MECHANICAL STRAIN-INDUCED REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION BY HUMAN VASCULAR SMOOTH MUSCLE CELLS.

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by

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ABSTRACT

The effect of mechanical forces in the development of certain pathologies, especially those associated with hypertension, is a phenomenon that has long been acknowledged but never fully investigated. The important force that changes with hypertensive patients, thus altering cardiovascular structure and function, is the actual pressure applied to the walls of vessels by the pulsatile flow blood.

The present thesis investigates the effect of cyclical mechanical strain on the expression of vascular endothelial growth factor (VEGF); a potent cytokine produced by human vascular smooth muscle cells (VSMC). The initial investigations observed the expression of VEGF mRNA and peptide over time and then the effect of increasing the magnitude of strain applied to the cells.

The second area of study was to identify the effect of strain on a candidate intracellular signalling pathway, the mitogen activated protein kinase (MAPK) cascade. The experiments observed the effect of strain on the level of phosphorylated MAPK within the cells and the actual activity of MAPK isolated from cultured human VSMC. The effect of a MAPK pathway inhibitor (PD98059) on the phosphorylated peptide activity was also analysed.

The data submitted in this thesis demonstrates that the expression of VEGF mRNA and peptide are increased in a dose-dependent manner when exposed to cyclical mechanical strain and that the level of phosphorylated MAPK increases likewise. Furthermore, the addition of a MAPK inhibitor not only reduces the level of MAPK activation, but also the expression of VEGF. Taken together, these data identify a potential mechanism whereby strain can directly effect vascular permeability by regulating the expression of VEGF peptide through the MAP kinase cascade.

ABBREVIATIONS

aFGF: acidic fibroblastic growth factor

AT_1: angiotensin II receptor type 1

AT_2: angiotensin II receptor type 2

BAE: bovine aortic endothelial cells

BASM: bovine aortic smooth muscle cells

bFGF: basic fibroblastic growth factor

CAKβ: cell adhesion kinase beta

CAT: chloramphenicol acetyl transferase

CL: corpus luteum

EC: endothelial cells

ECM: extracellular matrix

EGF: epidermal growth factor

EGF: epidermal growth factor

eNOS: endothelial nitric oxide synthase

ERK1: extracellular regulated kinase 1

ERK2: extracellular regulated kinase 2

ES: embryonic stem cells

FAK: focal adhesion kinase

FAT: focal adhesion targeting

FCS: foetal calf serum

FGF-2: fibroblast growth factor 2

Flt-1: fms-like tyrosine kinase (VEGF receptor 1)

Flt-4: VEGF receptor 3

FRNK: focal adhesion kinase related non-kinase

Grb2: growth factor receptor-bound protein 2

HEC: human endometrial carcinoma cells

JNK/SAPK: jun/stress activated protein kinase

KDR: kinase insert domain receptor (VEGF receptor 2)

MAPK: mitogen activated protein kinase

MAPKAPK: mitogen activated protein kinase activated protein kinase

MAPKK: mitogen activated protein kinase kinase

MAPKKK: mitogen activated protein kinase kinase kinase

MBP: myelin basic protein

MEK: mitogen activated protein kinase kinase

MEKK: mitogen activated protein kinase kinase kinase

NMA: non-muscle isoform of myosin A

NMB: non-muscle isoform of myosin B

NO: nitric oxide

PAI-1: plasminogen activator inhibitor 1

PDGF: platelet derived growth factor (two isoforms A and B)

PlGF: placental growth factor

PTK: protein tyrosine kinase

PYK2: proline-rich tyrosine kinase 2

REKS: Ras-dependent ERK-kinase stimulator

RTK: receptor tyrosine kinase

SH2: src homology 2 domain

SH3: src homology 3 domain

SM-1: smooth muscle myosin heavy chain 1

SM-2: smooth muscle myosin heavy chain 2

Sos: guanine nucleotide exchange factor 'Son of Sevenless'

TEM: transmission electron microscopy

TGFα: transforming growth factor alpha

TGFβ_1: transforming growth factor beta 1

TNF: tumour necrosis factor

tPA: tissue plasminogen activator

VEGF A: vascular endothelial growth factor A

VEGF B: vascular endothelial growth factor B

VEGF C: vascular endothelial growth factor C

VEGF D: vascular endothelial growth factor D

VEGF E: vascular endothelial growth factor E

VEGFR1: vascular endothelial growth factor receptor 1

VEGFR2: vascular endothelial growth factor receptor 2

VEGFR3: vascular endothelial growth factor receptor 3

VPF: vascular permeability factor

VSMC: vascular smooth muscle cells

VVO: vesiculovacuolar organelles

1) INTRODUCTION

1.1) General Introduction

1.1.1) Hypertension and Mechanical Stress

The effect of mechanical forces in the development of certain pathologies, especially those associated with hypertension, is a phenomenon that has long been acknowledged but never fully elucidated (1). The important force that changes with hypertensive patients, thus altering cardiovascular structure and function, is the actual pressure applied to the walls of vessels by the pulsatile flow blood. The increased blood pressure within the vasculature can exert its effect on the vascular tissue by increasing shear stress and cyclical mechanical strain. Shear stress is caused by the blood 'washing over' the cells in the vessel lining. It exerts a force that acts parallel to the direction of the flow of blood and predominantly effects the endothelium, which is in direct contact with the blood. The effect of shear stress on EC regulation/influence on vascular dynamics has been well documented and proven to induce autocrine and paracrine mechanisms for cell growth and function (2), (3), (4), (5), (6). The cyclical mechanical strain is different in that it manifests as a force running perpendicular to the direction of blood flow that acts on the VSMC within the vessel wall. Interestingly, it has been observed that in patients with hypertension, the mechanical strain on the arterial wall can increase to between 15 % and 30 % higher when compared to the strain experienced by a normotensive vessel (7), (3).

Essential or primary hypertension is associated with increased resistance in the vessels proximal to the capillaries, due to a narrowing of the lumen in the pre-capillary vessels. As such, these vessels have been termed resistance vessels (8). The vasculature responds to increased cyclical mechanical strain (hypertension), especially the resistance vessels, by the augmentation of the vascular media (see Figure 1 below) by VSMC hypertrophy

and/or hyperplasia (1), (9). This medial thickening increases chronic vascular resistance in hypertension. There are studies that support the idea that hypertension and neuro-humoral factors are involved in the hypertrophic/hyperplasic response *in vivo* (10), (11).

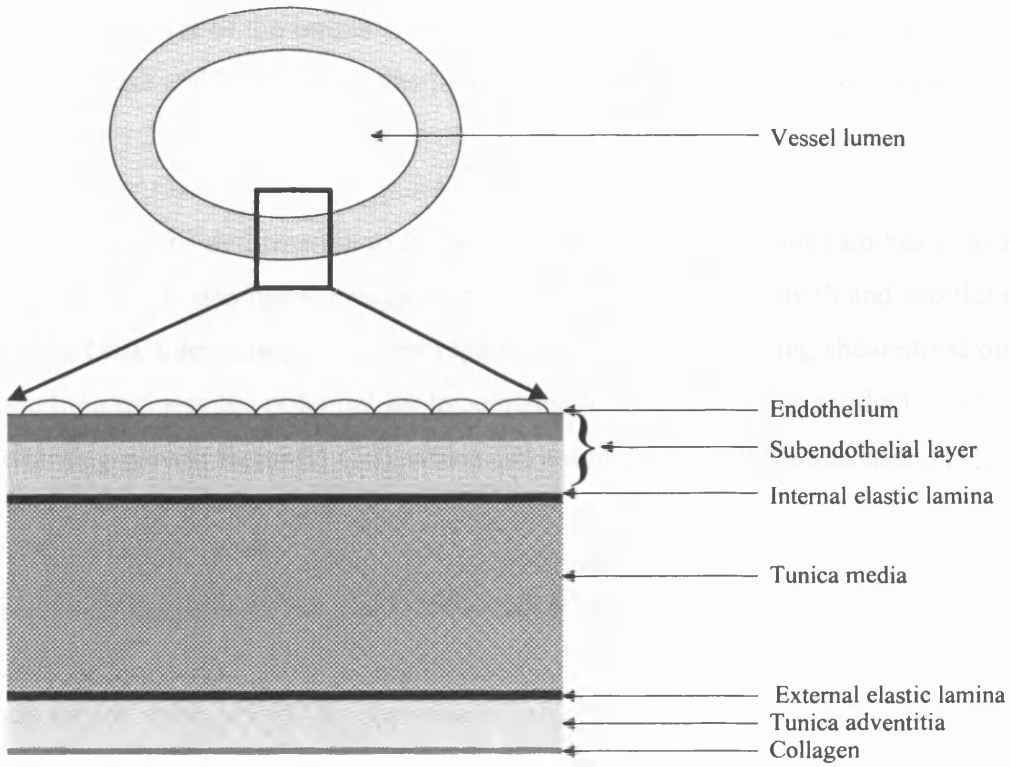

Figure 1- Schematic representation of a blood vessel, showing the tunica intima (consisting of the endothelium and subendothelial layer), the tunica media and the tunica adventitia.

The outermost layer of the vessel is called the tunica adventitia, and is comprised of connective tissue, mainly elastin and collagen, with fibroblasts, mast cells and macrophages. This layer contains a network of capillaries and lymph vessels, collectively called the vasa vasorum, which provide a blood supply to vessel. The adventitia is also the site of the perivascular nerve supply, and is kept separate from the media by a poorly defined external elastic lamina (12). The tunica media is comprised of smooth muscle cells and is the most prominent layer in resistance vessels. The smooth muscle cells regulate vascular tone and are the main area of structural change in hypertension. They follow a near helical arrangement around the lumen and are separated from the intima by

a well-defined internal elastic lamina (12). The intima is composed of three layers. The outer subendothelial layer consists of branching elastic fibres, whilst the subendothelial layer contains mainly collagenous bundles and elastic fibrils. Also, smaller amounts of smooth muscle and sometimes fibroblasts can be found in the subendothelial layer (12). The innermost layer of the intima is the endothelium and as discussed earlier, this is in constant contact with the blood in the lumen and is therefore continuously exposed to shear strain.

Research taking the deformation to the level of cyclical mechanical strain has indicated that physical force also plays a major role in promoting VSMC growth and proliferation (13), (14), (15). Interestingly, a recent report suggests that increasing shear stress on the endothelium reduces the potential for hyperplasia by stimulating the production of transforming growth factor β1 (16), which can act to inhibit cell proliferation (17).

1.1.2) Vascular Development and Mechanical Strain

In their natural state, VSMC are specifically orientated within the vessel to form a helical pattern at an angle of 20 °- 40 ° to the direction of the vessel. This corresponds to an angle of 50 °- 70 ° between the longer axis of VSMC and the resultant vector of the distending forces, which is at 90 ° to the direction of blood flow (18). This highly organised structure is an important factor for the viscoelastic properties of the vessel (19). During static culture of isolated VSMC *in vitro*, the cells display a random orientation, however the application of cyclical mechanical strain causes the cells to re-orientate to an invariant alignment at an angle of 61 °- 76 ° to the direction of strain. The degree of re-alignment has been shown to be proportional to the level of cyclical mechanical strain applied and also reversible (18), (20). These data indicate that the VSMC are probably not fixed but in fact may *in vivo* be responsive to the mechanical forces of blood pressure. This could represent not only an important mechanism for defence against increased force (hypertension) to retain haemodynamic performance, but also play a role in the

remodelling process that is the basis for the adaptive changes observed in resistance vessels in hypertension (12), (8).

The vasculature is dependent upon the stimulation of mechanical forces, not purely for alignment, but from its earliest development. At approximately three weeks gestation, an embryo has a simple network of poorly defined vessels. This is the time when the embryonic heart begins to beat and it is the effect of the blood washing back and forth through the vessels, i.e. shear stress, that stimulates the development of a complex vascular network. The embryo can survive for a short period without a heart but development of the vascular network remains retarded and ill defined. Furthermore, the effect of mechanical forces controls the development of the major vessels, in that if a major vessel is occluded during development, one of the nearby small vessels matures into the major vessel (21).

1.1.2.1) Mechanical Strain and Phenotypic Modulation

A common phenomenon of cultured VSMC is the reversion of the cells from a contractile, well differentiated cell to a synthetic, less differentiated phenotype by a process termed phenotypic modulation (22), (23). The phenotypic modulation is characterised by a reduction in expression of smooth muscle myosin heavy chain 1 and 2, termed SM-1 and SM-2 (the late markers of differentiation/maturation) and a reversion to expression of the non-muscle isoforms of myosin, NM-A and NM-B. Also, phenotypic modulation has been seen in VSMC vascular pathologies, where it causes a reduction in production of smooth muscle myosin, increased proliferation and increased protein/matrix secretion (23), (24). Reusch *et al* observed that rat aortic VSMC when exposed to cyclical mechanical strain increased their expression of SM-1 and SM-2, and decreased expression of NM-A and NM-B, suggesting the *in vitro* conversion back to the contractile phenotype (23). These cellular observations are in keeping with the morphological observations of Kanda and Matsuda, who demonstrated that strain in a three dimensional collagen matrix induced an abundance of myofilaments and dense

bodies that are typically found in differentiated VSMC (25). More recently, experiments studying the differentiation marker h-caldesmon demonstrated that intact aortas exposed to a low intraluminal pressure, equivalent to 10 mmHg, decreased expression of h-caldesmon. However, if the intraluminal pressure was increased to the physiological equivalent of 80 mmHg, the expression was normal (26). The presence of the endothelium was not required suggesting that the maintenance of the VSMC marker proteins occurs in response to cyclical mechanical strain.

The data above indicate that the mechanical forces experienced by VSMC are in fact responsible for the development of the vasculature and the maintenance of the differentiated, contractile phenotype of the cells. There also appears to be a graded response by the VSMC because static conditions cause regression to a non-contractile state, optimal physiological conditions maintain healthy, quiescent cells and hyperphysiological conditions (hypertension) cause cell hypertrophy and increased matrix synthesis.

1.1.2.2) Mechanical Strain and Extracellular Matrix Protein Synthesis

Increased extracellular matrix (ECM) can arise because of increased production and/or decreased breakdown by metalloproteinases. O'Callaghan and Williams demonstrated by zymography that metalloproteinase activity in the overlying media of VSMC exposed to cyclical mechanical strain did not increase compared to static cultures (27). Therefore the increase in ECM is due to deposition and not an alteration in the breakdown rate. The controlling influence for this increase was subsequently found to be a secreted factor, because when the media from stretched cells was introduced to static cultures, the static VSMC increased collagen synthesis (27). O'Callaghan *et al* previously noted that cyclical mechanical strain increased the expression of $TGF\beta_1$ mRNA in VSMC. Coupled with the observation that specific antibodies raised against $TGF\beta_1$ negated the ability of stretch-conditioned media to increase matrix synthesis in static VSMC, lead to the conclusion that $TGF\beta_1$ was a stretch induced mediator of ECM synthesis (27).

These data demonstrate an apparent paradox because on one hand mechanical strain maintains the differentiated phenotype of a mature VSMC. On the other hand, the application of cyclical mechanical strain also causes cell proliferation and ECM deposition, a trait normally associated with a dedifferentiated state. Likewise, Wilson *et al* (28) demonstrated that cyclical mechanical strain causes an upregulation of PDGF causing proliferation, which would be expected to promote a less differentiated state. There are three possible reasons for this discrepancy. Firstly that the cells undergoing proliferation and the cells expressing the mature markers are two separate populations in the same culture. Secondly that the composite response is caused by the varying degree of strain placed on the cells across the flexible membrane (23). Thirdly, an intermediate phenotype exists which remains differentiated whilst proliferating which is seen during vascular development in the embryo (29). It is likely *in vivo* that a mixture of these cell types co-exists to some degree, as would be found in situations such as foetal vascular development.

The forces exerted on the vasculature in a healthy normotensive individual serve to promote and maintain the vessels. However, it is widely documented that during hypertension, the permeability of the vascular wall increases as a manifestation of endothelial dysfunction, which plays a key role in the early pathogenesis of atherosclerosis (30).

1.1.3) Hypertension and Vascular Permeability

Schurmann and McMahon first observed that increased vascular permeability and the infiltration of plasma substances were important in the development of hypertensive pathologies (31). Selectively bred strains of spontaneously hypertensive rats have shown on numerous occasions that increased blood pressure causes an increase in vascular permeability (32), (33). Yamori *et al* identified that hypertension in their model caused an increase in vascular permeability that allowed the extravasation of fats which could

therefore be the initiation of atherosclerosis (33). Earlier studies had suggested that a circulating factor, causing the increase in vascular permeability, was produced in the body when the individual became hypertensive. Using the well-defined ischaemic kidney rat model that had been demonstrated to increase circulating renin levels, Sonkodi *et al* discovered that the increase in blood-brain barrier permeability that was previously attributed to increased levels of renin, also occurred in the clipped animals with normal serum renin (34). A potential mechanism for the change in vascular permeability was identified when it was found that hypertension caused an increase in the histamine forming capacity of the vessel wall in rat aortae (32). Similarly, Bowers *et al* demonstrated that if the intracranial pressure of sheep was elevated to a hypertensive state, the vessels of the pulmonary system increased their permeability (35). This would suggest that a circulating factor or increased neuronal activity increased vascular permeability at distal sites, and in support of this, Ross discovered that the increase occurs at sites where the endothelium is intact suggesting endothelial dysfunction rather than damage (30).

Taken together, these data suggest that the vasculature can detect increases in blood pressure and modify its response. With regard to changes in systemic vascular permeability, the reports thus far have proposed the existence of a circulating factor/s that becomes active once hypertension has developed. However, Surtees *et al* found that vascular permeability was increased in clip-nephrectomised rats before significant increases in blood pressure or arterial damage were recorded (36). This report suggests that the response to changes in the mechanical forces applied to the vessel wall is rapid and that the mechanism that initiates the increase in vascular permeability is very sensitive to a local environment. Research attention has primarily focused on documenting the vascular permeability changes associated with hypertension, however the mechanisms involved are largely unidentified.

In short, it can be seen that the human vasculature is continuously exposed to mechanical strain, whether it is shear stress (endothelium) or cyclical mechanical strain (smooth muscle). Within the last decade, experimental advances have identified that the

vasculature can sense these forces and react accordingly by re-aligning cells in the plane of the force and/or proliferating. The mechanical forces exerted by the flow of the blood are not only vital for the development and continuance of the vasculature, but also the release of circulating factors that influence endothelial function. Recently a potent endothelial permeabilising factor, vascular endothelial growth factor (VEGF) was described. Work in our laboratory had demonstrated that VEGF was produced by human VSMC (37). This suggested that the human VSMC was strategically placed to influence the permeability of the overlying endothelium via VEGF. We thus postulated that VEGF may be an important regulator of vascular endothelial permeability *in vivo* and that mechanical strain might promote the release of VEGF from human VSMC. This could provide a potential explanation for hypertension induced changes in endothelial permeability in hypertension. Thus, the aim of the research presented in this thesis was to assess the impact of cyclical mechanical strain on VEGF production by VSMC. Furthermore, to identify the potential intracellular signalling mechanism by which the human VSMC can sense an increase in strain and initiate an intracellular message to co-ordinate an appropriate response.

1.2) The Study of Chronic Cyclical Mechanical Strain

The study of the effects of cyclical mechanical strain has been hindered by difficulty in designing a suitable experimental model with which to undertake the investigations. Initial technology consisted of simple vacuum pump and valve equipment attached to 100 mm petri dishes (38) or rectangular sheets of elastic silicone membranes supporting a monolayer of cells attached to a motor and spring (39), (25). The one drawback with the latter system is that the pattern of stretch/relaxation is a smooth curve of troughs and peaks, rather than the physiological pattern of rapid strain and delayed relaxation, the latter being more typical of an arterial strain pattern.

More recently, the vacuum apparatus has evolved to become the Flexercell Stress Unit (Flexcell Corp. Durham USA), which is a vacuum/valve system regulated by a computer. This device allows cells from experimental animals or sample human tissues to be cultured on six well, flexible elastomer-based plates (Flex plate, Flexcell Corporation) with hydrophilic surfaces. The Flex plates are then incubated in a standard 5 % carbon dioxide tissue culture incubator in a specially designed vacuum manifold. A vacuum can then be repetitively applied to the plates, causing the bases to be deformed, under the regulation of a computer that controls the frequency, and degree of deformation applied to the plate. The level of strain administered to the plates results in mechanical deformation of the overlying VSMC. Real-time video studies performed by the manufacturers have showed that the VSMC remain attached to the plates during the application of strain (40). Thus, as the levels of stretch and relaxation can be altered to mimic different magnitudes of strain equating to different blood pressures, the direct impact of blood pressure on vascular cell biology can be simulated and studied. As well as manipulating the physical environment by changing the degree and timing of the strain applied to the cells, biochemical stress can be induced by altering the cell media to include paracrine, autocrine or humoral factors, such as angiotensin II. Further complementary studies can include metabolic manipulation of the cells by, for example, using hypo- or hyperglycaemic media.

The development of equipment to study cells in a biomechanically active state has enhanced the data provided by the 'static' cell culture system. Whilst not providing the perfect simulation of *in vivo* experimental conditions, the Flexercell Stress Unit provides a more complete picture of cellular function and its response to its environment, because VSMC in their natural state are continually exposed to cyclical mechanical strain.

Early developmental investigations that attempted to advance in vitro technology for the study of cyclical mechanical strain lead to the prototype Flexercell device (38). The stress unit consisted of a vacuum regulated by a simple timer, connected to a manifold head that could hold six 100 mm petri dishes. The timer could apply two levels of strain (contraction and relaxation) to the plates, the duration of which could be varied from 0.1–100 seconds. The pressure applied to the petri dishes achieved a maximal plate deflection of 1.5 mm, which corresponded to 0.13 % deformation of the internal surface. The analysis performed on the stretched fibroblasts showed that cyclical mechanical strain at a regimen of 25 seconds strain, 5 minutes relaxation for 3500 cycles induced changes in the synthesis of certain proteins, namely a significant reduction in tubulin (a cytoskeletal protein involved in phagocyte motility). Within a couple of years of this publication, Banes *et al* had refined the equipment and designed the Flexercell unit (38). This lead to a surge of data in numerous publications which began to elucidate the complex and varied responses of different types of cells to cyclical mechanical strain.

1.2.1) The Endothelium and Mechanical Strain

A major focus for the early studies was the effect of cyclical mechanical strain on the endothelial layer (EC) of the vasculature, as logically, it was assumed that these cells were exposed to the brunt of the forces. The data showed that bovine aortic EC, when exposed to a regimen of three cycles per minute (10 seconds stretched, 10 seconds relaxed), increased DNA synthesis and cell proliferation (41). When this regimen was investigated further (3 cycles/minute for five days), the EC exposed to cyclical

mechanical strain changed morphologically to become more polygonal in shape and increased synthesis of actin stress fibres (42). The stress fibres are thought to help with attachment *in vivo* to the vessel wall such that the EC are not dislodged by pressure changes and pulsatile flow. The EC did not however demonstrate any alignment or orientation towards a predicted direction of flow/shear stress. Previous investigations determining the effects of shear stress on EC alignment showed that the cells orientate themselves in line with the direction of flow (43), (44). Furthermore, the degree of alignment showed a direct correlation to the degree of shear stress applied (45), (46), (47). These data would suggest that endothelial proliferation is regulated by the degree of cyclical mechanical strain applied to the vessel and that the organisational alignment is dictated by the fluid shear stress.

