and cells stained with haematoxylin. Cells were counted at 40X magnification. **(A)** are representative photomicrographs. **a** represents CD14 staining on the lower membrane with **b** the corresponding secondary antibody alone. **c** represents Tie-2 staining on the lower membrane surface with **d** the corresponding secondary antibody alone. **e** represents CD34 staining on the lower membrane surface with **f** showing secondary antibody alone. **(B)** Show corresponding positively stained cell counts demonstrating CD14+ /Tie 2+ cells migrate through the transwell membrane. The average of five high power fields was calculated. Data is presented from three independent experiments as mean and SEM.

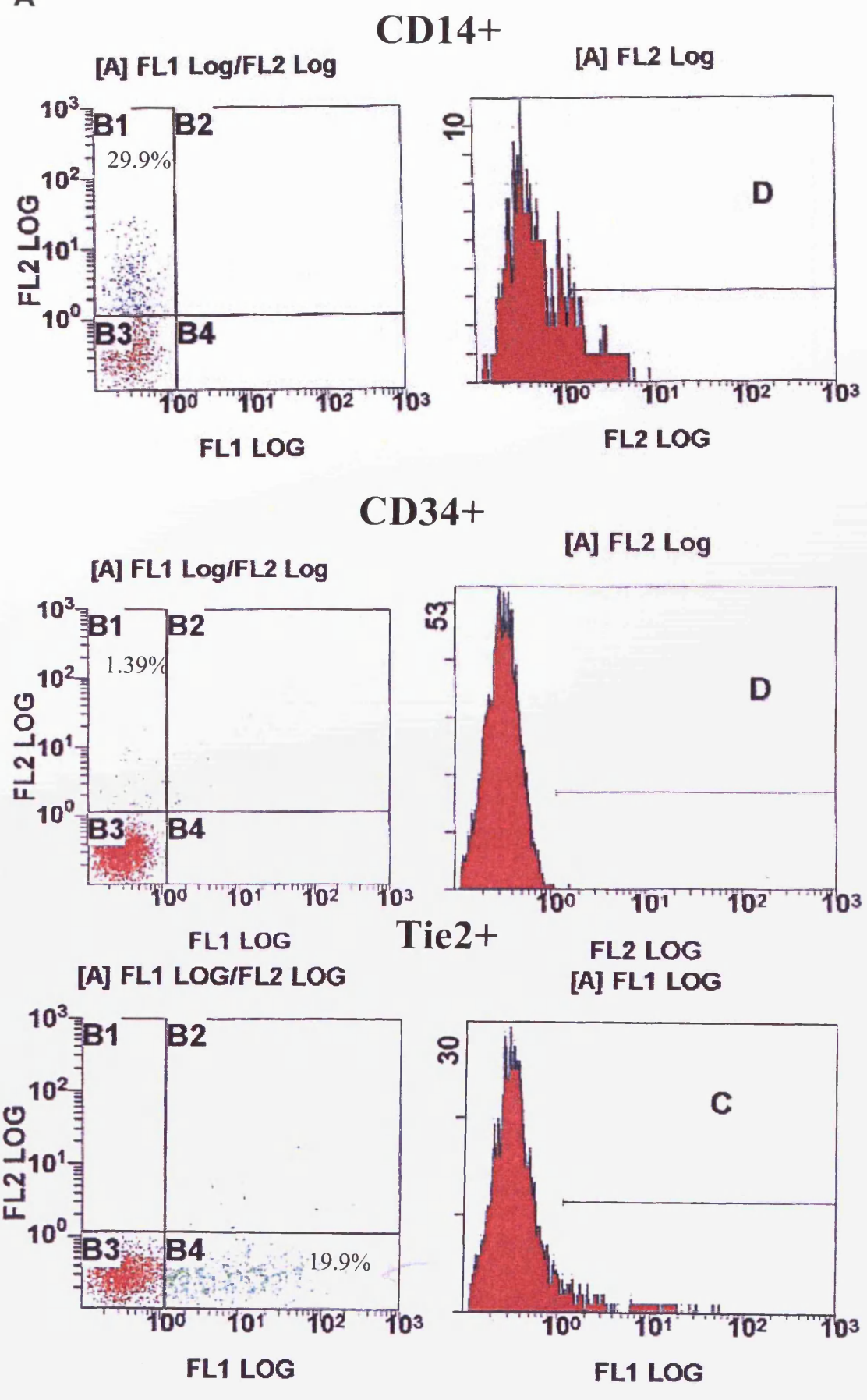
5.2.4 CD14+ cells co-express CD34 and the Tie- 2 receptor

In order to confirm the expression of CD34, CD14 and Tie-2 within the whole PBMC population and to further characterise this subpopulation of cells, flow cytometry analysis was performed as described in the Material and Methods chapter. Representative flow cytometry traces and quantitative results are shown in Figure 5.4 A. CD14 was expressed in 35.6% of cells whilst the CD34+ antigen was expressed in 1.03% of the whole PBMC population. The Tie 1 and Tie 2 receptor was found expressed on 3.1and 14.75% of cells respectively. These results can be seen in figure 5.4 B.

To determine the expression of Tie 2 in Tie 1, CD34 or CD14 populations, dual label flow cytometry was performed as described in the Material and Methods. As shown in Figure 5.5, 99% (range 98-100) of Tie-1+ cells co-expressed Tie -2. Furthermore, 3.01 % of cells co-expressed Tie-2 and CD34. Surprisingly, it was found 12.08% of Tie-2 cells also co-expressed the CD14 antigen. Examples of dual stained cells are shown in figure 5.5C and D.

Taken together these data show a sub-population of CD14 expressing cells in PBMNC that also express Tie 2.

A


CD14+
[A] FL1 Log/FL2 Log
B1
B2
29.9%
B3
B4
FL2 LOG
FL1 LOG
[A] FL2 Log
D
FL2 LOG
CD34+
[A] FL1 Log/FL2 Log
B1
B2
1.39%
B3
B4
FL2 LOG
FL1 LOG
[A] FL2 Log
D
FL2 LOG
Tie2+
[A] FL1 LOG/FL2 LOG
B1
B2
B3
B4
19.9%
FL2 LOG
FL1 LOG
[A] FL1 LOG
C
FL1 LOG