Upchurch *et al* employed a physiological regimen of 0–17 % elongation (equivalent to a maximal blood pressure of 76.2 mmHg) at 60 cycles per minute, and investigated the expression of prostacyclin by arterial and venous EC (48). *In vivo* prostacyclin is a potent inhibitor of platelet aggregation and also a very powerful vasodilator. This affords a degree of control of vascular homeostasis. Static venous EC exhibited the highest levels of prostacyclin suggesting that its function could be to reduce thrombus formation in low velocity areas of the vasculature. The data continued to show that prostacyclin levels were not only reduced in arterial EC but also further reduced in those EC exposed to cyclical mechanical strain (48).

Toshiaki Iba and Bauer Sumpio were two colleagues working with Al Banes' group in the development of the Flexcell apparatus. The earlier experiments (mentioned above) were refined by Iba and Sumpio such that a regimen more closely related to physiological conditions was developed. It was found that aortic endothelial cell proliferation (41), (49), prostacyclin activity (50), cytoskeletal protein synthesis (42), collagen (51) and endothelin (52) production were altered by cyclical mechanical strain *in vitro*. This regimen of 0.5 seconds stretch/0.5 seconds relaxation, 60 cycles/minute, clarified their earlier data. Using this physiological regimen, it was found that within fifteen minutes exposure to cyclical strain the EC had produced actin stress fibres that were aligned to the

direction of force. Furthermore, after 12 hours exposure, the EC had elongated and orientated in the same direction as the stress filaments (53). During the application of cyclical mechanical strain, Iba and Sumpio conceded that the level of stretch across the surface of the culture well was not uniform and decreased towards the centre of the membrane. The cells on the periphery of the well were exposed to between 7-24 % strain whereas the cells in the centre were exposed to less than 7 % elongation (54). A set of special plates were designed that had a protective ring protruding from the lid which separated the peripheral cells from the central cells. The overlying media was thus also kept separate which prevented any influence from autocrine factors. The cells were then exposed to cyclical mechanical strain for 1, 3 and 5 days and the levels of tissue plasminogen activator (tPA) and type-1 plasminogen activator inhibitor (PAI-1) were measured by immunocytochemical detection. It was found that those cells at the edge of the plate, i.e. the 7-24 % strain group, had a significant increase in immunoreactive tPA levels at 1, 3 and 5 days compared to baseline. The cells at the centre of the plate that were subjected to less than 7 % strain, showed no significant change in tPA levels. Moreover, actual tPA activity could only be detected in the media overlying the cells at the periphery experiencing the higher level of strain.

Despite these interesting observations that validated the technique, the EC was perhaps not the ideal cell to study. This is because the predominant strain that EC are exposed to is fluid shear stress as they line the lumen, not cyclical strain. This was suggested by data presented by Malek *et al*, which demonstrated that shear stress produced modifications in gene expression by EC whereas cyclical mechanical strain did not. Shear stress was found to increase the level of bFGF mRNA and decrease the expression of PDGF-B after nine hours exposure to fluid stress. However, cyclical mechanical strain did not significantly effect the expression of either peptide in EC (55). Investigations since then have shown that shear stress can upregulate the expression of genes such as the gap junction protein connexin 43 (56) and TGFβ-1 (16). Later research has indicated that shear stress can activate the p60src intracellular signalling cascade. This activates a complex intracellular signalling cascade that causes the phosphorylation of Ras-ERK peptides. The completion of this signalling cascade involves the phosphorylation of

multiple transcription factors (4), (2), which activate the expression of numerous genes within the nucleus. The complete picture of degrees of shear stress involved and which specific genes are activated have still to be determined.

1.2.2) VSMC and Mechanical Strain

Cyclical mechanical strain is the force that directly effects smooth muscle. An initial study performed on rabbit ear arteries that were excised and stretched using intraluminal springs showed that the uptake of radiolabelled proline and thymidine by the vessel media increased directly proportionally to the strain applied to the vessels (57). The proline and thymidine are used experimentally as markers for collagen and DNA synthesis respectively, in that an increase in uptake by the cells represents an increase in growth (proliferation) and collagen synthesis. Sumpio *et al* proceeded along this path by analysing the effect of cyclical mechanical strain on collagen production (40). Exposing porcine aortic VSMC to a regimen of low-frequency (3 cycles/minute) cyclical mechanical strain caused an increase in synthesis of both collagen and non-collagen protein. Rat neonatal aortic VSMC when exposed to cyclical mechanical strain for 48 hours increased DNA synthesis 3-fold and showed a 40 % increase in proliferation (28). A study performed by Kemp *et al*, which used the Flexercell apparatus to expose human VSMC to chronic cyclical mechanical strain, confirmed that the uptake of radiolabelled leucine and thymidine in VSMC was increased by the application of cyclical mechanical strain (58). This suggested an increase in cell protein and DNA synthesis in response to strain.

Wilson and co-workers analysed the potential synergy between cyclical mechanical strain and secreted factors in rat VSMC (28). Strain at physiological frequency (60 cycles/minute) induced a 4-fold increase in thymidine uptake, and the media from stretched cells could promote similar increases in static cells, suggesting the involvement of a secreted factor (28). Western blot resolution identified the presence of PDGF AA and BB isoforms. However, the addition of antibodies to PDGF-AA reduced the

mitogenic response of the cells by 75 %, whereas the PDGF-AB antibody only diminished the response by half (28). This report supports the hypothesis that there were a number of factors involved in the VSMC proliferatory response to cyclical mechanical strain. Sudhir *et al* demonstrated that angiotensin II could act synergistically to potentiate the mitogenic response of rat VSMC to stretch (59). The enhancement of response was all but abolished by the addition of antibodies against PDGF-AB. Interestingly a subsequent publication described the two-fold increased expression of the angiotensin II receptors (AT_1 and AT_2) in neonatal rat cardiac myocytes exposed to mechanical strain (60). This proposes a potential mechanism for the functional enhancement of angiotensin II via mechanical strain, notably upregulation of angiotensin II receptors. In support of this hypothesis, subsequent data utilising saralasin, a selective angiotensin II antagonist, demonstrated that cyclical mechanical strain induced rabbit aorta smooth muscle cell proliferation and PDGF expression could be substantially reduced by blocking angiotensin II action (61). This suggested that the stretch-induced proliferation of cultured rabbit aorta smooth muscle cells is mediated at least in part via either an increased production of angiotensin II by a local renin-angiotensin system, or increased 'potency' of angiotensin II. This increased potency is as a result of up-regulation of angiotensin receptors.

In human tissues, the picture is less clear. Whilst exposing VSMC to cyclical mechanical strain using the Flexcell in our laboratory, Kemp *et al* failed to demonstrate a role for an inducible secreted mediator such as PDGF, as the overlying media from stretched cells had no effect on static cultures. These studies suggest that VSMC growth is a direct response to cellular deformation during cyclical mechanical strain (13). These differences in autocrine/synergistic or independent mitogenic responses could be the result of differences in the species of origin (rat or human) or cell maturity (neonatal or adult).

A better understanding of the mechanism whereby VSMC sense mechanical strain was provided when Wilson *et al* demonstrated that specific integrin/matrix protein interactions are required to elicit a mitogenic response by VSMC in response to cyclical mechanical strain (62). During high frequency strain (60 cycles/minute) the addition of

the integrin binding peptide GRGDTP (RGD peptide) reduced the mitogenic response. These studies suggested that integrin/matrix interactions were important in the transduction of the strain response. Cells were transiently transfected with a full length 890 bp PDGF-A promoter bound to a CAT (chloramphenicol acetyltransferase) plasmid and subjected to strain in the presence or absence of RGD peptides. Those cells with no RGD peptide present, had a 3-fold increase in CAT activity, whereas those cells co-incubated with the RGD peptide had a significantly blunted strain-induced response (62). Furthermore, the addition of soluble fibronectin completely negated the strain response by interfering with the cellular interaction with the collagen coated plates (62). By transfecting the rat VSMC with serial truncations of a PDGF-A promoter CAT construct, Wilson *et al* identified GC-rich strain response regions within the promoter that contain response elements for the transcription factors Egr-1 and Sp-1. These factors are upregulated in VSMC within 24 hours of commencing the application of strain and therefore may bind the putative response element within the PDGF-A promoter (63).

Not all genes have their expression altered in a mechanically active environment. Fibroblast growth factor-2 (FGF-2) is a non-secreted peptide that is only released into the circulation by mechanisms induced by cell damage and therefore participates in the response to vascular injury (17). Cheng and co-workers demonstrated that at a level of cyclical mechanical strain usually found in normal blood vessel, the release of FGF-2 was not altered. However, the release of intracellular FGF-2 was closely modulated by cyclical mechanical strain and the peptide was only released when the cells were exposed to severe mechanical strain (64). This report suggests that VSMC are able to 'sense' and respond proportionately to different magnitudes of mechanical strain and that specific genes are upregulated at predetermined levels of strain. The fine tuning mechanism that selectively regulates the response to strain has yet to be elucidated.

1.2.3) Mechanotransduction of Cellular Stress

A major question that remains is how the external deformation of strain is transduced into an intracellular event. Smith *et al* studied the effect of cyclical mechanical strain on airway smooth muscle cell morphology using transmission electron microscopy (TEM) and fluorescent staining. They observed marked increases in the numbers and lengths of focal adhesions between the cell membrane and the substratum (65). This would indicate that the mechanotransduction of the stretch signal is transferred across the membrane via focal adhesions. Mechanical strain thus increases organization of cytoskeletal elements in cells exposed to strain and might also increase the efficiency of signal transduction from the extracellular matrix into the cell interior.

The importance of the extracellular matrix was highlighted by Reusch *et al*, who cultured neonatal rat vascular smooth muscle cells on different matrix protein coated plates prior to exposure to strain (66). They reported that cells grown on pronectin activated two intracellular signaling cascades (the ERK cascade and the JNK/SAPK cascade). However, the cell cultures on laminin only activated the JNK/SAPK cascade when exposed to cyclical mechanical strain (66). As was seen by Li *et al* earlier where the increase in proliferation of rabbit smooth muscle cells was in part due to a released factor acting in a paracrine manner, it has also been shown that the JNK/SAPK cascade can be activated in a similar fashion. Hamada *et al* demonstrated that conditioned media taken from stretched smooth muscle cells could increase the phosphorylated levels of JNK/SAPK increase because of activation of purinoceptors (67).

The studies highlighted represent a new and broadening field of mechanically active *in vitro* systems. The techniques, whilst not being 'whole organ' applications, have prevented the cells reverting to a non-contractile state and thus created a study environment where the VSMC are similar to the *in vivo* phenotype. Furthermore it has allowed the investigator to isolate and systematically analyse the effects of mechanical strain on a specific cell type. This provides an insight as to how each cell type may react to strain, and allows the effect of circulating factors to be scrutinised within the active

system. The question that has still to be answered is how the mechanical stimulus becomes an intracellular event. The studies have suggested that the nature and magnitude of the VSMC/extracellular matrix interaction is fundamentally important for the ability of the cell to sense mechanical strain. The contacts are mediated by the interaction of cell membrane-associated integrins with specific extracellular matrix proteins. The interaction is an attractive candidate as a key mediator of the intracellular signalling response to strain and co-ordination of the cell's biological response. This concept is discussed in the next section.

1.3) Mechanotransduction

The process of recognising and responding to specific mechanical stimuli, such as changes in cyclical strain, is critical for the growth, development and function of living cells. There are also many sensory functions including touch, hearing, baroreception, proprioception and gravity sensation, which require specialised mechanotransduction mechanisms. Recognition of the importance of mechanotransduction has prompted study of the mechanisms whereby a mechanical stimulus promotes an intracellular or nuclear event.

Extensive investigation has enabled the transduction to be separated into two related parts, the membranal phase and the cytosolic phase. The membranal phase consists of the interaction of specialised matrix receptors (integrins) with the membrane-associated proteins and the initial transduction of the extracellular stimulus across the membrane and into the cytosol. The complexity of the specific proteins involved in the different signaling cascades that are initiated in response to strain has made this a laborious task. The cytosolic phase involves a cascade of protein phosphorylation reactions, which eventually stimulates a protein to translocate into the nucleus to mediate the cells' response.

Maintenance of the structure and organisation of body tissues is dependent on the adhesion of cells to each other and the extracellular matrix (ECM). Numerous cell adhesion molecules have been described which have been divided into related groups by their basic molecular structure. The four major groups are as classified as follows:

1) The cadherins, which are simple transmembrane glycoproteins that bind cell to cell in a homophilic manner.
2) The immunoglobulin superfamily is so termed because the extracellular portion contains varying numbers of immunoglobulin like folded domains. This group of proteins is large and diverse because the extracellular portion is varied.

3) The selectins are different in that rather than entering into homophilic or heterophilic protein/protein interactions, the selectins bind to carbohydrates. This function is prevalent in the inflammatory response where for example L-selectins bind neutrophils and P-selectins bind platelets.
4) The final group of molecules is the integrins. This large family of proteins is of particular interest because they are involved in both cell/cell and cell/ECM adhesion. These adhesions are important as this enables the cell to detect deformational changes in its local environment.

The interactions of the integrins with the ECM activate a diverse array of intracellular signalling cascades. This provides a possible mechanism by which strain can induce cellular proliferation and changes in gene expression, by the activation of secondary messengers, such as the family of proteins called the mitogen-activated protein (MAP) kinases.

1.3.1) The Membranal Phase of the MAP Kinase Cascade.

There is an emerging consensus that when the cell surface receptor (integrin) is activated (either by binding of a ligand or by cellular deformation), a protein complex with the focal adhesion kinase (FAK) is formed. Once the extracellular domain of the receptor is stimulated, FAK becomes phosphorylated. This induces the binding of the mammalian adapter protein Grb2 (growth factor receptor-bound protein 2) via its SH2 domain to the intracellular domain of the receptor. The Grb2 protein has two other SH3 domains, which when the SH2 domain is bound, enforce the translocation of the guanine-nucleotide exchange factor Sos to the plasma membrane. Sos (son of sevenless) is the downstream effector protein of Sevenless, an RTK (receptor tyrosine kinase) that that controls photoreceptor development in the eye of Drosophila. This translocation brings the Sos protein into contact with the membrane bound, GTP-binding Ras protein. The Sos protein dissociates GDP from Ras, enabling it to bind GTP. The activated GTP-Ras complex recruits the Raf (68) protein to the plasma membrane, and whilst there, an unknown

mechanism activates the substrates collectively termed MAP kinase kinase kinases. The activated MAPKKK leaves the membrane and this is the beginning the cytosolic phase of signal transduction. The components of both the membranal phase and the cytosolic phase are discussed in more detail below.

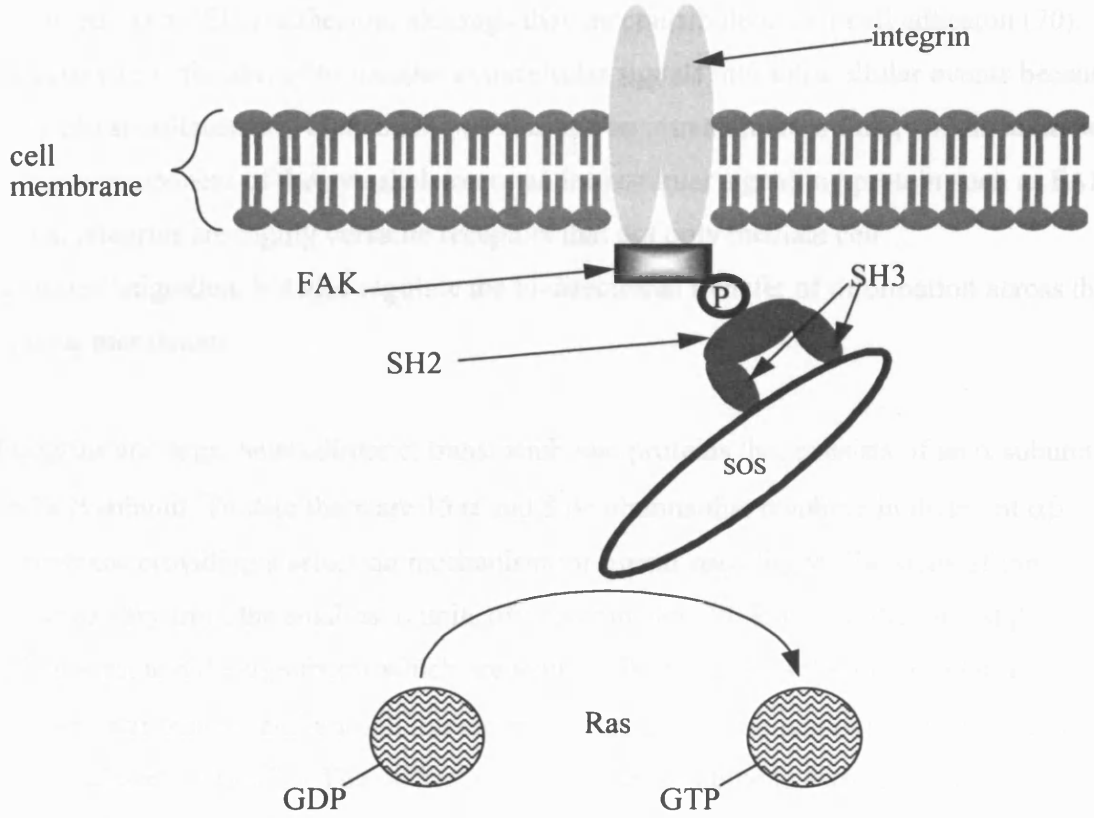

Figure 2: A schematic representation of the interaction between receptor tyrosine kinases and the Ras kinase.

1.3.1.1) The Integrin Family

Integrins are cell surface receptors that recognise three types of ligand, ECM proteins, plasma proteins and cell surface molecules. Cell adhesion is of fundamental importance to a cell, in that it provides anchorage, cues for migration and signals for growth and differentiation. Conversely, attached cells need to detach during specific events, such as

for migration or mitosis, and although the exact mechanism has still to be defined, integrins are suspected to play a part in these processes. There are two types of cell adhesion, those between two cells and those between a cell and the ECM (69), (70).

The integrins are a group of widely expressed cell surface proteins whose primary role is involved in cell-ECM adhesion, although they do contribute to cell-cell adhesion (70). Integrins have the ability to transfer extracellular signals into intracellular events because they can simultaneously bind ECM proteins and an intracellular protein, which could be either a component of the cytoskeleton or an intracellular signalling protein such as FAK. Thus, integrins are highly versatile receptors that not only mediate cell adhesion/migration, but also regulate the bi-directional transfer of information across the plasma membrane.

Integrins are large, heterodimeric, transmembrane proteins that consists of an α subunit and a β subunit. To date there are 15 α and 8 β subunits that combine in different $\alpha\beta$ complexes providing a selection mechanism for ligand specificity. The sizes of the subunits vary from the smallest β units of approximately 90 Kd up to the largest β subunit, β_4, and the α_1 subunit which are around 200 Kd. Each subunit has a large extracellular domain and a small intracellular cytoplasmic domain (with the exception of the β_4 subunit) (71), (72). The different subunits can combine to form at least 19 individual membrane proteins (see Figure 3). Integrins colocalise with intracellular proteins, such as vinculin, to form a focal point of contact between the extracellular matrix and intracellular kinase pathways (73). The composition of the extracellular matrix determines which integrins are included in the focal contact (69) and thus controls the specificity of the response.

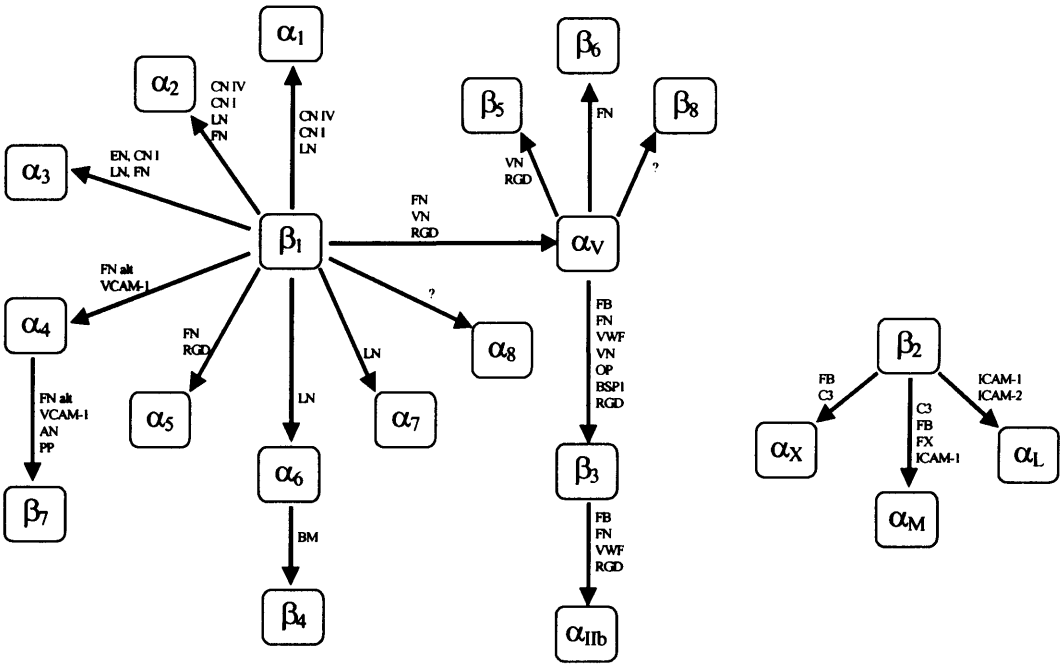

Figure 3: The Integrin Family. A diagrammatic representation of the subunit interactions and the ligand specificity, RGD specificity also indicated. Abbreviations: [FN, fibronectin], [FN alt, fibronectin alternatively spliced domain], [VN, vitronectin], [FB, fibrinogen], [LN, laminin], [VWF, von Willebrand factor], [CN, collagen], [OP, osteopontin], [BSP1, bone sialoprotein 1], [ICAM-1, ICAM-2, intercellular adhesion molecules], [FX, factor X], [BM, basement membrane], [C3, complement component C3bi], [EN, epiligrin], [AN, addessin], [PP, Peyers patch].

Investigations using α_5 deficient mice have established that homozygote embryos die *in utero* at 10-11 days of development. This was due to interruption of vasculogenesis (the formation of blood vessels from angioblasts- EC precursors) by preventing effective integrin-ligand interaction (74). A complementary study analysed the effect of fibronectin deficiency in mice, and found that the mutation proved lethal after a shorter gestation (75). Thus it was concluded that fibronectin and α_5 formed an important ligand/integrin complex regulating vasculogenesis *in utero* (69). VSMC in the media of normal arteries have been shown to express the $\alpha_1\beta_1$ integrin on their cell surface *in vivo*. However, when explants of vessels are cultured, the expression changes to predominantly $\alpha_2\beta_1$ integrins (76). It has been demonstrated that integrin expression can be influenced by growth

factors, therefore it is possible that this transformation is due to differences in cytokine concentrations *in vitro* compared to *in vivo* (77).

The cytoplasmic domains of the integrins have been shown to be critical in the formation of focal adhesions. The β subunit targets the integrin to a focal adhesion (78), (79), whereas the α subunit inhibits localisation (80), (81). When the integrin receptor is activated the inhibition of association with the focal adhesion (induced by the α subunit) is negated thus allowing the intracellular stimulus to be transferred (82). Two independent research groups identified that that integrins were involved with tyrosine phosphorylation when it was observed that thrombin activation of platelets induced phosphorylation in an integrin dependent manner (83), (84). Subsequent investigation identified a protein called $pp125^{FAK}$(see below), which was identified as a target molecule for the activated receptor (85).

1.3.1.2) Integrins and Disease

Integrins have been suggested to have an important role in a number of biological processes, such as adhesion to ECM, intercellular adhesion, maintenance of cell, morphology, cell migration, regulation of cell growth /differentiation, establishing tissue organisation/development, blood clotting and lymphocyte adhesion (71). Therefore normal development and homeostasis of multicellular organisms is dependent upon normal integrin function and interaction with FAK (a transducer of integrin stimuli). It can thus be concluded that integrin dysfunction cannot be underestimated in its importance in the development of certain pathological conditions.

The cell-to-cell interactions in the cardiovascular system are dynamic processes that require precise regulation. Most vascular cells express a number of different integrins, in that platelets express five integrins, EC have six, VSMC have three, neutrophils have three and monocytes and lymphocytes express eight and seven respectively (86). Cell proliferation, migration and matrix synthesis are important processes involved in

atherosclerosis, restenosis, myocardial ischaemia (causing the formation of collateral blood vessels) and medial thickening. These pathologies are regulated by the adhesive protein osteopontin, which mediates its adhesive and migratory effects via the $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors (87), (88), (89), (90), (91). In a normal vessel, VSMC express several integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_5\beta_1$, however at sites of cell accumulation and intra-plaque microcapillaries, the integrin $\alpha_v\beta_3$ (a regulator of angiogenesis) has been isolated (92), (76), (93). VSMC migration is required for wound healing (94) thus uncontrolled migration can turn renewal into pathological lesions i.e. intimal hyperplasia and vessel occlusion.

This confirms that integrins perform an important function during the development of an array of pathologies, which share similar basic mechanisms involving adhesion, inflammation and ECM production. In the areas of fibroproliferative and neoplastic disorders, it is uncertain as to whether the changes in integrin expression/levels is a cause of the disease or a secondary complication induced by alterations in the extracellular environment.

1.3.1.3) The Focal Adhesion Kinase Family

Protein tyrosine kinases (PTKs) are divided into two broad groups, separated by their basic structure, depending whether or not they span the cellular membrane. Membrane spanning PTKs include receptors for a variety of growth and differentiation factors, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), and are termed as receptor PTKs. The induction of an intracellular response is stimulated by the binding of a ligand to the extracellular domain of the protein causing tyrosine autophosphorylation of the intracellular domain. This in turn phosphorylates a cascade of exogenous substrates (reviewed by (95), (96)). The non-membrane spanning PTKs (or nonreceptor PTKs) lack an extracellular domain and thus utilise proteins from outside the cell, such as the ECM/integrin complex (97). There are two proteins in this group, FAK and PYK2.

1.3.1.3.1) Focal Adhesion Kinase- FAK or pp125^{FAK}

A focal adhesion is an integrin-mediated site of contact between the cell and the ECM (98). FAK or pp125^{FAK} (FAK for focal adhesion kinase), is the prototypical member of a small family of two non-membrane spanning PTKs, that is a 125 kDa peptide whose name was derived from its submembranal localisation (85). When the kinase was first isolated and cloned, immunoflourescent localisation detected FAK at cellular focal adhesions (85). The tyrosine phosphorylation of FAK is regulated by integrin adhesion to ECM proteins. Later investigation showed that FAK has either a ubiquitous distribution, e.g. neurones, or is localised away from a cellular focal adhesion site, e.g. astroglia where the FAK localises with the cytoskeleton (99), (100), (101).

The oncogenic PTK FAK, was first identified as one of a host of intracellular substrates to be phosphorylated by transfecting cells with a Rous sarcoma virus vector containing the tyrosine kinase pp60$^{v\text{-}src}$ (102). The cellular location of FAK, and its subsequent change of name from pp125, was made by two independent teams almost simultaneously. Schaller (with the team of investigators who initially found FAK) screened a chick embryo cDNA library to clone the gene and then employed immunohistochemistry to isolate the membranal location of FAK to the focal adhesions (85). Further research however, discovered that FAK was phosphorylated in growing cultures of BALB/c3T3 cells, but that trypsinisation of the cells stopped the phosphorylation. This activation could be restored within minutes when the cells were replated onto fibronectin (97). This discovery was the start of the elucidation of FAKs role in the transduction of extracellular stimuli via transmembranal proteins (integrins) into cytoplasmic signals (97), (103), (98).

FAK is a highly conserved protein between species, exhibiting approximately 90% overall amino acid homology between the *Xenopus*, avian, murine and human homologues, increasing to 97 % homology in the catalytic domain (97), (85), (104), (105), (106), (107).

FAK comprises of a central catalytic domain flanked by an NH_2- and C-terminal noncatalytic domains. The complete characterisation of the NH_2-terminus function has still to be completed although integrin binding is suspected. The C-terminal contains numerous binding sites for cytoskeletal and signalling proteins, as well as the focal adhesion targeting (FAT) sequence which is responsible for directing and/or anchoring FAK to cellular focal adhesions (108), (106). It does not contain any SH2 or SH3 domains, which are responsible for interactions with other PTKs, however an autophosphorylation site has been identified that is important for the formation of stable complexes with subsequent kinases (85), (109).

More recently, the existence of a protein containing just the carboxy-terminal of FAK has been described called pp41/43^{FRNK}, where FRNK represents FAK related non-kinase (110). The FRNK fragment is generated by alternative splicing and appears to act as a competitive inhibitor, competing for the protein that FAK requires for signalling (111). Conversely, whilst analysing FAK expression in human tissues, André and co-workers detected the protein in all tissues tested (highest in the brain) and found a 3.3 Kb N-terminally truncated form of FAK, exclusively expressed in the brain (104). A similar phenomenon is seen in embryonic stem cells where an N-terminally truncated form of the PDGFβ protein is expressed. This contains the catalytic and transmembrane domains but lacks the extracellular domain and is suspected to be functional during early development (112), (113).

1.3.1.3.2) Proline-rich Tyrosine Kinase 2- PYK2 or CAKβ

The other member of non-membrane spanning PTK family was isolated simultaneously by two independent research teams and called PYK2 and cell adhesion kinase β (CAKβ) (114), (115). The 4.5 Kb PYK2/CAKβ gene encodes a protein of 1009 amino acids and displays an overall 45% amino acid identity (60% in the central catalytic domain) with the FAK sequence (114), (115). Lev *et al.* demonstrated, by Northern analysis, that PYK2

was most abundant in the human brain. Further *in situ* analysis showed that PYK2 was expressed in discrete areas of the brain, with the highest levels being seen in the hippocampus, dentate gyrus and olfactory bulb. When PYK2 was precipitated with specific antibodies and migrated in an SDS gel, the peptide was found to have a molecular mass of 112 Kd. PYK2 can be rapidly activated, as was demonstrated in PC12 cells when the addition of carbachol induced phosphorylation within five seconds (114).

Unlike FAK, PYK2/CAKβ localises to sites of cell-cell contact and is thought to regulate signal transduction by cell contacts because the phosphorylation state of the peptide is not reduced by trypsinisation from matrix proteins (115). A complex series of experiments performed by Lev *et al* determined that PYK2 phosphorylation is indirectly regulated by changes in cytoplasmic calcium, via both pkC-dependant and pkC-independent mechanisms (114).

PYK2 has a simultaneous increase in phosphorylation levels with the adapter protein Shc, which is interesting because Shc has been shown to activate the Ras/MAPK signalling pathway (116), (117). Tyrosine phosphorylated Shc can bind to the SH2 domain of the Grb2 scaffold protein, whilst the guanine nucleotide releasing factor Sos is bound to the SH3 domain of the scaffold protein (118), (117). It was later discovered that PYK2 has an LNV sequence after Tyr 881 that is a canonical binding site for the SH2 domain of the Grb2 protein and that PYK2 formed a complex with Grb2 in stimulated PC12 cells (114). After a series of immunoprecipitation studies it was found that the Grb2 scaffold protein was the link because when phosphorylated PYK2 was bound to the SH2 domain, Sos was recruited to the SH3 domain and phosphorylated (114). Human embryonic kidney 293 cells were transfected with increasing concentrations of a vector producing PYK2. Analysis after 24 hours of the cell lysates showed that increasing concentrations of PYK2 expression caused myelin basic protein phosphorylation (a standard test for MAPK levels) to increase in a concentration dependant manner (114). Therefore suggesting that PYK2, a regulator of cell to cell adhesion signalling, could exert its effect via the MAPK cascade, which in turn can regulate gene expression by activating the AP-1 transcription factor (119).

1.3.1.4) The Grb2 Protein and its SH2 and SH3 Domains

In mammalian cells, the SH2 and SH3 domains are located on a peptide called Grb2 (growth factor receptor-bound protein 2) (118). The src homology 2 (SH2) and src homology 3 (SH3) domains, so termed because of their similarity to the src gene, provide an important link between an activated cell surface receptor and the primary intracellular target of a signalling cascade. The src oncogene (a gene associated with malignant growth) was first identified by Stehelin and co-workers from Rous sarcoma virus (120), when the aim of the investigation was to investigate the effects of transformation of oncogenes on cellular function. Research at this time into oncogenes was improving the knowledge of the scientific community in respect to the effect of oncogenes and their transformation effects in relation to intracellular communication and proliferation.

Grb2 has a molecular size of approximately 25 kDa and consists of a single SH2 domain about 100 amino acids in length, flanked by two SH3 domains of about 50 amino acids in length. Northern analysis by the team that identified and cloned Grb2 indicated that the peptide was widely expressed as every tissue analysed contained Grb2 mRNA. The levels were found to be particularly high in the brain, spleen, lung and intestine (118).

The functional activity of the SH2/SH3 domain containing proteins is to act as a scaffold, to orientate and hold kinase and target in the correct alignment for the continuation of the phosphorylation cascade. This function however has been found to be both excitatory (in the form of intracellular phosphorylatory pathways) and inhibitory by causing these domains to interact with one another. The inhibitory interactions between respective SH2 and SH3 domains can occur both as intramolecular and intermolecular reactions (121), (122).

Another function of the SH2/SH3 domains, especially the SH3 domain, is to control the location of the signalling proteins within the cell. Bar-Sagi *et al* demonstrated that in

mammalian fibroblasts the SH3 domains of Grb2 direct the protein the membrane ruffles (123).

When the extracellular domain of an RTK is stimulated, in this case the integrin/FAK complex, the receptor dimerises and undergoes autophosphorylation. This is characterised by the intrinsic ability of an enzyme/kinase to exchange factor, Sos (Son of sevenless) to induce the binding of GTP to, and phosphorylate itself or another protein of the same species. Once activated, the phosphorylated sites act as recognition sequences for the SH2 domain of Grb2. The flanking regions of Grb2, the SH3 domains then recruit the guanine nucleotide subsequent activation of Ras (124), (125), (126). It has been demonstrated that stable Grb2/Sos complexes require both of the SH3 domains of the scaffold protein (117), and that overexpression of the Grb2 protein induces an increase in Ras activity (127).

Despite differences in cellular localisation, there are a number of conserved binding sites for SH2 and SH3 between FAK and PYK2/CAKβ, suggesting that the same signalling molecules may be recruited into the cascades regulated by the two kinases.

1.3.2) The Cytosolic Phase of the MAP Kinase Cascade

1.3.2.1) Multiple MAP Kinase Cascades

The MAP kinase cascade is a series of highly ubiquitous serine/threonine kinases, which play a crucial role in signal transduction from the membrane to the nucleus. The cascade can be initiated at the membrane by activation of either an RTK (tyrosine kinase receptor) or a G-protein coupled receptor. This leads to the phosphorylation of the MAPKKK proteins, which in turn phosphorylates the MAPKK (MEK [ERK kinase]) group of kinases. These then phosphorylate the MAPK proteins, which unlike their predecessors require phosphorylation of both a threonine residue and a tyrosine residue that are

separated by a single amino acid. Therefore if MAPK is to fully be activated, both residues have to be phosphorylated by a dual specificity kinase, MAPKK (MEK).

MAP kinases are an important network of interacting intracellular proteins that transduce extracellular stimuli into an intracellular response. These signalling pathways exhibit a high degree of evolutionary conservation (128), (129). Each step of the MAP kinase cascade consists of a family of enzymes rather than a single protein, which allows for the development of a network of individual signals within the cell, causing specific cellular events. There are a varied number of MAP kinase intracellular signalling pathways that are becoming increasingly well documented. Initial research lead to the description of a number of signalling cascades in yeast and recent developments using the yeast sequences have elucidated new MAP kinase isoforms in mammalian cells. The different isoforms of MAP kinase respond to specific stimuli and have different intracellular substrates.

MAP kinases transduce an extracellular stimulus into an intracellular event by controlling the activity of several transcription factors, via phosphorylation of either stimulatory or inhibitory regulatory sites.

A common feature of all the MAP kinase isoforms is the requirement for phosphorylation of both threonine and tyrosine regulatory residues by a specific upstream protein kinase to be activated. Inactivating the MAP kinase by dephosphorylation can also regulate these intracellular signalling cascades. Activated MAP kinases are inactivated after dephosphorylated by protein phosphatases.

The common pathway identified is that after the membranal phase has been activated, MAP kinase kinase kinase, or MAP3K (these proteins include *Raf-1*, A-Raf, B-Raf, Mos and MEK kinase [MAP kinase/extracellular regulated kinase kinase] becomes phosphorylated and activates MAP kinase kinase (MAP2K and MEK). The activated MAP2K in turn phosphorylates MAP kinase, or MAPK (these proteins include the ERK's [extracellular regulated kinase], Jun kinase and SAP kinase). The final common

step in the cascade is when activated MAPK phosphorylates the MAP kinase activated protein kinase (MAPKAP kinase). The largest number of proteins has been found at the MAPKAP kinase level, and these can exert an effect within the nucleus, in the cytosol or at the cell membrane (130).

1.3.2.2) The MAP Kinase Cascade

The cytosolic phase of the MAP kinase cascade is a specific series of reactions that lead via sequential activation to the activation of MAPKAP kinase which then causes the intracellular effect (see Figure 4).

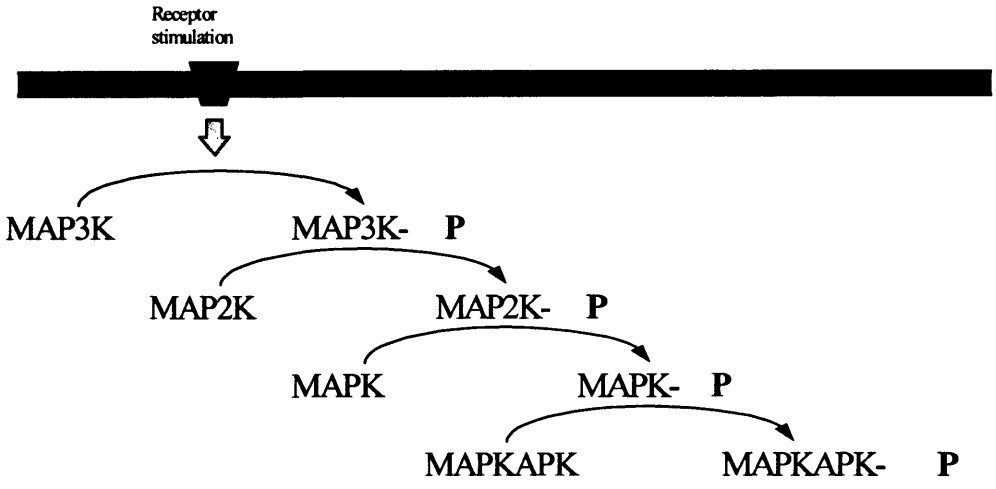

Figure 4: A schematic representation of the intracellular phosphorylation reactions within a cell during the cytosolic phase of the MAP kinase cascade

The most thoroughly studied MAP kinase signalling cascade is the extracellular signal-regulated kinase (ERK) cascade, so called because the MAP kinase proteins are termed ERK1 and ERK2. Early investigators found that ERK1 and 2 could undergo autophosphorylation on both the tyrosine and threonine residues, however the rate of reaction was very slow. This facilitated the development of the hypothesis that there were

upstream activators of this MAPK activity and thus proteins that could rapidly induce phosphorylation (131).

Although the final elucidation of the MAP kinase pathway was not sequential, for ease of reading, the following discussion will consider the components of the pathway from the membrane through the cascade of successive kinases. The discussion will follow the activation of Ras via the membranal phase of mechanotransduction, which in turn activates MAPKKK, MAPKK (MEK), MAPK and MAPKAPK.

1.3.2.2.1) The Ras Family: Ras, R-ras, R-ras2 and R-ras3

The *Ras* family of GTP-binding proteins is found anchored to the membrane and are responsible for the signal transduction of a wide variety of cellular events during cell growth and differentiation. Apart from the pathway of interest in this thesis, *Ras* - *Raf* - MEK - MAPK, this peptide has been shown to interact with numerous other intracellular signalling proteins. Targets include Racl (132), RhoA (133), RhoB (134), RalGDS (135), Cdc42 (136), Pak (137) and phosphatidylinositol 3 kinase (PI3-K) (138).

The presence of *Raf* was first noted to be required for *Ras* signalling by Williams *et al*, when it was demonstrated that $p21^{ras}$ transformed Sf9 cells needed *Raf* to induce transformation (139).

The *Ras* isoforms R-*Ras1*, *2* and *3*, perform similar functions within the cell. The different isoforms are identifiable by small alterations in sequence homology and more significantly differences in tissue expression.

The involvement of *Ras* in the MAPK cascade was first elucidated by Itoh *et al* when studying cytosolic fractions obtained from *Xenopus* oocytes (68). Using this cell free system, the effect of *Ras* on the levels of MAP2K and MAPK activation was assayed, and it was noted that addition of *Ras* increased the phosphorylation levels of the later two

peptides. Interestingly an unidentified peptide was also noted to be present, which was termed *Ras*-dependent ERK-kinase stimulator (REKS), although subsequently this protein would prove to be one of the MAP3K family (68).

1.3.2.2.2) MAP kinase kinase kinase: Raf, MEK kinase and Mos

The majority of research attempting to elucidate the specific members of the MAP kinase cascade was using the 'upstream' approach. Therefore although MAP3K is the first enzyme in the cascade, it was chronologically the last protein to be identified (it was already known that MAP2K was the upstream activator of the ERKs). The *Raf* proteins are a family (*Raf-1*, *A-Raf* and *B-raf*) of serine/threonine kinase that were previously known to be activated by many extracellular stimuli (140) and were therefore a good candidate for investigation.

The *c-raf-1* gene encodes *Raf-1*, which is a 72-74 kDa cytoplasmic protein with intrinsic serine-threonine kinase activity. Kyriakis *et al* stably transformed a cell line (F4) with v-*raf* (murine sarcoma virus 3611, which produces an N-terminal truncated *Raf-1* product) and were able to stimulate ERK activity in serum deprived media (141). Cytosolic cell fractions were assayed for their ability to phosphorylate myelin basic protein (MBP- a common MAP kinase substrate) from both the F4 (v-*raf* containing cells) and CL-7 (the parental NIH3T3 clone) after serum deprivation. The F4 cells, in the absence of agonist stimulation, exhibited vigorous endogenous MAP kinase activity compared to the basal level of activity obtained from the CL-7 cells. When isolated, the MBP kinase proteins were identified as two species with molecular weights of 44 and 42 kDa (ERK1 and 2). To characterise this response further, the cytosolic fractions were assayed for their ability to activate bacterially expressed ERK 1 (p44 MAP kinase). Extracts from CL-7 showed little MAP2K activity whereas the F4 cells displayed significant MAP2K, which when analysed by immunoblotting revealed a protein of 48 kDa which was neither an ERK nor *Raf*, and was subsequently identified as MAP2K (141). In addition, both cell lines had their MAP kinase activity blocked by the addition of specific phosphatases (protein

phosphatase 2A- PP2A), indicating that *Raf* was the upstream activator of MAP2K and the ERKs.

Shortly after this data was published, another group reported that *Raf* proteins isolated from cells infected with two forms of viral *Raf* (v-*Raf*) were able to activate PP2A treated MAP2K isolated from rabbit skeletal muscle (142).

Lange-Carter *et al* used identical sequences from Ste11 and Byr2 (two isoforms of yeast MAP3K) to design degenerate oligodeoxynucleotides so that the mammalian equivalent could be identified by PCR (143). Using these specific primers and a cDNA template derived from NIH 3T3 cell RNA, a 320 bp fragment was generated that was subsequently found to be similar in sequence to the two yeast isoforms. The PCR product was then used as a probe to isolated a 3260 bp MEKK sequence from a mouse brain cDNA library. When analysed, the sequence contained two possible start sites, which would generate a protein of between 672-687 amino acids in length (73-74.6 kDa). This is the expected molecular size of MEKK (78-80 kDa) as determined by SDS-polyacrylamide gel electrophoresis and immunoblotting (143).

A 7.8 KB mRNA that was expressed in the majority of cell lines encodes MEKK and mouse tissues analysed. High levels of basal expression were seen in the PC12 (pheochromocytoma) (144) and CHO (Chinese hamster ovary) cell lines and in the murine spleen, heart and kidney (143). Readily detectable levels of MEKK were also isolated from NIH3T3 and Rat1a cell lines and the lung and brain (143). Analysis of the rodent cell lines by immunoblot gave two immunoreactive species, one being the expected 78 kDa MEKK and the other a 50 kDa species. The level of intensity of the 50 kDa fragment varied between preparations, thus Lange-Carter *et al* concluded that this was a proteolytic fragment of the 78 kDa protein. However, this has still to be clarified. MEKK was identified as an isoform of MAP3K because when overexpressed in COS-1 cells, there was a four to five fold increase in MAP kinase activity. Further experiments using isolated MEK (MAP2K) showed that MEKK activated MAP kinase by activating MEK (143).

Williams *et al* sought to elucidate the interactions between *Raf-1* (two isoforms used, *v-Raf* and Raf22W), MAP kinase (ERK1/p44) and the membrane protein *Ras* (obtained using the recombinant bacculovirus $p21^{v\text{-}ras}$) (145). The two *Raf-1* isoforms encoded different products, in that *v-Raf* produces a 90 kDa protein and Raf22W produces the activated 38 kDa form of the protein. The interactions were observed by infecting cultured insect cells (Sf9) with the vector containing MAP kinase and then either individually or together, the vectors containing *Raf-1* and *Ras*. The investigation revealed that coexpression of MAP kinase with either *v-Raf* or $p21^{v\text{-}ras}$ induced a limited increase in MAP kinase activity. However, coexpression of MAP kinase with both *v-Raf* and $p21^{v\text{-}ras}$ lead to a very marked increase in kinase activity (145). Previous publications by Williams and co-workers (139) demonstrated that *Ras* activated *Raf-1*. Thus after considering the data from both studies, it was concluded that *Ras* was the final step of the membranal phase of the cascade, which then phosphorylated and activated *Raf-1* enabling it to continue the cytosolic phase of the pathway causing the activation of MAP kinase. Another vector used in the expression system contained a dominant-inhibitory mutant form of *Raf-1* (Raf-301). Interestingly, when this was coexpressed with $p21^{v\text{-}ras}$ it did not block the activation of MAP kinase, suggesting that there were other routes by which MAP kinase could be phosphorylated (145). The possibility of both *Raf-1* dependent and *Raf-1* independent pathways helps to explain the diversity and variety of stimuli and intracellular responses involved in the MAP kinase pathway. Furthermore it was observed that phosphorylated MAP kinase could phosphorylate and activate *Raf-1* (145). This ability of MAP kinase to stimulate itself is a viable mechanism by which the intracellular signal is amplified along the cascade causing such acute responses within the cell.