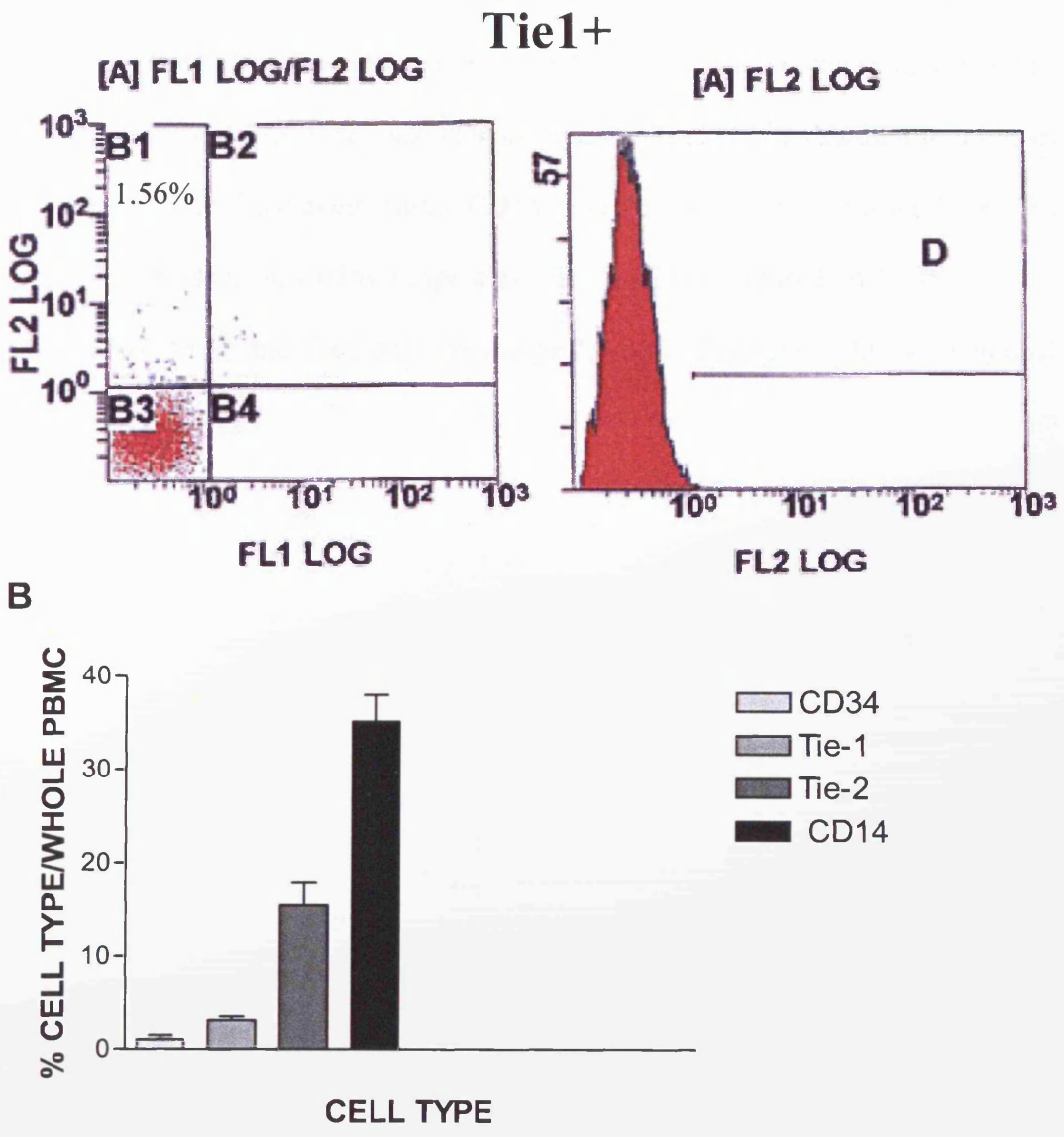
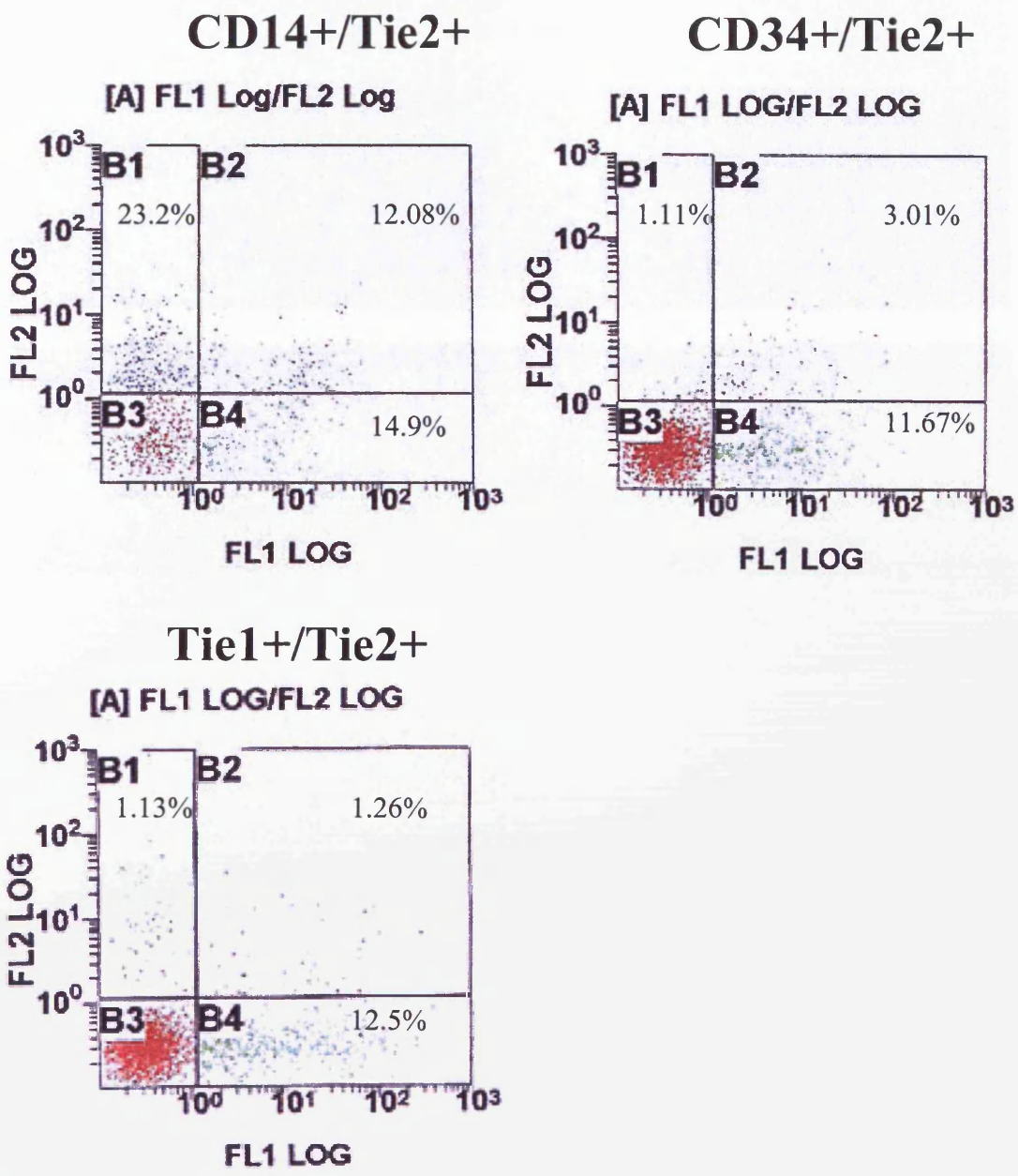


Figure 5.4. Expression of CD14, CD34 TIE and TEK on peripheral blood mononuclear cells

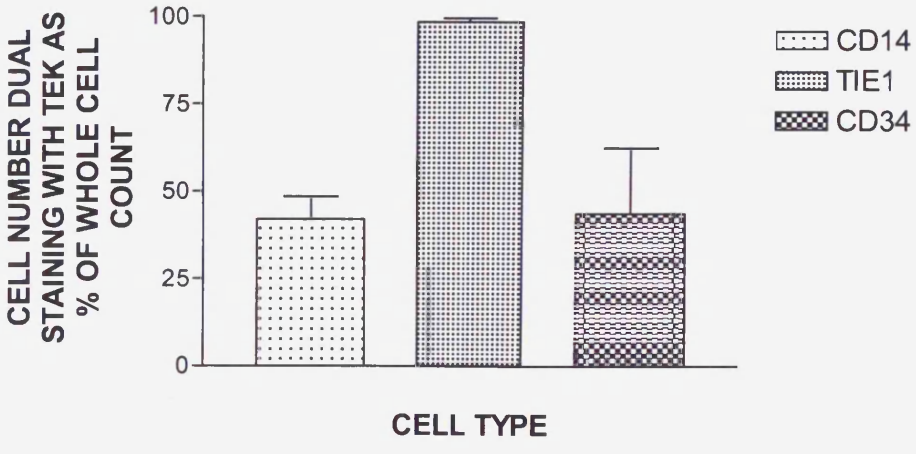
Peripheral blood mononuclear cells were isolated using density gradient centrifugation and $2x10^5$ cells incubated with monoclonal antibodies against CD14, CD34, Tie-1 and Tie-2 ectodomain. Quantitative evaluation of the whole PBMNC population was determined by flow cytometry using a combination of Cy3 labelled monoclonal antibodies against the extracellular domain of CD14, CD34, Tie-1 and

FITC monoclonal antibody against Tie-2 ectodomain. The number of positive cells was compared to isotype control. Representative results are shown in figure A. Figure B histogram from FACS data showing the average results of individual staining and is representative of three experiments. CD14 is expressed on 35.6% (range 29.9- 39.9), CD34 is expressed on 1.03% (range 0.1- 1.6), Tie-2 is expressed on 14.75 (range11.67-19.9) and TIE1 on 3.1% (range 2.7-3.9) of peripheral blood mononuclear cells.