More recently, a novel MAP3K that is stimulated by insulin has been identified by Haystead and co-workers (146). The novel peptide (termed as the insulin-stimulated MEK kinase or I-MEKK) has two isoforms with an M_r of 56 kDa that could phosphorylate and activate the 73 kDa MEK (an isoform of MAP2K). It was also found that PP2A inactivated the I-MEKK.

The MAP3K isoform of B-raf was isolated from a rat cell line (PC12) where previous research showed that c-Raf-1 expression in PC12 cells did not phosphorylate the ERKs, and almost none of the MAP3K activity of these cells was immunoprecipitated using antibodies raised against c-Raf-1 (147). NGF and EGF activated the protein. Shortly after this classification, a host of similar studies reported the phosphorylation of MEK via this peptide in PC12 cells. However whether or not both B-raf and *raf-1* can activate MEK, or just B-raf is still under debate (148), (149). This isoform of MAP3K has a very limited tissue distribution in that it was only detected in foetal brain and testis, nevertheless it had previously been observed in PC12 cells (150). A subsequent publication by Boulton and Cobb identified a novel MAPK isoform unique to the brain and testes (129). This would propound an intracellular signalling pathway unique to neural and testicular tissue, increasing the complexity and specificity of the MAPK family interactions.

1.3.2.2.3) MAP kinase kinase: MEK-1a, MEK-1b and MEK-2

The mammalian MAP2K family consists of three conserved serine/threonine kinases that display approximately 85% homology (151). MEK-1a is a 44 kDa peptide that only requires phosphorylation of its serine residues to become activated, and is capable of being phosphorylated by either MEKK or Raf-1, and can also autophosphorylate (152), (153). The phosphorylation of both serine 218 and 222 is necessary for full MAP2K activity (154), (155), (156). Although structurally related, the 41 kDa MEK-1b does not autophosphorylate, and displays no MAPK phosphorylating activity. Thus its biological role *in vivo* has yet to be elucidated (157).

1.3.2.2.4) MAP kinase: ERK1 and ERK2

There are two kinases, known as ERK1 and ERK2, which are acutely activated by a variety of extracellular stimuli and by oncogene products causing cell proliferation or

differentiation (158). In addition to regulating acute responses within the cytoplasm, they can also enter the nucleus and alter gene expression (159).

Under certain conditions, phosphorylation of only one of the ERK's has been observed (160), (161) suggesting that there is a selective mechanism that allows for the specific diversification of the signal through the members of the cascade. ERK1 and ERK2 were the first members of this protein kinase family to be identified (128), (162). Once purified and cloned, the enzymes of 44 and 42 kDa, were found to be 83% homologous, with the greatest differences located in the N-termini, preceding the protein kinase catalytic core (163), (162).

Northern analysis has indicated that ERK1 and ERK2 are ubiquitously distributed throughout the body, with the highest levels found in the brain and the spinal cord (129). There after the levels become more specific for each isoform. Outside the nervous system, ERK1 had highest levels in the intestine and lung whereas ERK2 had the greater expression levels in the muscle and heart. Both had low levels of expression in the thymus, liver, spleen, kidney, testes and in epididymal fat (129).

The phosphatase specific for ERK1 and ERK2, is an immediate early gene whose expression is increased by serum and growth factors (164).

1.3.3) Integrins, FAK, Ras and the MAPK Cascade

FAK is an important mediator of a variety of intracellular signalling pathways, as it appears to be the link between the membrane and the cytosol. When the integrin receptor is stimulated it activates FAK by phosphorylation of the amino acid Tyr-925. The Grb2 peptide binds to this amino acid, and once bound, the Grb2 protein can activate Ras and therefore stimulate the MAP kinase cascade (165). The description of the mechanism that stimulates and potentiates integrin/FAK activation are of important scientific and

therapeutic value. Conversely the inhibitory pathways could also provide vital information for the prevention of the progression of certain pathologies.

The completion of the MAP kinase cascade leads to the activation of specific nuclear proteins involved in the regulation of gene expression. They are termed as *trans*-acting factors, which then interact with sequences of the DNA called *cis*-acting elements.

The proteins stimulate an increase in the levels of transcription of the gene to which they have associated and are classified as either promoter or enhancer elements. Promoter elements increase the frequency of transcription of a gene, whereas enhancer elements can only increase the frequency of transcription of a gene when a promoter element is bound. The main difference between the two proteins is that promoter elements bind sequences in the promoter of the gene and enhancer elements are found up to 1000 bp upstream. Sequence specific DNA binding proteins have molecular weights between 35-80 kDa and bind to the DNA as either homo or heterodimers to palindromic sequences. There are at least four functional domains within the protein, a transcriptional activation domain, a dimerisation domain, a DNA binding domain and a stimulatory domain, which is the target for the upstream activator. Most of the proteins bind to the DNA as either homo or heterodimers to palindromic sequences.

The MAP kinase cascade is known to regulate promoter elements, such as AP-1 (c-Fos and c-Jun) and SP-1 and these proteins are called mitogen activated protein kinase activated protein kinases (MAPKAPK) (166), (167).

The ERK signalling pathway has been shown to be involved in both stimulation and/or response to a variety of cytokines, some involving integrin stimulation (62). Thus, in trying to elucidate a mechanism in which cyclical strain is sensed and the stimuli passed to the nucleus, the ERK/integrin pathway became a strong candidate for investigation.

1.4) Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF), which is also referred to as either vascular permeability factor (VPF) or vasculotropin, is a family of secretory proteins that increases endothelial cell growth and potently increases endothelial permeability (168). The VEGF proteins were first identified on the basis of their ability to promote fluid and protein extravasation, when conditioned media taken from tumour cells increased the leakiness of subcutaneous blood vessels of guinea pigs (169), (168), (170). Senger et al identified and purified a protein with a molecular weight (M_r) of between 34,000-42,000 kDa, and as little as $5x10^{-12}$ M (200 ng) increased vascular permeability to the same degree as $4x10^{-9}$ M (1.25 μg) histamine. This suggests that on an equimolar basis, VEGF increases permeability about 50,000 times more potently than histamine (168), (170), (169).

At about the same time VEGF's angiogenic function was recognised by two research teams working independently. Connolly *et al* released two novel reports that presented data to suggest that this recently isolated permeabilising factor was also able to cause a mitogenic response in bovine aortic endothelial (BAE) cells (169) (171). This paper also identified the possibility of isoforms of the peptide, in that the team identified subunits with varying sizes from M_r 18-24 kDa. These differences were postulated to arise because of alternate glycosylation or proteolysis although any differences between the isoforms in the ability to enhance permeability or induce EC proliferation was not specified (171).

Ferrara *et al* demonstrated that conditioned media taken from bovine pituitary folliculo-stellate cells (FC) could induce a mitogenic response in adrenal-cortex-derived capillary endothelial cells (172). This observation was interesting because at this time, adrenal-cortex-derived capillary endothelial cells had shown a mitogenic response to either aFGF or bFGF, but not to EGF, TGF-α, TGF-β, PDGF, insulin or TNF (173). Further purification and HPLC analysis revealed a protein of approximately 23,000 kDa, with a

unique N-terminal amino acid sequence, compared to the previously described growth factors. Gas phase microsequencing showed a single N-terminal with the first five residues as follows: Ala-Pro-Met-Ala-Glu. Analysis of the known protein sequences using computer data banks indicated no protein with any significant homology (172). Ferrara and co-workers studied the bioactivity of the novel growth factor, by analysing its proliferative effects on different cell types. Of the cell lines analysed, appreciable activity was only observed in the cell types of vascular endothelial origin, such as foetal and adult bovine aortic EC, bovine brain capillary EC and human umbilical vein EC. The concentration of VEGF required to elicit a mitogenic response (assuming a dimer M_r of approximately 45,000) was between 0.55 and 26 pM (172). In contrast, adrenal cortex cells, lens epithelial cells, corneal EC, fibroblasts and keratinocytes did not exhibit any significant mitogenic response to the novel growth factor (169), (172). A subsequent publication by Ferrara's group isolated and cloned VEGF A_{165} from a FC cDNA library (174). The clone was isolated using a 59 bp probe raised against the NH_2-terminal amino acid sequence (position 2-21) of VEGF. The cDNA inserts for the human and bovine genes (which display an amino acid homology of over 95 %) were cloned into a vector containing the cytomegalovirus (CMV) promoter (a high copy number promoter). The media conditioned by human 293 cells transfected by the VEGF vectors promoted proliferation of capillary EC, whereas the media from untransfected cells or cells transfected by the vector alone had no mitogenic effect. The presence of VEGF A_{121} and $_{189}$ was also detected although the explanation and reason for their presence was unknown (174).

The property of VEGF which facilitated its extraction and purification, was its ability to bind to heparin (168), (172). Heparin sulphates are fundamental components of the extracellular matrix (ECM), and it is believed that they are involved in the interactions between target cells and heparin binding growth factors (172).

1.4.1) Vascular Permeability

Proteins that mediate permeability have been separated into two groups. The first are those which function by recruiting circulating neutrophils to interact with the endothelium and are termed as chemoattractant mediators, such as interleukin-8. The second type acts directly on the endothelium and is thus called direct-action mediators, such as bradykinin and histamine. VEGF acts as a direct mediator of permeability and differs from other agents such as serotonin, histamine, kinins, prostaglandins and leukotrienes that only affect vascular permeability in the presence of injury or inflammation (175). In contrast VEGF increases permeability, both rapidly and reversibly, without the requirement of injury or inflammation (168), (170). A single intradermal injection caused an increase in vessel permeability within five minutes, however, after twenty minutes only a low level of residual increased vascular permeability was present (168). When studied by light and electron microscopy, the EC challenged with VEGF show no physical damage or change (168), (171). The change in vascular permeability is achieved without causing mast cell degranulation or endothelial cell damage (170). More recent investigations have demonstrated that the vascular permeabilising response is biphasic, in that VEGF produces a transient increase lasting no more than a few minutes coupled with a chronic sustained increase in permeability that is present twenty-four hours after exposure to VEGF (176). The chronic response was later attributed to calcium influx as addition of a known inhibitor of calcium influx ($NiCl_2$) halted the increase in permeability (177).

A variety of models for the mechanism by which VEGF increases vascular permeability have been suggested, the first is that VEGF causes separations at endothelial cell junctions (170). Another is that cytoskeletal changes lead to an in cellular tensigrity thus causing an increase in permeability (178). A further study investigating the VEGF expression during embryonic development found persistent peptide expression in the cells adjacent to fenestrated endothelium (179) and yet another found increased VVO (vesiculovacuolar organelles) action in VEGF treated areas (180). VVO's are grape-like clusters of interconnected membrane bound vesicles which are found in the cytoplasm of

EC. Qu *et al* observed that EC's exposed to elevated levels of VEGF had increased concentrations of VVO's and that the endothelial layer was fenestrated (180).

A detailed study of the mode of permeabilising action was performed by Roberts and Palade (181). Vessels were treated by an intradermal injection of recombinant VEGF A_{165} and closely analysed by microscopy. It was found that the peptide induced extensive opening of endothelial intercellular junctions, caused the development of single diaphragmed fenestrae and transendothelial channels and lead to the formation of aggregated dilated plasmalemmal vesicles (VVO's). Compounds such as histamine and serotonin cause an increase in vascular permeability by opening endothelial intercellular junctions solely in the post-capillary venules and do not cause fenestrations, however VEGF caused fenestrations in capillary and venular EC's (the microvascular beds where fenestrated EC's are not normally present). These effects were indicated to be the direct effect of VEGF administration because dosing with heat inactivated VEGF A_{165} or the simultaneous administration of monoclonal antibodies raised against VEGF A_{165} inhibited these phenomena (181).

Interestingly, VEGF was shown to induce blood flow directly by nitric oxide (NO) mediated vasodilatation (182) and continuing this theme, Murohara *et al* illuminated a possible intracellular mechanism by which VEGF exerts its permeabilising actions via NO. Analysis of *in vivo* protein extravasation assays demonstrated that the VEGFR1 receptor (see below) when stimulated by the related placenta growth factor, failed to induce permeability. However VEGF action increased permeability by activating NO in EC. The effect of VEGF was enhanced by the addition of NO donors, suggesting in part a potential intracellular signalling event that could regulate the permeabilising action of VEGF (183). Feng *et al* completed the jigsaw by demonstrating that VEGF activated endothelial NO synthase (eNOS) via VEGFR2 in cultured bovine retinal EC. The data presented indicated that eNOS activated the caveolin-1 protein, which caused the formation of caveolae. These are small membranal invaginations that close over and detach to form small vesicles called pinosomes (184) (185). A simultaneous publication

from Vasile and co-workers confirmed the presence of caveolin-1 and VVO's in bovine EC exposed to VEGF, both *in vitro* and *in vivo* (186).

VEGF's ability to increase vascular permeability has been linked to the mitogenic activity, because extravasation of plasma fibrinogen causes clots which form an extravascular matrix that favours and supports the growth of new vessels (187), (188). In keeping with the above information and the data presented earlier from Murohara *et al*, Papapetropoulos *et al* demonstrated that the angiogenic effect of VEGF on EC was also mediated via NO. Although short-term exposure to VEGF caused tyrosine kinase release of NO, long term exposure (at least twenty-four hours) caused an upregulation of endothelial NO synthase (189). Further observation revealed that VEGF stimulated the NO-dependent formation of networks of EC when cultured in collagen gels, suggesting that NO is involved in VEGF induced EC proliferation and organisation, as well as vascular permeability.

Although it has been demonstrated that VEGF can cause an increase in endothelial permeability and proliferation, the mechanism by which the two signals are differentiated within the EC has still to be defined. A report submitted by Wu *et al* presents data that indicates that VEGF activates the KDR receptor (see Section 1.4.3.2) and induces permeability utilising nitric oxide and protein kinase C, however the full mechanism has still to be elucidated (190).

1.4.2) VEGF Isoforms

The VEGF family consists of five genes, termed VEGF A to E. Differential splicing these genes has lead to the production of at least nine, disulphide-linked, heparin binding dimeric glycoproteins. The interactions of the isoforms and there specific receptors are discussed below.

1.4.2.1) VEGF A

VEGF A is a family of peptides, which are widespread throughout the body and well characterised. Four of the species, VEGF A_{121}, VEGF A_{165}, VEGF A_{189} and VEGF A_{206} (which are 121, 165, 189 and 206 amino acid residues in length respectively) are produced by differential splicing of the eight exon VEGF A gene (174), (191), (192), (193). The two shorter constructs (VEGF A_{121} and VEGF A_{165}) were found to be freely soluble and released from the cell (194). These proteins can be deglycosylated to form monomers, which can have either of two molecular weights, VEGF A_{165} has monomers of 23K and 18K and VEGF A_{121} can form monomers of 18K and 14K (195). The transcript of VEGF A_{121} was isolated from a human placental cDNA library (196). The two larger species VEGF A_{189} and VEGF A_{206} remain cell associated because of a molecular signal in codon 6 of the mRNA which encodes a highly basic 24 amino acid sequence that promotes tight binding to heparin proteoglycans on cellular surfaces and ECM (197). The specific biological function of these larger isoforms is still unknown although cleavage of VEGF A_{189} with plasmin produces a 110 amino acid fragment, which is biologically active (198). Thus the longer isoforms may be retained by extracellular matrix associated heparin-sulphate moieties as a storage mechanism until enzymatic cleavage and release.

Subsequent work has identified the mRNA of a further splice variant from the VEGF A gene. Anthony *et al* performed reverse transcription polymerase chain reaction analysis on the mRNA isolated from human placental tissue and cultured placental fibroblasts (obtained during the first trimester of pregnancy). This identified a splice product encoding a 145 amino acid sequence, although the expected peptide product has still to be isolated (199). This observation was supported by further PCR analysis of both endometrial and placental mRNA showing the existence of VEGF A_{145} (200), (201). A report, which studied the expression of growth factors in the avian embryo, identified quail homologues of VEGF A_{189}, VEGF A_{165}, VEGF A_{121} and VEGF A_{145}, which given the limited number of growth factors identified in birds, supports the existence of this new species (202). Until very recently, the VEGF A_{145} species was thought to be

expressed purely in placental tissues, when Whittle *et al* demonstrated that this isoform represented 18 % of the total VEGF mRNA expressed in human kidneys (203). Furthermore, 27 % of the total VEGF product consisted of a novel splice variant termed VEGF A_{148}.

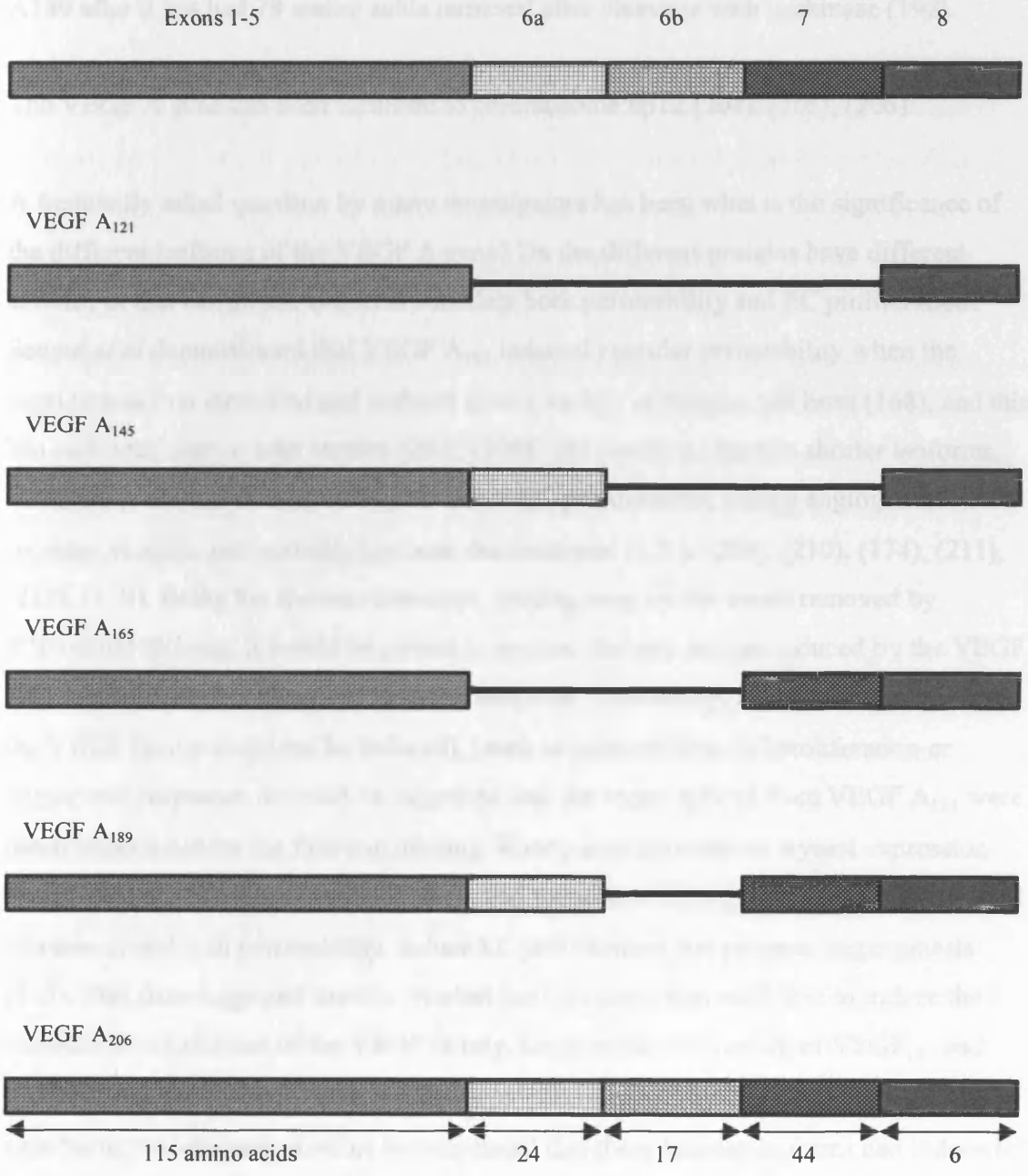

Figure 5- A schematic representation of the differential splicing of the VEGF A gene.

This latest isoform lacks exon six and contains exon seven with a 35 bp deletion, which after the removal of the signal peptide would produce a peptide of 148 amino acids in length. It is postulated that the species would be mitogenic as it contains the first 110 amino acids. It is this section of the peptide that confers the mitogenic activity to VEGF A189 after it has had 79 amino acids removed after cleavage with urokinase (198).

The VEGF A gene has been localised to chromosome 6p12 (204), (205), (206).

A frequently asked question by many investigators has been what is the significance of the different isoforms of the VEGF A gene? Do the different proteins have different actions, in that can all the isoforms stimulate both permeability and EC proliferation? Senger *et al* demonstrated that VEGF A_{165} induced vascular permeability when the peptide was first identified and isolated from a variety of tumour cell lines (168), and this has also been seen in later studies (207), (208). The ability of the two shorter isoforms, VEGF A_{121} and $_{165}$ to equipotently stimulate EC proliferation, induce angiogenesis and increase vascular permeability has been demonstrated (171), (209), (210), (174), (211), (212), (179). Being the shortest transcript, varying only by the exons removed by differential splicing, it would be correct to assume that any actions induced by the VEGF A_{121} would also be induced by the larger isoforms. Conversely, if one of the actions of the VEGF family could not be induced], [such as permeability, EC proliferation or angiogenic responses, it could be suggested that the exons spliced from VEGF A_{121} were those responsible for the function missing. Kondo *et al* constructed a yeast expression system that produced human VEGF A_{121}, and found that the shorter when purified, could increase vessel wall permeability, induce EC proliferation and promote angiogenesis (213). This data suggested that the shortest isoform alone was sufficient to induce the characteristic functions of the VEGF family. Large scale purification of $VEGF_{189}$ and $VEGF_{206}$ has not yet been achieved, however data obtained by Park *et al* using cells expressing recombinant proteins has suggested that these heavier isoforms can induce EC proliferation as their primary function (214), (194). These two isoforms were localised to the subepithelial ECM where they were released into a bioactive form by heparin or

plasmin (215). The ECM was proffered as an important store of VEGF that when activated could release active VEGF A_{189} and VEGF A_{206}, and have a marked angiogenic potential. Thus this data would indicate that the expected hypothesis proposed by Kondo was wrong. It would appear that a full complement of exons causes a reduction in the ability of the peptide to induce the full range of actions normally seen from this group of peptides.

The functions of the predicted VEGF A_{145} species were analysed using two expression systems. The first system employed a bacculovirus vector to express the VEGF A_{145} DNA in Sf9 insect cells and the second used the mammalian bicystronic expression vector MIRB to transfect BHK-21 cells (216). When expressed the protein displayed similar actions to those of VEGF A_{121} and $_{165}$. VEGF A_{145} promoted angiogenesis, induced EC proliferation and increased vascular permeability. The expressed peptide displayed a cognate affinity for heparin as VEGF A_{165} and therefore it was postulated that the ECM binding affinity would be similar. VEGF A_{145} is a secreted protein, however it does bind to specific heparin sulphate moieties and was found to bind to the ECM of corneal EC and was still active (216).

Although VEGF A has been shown to increase permeability via the Miles assay, it has been suggested that this technique is not a true measure of permeability. In this technique a high molecular weight dye, such as Evans blue-albumin, is injected into the blood stream prior to the intradermal administration of the test substance. VEGF is a potent vasodilator and therefore causes increased blood flow and pressure in the microvasculature. Hence, the dye leakage observed in early experiments could be extravasation caused by increased microvascular blood flow rather than increased endothelial permeability. Recent work in our laboratory has studied the effect of VEGF on individually perfused frog mesenteric microvessels *in situ* and demonstrated that the changes in vessel wall permeability are directly influenced by VEGF, independently of increased blood flow (217).

This still leaves the question of why are multiple species of VEGF produced, especially as all stimulate angiogenesis and EC proliferation? Angiogenesis occurs under adverse conditions, such as wound healing, and as many cell types produce multiple forms of VEGF (174), (191), (192), (193), therefore it could be suggested that each species offers different advantages under different circumstances. If this were true, the interesting point of study is the mechanism that controls the specific VEGF gene expression and the subsequent differential splicing of the mRNA.

1.4.2.2) VEGF B

Of the remaining species of VEGF, two isoforms are produced by the recently identified VEGF B gene, which produce proteins of 167 and 186 amino acids in length (218), (219). The VEGF-B genes were isolated simultaneously by two teams of researchers. Olofsson *et al* identified the VEGF B gene but initially was only able to isolate the smaller of the two proteins (VEGF B_{167}). However they found that VEGF B was as ubiquitous in its distribution as the VEGF A gene and that both the species were coexpressed in many tissues, especially the heart, skeletal muscle, pancreas and prostate. Examination of murine tissue by *in situ* hybridisation concluded that VEGF B was predominantly expressed in developing foetal muscular tissues. After secretion the VEGF-B protein binds to the ECM and remains cell associated. The study also discovered that simultaneous transfection into human embryonic kidney cells (293EBNA) with $VEGF_{165}$ lead to the formation of cell surface associated disulphide linked VEGF B-VEGF heterodimers (219). The *in vivo* functional significance of these heterodimers has still to be elucidated. Grimmond and co-workers found not only the VEGF B_{167} but also the larger splice variant of VEGF B_{186}. A genomic restriction map shows that the VEGF B gene consists of eight exons (as does the VEGF A gene) and that the larger isoform VEGF B_{186} contains the entire message. The smaller isoform VEGF B_{167} has had the 101 bp fragment of exon 6 removed by differential splicing. VEGF A has been found to be overexpressed in highly malignant tumours and tumour cell lines, however after Northern

analysis of 11 glioblastomas, 13 metastasising and 12 nonmetastasising breast carcinomas, an elevated level of VEGF B was not observed (218).

More recent work by Aase *et al* has identified prominent expression of VEGF B in developing murine embryos. During the first two weeks of gestation, the highest levels of VEGF B were expressed in the developing myocardium. This level of expression persisted until the later stages of development where weaker signals were also isolated in the muscle and smooth muscle cells of the larger vessels (220). Furthermore, Bellomo and co-workers confirmed this theory using VEGF B knockout mice (VEGF B (-/-)). Unlike VEGF A (-/-) mice that die during embryogenesis, VEGF B (-/-) mice are healthy and fertile. However, the mice present with hearts that are reduced in size, display vascular dysfunction after coronary occlusion and demonstrate impaired recovery after experimentally induced myocardial ischaemia (221). Taken together, these data suggest a role for VEGF B in the development of the foetal heart and vasculature, acting in a paracrine manner due to its seeming localised response.

The VEGF B gene has been localised to chromosome 11q13 (222), (218).

1.4.2.3) VEGF C

The VEGF C gene encodes a 317 amino acid protein and was discovered by Joukov *et al* whilst studying the Flt4 receptor, a member of the RTK family (see section 1.4.3.3) and related to the known VEGF receptors (223). The receptor Flt4 is a lymphatic endothelial cell specific protein, which does not bind the previously identified VEGF species and is expressed mainly during development (223). The ligand for the receptor was isolated from media and the amino acid sequence was determined. This enabled a set of PCR primers to be designed; specific for the N-terminal sequence of the Flt4 ligand, and the cDNA was obtained by amplification of cDNA from a human prostatic carcinoma (PC-3) library. The cDNA obtained was then employed as a probe for electrophoretic analysis. The protein was found to exhibit 30 % homology to VEGF A, 27 % homology to VEGF

B and 25 % homology to PlGF (223). The VEGF C mRNA was predominantly expressed in the heart, placenta, muscle, ovary and small intestine, with little or no expression found in the brain, liver or thymus. Studies using bovine capillary endothelial cells (BCEC) indicated that stimulation with VEGF C promoted proliferation and migration of these cells, which is interesting because BCEC do not express the Flt4 receptor on their cell surface (223). The proliferatory response caused by VEGF C in bovine EC was found to be synergistic when the cells were co-cultured with PDGF suggesting that the angiogenic response may depend on the presence and concentration of other cytokines in the environment of the responding endothelial cell (224). The widespread distribution of VEGF C in foetal tissues indicates that this isoform is vital for the formation of venous and lymphatic vasculature during embryogenesis. The constitutive production of VEGF C in adults and the restriction of the receptor Flt4 (VEGFR3) to the lymphatic system suggests that the peptide is involved in the maintenance of the differentiated functions of the lymphatic endothelial cells (223) (225).

Recent work in our laboratory has demonstrated that VEGF C increases vascular permeability in frog mesenteric vessels. The effect on permeability was detected twenty minutes after the addition of VEGF C and furthermore, the chronic effect on permeability was still significant twenty-four hours post-dosing (226). It was also observed that VEGF C caused a similar increase in vessel diameter as previously found by VEGF A exposure (226).

The VEGF C gene has been localised to chromosome 11q13 (222).

1.4.2.4) VEGF D

The murine homologue of VEGF D was identified after c-fos induction and was termed FIGF (227). The human variant was identified as a member of the VEGF proteins because it displayed 23.3 % identity with VEGF C (228). Subsequent analysis confirmed high levels of expression of VEGF D in murine lungs, both foetal and adult,

corroborating the hypothesis that this species is involved in regulation of angiogenesis and neovascularisation in this tissue (229). The new VEGF D gene encodes a peptide of 354 amino acids in length (228). The upstream promoter of the new gene contained an AP-1 binding site that is consistent with the fact that VEGF D is regulated by c-fos, a component of the AP-1 transcription factor.

The VEGF D gene has been localised to chromosome Xp22.31 (230).

1.4.2.5) VEGF E

This member of the VEGF family was recently identified from the genome of the ovine Orf virus. The 20 kDa peptide displays approximately 25 % amino acid homology with the human VEGF A proteins, and similarly to the VEGF A_{121} isoform, it contains no heparin binding capability. It is accepted that the lack of heparin binding in the VEGF A_{121} causes the reduction in its mitogenic activity by 100-fold. The same is not so with VEGF E, where even with no heparin binding ability, its mitotic activity was found to be as potent as that of VEGF A_{165} (231), (232).

1.4.3) VEGF Receptors

Simultaneous reports published data that indicated that the VEGF EC specificity was due to distinct receptors presented on the endothelial cell surface (233), (234), (235), (236). Subsequent work localised the receptors to the surface of EC but not fibroblasts or smooth muscle cells (233), (237). The known VEGF receptors are part of a group of kinases known as the conventional receptor tyrosine kinases (RTK) group. They are so called because they are very specific and have closely related kinase domains that can only phosphorylate tyrosine residues, unlike other groups of protein kinases that can phosphorylate both serine and threonine. RTK's were initially isolated from retroviral oncoproteins, but are now widely recognised for their roles in transducing growth and differentiation signals. There are three identified subclasses in this family, subclass I has

the distinct characteristic of two sequential cysteine rich repeats in its extracellular domain, such as the EGF receptor. The second class of receptor is thought to function as a heterotetrameric structure, with two extracellular α subunits and two β subunits that carry the kinase domains. This subclass also displays two cysteine rich repeats, one in each subunit, such as the insulin and IGF-1 receptors.

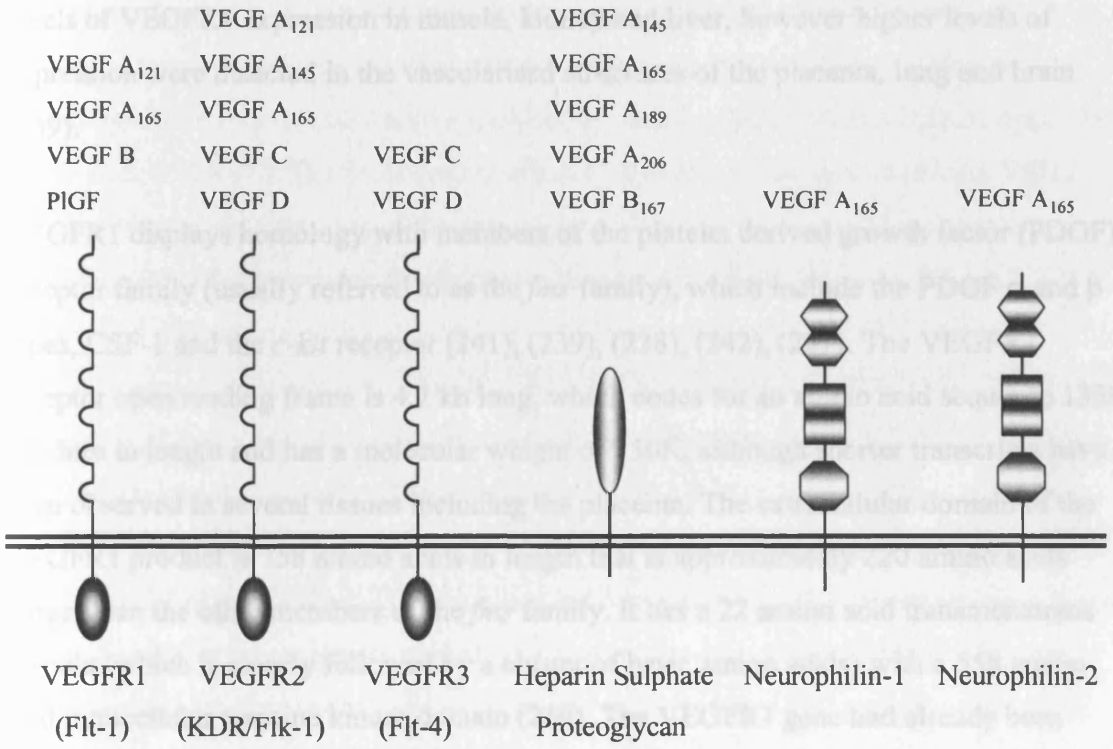

Figure 6- A schematic representation of the VEGF receptors and their identified ligands.

The type III RTK's are a single membrane spanning protein, like the type I, however they characteristically lack the cysteine rich repeats and have conserved cysteine residues in preference (238), (239).

There are three receptors identified, the *fms*-like tyrosine kinase, or Flt-1 (termed the VEGFR1), the kinase-insert-domain-containing receptor, or KDR/Flk1 (termed the VEGFR2) and the Flt-4 (termed the VEGFR3).

1.4.3.1) VEGF Receptor 1 (VEGFR1): Flt-1

The gene for VEGFR1, or the fms-like tyrosine kinase (Flt-1), was identified by random screening of a cDNA library before any ligand or function was assigned (239). A 123 bp fragment of the gene was donated by another research team (240) and Shibuya *et al* using this as a probe retrieved the complete gene for analysis. *In situ* hybridisation revealed low levels of VEGFR1 expression in muscle, kidney and liver, however higher levels of expression were detected in the vascularised structures of the placenta, lung and brain (239).

VEGFR1 displays homology with members of the platelet derived growth factor (PDGF) receptor family (usually referred to as the *fms* family), which include the PDGF α and β types, CSF-1 and the *c-kit* receptor (241), (239), (238), (242), (237). The VEGFR1 receptor open reading frame is 4.2 kb long, which codes for an amino acid sequence 1338 residues in length and has a molecular weight of 150K, although shorter transcripts have been observed in several tissues including the placenta. The extracellular domain of the VEGFR1 product is 758 amino acids in length that is approximately 220 amino acids longer than the other members of the *fms* family. It has a 22 amino acid transmembrane domain (which is closely followed by a cluster of basic amino acids) with a 558 amino acid intracellular tyrosine kinase domain (239). The VEGFR1 gene had already been isolated some years earlier to chromosome 13 (13q12), as part of a cluster of RTK genes (240), (241).

Research involving *in situ* and cell-fractionation studies have shown that maximal VEGFR1 expression is in vascular endothelial cells (243), (244). So as to ascertain whether or not VEGFR1 (Flt-1) was activated by VEGF, the complete transcript was injected into *Xenopus laevis* oocytes. Previous experiments assessed growth factor receptor activation using a calcium efflux assay (PDGF and FGF). The addition of VEGF to the oocytes induced a five fold increase in calcium efflux in those cells injected with VEGFR1, indicating that this peptide is one of the receptors for VEGF (242).

It has been demonstrated that the related protein PlGF only interacts with this receptor (245), (246), (247). In competition assays, VEGF and PlGF competed efficiently for VEGFR1 binding, although VEGF displayed a higher affinity.

A recent report has surprisingly identified functional VEGFR1 (and VEGFR2) peptides expressed on the surface of uterine VSMC (248). The expression of VEGF receptors on cell types other than EC has previously been reported, however the cell types were mostly tumour-derived and the receptors were not shown to be functional. *In situ* hybridisation, Northern and Western analysis in uterine VSMC detected the receptors but not in colonic VSMC. The proliferative effect of VEGF on the cultured uterine VSMC was as potent as the response of the control EC (248). The reason for this tissue type specific expression by uterine VSMC has still to be elucidated; however the cyclical growth and denudation of the uterine lining during menstruation may facilitate the requirement of these receptors. Thus enabling a rapid mitogenic response, of similar magnitude, in both vascular smooth muscle and endothelium, whether induced by uterine wall thickening or as a result of fertilisation.

The VEGFR1 gene was found to be located at chromosome 13q12 by two research teams simultaneously using *in situ* hybridisation and v-ros probing (241), (240), (243). Data from a number of reports has indicated that this receptor is the cell surface target for all the VEGF A isoforms (242), (245), (249).

The importance of the VEGFR1 was highlighted by Fong *et al* who developed a mutation in the gene of mice embryos. A vector was designed to generate a mutation in ES cells by deleting the exon encoding the signal peptide of the VEGFR1 gene and replacing it with the *lacZ* and *neo*^{r} genes. This resulted in a shift mutation that caused the gene products to be translated out-of-frame. The transformed ES cells were aggregated with mice embryos in the morula stage of development, to produce the chimaeric offspring. The heterozygous (VEGFR1/VEGFR1^{lacZ}) embryos were normal and fertile, but the homozygous (VEGFR1^{lacZ}/ VEGFR1^{lacZ}) offspring had growth arrested at the mid-somite stage. Examination of the homozygotes showed that they had failed to develop an

organised vascular network in the yolk sac. Further investigation revealed that within the foetal head, instead of finding normal development of individual small vessels, large fused vessels were forming containing internally localised groups of EC. A similar pattern was observed in the heart, in that VEGFR1 deficient embryos could produce EC, but a lack of VEGFR1 signalling caused a fatal impairment of endothelial organisation. Even when studied at an earlier age, the blood islands were incorrectly developed. Rather than being discrete structures of haematopoietic progenitors contained within angioblasts, the homozygotes present intermixed bundles of cells. Analysis of the other known endothelial RTK's (VEGFR1, VEGFR2, VEGFR3, Tie-1 and Tie-2) by RT PCR demonstrated that the introduction of the vector into the homozygotes had only impaired the expression of VEGFR1. These data suggest that VEGFR1 is essential for the organisation of the embryonic vasculature, controlling cell adhesion and EC interaction with the ECM (250). Subsequent publications have confirmed that VEGFR1 is involved with stimulation and regulation of cell migration, whilst having little effect on cell proliferation (249), (251), (252). These data suggest that this receptor alone is not able to transduce the array of responses stimulated by VEGF binding at the cell surface, again giving rise to multiple family members, each providing different responses to ligand binding. When combined with the different species of VEGF peptide, the specificity and diversity of the response can be strictly regulated using multiple receptor types.

Alternative splicing of the VEGFR1 mRNA generates two protein products, the full-length receptor and a truncated soluble form of the VEGFR1 (sVEGFR1). This truncated protein consists of all the extracellular domains of the normal receptor, but lacks the transmembrane spanning region and the intracellular domain. This endogenously encoded sVEGFR1 binds VEGF A with the same high affinity of the membrane presented receptor. More interestingly, introduction of the soluble VEGFR1 inhibits VEGF induced mitogenesis of human umbilical vein EC (253). More recently it was demonstrated that that an intravitreal injection of VEGFR1 could reduce retinal neovascularisation, which is associated with ischaemic ocular diseases (254).

1.4.3.2) VEGF Receptor 2 (VEGFR2): KDR/Flk1

Utilising the unusual property of all type III RTK in that the conserved kinase domain contains a unique insertion identified the novel second VEGF receptor (Flk1 or VEGFR2). The VEGFR2 was isolated from a human EC cDNA library using polymerase chain reaction technology (255). Northern analysis using a probe constructed from the first 908 bp of the cDNA identified a single 7 Kb band in bovine aortic EC. This band was not detected in human foreskin fibroblasts or bovine VSMC (255). The report also proposed that the gene was located on human chromosome 4, although a more specific location was not identified.

A study published shortly after the paper by Terman *et al* discussed the cloning of VEGFR2 from mouse cell populations enriched for hematopoietic stem and progenitor cells (256). The murine sequence is 5.4 kb in length, but flanked by untranslated sequences of 0.2 and 1.1 kb at the 5' and 3' ends respectively (about 6.7 kb in total), which is of a consistent size to the fragment found by Terman *et al*. The complete receptor has an extracellular domain of 762 amino acids that terminates with a 22 amino acid hydrophobic transmembrane sequence. The intracellular domain is divided into two distinct kinase regions, which are separated by a 67 amino acid sequence. It was this characteristic that provided the receptor with its initial name if the kinase insert domain receptor (KDR). At 14 dpc VEGFR2 mRNA was detected in murine brain, heart, kidney, lung, liver, stomach and intestine by Northern analysis. In adult tissues the receptor mRNA was detected in heart, kidney, brain and lung (256). When these data were presented the function of the protein as a VEGF receptor was not known, thus the authors suggested that the receptor in regulation and/or development of the hematopoietic system.

The identification of the human VEGFR2 was achieved by screening an umbilical vein EC cDNA library with KDR-derived probes from the previously described sequence. The recent report by de Vries *et al* that identified VEGFR1 (Flt1) as a VEGF receptor gave rise to the idea that VEGFR2 was also a VEGF receptor because of the structural similarities (237). The human VEGFR2 has a sequence just over 4 kb long encoding a

1356 amino acid residue, marginally different to its murine homologue, with a 19 amino acid transmembrane spanning sequence. The VEGFR2 gene was cloned into a modified pcDNA1 expression vector and transfected into CMT-3 (monkey kidney) cells. Radiolabelled VEGF (^{125}I-VEGF) was incubated with the transfected cells and the ^{125}I-VEGF bound to the cells. The addition of PDGF did not compete for the ^{125}I-VEGF binding site nor did the ^{125}I-VEGF bind to cells transfected with the empty vector (237). This data was supported by a later report that confirmed the hypothesis that VEGFR2 was a VEGF receptor. Quinn *et al* demonstrated that that the murine VEGFR2 bound VEGF, underwent autophosphorylation and mediated VEGF induced calcium efflux into *Xenopus* oocytes injected with VEGFR2 mRNA (257). In situ hybridisation of murine embryos provided the interesting data in the report. This revealed that VEGFR2 expression was high in the vascularised organs, such as the brain, perineural vascular plexus, heart and lung], [however closer examination showed that VEGFR2 expression was exclusive to the vascular EC of these structures. A report submitted by Rooman and co-workers identified the origin of expression of VEGFR2 in the pancreas. The EC of other tissue types demonstrate a basal distribution of VEGFR2 such that the receptor is in close proximity to the secreting cell. However, the EC of the pancreatic ducts exhibit apical membrane expression of the receptor indicating that the cells respond to VEGF present in the lumen of the ducts (258).

The VEGFR2 gene was indicated to be expressed on chromosome 4 (255) and was then initially located at 4q31.2 (259). Subsequently, the same research team published a report stating that the actual location of VEGFR2 is 4q11-q12 (260). The explanation for the incorrect identification of loci in the previous paper is unknown, however the development of more efficient probes with less background and the recent identification of a cluster of tyrosine kinase genes at 4q31-q32 is thought to be a contributing factor.

The first VEGFR2 dominant-negative knockout model was employed by Millauer *et al* where they produced a replication deficient viral vector based on the mouse sarcoma virus containing a mutant VEGFR2 (261). NIH3T3 were superinfected with the mutant VEGFR2 (mV2) virus and even though the cells produced the normal level of wild-type

VEGFR2 expression, the over expression of mV2 caused a six-fold increase in the number of receptors presented on the cell surface. Nude mice were implanted with either C6 rat glioblastoma cells, or a mixture of C6/VEGFR2 or C6/mV2 virus producing cells and the resultant tumours were resected at between 10-20 days post implantation. C6 cells produce aggressive subcutaneous tumours in mice and upon resection, the mice implanted with just C6 cells or C6/VEGFR2 had well defined tumours developing. Those mice infected with C6/mV2 had dramatically reduced tumour development (261). This experiment to block the VEGFR2 mediated signal transduction is not only of potential therapeutic value, but it also links the receptor to possible roles in the regulation of angiogenesis and vasculogenesis.

The involvement of VEGFR2 in foetal development was further illuminated when Shalaby *et al* constructed an ES cell mutation, which replaced the VEGFR2 gene with a promoterless *β-galactosidase* gene from *Escherichia coli*. The correctly targeted ES cell clones were aggregated used to form aggregated chimaeras. The heterozygous F_1 and F_2 mice were normal, but in over one hundred newborn animals, no homozygous offspring were found from heterozygous crosses. This indicated that a $VEGFR2^{-/-}$ genotype was lethal in utero. When investigated it was discovered that at 10.5 dpc most of the homozygous embryos had been resorbed, although they could be recovered at 9.5 days. The examination of embryos at 9.5 days showed that the homozygotes had no organised blood vessels and had extensive necrotic regions, presumably because of circulatory failure. When analysed at 8.5 days, before the onset of necrosis, intravascular blood cells were readily identified in the heterozygous embryos, whereas the homozygous yolk sacs had no blood vessels and had only very rare committed haematopoietic progenitors (which were abundant in their heterozygous littermates). At 7.5 days VEGFR2 expression was easily detectable in heterozygote mesenchymal aggregates that are the developing blood islands. These aggregates were absent in the homozygous embryo and the mesoderm is an underdeveloped single cell layer. The presence of *lacZ* staining in the homozygotes suggests that the cells are signalled to initiate VEGFR2 expression but in the absence of a functional receptor, the cells cannot differentiate to form blood islands. Analysis of the other known endothelial RTK's (VEGFR1, VEGFR2, VEGFR3, Tie-1

and Tie-2) by RT PCR demonstrated that the introduction of the mutation into the homozygotes had only impaired the expression of VEGFR2. Therefore, the complete absence of blood islands and developing blood vessels in the homozygous embryos, which renders them deficient in VEGFR2, indicates that this receptor is necessary in the early development of the vasculature. Whilst the endothelial precursors were observed forming, a mature endothelium did not develop. Another role of the VEGFR2 may be in the development of blood, as there were no haematopoietic progenitors found in the homozygous embryos (262).