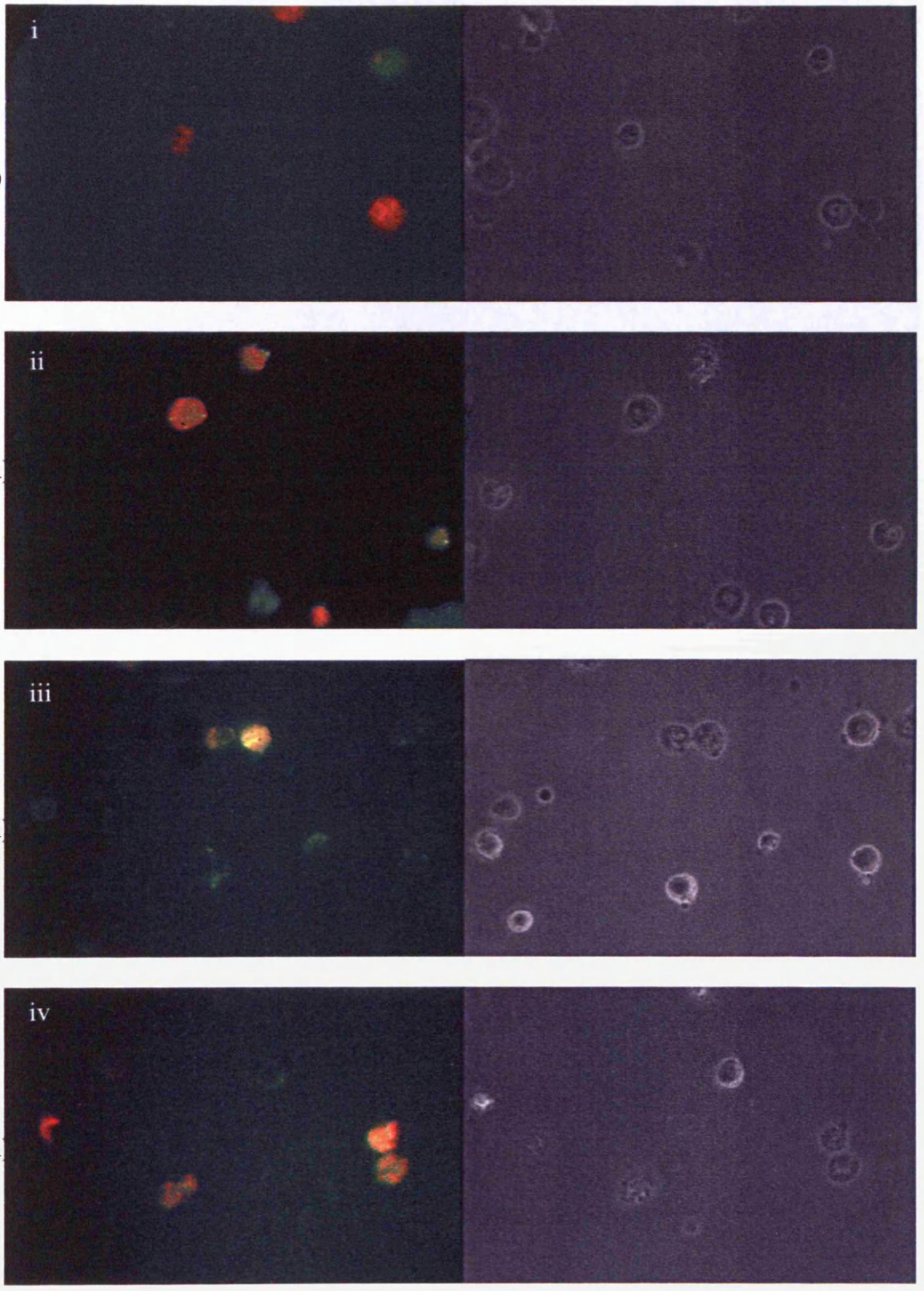
A


CD14+/Tie2+
[A] FL1 Log/FL2 Log
B1
B2
B3
B4
23.2%
12.08%
14.9%
FL2 LOG
FL1 LOG
CD34+/Tie2+
[A] FL1 LOG/FL2 LOG
1.11%
3.01%
11.67%
Tie1+/Tie2+
[A] FL1 LOG/FL2 LOG
1.13%
1.26%
12.5%

B


CELL NUMBER DUAL STAINING WITH TEK AS % OF WHOLE CELL COUNT
100
75
50
25
0
CD14
TIE1
CD34
CELL TYPE

C


i
CD14 (red)
Tie-1 (green)
ii
CD14 (red)
Tie-2 (green)
iii
Tie-1 (red)
Tie-2 (green)
iv
CD34 (red)
Tie-1 (green)

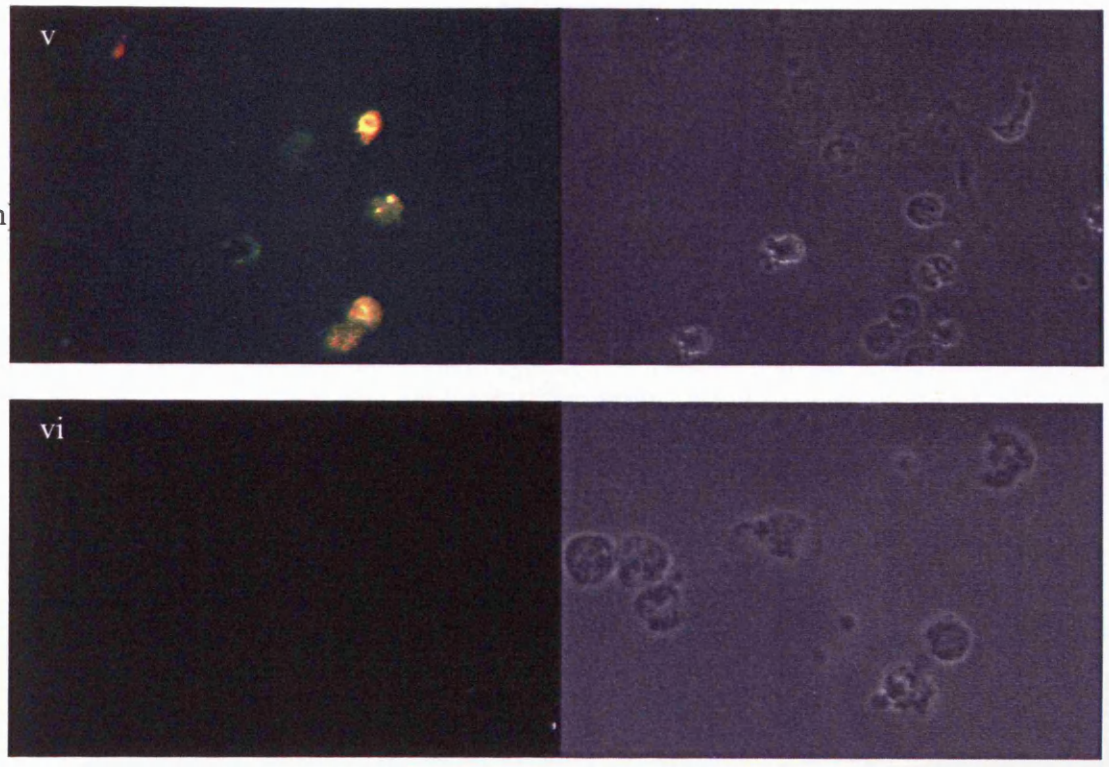


Figure 5.5. Co-expression of CD14, CD34 TIE and TEK on peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated using density gradient centrifugation and $2x10^5$ cells incubated with monoclonal antibodies against CD14, CD34, Tie-1 and Tie-2 ectodomain. Quantitative evaluation of the whole PBMNC population was determined by flow cytometry using a combination of Cy3 labelled monoclonal antibodies against the extracellular domain of CD14, CD34, Tie-1 and FITC monoclonal antibody against Tie-2 ectodomain. The number of positive cells was compared to isotype control. Representative results are shown in figure **A**. Figure **B** Histogram from FACS data shows the average results of dual staining and is representative of three experiments. Data is shown as mean +/- SEM for three independent experiments. Figure **C** Representative fluorescent photomicrograph of cultures of freshly isolated PBMC subjected to immunocytochemistry. Representative fluorescent photographs (X40 magnification) of cultures of freshly isolated PBMC

with equivalent phase contrast images below. CD14+/34+ cells stain red (Cy3 conjugated CD14/34 MoAB), whilst Tie-2+ and Tie-1+ cells stain green (FITC conjugated MoAB). Dual stained cells are represented in yellow. i) CD14 & Tie-1 ii) CD14 & Tie-2 iii) Tie-2 & Tie-1 iv) Tie-2 & CD34 v) CD14 & CD34 vi) Control secondary antibody only representative image for PE, Cy3, FITC.