This receptor binds all species of VEGF A *in vivo*, however, VEGF A_{121} binds preferentially to this receptor instead of VEGFR1 (263). This receptor also binds and is autophosphorylated by VEGF C *in vitro*, although the physiological significance of this has yet to be elucidated. The latest addition to the family, VEGF E preferentially binds and activates this receptor.

The data reviewed in the last two sections indicates that VEGFR1 and VEGFR2 play complimentary roles in the angiogenic response. Activation of VEGFR1, in cells devoid of VEGFR2, induces cell migration but does not cause a mitogenic response. However, when VEGFR2 is stimulated in cells devoid of VEGFR1, the primary response is proliferation, although a migratory response has been observed (251), (264). Moreover, activation of these VEGF receptors results in the synthesis of proteases that are necessary for the degradation of the vessel basement membrane during angiogenesis (265), (266), (267).

1.4.3.3) VEGF Receptor 3 (VEGFR3): Flt-4

As with the other VEGF receptors, the identification and location of the VEGFR3 was documented prior to its function. The VEGFR3 was isolated from the HEL human erythroleukemia cell line, using PCR cloning, and was identified as a novel member of the class III RTK's because it exhibited a 79 % homology in its kinase domain with that

of VEGFR1 (268). This report only achieved a partial clone of the kinase domain, however this section of the protein was split into two regions, separated by a 65 amino acid insert, which is consistent with other KDR peptides (256). The expression of the receptor was found to be very high in the human placenta, kidney and lung, and surprisingly, very low in the brain. Analysis of somatic cell hybrids mapped the human VEGFR3 gene to the chromosomal location of 5q33 (268), (269). Further investigation using Northern analysis revealed the existence of a splice variant of the VEGFR3, this shorter transcript encoded a peptide that was 65 amino acids shorter in the carboxy-terminal tail than the initial protein. There was some degree of differential expression between tissues, however the physiological significance is still not understood (270).

A recent, more detailed study of VEGFR3 expression was conducted by Kaipainen *et al*, and as with other reports concerning VEGF receptor expression, the peptide was not detected in early murine foetal life (up to 7.5 days) (271). After 8.5 days however, VEGFR3 expression was detected in the head mesenchyme and veins of the embryo. In contrast the aorta, heart and blood islands of the yolk sac had little or no VEGFR3 expression. When the foetus had developed further at 11.5 days, the restriction of VEGFR3 expression to the venous system was even more marked, interestingly, Tie1 expression was similarly limited to the arterial system at this developmental stage. When the 14.5 and 16.5 day embryos were studied, the large arteries and veins, and small, red blood containing vessels had no VEGFR3 expression in the endothelium. The lymphatic system is proposed to develop by sprouting from the existing vasculature, when the large central veins sprout to form primordial lymph sacs that eventually develop into lymph vessels (272). Kaipainen *et al* detected high levels of VEGFR3 in sac like structures in the jugular and axillary area of 12.5 day offspring, which was consistent with the theory of Sabin. The union of duct primordia forms the main lymphatic duct (thoracic duct) and analysis of the 14.5 embryo produces a VEGFR3 signal in the primordia. When the foetus is 16.5 dpc (days post coitum) the thoracic duct has formed and has high levels of VEGFR3 expression. Analysis of adult tissues confirmed that the expression of this receptor was restricted to the lymphatic system. *In situ* hybridisation confirmed the expression of VEGFR3 in lymphatic EC and in the sinuses of human lymph nodes. The

receptor also had high levels of expression in growing EC lined lymph channels and lymphangiomas- benign tumours of connective tissue stroma (271).

An initial study hypothesised that as the VEGFR3 gene encoded a protein that displayed structural similarities to the two known VEGF receptors, VEGFR1 and VEGFR2, it was a VEGF receptor (273). The expression of the three VEGF receptors on the surface of EC, exhibited both similar and individual patterns of manifestation that tended to differ in the latter stages of foetal development and adult life (274). An expression vector was constructed to express the VEGFR3 and this was transfected into NIH3T3 cells. After the cells expressing the VEGFR3 vector were identified by puromycin selection, 30 ng/ml of VEGF A was given to quiescent cells, and the level of VEGFR3 phosphorylation was analysed from immunoprecipitates probed with antibodies raised against phosphotyrosine. The data presented in the report found no induction of phosphorylation of the VEGFR3 by exposure to VEGF A, and the author proposed the receptor transmitted a signal for an as yet, unidentified growth factor (273).

In essence, Pajusola *et al* were both correct and incorrect, as two papers, published simultaneously, demonstrated. The first report presented a novel protein, isolated from a human glioma cell line, that not only bound to VEGFR3 with high affinity, but also stimulated tyrosine phosphorylation and induced a mitogenic response in human lung EC (275). Upon closer analysis, the novel peptide was found to be secreted and displayed 32 % amino acid homology with VEGF A, although it did not bind the either of the known VEGF receptors (VEGFR1 and VEGFR2). The protein was therefore called VEGF-related protein (VRP), and expression was observed in several adult tissues, including the heart, placenta and ovary and in foetal lung and kidney (275). The second report identified the same protein, but deeper analysis led the group to name the peptide VEGF C (223).

VEGFR3 predominantly binds VEGF C, and is expressed on foetal venous and lymphatic tissue, and adult lymphatic tissue (223), (276), (271), and more recent reports indicate that VEGFR3 also binds VEGF D (277).

1.4.3.4) VEGF A_{165}-Specific Receptor: Neurophilin 1 and 2

More recent reports have identified another VEGF receptor on the endothelial cell surface, which has a lower mass than VEGFR1 and VEGFR2 and is isoform specific for VEGF A_{165} (278), (279). It has been demonstrated that the binding of VEGF A_{165} to the receptor is mediated by the carboxyl-terminal part of the peptide produced from exon 7 of the differentially spliced VEGF mRNA (279), (280). Affinity purification identified the receptor as neurophilin-1, a receptor previously associated with the semaphorins, which are a group of chemorepellant factors that act to rebuff nerve growth cones (281). Cloning of the receptor lead to the identification of a closely related gene which expressed the neurophilin-2 receptor (281), (282).

The neurophilin receptors have very short intracellular domains and when exclusively expressed in cells with no other VEGF receptors, stimulation of the neurophilins caused no response. Soker *et al* proposed that these peptides acted as co-receptors, and demonstrated that VEGF A_{165} binds more efficiently to VEGFR2 in cells expressing neurophilin-1 and that the migratory response is potentiated by the presence of both receptors (282). As with the other VEGF receptors, the neurophilins have been shown to be vital for embryonic vascular development. Gene knockout studies demonstrated that murine embryos terminate when lacking a functional neurophilin-1 receptor as the cardiovascular system fails to develop (283).

1.4.4) Site of synthesis of VEGF

The initial isolation and characterisation of VEGF led early researchers to conclude that the protein was produced only during specific pathological conditions, such as in tumours. It was assumed that the abnormally high vascular permeability and tumour-associated ascites were as a result of VEGF. As more information came to light concerning the sequences and biological properties of VEGF, the specificity and

sensitivity of the methods of detection increased, indicating that VEGF was widely distributed throughout the body and that the highest levels were found in highly vascularised organs (284), (285). VEGF appears to play an important role in angiogenesis in a number of tissues.

A complex study by Bacic *et al* examined the precise quantitative expression of VEGF in rat tissues to determine the relative abundance of each of the isoforms of the VEGF A gene (286). An RNase protection assay was used to directly quantify the levels of VEGF A isoform expression in five adult rat tissues, brain, kidney, lung, spleen and heart. The longest isoform VEGF A_{206} was not detected in any of the tissue types analysed. Of the remaining three species, VEGF A_{189} was the predominant form in the heart and lung, accounting for over 60 % and 50 % of the total VEGF mRNA respectively, but was the least abundant in the other tissues (between 6-15 %). VEGF A_{165} was predominant in the brain and kidney accounting for over 65% and 50 % of the VEGF mRNA expression respectively, but was the least represented in the heart and lung. The two smaller species, VEGF A_{165} and $_{121}$ were present in equimolar amounts in the spleen making approximately 46 % each of the total RNA. The smallest transcript of VEGF A_{121} was also detected in the kidney (38 %) and lung (27 %), but was least abundant in the brain and heart (15 %). Although these data do not provide any conclusions, they do indicate a differential expression of isoforms in specific tissues and imply that VEGF A_{165} is not the primary species, as was once thought.

1.4.5) VEGF and Vascular Smooth Muscle

One of the properties of large vessels is their ability to retain the integrity of the endothelial lining after an injury to the intima, providing that the injury is not too severe. Endothelial dysfunction is thought to be the key to the development of certain vascular pathologies such as atherosclerosis (173), (195). A set of experiments performed by Ferrara *et al* isolated VEGF mRNA from bovine aortic smooth muscle cells (BASM). This however did not mean that a functional peptide was produced, thus they examined

whether BASM produced a VEGF-like mitogen. The media from conditioned BASM was concentrated and passed through a heparin-sepharose affinity column from which a fraction was eluted, in the presence of 1 M sodium chloride (previously used to isolate VEGF (172)), which exhibited mitogenic activity. The presence of VEGF peptide was conclusively established when the mitogenic activity of the eluted sample was negated using antibodies raised against recombinant VEGF A_{165} (195). The production of VEGF by VSMC supports the hypothesis that the peptide could function as a paracrine factor to maintain endothelial integrity, i.e. to regulate endothelial proliferation and permeability.

Initial research data suggested that the predominant species of VEGF A produced was VEGF A_{165}, however as discussed above different tissues expressed different isoforms. As Bacic *et al* demonstrated previously in rats (286), the VEGF mRNA species vary in dominance dependent upon the tissue type analysed. The explanation for this misinterpretation was that early purification techniques favoured the isolation of this species because VEGF A_{206} and $_{189}$ were not secreted into the media and VEGF A_{121} does not bind well to heparin or cation exchange resins (194). This information taken as a whole would suggest that VEGF is produced by all vessels and at high levels in vascularised tissues and organs.

1.4.6) Physiological Roles of VEGF

1.4.6.1) Foetal Development

During gestation, angiogenesis and neovascularisation occur in both pre-embryonic and extraembryonic tissue. In the pre-embryonic tissue, the vascular system is the first functional organ system (202) which begins to develop in the neurula at approximately 17 days, with the formation of blood islands (an accumulation of mesenchyme cells [connective tissue from the mesoderm] in the angioblast). It has been observed that these islands are predominantly located in the mesoderm layer close to the endoderm (the

innermost of the three embryonic germ cell layers). This has been explained by the hypothesis that there is an endodermally secreted growth factor, which induces the mesoderm to differentiate so specifically (21). The neurula heart begins to beat at around 21 days, which causes the blood to wash back and forth through the ill-defined vasculature but the vessels slowly develop a patterned flow. Vascular development is highly dependent upon blood flow at this stage, implicating the need for shear and cyclical stress to enable orientation and maturation of the vessels. Although the embryo will survive a short period without a heart, in the absence of blood flow, the vascular network remains retarded and undifferentiated. This has been demonstrated experimentally when it was discovered that if blood flow through a developing major vessel is occluded, one of the surrounding network of small vessels matures into the major vessel (21).

The importance of VEGF and its receptors in the development of the foetal vasculature has been suggested (287), (257). This hypothesis was supported by two recent papers, published simultaneously, demonstrating that VEGF inactivation during embryonic development was lethal (288), (289).

Carmeliet *et al* designed a research model that employed embryonic stem (ES) cells in which inactivation of one ($VEGF^{+/-}$) or both ($VEGF^{-/-}$) alleles was accomplished by replacing the third exon of the VEGF gene with the neomycin phosphotransferase (neo) gene. The fusion of these heterozygous or homozygous VEGF-deficient ES cells with tetraploid embryos (T-ES) lead to the formation of embryos that could be studied for the effects of VEGF gene knockout on foetal development (289). At 8.5 days post-coitum (dpc) all three genotypes ($VEGF^{+/+}$, $VEGF^{+/-}$ and $VEGF^{-/-}$) appeared normal, however at 9.5 dpc the majority of the $VEGF^{+/-}$ and $VEGF^{-/-}$ embryos were exhibiting poorly developed aortae, with the $VEGF^{-/-}$ having abnormally enlarged vascular structures within necrotic tissue. At 10.5 dpc all of the heterozygous and homozygous VEGF-deficient ES cells appeared dead. The data obtained from this study indicated that a deficiency in normal levels of VEGF retarded early vascular development, by impairing vasculogenesis (differentiation of blood islands), angiogenesis, lumen formation and

large vessel development (289). At both 8.5 and 9.5 dpc the $VEGF^{-/-}$ genotype produced a more severe phenotype than the $VEGF^{+/-}$, however as no viable F_1 $VEGF^{+/-}$ offspring were produced the genotype appears to be lethal.

These conclusions were supported by data published by Ferrara *et al* who employed a targeting vector containing the VEGF gene, with the third exon of the gene replaced by a neomycin resistance gene (neo^r). Controls for the experiment were subjected to similar disruptions in both alleles of the c-pml gene (encodes the thrombopoietin). After 48 hours incubation, the wild type and $c\text{-}pml^{-/-}$ ES cells had secreted similar amounts of VEGF. The $VEGF^{+/-}$ cells secreted about half that of the controls and the $VEGF^{-/-}$ produced no VEGF (288). The interesting data from this study was obtained when it was discovered that $VEGF^{-/-}$ ES cells exhibited a marked reduction in ability to form tumours in female Beige nude mice. The mice were injected subcutaneously with approximately 1×10^7 cells, and after four weeks, the tumours removed from the wild type and $c\text{-}pml^{-/-}$ mice were at least ten times larger than the tumours obtained from the $VEGF^{-/-}$ injected animals. The number of vessels found in the $VEGF^{-/-}$ animals was greatly reduced compared to that of the wild type and $c\text{-}pml^{-/-}$ animals, and displayed a much less complex branching pattern. No viable heterozygous mice were obtained after analysis of over 100 offspring. PCR analysis of the aberrant embryos indicated that they were all positive for the neo^r gene, also physiological examination showed a developmental delay in the heart region (common atrium and primitive ventricle) and retarded maturation of the dorsal aortae and ventricular wall. These data indicate that the loss of a single VEGF allele is fatal at 11-12 dpc, which supports the hypothesis, suggested by Carmeliet *et al* that VEGF is critical in foetal development (289).

These two studies demonstrate the importance of VEGF during foetal development. A complimentary study was performed examining the effect of over expression of VEGF to the avian embryo (202). The cDNA of the VEGF A_{121} gene was cloned into the replication-competent avian retrovirus (RCAS) expression vector, to form the qVEGF A_{121}-RCAS construct and transformed into chicken embryo fibroblasts (CEF). A control

vector was produced using the same protocol but expressing the heat stable alkaline phosphatase from human placenta (HSAP-RCAS). Fertilised eggs of Japanese quail were incubated for three to four days, before the virus producing CEF microaggregates were transplanted into the developing wing bud. At ten days, the embryos were either dissected to assess changes in the vasculature, or injected intravascularly with dye to evaluate changes in permeability. Microscopy examination of the vascular beds from 10 dpc embryos showed that the qVEGF A_{121}-RCAS embryos had highly developed vascular plexus, with far greater density that those observed in the HSAP-RCAS embryos. However, this dense vasculature evolved around the blueprint of the normal plexus and did not alter the overall gross anatomy. The changes in vascular morphology were strictly limited to the site of transplantation indicating that even the secreted isoforms of VEGF may only be active over a limited area. Over expression of VEGF also induced hyperpermeability causing chronic oedema in the wing bud. The gross morphology of the wing and the time course of development were not impaired unless the oedema incurred was too severe. The data suggests that as over expression of VEGF does not alter the genetically predetermined development of the foetus. Furthermore it does not induce vascular tumours (hemangiomas) and would indicate that VEGF itself it not oncogenic by itself, thus other tumour inducing factors regulate tumour development (202).

1.4.6.2) Ovarian Angiogenesis

Reproduction in mammals depends upon the cyclical development of a receptive endometrium produced by the proliferation and differentiation of epithelial cells. Humans (like other primates) shed the receptive uterine lining at menstruation and must therefore undergo rapid repair of the sacrificed surface such that implantation can occur. A major phenomenon of the redevelopment of the endometrium is the process of angiogenesis, involving the proliferation, maturation and migration of EC's (290).

Angiogenesis is important in the development and differentiation of the corpus luteum (CL). The glandular mass of the CL is formed when a mature ovarian follicle discharges

its ovum, and if fertilisation occurs, the CL continues to grow. The thecal vessels (those vessels in the capsule surrounding the ovum) grow into the ruptured follicle and form a complex vascular network. These new capillaries are highly permeable which helps to facilitate the supply of large proteins (low density lipoproteins) which are essential for the delivery of the large quantities of cholesterol that are required for progesterone production during the late luteal phase and early pregnancy (291). Angiogenic proteins have been identified in the ovary, such as bFGF, however these factors do not fill the requirements for the diffusable protein needed. The ovaries were removed from rats that displayed two consecutive four-day cycles and *in situ* hybridisation for VEGF was performed. The data obtained showed that VEGF was predominantly expressed on the CL and was the first evidence to suggest that VEGF was involved in a non-pathological, major physiological process (285).

Subsequent data from other researchers provided a clearer picture of the involvement of VEGF in the ovary. Ravindranath *et al* analysed the mRNA of primate ovaries, by *in situ* hybridisation and Northern analysis, which was extracted from tissues in the early, mid and late luteal phases of the menstrual cycle. Both protocols of mRNA analysis indicated that the level of VEGF expression increased as the cycle progressed, and was prevalent in those structures that became vascularised. This was thought to also induce selection of the preovulatory follicle, as well as promoting neovascularisation of the CL. It still has to be determined if the reduction of VEGF levels at the time of menses is due to luteolysis or vice-versa (292). The blockade of VEGF in the ovary was shown not only to inhibit ovarian angiogenesis, but also to compromise luteal function. Fraser *et al* administered an antibody to VEGF at the time of ovulation to marmosets and noted from ovarian sections that angiogenesis was impaired and that progesterone secretion was reduced by 60 % in the anti-VEGF treated animals compared to the control animals (293).

An early study (199) isolated three of the previously identified species of VEGF and a novel fourth. RT PCR identified VEGF A_{189}, VEGF A_{165} and VEGF A_{121} and the fourth isoform encoded a protein of 145 amino acids in length. As this splice variant had not been isolated from other cell types it was assumed to be a tissue specific species (the

peptide for which has yet to be isolated). Conditioned serum-free media from the human endometrial carcinoma cell lines (HEC 1A and HEC 1B) displayed mitogenic activity by stimulating tritiated thymidine uptake by EC's. Passing the media through a heparin affinity column negated this activity. The combination of these two factors is characteristic of VEGF. The longer isoform of VEGF A_{189} remains cell associated and is not secreted, thus it was expected that immunohistochemical staining for this species would be localised in the nucleus. However analysis showed no specific localisation and Charnock-Jones *et al* surmised that the VEGF A_{189} was retained in the cytoplasm. Investigation by *in situ* hybridisation revealed very high levels of VEGF mRNA in the menstrual endometrium, and was explained by the fact that at menstruation the uterine lining becomes severely hypoxic (a condition that powerfully stimulates VEGF expression). This was the first study to show that VEGF was produced by normal endometrium as well as endometrial cell carcinomas.

1.4.6.3) Wound Healing

The roles of VEGF in endothelial growth and vasculogenesis are well documented throughout this thesis. Vascular damage and maintenance of the vasculature would require a potent and well regulated mitogen such as VEGF and therefore, the role of VEGF in wound healing has been investigated.

Wartiovaara *et al* analysed peripheral blood samples umbilical cord blood CD34+ cells. The data presented indicates that VEGF A was expressed in all of the cell fractions investigated, and VEGF C expression was limited to platelets and T cells (294). Further investigation demonstrated that activated platelets released both VEGF A and VEGF C simultaneously with beta-thromboglobulin, suggesting that VEGF A and VEGF C reside in the alpha-granules of the platelets (294). The release of the VEGF peptides by activated platelets strongly supports the involvement of this cytokine in vasculogenesis during wound healing.

1.4.7) Pathophysiological Roles of VEGF

Previous discussion has demonstrated that VEGF plays an important role in the stimulation and regulation of proliferation/permeability in a variety of organ systems. As this cytokine is involved in a number of diverse biological functions, its over- and under-expression has been associated with many pathologies. Angiogenesis itself is a cascade of events, which includes the migration and proliferation of EC, the catabolism of the basement membrane, the proteolytic degradation of the extracellular matrix prior to its re-assembly and endothelial tube formation. It is a necessary biological process for development, ovulation, placental maturation and wound healing, therefore, exclusive of these processes, the vasculature is usually quiescent and rarely proliferates. However, insufficient angiogenesis after vasculature damage can lead to such problems as ulcers that do not heal and myocardial infarction. Conversely, excessive angiogenesis is a major complication in other pathologies such as cancer, diabetes (retinopathy) and atherosclerosis (295). The following section introduces the data and clinical possibilities drawn from the information, linking the protein VEGF with different pathologies.

1.4.7.1) Vascular Pathologies

Initial research investigating a potential role for VEGF in vascular pathologies was of a positive nature, which sought to utilise the angiogenic properties of the peptide family. A study by Mezri *et al* investigated the possibility of VEGF gene transfer to induce focal angiogenesis and reduce tissue ischaemia. Human VEGF A_{165} was inserted into a herpes simplex virus type 1 (HSV-1) and the resultant construct (HSVhvegf) produced biologically active VEGF when transduced into NIH 3T3 cells, as assayed by microvascular EC mitogenesis (296). To study the impact *in vivo*, either the active HSVhvegf or the control HSVlac virus (containing the *E. coli* β-galactosidase gene) were transduced into BLK-CL4 fibroblasts. The transduced 3T3 cells were then suspended in a basement membrane extract to form a gel. Both the gels were injected subcutaneously into syngenic (phenotypically identical) C57BL/6 mice and when resected after seven

days, the HSVhvegf plugs showed a strong angiogenic response, unlike the HSVlac plugs. The data in the presentation suggests that the *in vivo* administration of VEGF via gene transfer techniques, could provide a viable gene therapy for the treatment of tissue ischaemia related pathologies (296).

The potential for VEGF to be used as a therapeutic agent with vascular procedures was investigated by Asahara *et al* shortly after Mesri's submission. Currently, most techniques employed to reduce restenosis after balloon angioplasty involve the direct inhibition of VSMC growth. Asahara and co-workers investigated the hypothesis that induction of endothelial proliferation to balloon-injured arterial endothelium would attenuate intimal hyperplasia (297). The investigation studied the effect of balloon angioplasty on two populations of Sprague-Dawley rats, one underwent the standard procedure followed by treatment with 100 μg VEGF for thirty minutes, whilst the control population were treated with 0.9 % saline. Analysis at two weeks showed that the VEGF treated animals had far more advanced EC repair compared to that of the saline treated animals. This degree of superior reendothelialisation was sustained until the second analysis and four weeks. This positively corresponded with a significant attenuation of neointimal thickening in the VEGF treated animals. The investigation noted that at two weeks, the repair of the endothelium was 80 % complete. Thus Asahara reports that a single, direct application of VEGF to the site of an angioplasty will not only promote the recovery of the EC but also reduce the level of neointimal proliferation (297).

An investigation by Takeshita *et al* demonstrated the range of possibilities that VEGF could fulfil (298). Rather than constructing a viral vector or plasmid vehicle, Takeshita administered naked DNA, encoding the three smaller isoforms of VEGF, by applying the peptide directly onto the hydrogel polymer coating of an angioblast balloon. The VEGF angioplasty balloons (or balloons coated with Lac Z for the control) were delivered to the iliac artery of rabbits with operatively induced hind limb ischaemia. It was found that this administration could induce collateral artery development at the site of the angioplast. Previous investigation showed that the transfer was effective in approximately 0.5 % of the arterial cells, and the strong angiogenic response suggests that the protein expressed

has a potent biological consequence as the copy number of transfectants is low. When analysed by RT-PCR, the duration of expression of the VEGF gene is around thirty days, however this was sufficient to induce collateral vessel growth. The relatively short lifetime of the VEGF transfect makes it ideal for use as a therapeutic tool. It represents a safety switch which allows enough time for the potent peptide to exert its effect, whilst ensuring that the recipient is not exposed indefinitely to the high level of VEGF that in itself could cause many problems (298).

These studies indicate the potential benefit that VEGF could have in pathologies that reduce or retard the vasculature in the body. The administration of VEGF transcripts to an afflicted area promotes vascular development and growth, suggesting that with further research VEGF could rectify *in vivo*, inborn errors in vascular development.

The promotion of vasculogenesis is only one side of the story as there are also pathologies involving the other extreme, the vasculoproliferative disorders, such as retinal neovascularisation in diabetic retinopathy. The presence of elevated levels of intraocular VEGF in patients with pathologies such as diabetes mellitus, prematurity induced retinopathy, retinal vein occlusion and rubeosis iridis has long been established (299), (300). However, the presence of VEGF as a requirement for the development of the disease state was not elucidated until later. Aiello and co-workers constructed VEGF-neutralising proteins by joining the extracellular domain of either the human VEGFR1 or murine VEGFR2 peptide to an immunoglobulin (IgG). These constructs, using a similarly designed control peptide that did not bind VEGF, were used to see if *in vivo* inhibition of VEGF activity could reduce retinal neovascularisation in a murine model of ischaemic retinopathy (254). The *in vivo* effect was monitored using animals with bilateral retinal ischaemia, where one eye received an intravitreal injection of the VEGF construct and the other received the control construct. After five days the animals were culled and the eyes embedded in paraffin before sections were analysed by histological examination and it was noted that the VEGF construct reduced the level of neovascularisation in all animals by 47 % ± 4 % (254). The potency of total genomic

inactivation of VEGF was found to have an even more pronounced effect proving lethal after approximately eight days (289), (288), as previously discussed.

Northern blot analysis and *in situ* hybridisation have demonstrated the range of tissues which express VEGF (301), and earlier discussion has highlighted the frequency of events (especially during foetal development) of which neovasculogenesis is a fundamental part. Thus given the potency and widespread distribution of the VEGF isoforms, their involvement in the development or exacerbation of vascular pathologies could not be excluded.

Thus it could be hypothesised that the level of blood pressure determines VEGF production by VSMC. Excessive synthesis and secretion of VEGF by VSMC could then cause a profound localised increase in endothelial permeability, allowing cellular (monocytes) and protein extravasation, leading to the fatty streaks associated with the early stages of atherosclerosis. As the plaque develops in size, it begins to occlude the blood flow and reduce oxygen tension (302). Hypoxia increases expression of both the VEGF peptide and its receptor (303), (304), which in the latter stages of development, could assist any increase in endothelial permeability or increase in vascularisation associated with the plaque site.

1.4.7.2) Cancer Angiogenesis

The existence of a vascularising protein within tumour cell lines has long been recognised (187), although the identification and full potential of VEGF came much later (305). The paper presented by Dvorak *et al* noted the high levels of locally secreted plasmin in malignant cells and proposed that this may be of significance during fibrin deposition (187). However, this poses an interesting scenario when it is presented with the fact that plasmin can cleave VEGF A_{189} to produce a biologically active 110 amino acid peptide (215). The involvement of growth factors, which usually induce non-pathological cell division, in tumour development is a logical assumption. *In vitro* studies

supported this theory, and the first *in vivo* study to link VEGF to tumour growth was published by Plate *et al* (306). The study was performed by *in situ* hybridisation analysis on sections of human malignancies (glioblastoma) using a ^{35}S-labelled antisense RNA probe. The data presented by Plate *et al* showed that not only was the level of VEGF vastly upregulated in glioblastomas, but also that the VEGFR1 (Flt1) receptor, although not expressed by normal brain EC, was produced by the malignant sections. This was the first strong evidence to suggest that VEGF acted as a paracrine growth factor in tumour angiogenesis (306). Shortly after this publication, a complementary study by Kim *et al* (307) was performed which looked at the effect of inhibiting VEGF production of tumours *in vivo*. Human rhabdomyosarcoma, glioblastoma multiforme or leiomyosarcoma cell lines were injected into nude mice, and the growth of resultant tumours could be inhibited when the mice were treated with VEGF-specific monoclonal antibodies. Another interesting observation was that the VEGF monoclonal antibodies had no inhibitory effect *in vitro* (307).

A subsequent study performed by Warren *et al* (308) took this idea a stage further. When the levels of VEGF message were analysed in 8 human colon carcinoma cell lines and in 30 human colorectal cancer liver metastasis, they were found to be profoundly elevated. This coincided with an increase in the expression of the VEGFR1 (Flt1) and VEGFR2 (KDR) receptors on the endothelial cells. When treated with an antibody raised against VEGF, the growth of the tumours could be inhibited in a dose- and time-dependent manner (308).

These two publications, along with numerous others, confirm the involvement of VEGF in tumour growth and angiogenesis. As later research techniques developed, antisense procedures were able to further demonstrate the important role of VEGF in tumour progression. Saleh and co-workers transfected antisense-VEGF cDNA into rat C6 glioma cells and the stable transfectants had reduced VEGF expression under hypoxia (a known inducer of VEGF expression). The C6 glioma cells and the antisense glioma cells were then implanted into nude mice and the growth of the transfected cells was greatly retarded compared to that of the glioma cells. The inhibition of tumour development was

confirmed when analysis of the antisense tumours revealed that they had fewer blood vessels and a higher degree of necrosis (309). The potential for VEGF to play other roles in tumour development was explored by Gabrilovich *et al* who investigated the immunological impact of the peptide. A possible mechanism by which tumour cells avoid the host's immune response is to reduce the level of tumour antigen presentation by host professional antigen-presenting cells (APCs), such as dendritic cells, macrophages and B cells. The study was performed on twelve tumour cell lines (breast and colon adenocarcinomas) in which the functional maturation of CD34 stem cells into antigen-presenting dendritic cells was monitored by fluorescent labelling and cell surface peptide expression analysis. Data presented in the paper indicates that VEGF expressed in the tumour cell lines inhibits the development of fully functional, mature dendritic cells. This reduces the ability of the host to mount an immune response to the tumour cells, suggesting that VEGF can promote tumour development not only by promoting vasculogenesis but also by inhibiting local immune responses (310).

1.4.7.3) Diabetic Retinopathy

In neovascular retinopathies such as proliferative diabetic retinopathy, there is initially extensive proliferation of the vasculature. Vitreous haemorrhage or fluid exudation from fragile new vessels eventually causes blindness, although eventually, the disease becomes inactive and further sight loss ceases (311), (312). This has focused research towards the identification and elimination of the factor/s involved in the development of the pathology. Interestingly it has been noted that prior to neovascularisation, the retina becomes ischaemic (313), which is a condition known to induce expression of VEGF.

A study performed by Adamis and co-workers investigated the difference in the levels of VEGF found in vitreous samples collected from patients with diabetic retinopathy compared to control patients without any proliferative disorder. Undiluted vitreous samples were taken from twenty patients, and the levels of VEGF were assayed by immunofluorometric techniques. The data clearly demonstrates an increase in the levels

of VEGF in those patients with proliferative diabetic retinopathy compared to the samples without the proliferative disorder (p= 0.006). Moreover, there was a statistically significant correlation between high levels of VEGF and vitreous haemorrhage (300), as would be expected as new vasculature tends to be fragile. The median levels of VEGF detected in the samples was found to be in excess of the known concentration required to induce maximal EC proliferation *in vitro* (197).

This work was closely followed by a report from Aiello *et al*, which used a larger population of patients undergoing intraocular surgery. Samples were obtained from 164 patients and after the level of VEGF was determined by radioimmunoassay, the proliferative effect *in vitro* was measured and the effect of VEGF-neutralising antibodies was investigated (299). The data showed a significant increase in VEGF expression in the samples obtained from patients with active retinal neovascularisation. Furthermore, the levels of VEGF in those patients that had undergone laser photocoagulation were less than those with the active disorder, suggesting that the reduction in the number of ischaemic vessels reduces the production of angiogenic factors. The samples of vitreous from the patients with diabetic retinopathy induced retinal EC growth *in vitro* as assessed by DNA content. Addition of an antibody raised against VEGF reduced the EC proliferation by over 65 %. This clearly demonstrated that VEGF was released in response to the hypoxic conditions within the eye and that the cytokine could promote vascular proliferation, a conclusion substantiated by *in situ* analysis in later investigations by other researchers (314). Subsequent work by the author demonstrated the same inhibition of the VEGF response by the addition of soluble VEGF receptor proteins *in vivo* (254). The research utilised the murine model of ischaemic retinopathy using the soluble portion of the human Flt or murine Flk receptor. Surprisingly, the human Flt peptide inhibited neovascularisation in all animals treated, whereas the murine Flk peptide was only effective in 95 % of animals treated. Moreover, these responses were evident after a single injection and were dose dependent with neovascular suppression attained at a 200 ng dose (254).

1.4.7.4) Kidney Dysfunction

The kidneys perform two major functions. First, they excrete most of the end products of metabolism and second they control the concentrations of many of the constituents of the body's fluids. The kidneys are vitally important for maintaining homeostasis of blood constituents and preventing protein loss to the urine, and except during kidney dysfunction, proteinuria does not occur.

Each kidney is made up of about one million nephrons, each capable of producing urine. Nephrons can be split into two groups that differ only in location and length of loop of Henle. The nephron is supplied with blood via the afferent arteriole, which furnishes a glomerulus. The glomerulus consists of a network of up to fifty parallel branching capillaries covered by epithelial cells and encased in the Bowman's capsule. The small capillaries of the glomerulus cause an increase in blood pressure, which causes fluid and solute extravasation from the vessel into the Bowman's capsule. The high permeability of the glomerulus has been attributed to the existence of an extremely fenestrated endothelial layer. The glomerular filtrate contains a concentration of negative ions that is 5 % higher than plasma, positive ions are 5 % lower and the protein level in the filtrate is approximately 0.03 % that of plasma (315). Although very permeable to plasma solutes the glomerular filtrate has a similar composition to plasma except for the low level of protein.

The presence of VEGF in rat and mouse kidney has been recognised (179), (284), however an interesting study by Brown *et al* analysed the location of VEGF in human kidney (316). The location of VEGF was determined by *in situ* hybridisation, immunohistochemistry and Northern analysis in grossly and microscopically normal-appearing sections of tumour nephrectomy specimens. The data presented in the paper indicated that both VEGF mRNA and peptide were readily detected in normal adult kidney whilst *in situ* hybridisation and immunohistochemistry localised the VEGF to the glomerular epithelium. Although diffuse signal can be detected throughout the kidney, no signals have been detected as convincingly as in the glomerulus (316), (317), (179),

(284), (318). Investigators believed that the glomerular basement membrane and endothelium were responsible for the regulation of glomerular permeability, however this data suggests a mechanism by which the glomerular epithelium may control filtration.

Initial researchers were confused by the notion that such a potent permeabilising agent could be highly expressed in the kidney, yet not cause dysfunction via proteinuria (168), (284), (317). As previously discussed, VEGF can induce hyperpermeability by the formation of fenestrations (179), (181). Fenestrations are tiny holes that have a diameter averaging 60 nm, which is often transected by a diaphragm formed by a radial array of fibres that converge at the centre of the pore. These fenestrations enable water, non-protein ions and very small proteins (below a molecular weight of approximately 6000-10000 kDa, i.e. insulin) to freely pass into the glomerular filtrate. The small openings of the fenestrations allow only small proteins and solutes to pass through, whilst protein efflux and escape of cells has been shown to be caused by separating EC junctions (181). However, research also indicates that VEGF induces the formation of EC junctions and increases VVO action (178), (180). The lack of these structures may be because of tissue type specificity of reaction to VEGF as a regulatory mechanism to prevent renal dysfunction. By neutralising the glomerular epithelial cell ability to form EC junctions and VVO's in response to VEGF, the development of proteinuria and glomerulosclerosis will then be regulated by injury to the epithelium. Larger proteins are also repelled from the pore walls by the strong negative charge exhibited by the complex proteoglycans that line the pore. These two factors prevent virtually all protein larger than about 70000 kDa (roughly the size of albumin) from passing into filtrate.

The importance of VEGF to the developing kidney was assessed by Tufro *et al*, who demonstrated that rat kidney organ cultures in VEGF free media failed to vascularise (319). The data suggests that in standard kidney cultures, the level of VEGF expression is down regulated, but in the embryonic kidney, VEGF stimulates vasculogenesis and tubulogenesis, ensuring renal development (319).

1.4.7.5) Pathologies of the Reproductive Tract

A study by Gordon *et al* compared the distribution of ovarian VEGF between premenopausal and postmenopausal women. Data from the study confirmed previous findings in that normal premenopausal ovaries had high levels of VEGF in healthy follicles, primarily in the thecal cell layer, minimal expression in the granulosa cell (outer) layer. In the samples obtained from postmenopausal women, VEGF expression was only detected in the ovaries during the development of epithelial inclusion cysts and serious cystadenoma. The luminal epithelium, smooth muscle and pericytes lining the blood vessels of the fallopian tubes from both pre- and postmenopausal woman exhibited VEGF specific staining (320).

Endometriosis is a condition characterised by the growth of ectopic endometrium in the peritoneal cavity. Elevated levels of VEGF had been observed in the peritoneal fluid of women with endometriosis (321) and McLaren subsequently observed that tissue macrophages present in the ectopic endometrium were secreting VEGF (322). Media conditioned by these peritoneal fluid macrophages was found to induce proliferation in cultures of HUVECs, which was VEGF dependent as the addition of VEGF-specific antibodies could negate the proliferative effect (322). During the luteal phase of the menstrual cycle, the expression of VEGFR2 was increased, which coincided with EC migration in response to VEGF. The study reports that patients with endometriosis have significantly increased VEGF peptide and VEGFR2 expression, which in turn promotes a stronger EC migratory response at specific times during the menstrual cycle (322).

Preeclampsia is a multisystem disorder characterised by hypertension and proteinuria. It has been postulated that it is a disease of the endothelium caused by circulating factors as Brockelsby *et al* have demonstrated that myometrial vascular function is altered on exposure to plasma from women with preeclampsia. The level of expression of VEGF is increased in this condition (323) and incubation of vessels from pregnant women with VEGF reduced endothelium-dependent relaxation in a manner that mimicked the reduction induced by the plasma from women with preeclampsia (324).

1.4.7.6) Endothelial Dysfunction

Abnormalities in EC function and VSMC proliferation are key factors in the pathogenesis of atherosclerosis. Endothelial dysfunction during the early development of an atherosclerotic lesion is characterised by an increase in permeability of the endothelium to circulating macromolecules. The site at which the increase in permeability occurs appears to be morphologically intact supporting the argument of dysfunction as opposed to endothelial damage (30).

VEGF was investigated for its potential involvement in this pathological process as it increases vascular permeability and induces VSMC proliferation, the key processes in the early stages of atherosclerosis. As its site of production is the smooth muscle itself, it is now accepted that VEGF synthesis is upregulated during VSMC proliferation, which then acts in a paracrine manner to influence the endothelium (37).

Once the atherosclerotic process has been initiated, VSMC migrate from the medial layer of the blood vessels to the intima, stimulating further the muscle cell proliferation. Interestingly, VEGF has been shown during a number if investigations to induce cell migration (325), (326), (327). Taken together, these data indicate a potential mechanism by which the VEGF peptide can induce endothelial dysfunction.

Conversely, after balloon angioplasty, endothelial damage induces VSMC proliferation and intimal hyperplasia. To investigate possible methods of reducing the EC damage and resultant dysfunction, Asahara *et al* examined the effect of local administration of VEGF to the site of damage. After a single, thirty-minute incubation with 100 μg VEGF, the level of reendothelialisation was higher than the saline controls. Furthermore, the neointimal thickening was significantly reduced in the VEGF treated group compared to the control group (297). This report suggests a different mechanism by which VEGF can

in specific circumstances be vasculoprotective, by preventing endothelial dysfunction and neointimal hyperplasia (297).

1.4.8) Factors effecting the production of VEGF and its receptors

1.4.8.1) Hypoxia and Reactive Oxygen Species

The strong stimulatory effect of hypoxia on angiogenesis has been well documented and was first indicated by Knighton *et al* (302). The report was designed to investigate the unique abrupt initiation and termination property of the wound healing process. The data indicate that an angiogenic response can be initiated in response to hypoxia, and that the process is terminated when normal physiological levels of oxygen are re-established. The hypoxic conditions of a healing wound, approximately 2 % oxygen, potently stimulated macrophage-directed angiogenesis. However at a partial pressure similar to that of arterial blood, approximately 10 % oxygen, or that of tissue, approximately 5 % oxygen, no mitogenic response was observed (302). The link between this mitogenic response to hypoxia, the necrotic conditions of a tumour core and the potential role of VEGF was not realised for a number of years.

It was well documented that an inefficient vascular supply and the subsequent reduction of available oxygen would induce neovascularisation so as to meet the requirements of the tissue. Shweiki *et al* proposed that there were specific factors that could stimulate growth in the hypoxic conditions of tumours and healing wounds, and termed these undefined substances as hypoxia-inducible angiogenic factors (304). Research focused upon one type of human tumour, glioblastoma multiforme (a malignant primary intracranial brain neoplasm), because the development of the vasculature within these rapidly growing tumours is often incapable of satisfying the perfusion requirements of the tumour. Analysis of freshly resected specimens was performed by *in situ* hybridisation using VEGF-specific, ^{35}S-labelled antisense riboprobes. The expression of VEGF mRNA in the tumours was restricted to a band of cells, arranged in a stripe-like pattern along the

edge of the necrotic regions. The highest levels of mRNA expression were seen in those cells nearest to the necrotic regions, probably because these cells were experiencing the most severe hypoxic conditions. Another interesting characteristic of glioblastoma multiforme tumours is the high level of leakiness of the blood vessels, which could be induced by the large amounts of VEGF secreted by the tumour cells (304). A later study concluded that both VEGF gene and mRNA for VEGFR1 and VEGFR2 were increased in response to hypoxia (303). Furthermore, the overall induction of gene and receptor has subsequently been reported in different guises (328), (329), (330), (331), (332), (333), (334).

The subsequent research concerning the hypoxic induction of VEGF mRNA was focused toward the isolation of the DNA sequences that regulated the response to low oxygen concentrations. Minchenko *et al* performed transient transfection assays in which successive flanking sequences of the VEGF gene (2.5 Kb upstream and 4.2 Kb downstream) were digested and inserted into a promoterless expression vector. After exposure to cobalt, hypoxic or normoxic conditions, the vector expression was analysed. After the digested fragments were assayed for their responsiveness to hypoxia, more specific sequences were generated by PCR such that the exact location and sequence of the hypoxia responsive elements could be determined. The 5' flanking region was a 100 bp fragment located approximately 800 bp upstream of the start site. Whereas the 3' hypoxia regulatory element was a 160 bp fragment located about 60 bp downstream from the polyadenylation site (335). A later report submitted by Liu *et al,* utilising similar techniques, further restricted the specificity of the 5' hypoxia responsive element to a 28 bp sequence that was sufficient to illicit the expression response (336). Previous studies have indicated that the expression of the AP-1 proteins (*c-jun* and *c-fos*) is upregulated under hypoxic conditions. Thus, as three potential AP-1 binding sites have been identified in the VEGF promoter, it is not unreasonable to speculate their involvement in the upregulation of the gene in hypoxia (337), (338), (191). However the data presented by Liu *et al,* suggests that the AP-1 sites may not be employed as the 28 bp enhancer element provides a sufficient response to hypoxia when attached to a reporter gene (336).

1.4.8.2) Growth Factors

The term growth factor refers a large group of polypeptides, which after interacting with a cell induce a chain of events that promote DNA synthesis and mitosis. These proteins exert their effect at low concentrations (approximately 10^{-9} to 10^{-11} M) and have been separated into two classes dependant upon their activity. The first class is referred to as direct angiogenic growth factors because they promote cellular proliferation both *in vivo* and *in vitro*, i.e. key regulators of angiogenesis. In contrast, the second class of growth factors called indirect angiogenic factors, promote angiogenesis *in vivo*, but have no effect *in vitro*, probably because they require other factors to exert their influence (339), (17). Growth factors differ from the classic endocrine hormone because the latter are usually produced by specific glands and released into the circulation to act upon the target organ. In contrast, growth factors are synthesised in multiple tissue/cell types and then released to act locally within the tissue of origin. This property of growth factors lead to the development of the theories of paracrine and autocrine action, where paracrine factors interact with cells adjacent to the cell of synthesis and autocrine factors are expressed by and interact with the same cell.

The ability of growth factors to induce a mitogenic response is regulated by both the specificity of the angiogenic protein and the number of cell types that respond to a specific growth factor. Whilst some growth factors will induce a response in a variety of cell types, such as fibroblast growth factor which is a mitogen for both EC and VSMC, other growth factors will only cause proliferation in one cell type, such as VEGF. Conversely, some cell types respond to a variety of factors, such as 3T3 cells, whereas others such as erythroblasts are highly selective as to which growth factors they will respond (17).

1.4.8.2.1) Basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor as the name implies, promotes fibroblast proliferation and more recently has been demonstrated to be a potent inducer of EC, chondrocyte, VSMC and melanocyte proliferation (339), (340). Interestingly, bFGF was isolated as a result of its ability to bind immobilised heparin. Thus as bFGF interacts with heparin-containing proteoglycans found in many tissues, it becomes secured and acts as a paracrine and/or autocrine factor at the site of synthesis (340). After the initial purification of bFGF, it was discovered that bFGF was actually two closely related distinguishable by their pH. The first peptide, termed bFGF, was found to be widely distributed in adult and foetal tissue, and to exert a powerful mitogenic response especially in capillary and large vessel EC. Its expression has also been detected in a wide variety of normal and tumourgenic cell lines. The second peptide, called acidic fibroblastic growth factor (aFGF), was found to be restricted in both action and expression to the cells of the central and peripheral nervous system (17). An interesting characteristic of both bFGF and aFGF is that they lack the secretory sequence normally found in the amino termini of secreted proteins, suggesting that these peptides are not usually discharged from the cell. This would suggest that the proteins are only introduced to responsive cells as a result of damage to the cell responsible for the production of bFGF or aFGF (341), (342).