5.3. CD14+ CELLS AND ANGIOGENESIS

Data presented in Sections 5.1 and 5.2 indicate a population of CD14+/ Tie 2 + cells and that some CD14+ cells have .the capacity to migrate in response to Ang 2. CD14+ cells may also be recruited for vasculogenesis (82, 94, 95, 149). Therefore to investigate CD14+ cells in more detail, further experiments were performed on the CD14+ population within PBMNC.

5.3.1. Isolation of CD14+ cells and characterisation

CD14+ cells were isolated according to published methods (18) as described in the Materials and Methods chapter. Immunofluorescence of isolated cells was performed to confirm phenotype. Cells were counted as described in the materials and methods. As seen in Figure 5.5, 90.3% of cells isolated expressed CD14 antigen. Indirect immunocytochemistry was performed with FITC conjugated Tie-2 monoclonal antibody in conjunction with TRITC labelled CD14 monoclonal antibody. Dual staining was noted in 39.7 % of cells. 99.5% of Tie-2 expressing cells co-expressed CD14. Results are shown in figure 5.5.

These results suggest the CD14+/Tie-2 + cellular subset described in section 5.1.4 was present within this isolated population. Cell viability was confirmed using trypan blue exclusion.

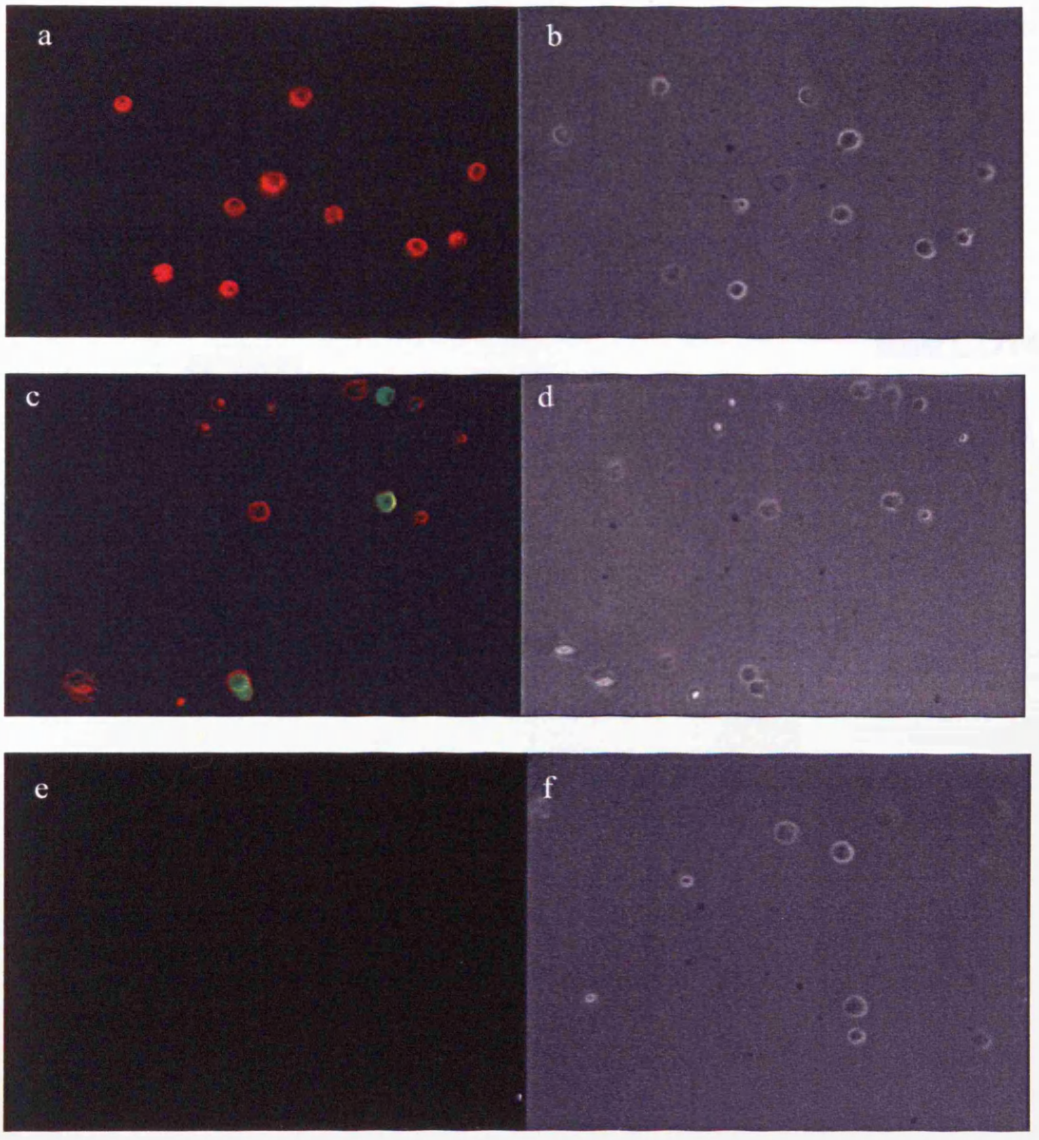


Figure 5.6. Isolated CD14+ cells co-express the Tie-2 receptor.

CD14+ cells were isolated using standard method as described in the Material and Methods. Cell surface expression of CD14 and Tie-2 was assessed by indirect immunofluorescence. Fig 5.6a demonstrates representative fluorescence photomicrographs of CD14 staining within the whole PBMC population 20X magnification) with corresponding phase contrast b. Figure 5.6c represents dual staining for Cy 3 conjugated CD14+(red staining) and FITC conjugated Tie-2+ cells (green staining). Figure 5.6d represents the corresponding phase contrast photograph 20X magnification). Figure 5.6e shows control secondary antibody only

representative for PE-Cy3 and FITC with Figure 5.6f representing the phase image of e.

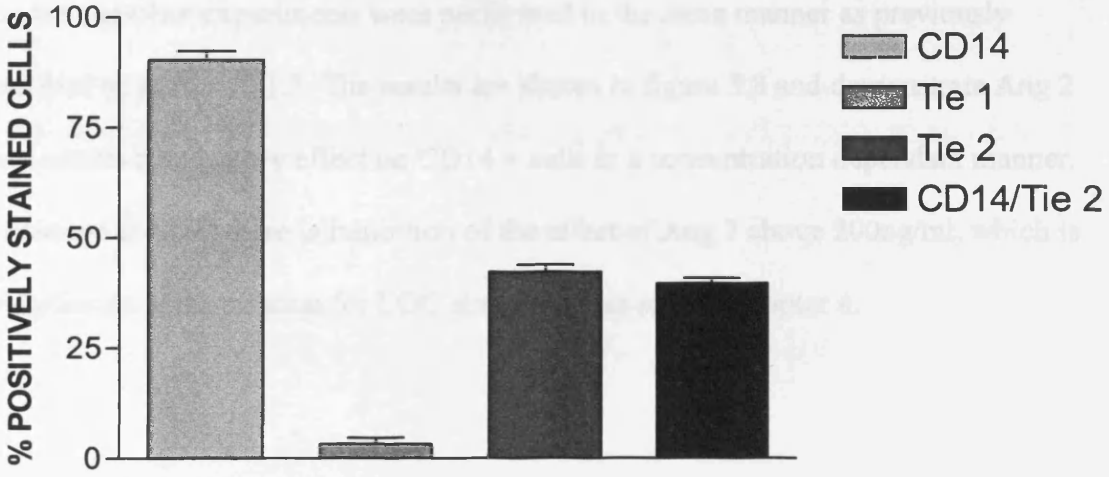


Figure 5.7. Expression pattern of the surface markers on isolated CD14+ cell.

Cells were counted from 10 random high power fields (40X magnification) and the percentage of fluorescent cells calculated. 90.3% (range 83.3-95%) of isolated cells are CD14+ using indirect immunofluorescence. Tie-2 was expressed in 39.7% (range 36-42.1%) of these cells. Data is presented from three independent experiments as mean and SEM.

5.3.2. Angiopoietin 2 stimulates the migration of isolated CD14+ cells in a concentration dependant manner

Further experiments were performed to establish whether CD14+ isolated cells migrated in response to Ang 2 as demonstrated in the whole PBMNC population. Boyden chamber experiments were performed in the same manner as previously described in section 5.1.3. The results are shown in figure 5.8 and demonstrate Ang 2 has a positive migratory effect on CD14 + cells in a concentration dependant manner. In contrast to LOC there is inhibition of the effect of Ang 2 above 200ng/ml, which is the optimum concentration for LOC stimulation as seen in chapter 4.

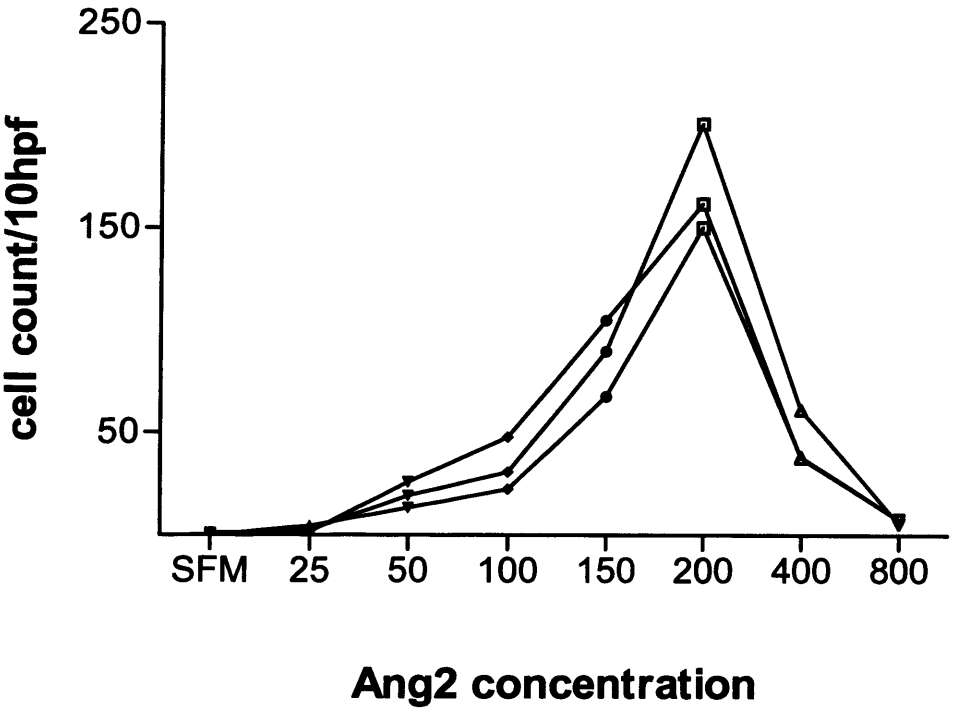


Figure 5.8. Angiopoietin 2 stimulates migration of isolated CD14+ cells in a concentration dependant manner.

Migration was measured using a modified 24 well Boyden chamber with cell culture inserts containing 8μM pores (Becton Dickinson, Cowley, UK). To the lower chamber was added either, 750μl migration medium, 750μl migration medium containing 25,50,100,150 200,400 or 800ng/ml Ang 2. To the upper chamber were added $0.75x10^5$ CD14+ cells in 500μl of migration medium (concentrations as determined in chapter 4).. The cell culture insert was fixed in 70% ethanol at -20°C for 1 hour and then the lower part of the membrane was stained using haematoxylin and eosin before mounting on slides. Migratory CD14+ cells were counted at 40X magnification only cells that complete nuclei were counted and an average taken from ten high power fields of view used. Data is presented from three independent experiments as mean and SEM.

5.3.3. Inhibition of Angiopoietin 2 induced migration by the Tie- 2 ectodomain.

To determine if the Ang 2 mediated migration of CD14+ cells required Tie 2 binding site of the ligand, migration experiments were repeated in the absence and presence of the Tie- 2 ectodomain/Fc fusion protein (concentrations as determined in chapter 4). The Tie- 1 Fc fusion protein was used at the same concentration for control.

As shown in figure 5.9, Tie 2-Fc inhibited any increase in migration in response to Ang 2. In contrast the Tie 1/Fc failed to inhibit Ang 2 stimulated migration of CD14+ cells above basal levels. This data demonstrates the soluble Tie-2 ectodomain binding of Ang 2 reduces the ability of the ligand to stimulate migration suggesting any Ang 2 induced migration is dependant on the ability of the Tie-2 ectodomain binding site on Ang 2.

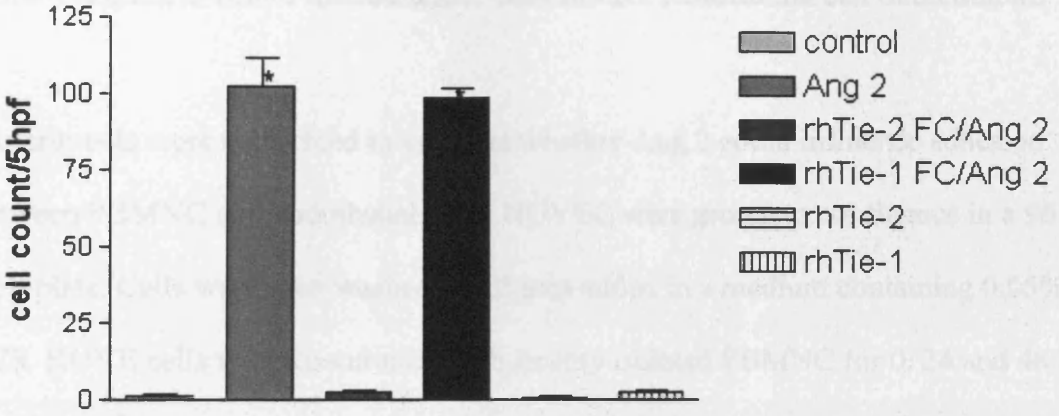


Figure 5.9. Angiopoietin 2 induced migration is blocked with the Tie- 2 ectodomain.

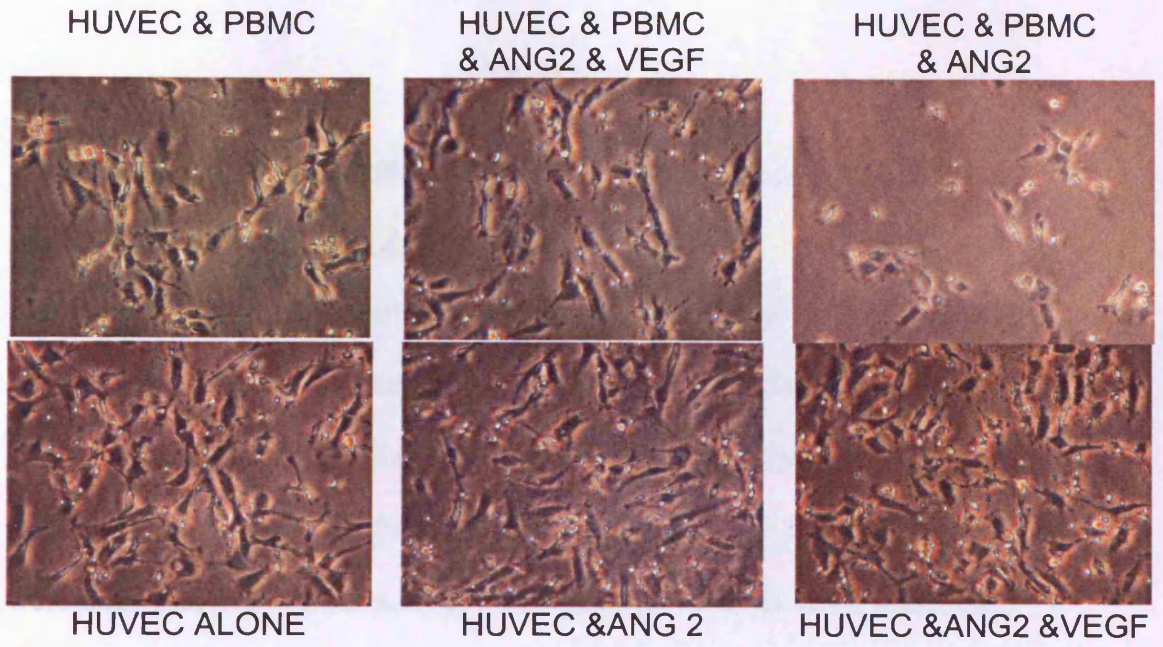
Experiments were performed to examine the effects of competitive inhibition with the Tie- 2 ectodomain. $0.75X10^5$of isolated CD14+ cells resuspended in 500µl of migration medium and seeded into the upper well of the Boyden Chamber. In the lower chamber 200ng/ml of Angiopoietin 2 with either Tie 2/Fc or the Tie 1/Fc (control) ectodomain at a concentration of 20µg/ml were added to the same medium having been pre-incubated at room temperature for 30 minutes. Inserts were removed at 6 hours, washed, stained and 10 high power fields counted. Similar results were obtained from three independent experiments. Data is presented from three independent experiments as mean and SEM,* $P<0.001$ compared with migration the absence of ligand and fusion proteins using Student t-test.

5.3.4. Peripheral blood mononuclear cells induce endothelial cell detachment.

Experiments were performed to examine whether Ang 2 could influence adhesion between PBMNC and endothelial cells. HUVEC were grown to confluence in a 96 well plate. Cells were then washed, cytokines added in a medium containing 0.05% FCS. HUVE cells were co-cultured with freshly isolated PBMNC for 0, 24 and 48 hours at 37^0C, 5% CO_2. Surprisingly, as shown in Figure 5.10, PBMNC caused dissociation of endothelial cells from the matrix with the maximum effect seen at 24 hours, until at 48 hours nearly all cells had detached.

Further experiments were performed to assess the effect of VEGF and Ang 2 on PBMC induced endothelial cell dissociation. Co-culture experiments were repeated adding VEGF at 10ng/ml and Ang 2 at 200ng/ml to the culture medium. Angiopoietin 2 potentiates the PBMNC induced endothelial cell loss by 60.8 % at 24 /hours ($P<0.0001$). VEGF conferred some protection against Angiopoietin 2 enhanced detachment with EC dissociation reduced only by 2.4 % ($P<0.001$) compared to control at 24 hours. These data are shown in figure 5.10B.

Trypan blue exclusion showed 90% of detached HUVEC were non-viable and therefore detachment was a result of, or resulted from, endothelial cell death.


HUVEC & PBMC
HUVEC & PBMC & ANG2 & VEGF
HUVEC & PBMC & ANG2
HUVEC ALONE
HUVEC &ANG 2
HUVEC &ANG2 &VEGF

A

B

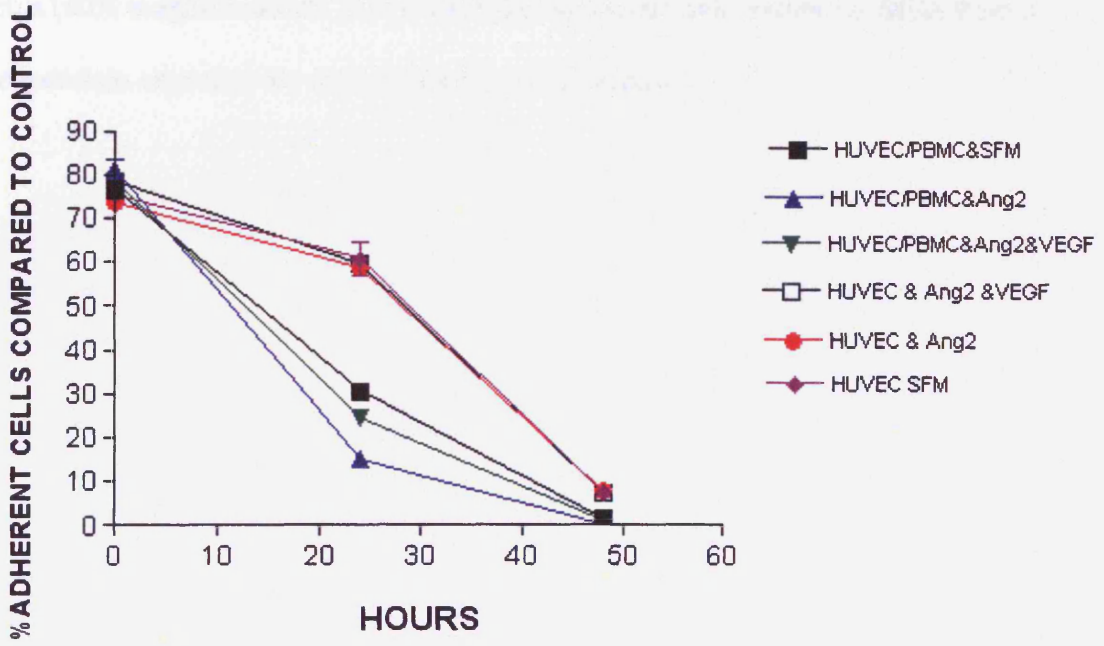

% ADHERENT CELLS COMPARED TO CONTROL
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
HOURS
HUVEC/PBMC&SFM
HUVEC/PBMC&Ang2
HUVEC/PBMC&Ang2&VEGF
HUVEC & Ang2 &VEGF
HUVEC & Ang2
HUVEC SFM

Figure 5.10 : PBMNC suppress endothelial cell adhesiveness, the effect being potentiated by Angiopoietin 2.

Density gradient centrifugation was used to isolate PBMNC. HUVEC (passage 2) were grown to 90% confluence in 6 well plates medium removed, cells washed, and serum free medium added alone or with VEGF(10ng/ml) and Ang 2 (200ng/ml) or both in combination. $1x10^3$ PBMNC/well were plated onto the HUVEC. Cultures were incubated at 37^0C and adherent cells counted at 12, 24 or 36 hours. **(A):** Representative phase contrast photomicrographs of co-cultures 12 hours. **(B):** Quantification of HUVEC dissociation in the presence of PBMNC and VEGF/Ang 2. Non-adherent and remaining adherent cells were counted from 5 random high power fields (40X magnification). This data represents mean cell counts +/- SEM from 3 independent experiments each performed in triplicate.

5.3.5. CD14+ cells induce endothelial cell detachment.

As already discussed, the PBMNC is a heterogeneous population of cells inclusive of circulating natural killer cells. Initial experiments in section 5.2.2 established a functional interaction between CD14+ cells and Angiopoietin 2. This section of work aimed to establish whether CD14+ cells within the PBMNC population could be responsible for the observed effects on EC dissociation.

CD14+ cells were isolated from PBMNC as described in section 5.2.
Co-culture experiments were repeated, in the same manner as in section 5.2.3, this time using freshly isolated CD14+ cells. Figure 5.11 is representative of three independent experiments and show CD14+ cells induce endothelial cell loss in a time and CD14+ cell number dependent manner. 1 $x10^2$ CD14+ cells/well produced a reduction in adherent HUVEC number of 41.7% at 12 ($n=3$, $P<0.009$ paired 't'-test) and 66 % at 24 hours ($n=3$, $P<0.001$ paired 't'-test).

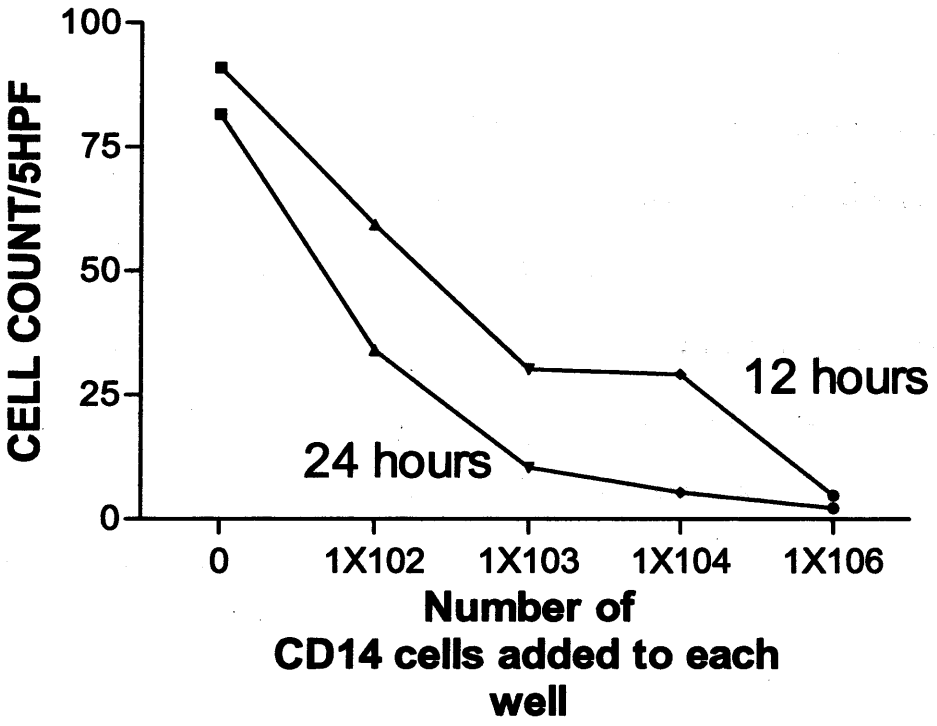


Figure 5.11: Endothelial cell loss following co-culture with isolated CD14+ cells is time and cell number dependent.

HUVEC (P2) were grown to 90% confluence in 6 well plates. Freshly isolated and washed CD14+ cells were added to serum starved HUVEC (for 2 hours) in varying quantity. Cultures were incubated at 37^0C for 12 and 24 hours when dissociated cells were removed and the number of remaining adherent cells counted. Five random fields were counted. Cell viability was determined using trypan blue exclusion. CD14+ cells induced detachment of HUVE cells in a concentration and time dependant manner. From these experiments $1ix10^2$ cells and 24 hour incubations were used. Data is shown for a single experiment and is representative of three.

5.3.6. Effects of Angiopoietin 2 on CD14+ cell effects on endothelial cell detachment.

Having established Ang 2 stimulates CD14+ migration and, furthermore, CD14+ cells induce endothelial loss, experiments were performed to determine whether Ang 2 could potentiate the effect of CD14+ on HUVEC.

Co-culture experiments using freshly isolated CD14+ cells were performed as previously described (section 5.2.5). $1x10^2$ CD14+ cells were seeded onto a 90% confluent lawn of HUVEC in medium 19-9 supplemented with FCS in the presence or absence of Ang 2 at 200ng/ml and /or VEGF at 10ng/ml.

Figure 5.11 shows Angiopoietin 2 has a non-significant effect of CD14+ cells on HUVE loss with a reduction in adherent cell count by 6.73 % ($n=3$, $P< 0.007$). VEGF confers some EC protection with a reduction in adherent cell count of only 28.2% ($n=3$, $P< 0.001$). This VEGF effect was maintained in the presence of Ang 2 with no significant increase in endothelial cell dissociation.

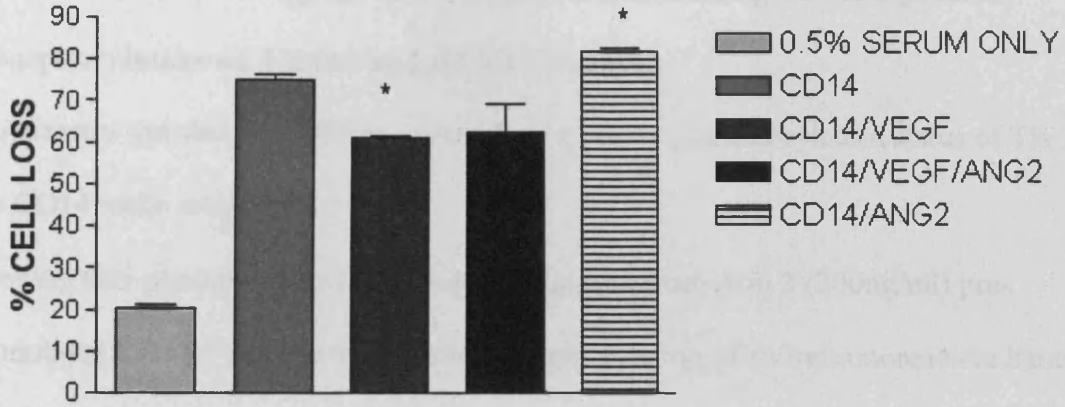


Figure 5.12. Effect of Ang 2 and VEGF on CD14+ dependent endothelial cell dissociation.

Co-culture experiments were repeated in the presence of CD14+ and Ang 2 . The average of 5 random fields was calculated and is shown as a percentage of the number of adherent HUVE cells compared to serum free monoculture. Data is presented from 5 independent experiments as mean and SEM. * < P0.001 compared with adherent cells in the absence of cytokines.

5.3.7. The Effect of Angiopoietin 2 (A2) pre-conditioning on Tie-2 protein phosphorylation on CD14+ and HUVE (H) cells.

To directly test this, the effects of Ang 2 on tyrosine phosphorylation status of Tie 2 on CD14+cells was tested.

Probing anti-phosphotyrosine immunoprecipitates from Ang 2 (200ng/ml) pre-stimulated CD14+ showed an increase phosphorylation of an immunoreactive band corresponding to the size of the Tie-2 receptor compared to Ang 2 pre-conditioned HUVEC. This was repeated twice and suggests Ang 2 acts an activating ligand for Tie-2 on CD14+ cells that may be responsible for inducing migration and slight reduction in EC loss.

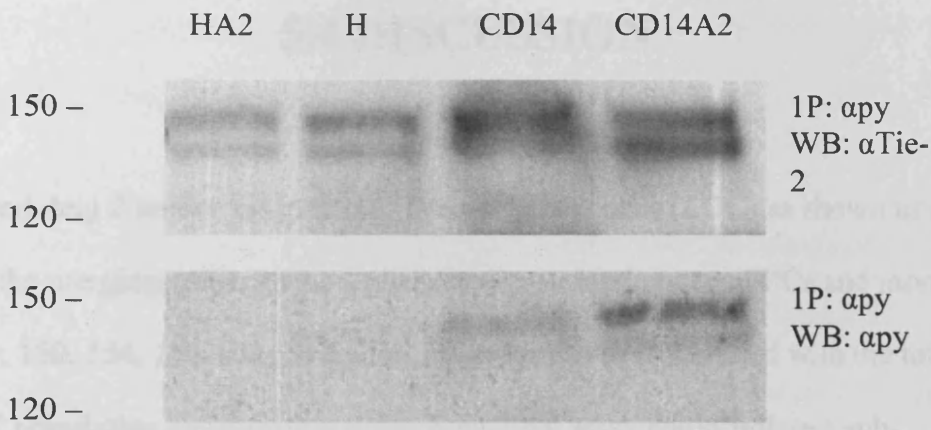


Figure 5.13. The Effect of Angiopoietin 2 (A2) pre-conditioning on Tie-2 protein phosphorylation on CD14+ and HUVE (H) cells.

90% confluent HUVE and CD14+ cells were pre-conditioned with Ang 2 for 15 minutes or control medium only. Following incubation cells were washed in PBS and lysed. Immunoprecipitation (IP) was performed with an anti-phosphotyrosine antibody on agarose beads (α Py), of the cell lysates were performed for 3 hours at 4^0C. The immunoprecipitates were washed and analysed by western blotting (WB). The blot was stripped and re-probed with an anti-Tie-2 antibody to identify the Tie-2 protein. The bands according to Tie-2 are marked accordingly. This is a representative immunoblot of 2 independent experiments.

5.4 DISCUSSION

VEGF and Ang 2 induce migration of late outgrowth cells (LOC) as shown in chapter 4. With the merging evidence for a phenotypic overlap between EPCs and monocytes (95, 149, 150, 154, 156-158), migration experiments were repeated with the total PBMNC population to determine if this functional assay could isolate a sub-population that may represent a less differentiated EPC or the early outgrowth cells described in chapter 3. The results described in this chapter show a large population of the total PBMNC migrate in response to Ang 2 and , therefore, with an inferred Tie 2 expression.

During the course of this work, three groups described a subset of PBMNCs, namely CD14+, differentiate into endothelial cells. They demonstrated a clear progression from a monocyte to endothelial cell phenotype within these CD14+ cultures (95, 149, 150, 154, 156-158). Using this information, immunocytochemistry was utilised to determine whether the cells within the PBMNC population that migrate in response to Ang 2 , were actually CD14+ cells and revealed the majority of Ang 2 responsive cells in this study were indeed CD14+ . Given the evidence that CD14+ cells could go onto assume endothelial characteristics, it was anticipated these cells would be the primary cells recruited from the blood to sites of angiogenesis to bind to endothelium and undergo differentiation to endothelial cells. Therefore the ability of angiogenic factors to stimulate migration and adhesion of isolated CD14+ cells from fresh blood was studied .

In order to investigate this subgroup of cells further pure CD14+ cells were isolated using established techniques (159) and when Ang 2 migration experiments were

repeated , CD14 + cells were seen to migrate in response to Ang 2. The presence of the CD14/Tie2 phenotype was confirmed using FACS.

To determine the phosphorylation of the Tie 2 receptor it was necessary to precipitate this protein. Several attempts at analysis showed varying levels of tyrosine phosphorylation. This could either be due to failure of the technique or varying levels of Tie-2 /ligand binding. Future work needs to investigate the phosphorylation state of the Tie2 receptor after EPC pre-conditioning with Ang 2 by optimising the IP conditions further or improving cell separation techniques to allow ex vivo expansion of a less heterogeneous population.

It was noted in chapter 3 of this thesis, when early (<24 hour outgrowth) EPC were plated with mature endothelial cell monolayer, the latter dissociated from the gelatin matrix and underwent apoptosis. Experiments in the present chapter using freshly isolated total PBMNC demonstrated large losses of EC from culture dishes in the presence of PBMNC. Furthermore, a similar effect was observed using the CD14+ sub-fraction of PBMNC. The precise mechanism responsible for the effects of CD14 cells observed in this work requires further study. However, it is possible that the CD14+ cells could induce endothelial cell apoptosis as has been described by others (19 (82, 156, 160)).It is surprising, though, that such a large percentage of EC were lost in response to the CD14+ cell co-culture..

It has been suggested CD14+ monocytes have the capacity of assuming an endothelial phenotype (6,94). These findings have recently been confirmed by Gulati et al who explored the lineage relationship between early EPCs and late outgrowth cells in

culture. It was concluded that EPCs were derived mainly from a CD14+ sub fraction of PBMNC whereby LOC were derived exclusively from the CD14- fraction (95, 150). Furthermore, LOC exhibited a markedly greater capacity for capillary morphogenesis in vitro and in vivo models (95). It could therefore be plausible from the findings in this chapter, that Ang 2 and VEGF recruit CD14+/CD34+/Tie-2 + or early outgrowth cells to areas of angiogenesis. Although Ang 2 and VEGF were found to have modest effects on CD14+ induced endothelial detachment in the present study, it is possible that the balance of these agonists could control whether the recruited cells induced vessel regression or vessel growth. Indeed Lobov et al (26) have reported in a mouse model that Ang 2 stimulates recruitment of CD14+ cells to microvessels of the eye and induces vessel regression the effect being suppressed by VEGF.

The mechanism of CD14+ cell induced EC loss, potential role of endothelial programmed cell death and possible involvement in vessel regression warrant further investigation. Furthermore, confirmation of these findings in vivo is necessary to further investigate this potential model of angiogenesis, and so contribute to our understanding of EPC function.

CHAPTER 6

6.1 CONCLUSIONS AND FUTURE RESEARCH

It is now well established that bone-marrow derived EPCs are present within the systemic circulation and are augmented in response to certain cytokines (5, 10-12, 14, 57, 64, 72, 161), tissue ischaemia (3-6) and various therapeutic agents (2, 8, 12, 17, 76, 77, 88, 162) to home to sites of neovascularisation. This in turn has lead to a plethora of research attempting to define the cell type further and determine the underlying molecular process governing their function.

During the course of this study , it became clear that it is not simple to identify an EPC. Work detailed in Chapter 3 characterised the EPC derived using commonly utilised methods for EPC isolation and expansion prevalent at the time this study was initiated (51, 53, 63, 72).). The conditions used in this study were those described in the literature by Asahara in his original work, which were subsequently adopted by other researchers (6, 51, 57, 77, 89, 121). All studies of morphology and phenotype were reproducible throughout the study.

Several sources of EPC exist including haematopoietic stem (163) and myeloid cells which may differentiate into EC under cultivation selection pressure, or other circulating progenitor populations by transdifferentiation depending on environmental cues (68, 164). It is not surprising, therefore, that a heterogeneous population exists in culture and therefore, there is no consensus as to a definitive antigenic phenotype for EPCs. Thus in order to conclude that a cell is an EPC in *in vitro* studies it seems reasonable to expect a multi-antigen assessment in conjunction with one or more functional assay (156, 160). Continuous cultivation of cells resulted in an increase in endothelial marker expression. Noteworthy, is the appearance of the

Tie-1 receptor following one week in culture, in comparison the Tie-2 receptor was present from immediate isolation. VEGFR was present in high concentration from the outset whilst VECad is upregulated after five weeks outgrowth. When cells were expanded *ex vivo*, the late outgrowth cells demonstrated an endothelial –like ability to form cords structures in three dimensional matrices consist with the literature (9, 51, 62, 72).

At the outset of this work there was little known about the 'homing' mechanisms of EPCs. It is established the homing of cells is a multi-step process, primarily involving the migration, adhesion and differentiation of cells (69). Data presented in this work demonstrates that the angiogenic factors VEGF and the Angiopoietins 1 and 2 are important modulators of this process In the field of angiogenesis, much work has focussed on the role of VEGF and Angiopoietin 1 and 2 as critical regulators of vascular assembly (47, 73, 152, 165) with upregulation of VEGF and Angiopoietin 2 expression at leading tips of sprouting capillaries and areas of vessel co-option (72). This, along with the findings in this study suggest it may be plausible these factors are responsible for recruiting EPCs to these areas of neovascularisation and may subsequently be involved in anastomosis of developing vessels (41, 140, 142).
The interaction between EC and EPCs is partially mediated by the integrins and ICAM-1. Future work would aim to strengthen these findings using gene array analysis and quantitative PCR. This novel data adds to the poorly understood area of EPC homing. Future *in vivo* work is necessary to confirm these *in vitro* findings.

In Asahara' original work 90% of EPCs derived from non-selected PBMNC expressed CD14, a monocyte marker.(51) and furthermore, it became evident during the course of this work a phenotypic overlap exists between EPC and monocytes (149, 157) with monocytes having the ability to differentiate into endothelial like cells under angiogenic conditions (158).Three studies brought this into sharper focus (149, 154, 157). In all three studies, the authors demonstrated a clear progression from a monocyte to endothelial cell phenotype in their CD14 + culture. Given this observation(154) is has been suggested these are the primary cells recruited from the blood to sites of angiogenesis it would then be anticipated these cells may bind to the endothelium and either undergo differentiation into endothelial cells or form capillaries by vacuolisation and coalescence with multiple cells (156, 160). It was therefore logical to test the ability of angiogenic factors to stimulate migration and adhesion of these cells from fresh blood. Based on the findings described in chapters3 and 4 I went on to examine Ang 2 responsive cells in fresh PBMNC and found a subset of CD14/Tie-2 cells were identified within the PBMNC population that functionally responded to VEGF and Ang 2. Furthermore, it was found that CD14+ cells induced endothelial detachment, probably involving endothelial cell death, and this was modestly stimulated by Ang 2 and inhibited by VEGF in culture. Whilst it is clearly necessary to define in much greater detail the molecular basis and physiological relevance of these findings it is possible that they may be important *in vivo*. Increased Angiopoietin 2 production by endothelial cells at sites of vascular remodelling could induce recruitment of EPC and CD14 cells (41, 47, 73, 152, 165). If CD14 cells initiate endothelial apoptosis, as indicated by Gulati (166) the CD 14 cells could induce vessel regression. In the presence of VEGF the pro-apoptotic effects of CD14 + cells could be suppressed leading to a decrease in vessel regression

and promotion of EPC differentiation and possibly contributing to new vessel growth.. Evidence supporting this hypothesis comes from the work of Moldovan et al who suggested monocyte recruitment was for the sole purpose of endothelial regression followed by 'true' EPC recruitment for vascular remodelling (82, 167). Careful in vivo analysis of recruitment of different cell populations to areas of vessels remodelling will be required in future work to test this hypothesis. Along with studies to determine whether monocyte derived endothelial cells can develop a functional behaviour as described for vascular endothelial cells and contribute to neovascularisation by a direct vessel forming role (157).

Based on the preponderance of evidence one can conclude the population of circulating cells with an endothelial phenotype, or potential for assuming an endothelial phenotype is heterogeneous and includes monocytes (CD14+) capable of phenotypic and functional differentiation and a subset of CD34+ 'true' EPCs (168), along with a population are LOC which when expanded *ex vivo* undergo phenotypic and functional differentiation that would be isolated and expanded to therapeutically enhance neovascularisation on re-introduction into the circulation.

In conclusion, the biology of EPCs from the bone marrow is not well understood, but considering their initial description following Asahara's seminal work in 1997 the progress has been substantial. A number of key regulators have been identified, including VEGF and Angiopoietins in this study, and now with the understanding that there is not a single EPC precursor, further work can focus understanding these phenotypic variations and hence EPC biology.

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