A report submitted by Stavri *et al* presented data that demonstrated that incubation of rabbit VSMC with 10 ng/ml bFGF could promote a 25-fold increase in VEGF mRNA expression within 12 hours (339). The paper also illustrates the synergistic relationship between bFGF and hypoxia, in that similar increases in VEGF expression could be seen under hypoxic conditions with only 3 ng/ml of bFGF. This report was supported by an ensuing *in vivo* publication investigating the levels of bFGF and subsequent VEGF expression in human patients with unstable angina. The data suggested that the pericardial fluid taken from angina patients had higher levels of bFGF (and VEGF) than patients with nonischaemic heart disease because of myocardial ischaemia (343).

1.4.8.2.2) Transforming Growth Factor-β (TGF-β)

Transforming growth factor–β (TGF-β) is a multifunctional polypeptide that regulates the proliferation and differentiation of various cell types in the body. TGF-β is a member of the group of proteins referred to as the epidermal growth factor (EGF) family. The first species to be identified was isolated from murine submaxillary glands and extracts obtained were found to accelerate maturation of various epithelia, causing premature eyelid opening and incisor eruption in newborn mice. This factor was called EGF (344). During unrelated investigations, a group of researchers isolated an agent in pregnant women that inhibited gastric acid secretion. Sequence analysis of this protein revealed that it displayed significant amino acid homology to murine EGF, and exhibited identical biological activities both *in vivo* and *in vitro*. Thus the newly termed urogastrone was deemed to be a human homologue of EGF (345). Unlike other growth factors that are ubiquitous in expression, EGF peptide is only expressed in the submaxillary gland, the kidney and (in humans) the Brunners gland in the gut.

It was observed that when fibroblast cultures were infected with retroviruses, such as moloney sarcoma virus (MSV), the resultant cells formed aggressive tumours in host animals without the presence of EGF. The explanation for this was that the MSV infection caused the production of an EGF-like peptide that acted in an autocrine manner to induce tumourgenesis. When the media from MSV infected cells was analysed, a protein related to EGF was isolated and found to display almost identical biological properties. This novel 6 kDa polypeptide was called TGF-α (346). Conversely to the distribution of the prototypical member of the family EGF, TGF-α expression was found to be widespread throughout a variety of foetal and adult tissues as well as a number of tumour cells.

During the course of the purification of TGF-α it was observed that the earlier fractions, which contained the majority of the EGF-like activity, could induce proliferation of fibroblasts in a semi-solid medium. The ability of cells to proliferate without attachment to a solid substrate (called anchorage-independent proliferation) was considered to be a

definitive characteristic of tumourgenic (or transformed) cells. Thus it was concluded that there must have been a peptide secreted from the MSV cells that could cause 'normal' cells to switch to anchorage-independent proliferation. This novel protein was purified from the media containing TGF-α and called transforming growth factor β (TGF-β). As further purification of TGF-β occurred, it was noticed that the induction of anchorage-independent proliferation in normal fibroblasts could only be achieved in the presence of both TGF-β and TGF-α, neither had the ability alone. The hypothesis then developed suggesting that TGF-β regulated cellular proliferation, as well as other phenomena, by controlling the effects of other growth factors (347).

The positive effect of TGF-β on VEGF expression was observed when quiescent cultures of fibroblastic and epithelial cells (mouse embryo-derived AKR-2B and human lung adenocarcinoma A549) were treated with TGF-β (314). Whilst qualifying the effect of TGF-β on the regulation of VEGF, EC were exposed to the peptide and no VEGF mRNA was detected in the cells. This lead to the conclusion that the angiogenic effect observed in the EC of blood vessels was mediated by the paracrine induction of VEGF by surrounding cell types, induced by TGF-β (314). This hypothesis was supported and further investigated by Brogi *et al*, who studied the effect of TGF-β and PDGF-BB (see below) on VEGF expression in VSMC. The data presented shows that these indirect cytokines are likely to exert their angiogenic effect by inducing VEGF expression in cells near to the EC, i.e. smooth muscle (348).

1.4.8.2.3) Platelet-Derived Growth Factor (PDGF)

The identification of PDGF was achieved when it was noted that VSMC were induced to proliferate when cultured in serum (the soluble fraction of clotted blood) and that the rate of proliferation was markedly reduced in plasma (the non-cellular fraction of whole blood). This lead researchers to speculate that a growth factors was released into the serum during the clotting process and subsequent investigation determined that the mitogenic activity of serum could be restored by the addition of an extract obtained from

platelets. This extract was termed platelet-derived growth factor. The purification of the peptide proved to be an arduous task because PDGF was very potent and only expressed in small quantities (349), (350).

PDGF is a dimeric protein comprising of two polypeptide chains of approximately 12 kDa each. The polypeptide chains were found by sequence analysis, to be closely related and were subsequently called PDGF-A and PDGF-B. These two chains could combine to create three isoforms, either a PDGF-A homodimer, a PDGF-B homodimer or a PDGF-A/ PDGF-B heterodimer.

Following the characterisation of the cDNA of the two chains, the tissue expression was further investigated and it was seen that PDGF was not restricted to platelets and megakaryocytes as first suggested. The peptide has been isolated in EC, neonatal VSMC, placenta, macrophages and the central nervous system. This investigation also illuminated certain tissue specificities between the PDGF isoforms, in that PDGF-B was predominant in the placenta and many adult tissues and PDGF-A was the foremost isoform in the brain and foetal tissues.

Williams *et al* performed a series of experiments investigating the effect of serum (which contains a host of cytokines) and PDGF on VEGF mRNA expression in human VSMC (37). This enabled the investigators to ascertain whether on not the human VSMC produced and increased level of VEGF during proliferation, and if so could this growth effect EC function/development.

VEGF is known to be induced by TNF-α, bFGF, interleukin 1 (166), epidermal growth factor (351), PDGF (352), TGF-β (353), phorbal esters (191), cAMP analogues (354) and hypoxia (304), (329).

1.5) Summary

Hypertension is associated with increased vascular endothelial permeability but the mechanism for this is unclear. VEGF is one of the most potent vascular permeability factors ever identified. VEGF is produced by vascular smooth muscle cells and is thus strategically situated to directly influence the permeability of the overlying endothelium.

Hypertension induces mechanical stress on the vascular wall and the vascular smooth muscle cells experience much of this cyclical mechanical strain. To date, very little is known about the effects of mechanical strain on human vascular smooth muscle cells. It is conceivable that mechanical strain could influence VEGF production by human VSMC and thus provide a link between vascular wall stress and the subsequent increase in vascular endothelial permeability in hypertension.

If this were the case, then it would be important to define how the human VSMC senses variations in cyclical mechanical strain and define the intracellular signalling pathways that may be linked to the proposed strain-induced regulation of VEGF production. In this latter regard, current evidence suggests that integrin dependent activation of the MAP kinase cascade is likely to be important. These observations and hypothesis form the basis of the work reported in this thesis.

1.6) Hypothesis

1- Human vascular smooth muscle cells synthesise VEGF mRNA and that the expression of VEGF mRNA is increased in response to cyclical mechanical strain *in vitro*.

2- The increase in VEGF mRNA is associated with an increase in VEGF peptide production.

3- The level of VEGF mRNA and peptide produced by human VSMC in culture is responsive to cyclical mechanical strain applied in a 'dose dependent manner'.

4- The mitogen activated protein kinase cascade in human vascular smooth muscle is activated in response to cyclical mechanical strain.

5- The strain-induced expression of VEGF is dependent on strain-induced MAPK activation.

2) METHODS AND MATERIALS

2.1) Cell Culture

VSMC were grown from healthy human saphenous vein, procured at the time of varicose vein surgery, where the section used is the healthy flanking regions adjacent to the varicose vein. The vessel was obtained directly from the operating theatre in a petri dish containing minimal essential media (MEM [Sigma, Poole, UK]) and whilst in this, excess tissue and fat was removed from the vessel wall.

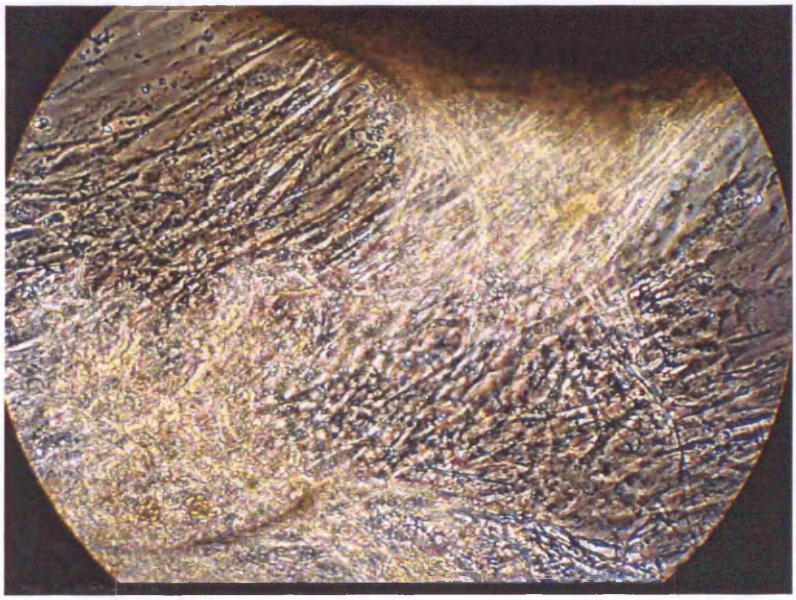

Figure 7- A photograph of tissue explant producing human VSMC *in vitro*, in 100 mm culture dish.

The vessel was then rinsed twice in MEM and transected longitudinally so that the endothelium could be abraded using a razor blade. The vessel was then transferred into

modified Hams F12[1] growth media with 20 % FCS (foetal calf serum [Gibco BRL, Paisley, UK]) and cut in small sections. These explants were then individually cultured in 100 mm culture dishes in a maximum of 3 ml 20 % FCS for at least seven days at 37°C in a humidified incubator in 5 % carbon dioxide. These primary cultures then underwent partial media changes, so as not to dislodge the growing sections, every 2-3 days until steady growth was achieved, and then complete media changes were employed.

VSMC gradually grew from the explants and after approximately three weeks the primary cultures were confluent. These cultures were harvested and the resultant VSMC (second passage) were grown to confluence on six-well, flexible bottomed plates [Flexcell, MacKeesport, USA] in modified Hams F12 media, containing 15 % FCS, at 37°C in 5 % carbon dioxide. The cells were then starved in modified Hams F12 media, containing 1 % FCS for 48 hours so as to achieve quiescence prior to exposure to cyclical strain.

2.2) Chronic Cyclical Mechanical Strain (CMS)

The cyclical strain unit, as previously described, consists of a vacuum unit connected to a regulator solenoid valve that is controlled by a computer with a timer program. When a precise vacuum level is applied to the vacuum manifold system, the flexible bottoms of the culture plate wells are deformed which results in elongation of the VSMC. When the maximal strain is released the plate bottoms return to a 'resting' level. As the degree of strain applied can be controlled precisely, the deformation at the two strain levels can be adjusted to approximate the magnitude of stretch that would be produced *in vivo* by different levels of systolic and diastolic blood pressures.

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[1] Hams F12 [Sigma, Poole, UK], containing 0.5% chick embryo extract, and 1% concentrations of reconstituted 10 mg/ml stock solution penicillin/streptomycin and 200 mM stock solution L-glutamine [Gibco BRL, Paisley, UK]

Banes *et al* hypothesised a mathematical expression to explain the action of cyclical mechanical strain on cells in culture (Flexercell Strain Unit Operator's Manual [Flexcell, MacKeesport, USA]). The response of the cell exposed to strain (R) is dependent upon the amplitude of strain applied (A), the duration of A (t1), the time between cycles (t2), the duration between supercycles (t3) and the number of duration cycles applied to the cells (C). Also within the equation, the strain rate as deformation reaches maximal A (e*1) and declines from maximal (e*2), the effect of shear strain (T), if this is a factor, the characteristics of the substrate (s) and the frequency of cycle repetition (n) are included as factors influencing the cells response. The full equation is as follows:

$$R = f [A, (t1,t2,t3), C, (e*1, e*2, T), s] n$$

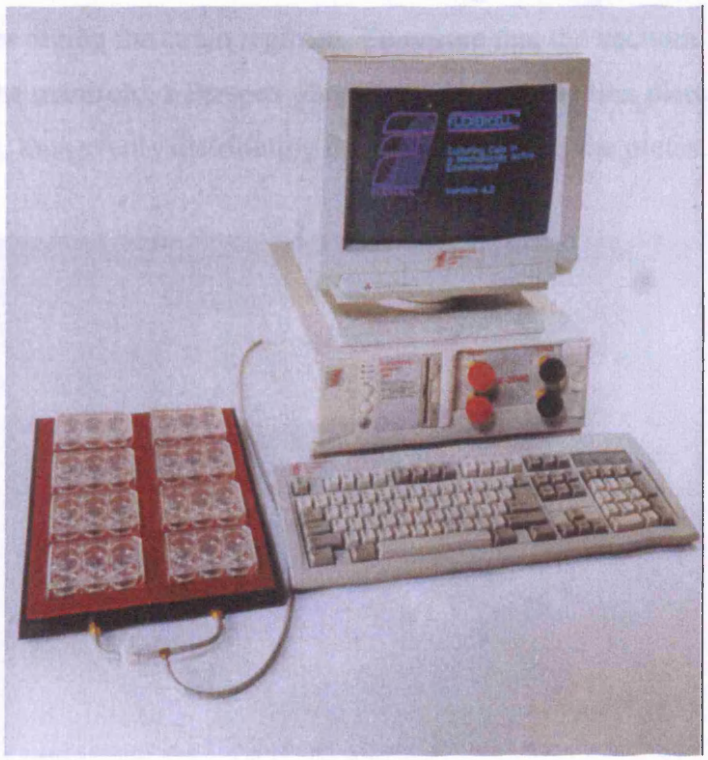

Figure 8- The Flexercell Strain Unit showing the vacuum manifold containing the Flexercell plates.

It has been determined that the application of 0.133 kPa vacuum pressure produces a degree of strain that is equivalent to 1 mmHg of blood pressure (Flexercell Strain Unit Operator's Manual [Flexcell, MacKeesport, USA]).

Thus to obtain the strain on the plates that would be exerted by a normal blood pressure of 120/80 mmHg, maximal strain must be set to 15.96 kPa and 'resting' strain set to 10.64 kPa (see Flexercell Strain Unit Operator's Manual [Flexcell, MacKeesport, USA]). The time of application of these levels of CMS were controlled so as to produce a 'heart rate' of approximately 60 bpm, such that the maximal strain was applied for 0.3 seconds and the resting strain was applied for 0.6 seconds, thus mimicking the 'normal' duration of systole and diastole.

The photograph above illustrates the strain unit, showing the computer and flex manifold that holds the plates during the strain regimen. To ensure that the vacuum is maintained uniformly across the manifold, a Perspex plate was laid over the flex plates with a 5-10 pound weight on it, thus evenly distributing the weight upon all the plates.

Figure 9- A photograph of the six well Flex plate.

2.3) VEGF ELISA Method

VEGF peptide concentrations were measured using a commercial ELISA kit [R & D Systems Europe, Oxon, UK] which uses antibodies specific for VEGF A, raised against insect cell *Sf*21-expressed recombinant human VEGF A_{165}. This technique is able to detect VEGF concentrations as low as 5.0 pg/ml. However, because a soluble form of the Flt-1 has been detected in some biological samples (as stated by the manufacturer) the possibility of interference with the readings cannot be excluded. Where VEGF peptide was analysed, media was collected from all six wells of the appropriate plate at the designated time point and pooled. This was aliquoted into 1.5 ml eppendorfs and stored at -20°C until analysed (this was to avoid repeated freeze/thaw cycles).

The assay is a standard sandwich capture ELISA, which uses a plate precoated with the monoclonal antibody raised against VEGF. The standards and samples for analysis are added and after washing the detection polyclonal antibody is added. The detection antibody is conjugated to horseradish peroxidase (HRP) and when the substrate solution (hydrogen peroxide and tetramethylbenzidine) is added to the wells, a colour develops in proportion to the amount of VEGF captured during the previous steps. The optical density is measured and a standard curve is prepared with the data from the known standards. This curve is then used to determine the unknown concentration of the samples.

The assay was performed using the Quantikine Human VEGF ELISA Kit (R & D Systems Europe, Oxon, UK) as directed in the accompanying protocols booklet, and the optical density was measured at a wavelength of 450 nm (Dynatech Plate Reader [Dynatech, Billingshurst, UK]). An intra-assay precision of 4.8 % was calculated by assaying 5 samples of known concentration in a single assay. An inter-assay precision of 5.8 % was a calculated by assaying the same ten samples in three separate assays. The recovery of VEGF from cell culture supernatant was greater than 90 %. This was

calculated by "spiking" the media with pure $VEGF_{165}$ peptide [R & D Systems Europe, Oxon, UK] and assaying its recovery.

2.4) RNA Preparations

The VSMC were washed twice with 1.5 ml/well cold PBS [Sigma, Poole, UK] and then lysed by adding 1 ml Solution A[2] to each plate. The lysed cells were then abraded with a cell scraper and the lysate pooled in an eppendorf containing 120 µl chloroform/indole acetic acid (IAA) [Sigma, Poole, UK], mixed at a ratio of 24:1. The lysate was then vortexed for ten seconds and stored on ice for twenty-five minutes. After centrifuging at 12,000 rpm for twenty minutes at 4°C, the aqueous layer was decanted into a fresh eppendorf containing 800 µl 100% ethanol and stored at -70°C for at least two hours to precipitate the RNA (optimal precipitation obtained after over night incubation). The centrifugation step was repeated and the resultant RNA pellet washed twice with 500 µl cold 75% ethanol, centrifuging at 12,000 rpm for ten minutes each time. The pellet was dried completely before being dissolved in 21 µl DEPC (diethyl pyrocarbonate [Sigma, Poole, UK]) treated water at 65°C for fifteen minutes. 5 µl of this RNA solution was used to measure the absorbence at a wavelength of 260 nm (Pharmacia Biotech Ultraspec III [Pharmacia, St Albans, UK]) to determine its concentration so that equal loading of total mRNA on the Northern gel could be achieved.

2.5) VEGF Isoform Specific PCR

This technique was employed so as to ascertain which isoforms of VEGF A were produced by the human VSMC. After RNA had been extracted from the cells, the

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[2] 50% phenol [Fisher Scientific, Loughborough, UK], 2M guanidine thiocyanate [Sigma, Poole, UK], 12.5 mM tri-sodium citrate [ICN, Thame, UK] and prior to use add 10 ml 2M sodium acetate [Fisher Scientific, Loughborough, UK] and 1.57 ml β-mercaptoethanol [Sigma, Poole, UK].

samples were reverse transcribed to obtain cDNA. The RT reaction was then amplified by polymerase chain reaction (PCR) and the generated fragments separated by gel electrophoresis. The sequence below illustrates the PCR primer locations and the size and sequence of the fragments obtained (207). The optimal RT and subsequent PCR conditions used for the amplification were as follows.

2.5.1) Reverse Transcription

5X RT buffer	4 µl
0.1 M DTT	2 µl
10 mM dNTP's	1 µl
MMLV Rtase (200U/L)	1 µl
RNAguard	0.3 µl
Oligo dT (500µg/ml)	1 µl
dd H_2O	87 µl
RNA	2 µl

Prior to use, the RNA sample was heated to 70°C for ten minutes and quenched on ice. The reaction mixture was heated to 42°C for two minutes to equilibrate. The RT enzyme was then added and the reaction was incubated at 42°C for fifty minutes, after which time the reaction was terminated by heating at 70°C for fifteen minutes.

2.5.2) VEGF Isoform PCR

The cDNA was amplified using the two primers indicated on the above VEGF sequence map, which also indicates the regions excised when the mRNA is differentially spliced to generate the individual isoforms. The primer pair should generate fragments of 589, 659 and 734 bp in length, which correspond to VEGF A_{121}, VEGF A_{165} and VEGF A_{189} respectively. The larger isoform of VEGF A_{206} is not represented in the sequence because

the additional insert it contains between exons six and seven is not on the basic full length sequence. The PCR reaction mixture was made as follows:

10X PCR buffer	10.0 µl
10 mM dNTP's	2.0 µl
Primer 1	0.5 µl
Primer 2	0.5 µl
cDNA	4.0 µl
dd H_2O	82.6 µl
Taq	0.4 µl

After all the reagents were added to the reaction, the eppendorf was briefly vortexed and pulse centrifuged prior to incubation. The amplification was performed using a DNA thermal cycler (Perkin Elmer 480, [Perkin Elmer, UK]) for 35 cycles as follows:

- **Denaturation-** 1 minute at 94°C
- **Annealing-** 1 minute at 64°C
- **Extension-** 1.5 minutes at 72°C

The PCR fragments were then separated by electrophoresis using a 2 % agarose gel and their authenticity was confirmed by Southern hybridisation.

1 GCGCAGACAG TGCTCCAGCG CGCGCGCTCC CCAGCCCTGC CCGGCCTCGG

51 GCCGGGAGGA AGAGTAGCTC GCCGAGGCGC CGAGGAGAGC GGCCGCCCC

101 ACAGCCCGAG CCGGAGAGGG ACGCGAGCCG CGCGCCCCGG TCGGGCCTCC

151 GAAACC

SIGNAL PEPTIDE

157 ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT

193 GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC

229 CAG GCT

MATURE PEPTIDE

235 GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC

271 GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC

307 TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC

343 CAG GAG TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG

379 CCA TCC TGT GTG CCC CTG ATG CGA TGC GGG GGC TGC

415 TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC ACT GAG

451 GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA

487 CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC

523 CTA CAG CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA

559 GAT AGA GCA AGA CAA GAA AA***A AAA TCA GTT CGA GGA***

595 ***AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC***

631 ***CGG TAT AAG TCC TGG AGC GTT CCC TGT GGG CCT TGC***

667 ***TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG***

703 ***CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG***

739 ***CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT***

775 ***ACT TGC AG***A TGT GAC AAG CCG AGG CGG TGA

805 GCCGGG CAGGAGGAAG GAGCCTCCCT CAGGGTTTCG GGAACCAGAT

851 CTCTCACCAG GAAAGACTGA TACAGAACGA TCGATACAGA AACCACGCTG

901 CCGCCACCAC ACCATCACCA TCGACAGAAC AGTCCTTAAT CCAGAAACCT

951 GAAATGAAGG AAGAGGAGAC TCTGCGCAGA GCACTTTGGG TCCGGAGGGC

1001 GAGACTCCGG CGGAAGCATT CCCGGGCGGG TGACCCAGCA CGGTCCCTCT

1051 TGGAATTGGA TTCGCCATTT TATTTTTCTT GCTGCTAAAT CACCGAGCCC

1101 GGAAGATTAG AGAGTTTTAT TTCTGGGATT CCTGTAGACA CACCCACCCA

1151 CATACATACA TTTATATATA TATATATTAT ATATATATAA ATTAA

Figure 10- Complete VEGF sequence (coding for VEGF A_{189}) used for the generation of PCR products, where the PCR primers are double underlined, and the exons excised for VEGF A_{121} are in bold italics and those excised for VEGF A_{165} are single underlined

2.6) Intracellular Protein Extraction

The media was aspirated from the stretch plates after MAP kinase induction, and the VSMC were frozen for two-three minutes with liquid nitrogen to prevent the intracellular messengers being dephosphorylated. After this, the excess liquid nitrogen was tipped off and the plates were allowed to slowly thaw on ice. Once thawed, 30 μl homogenising buffer[3] was added to each half plate, and the VSMC were disrupted with a scraper. Immediately prior to use, add 20 μl of 100 mM stock PMSF (serine protease inhibitor [Sigma, Poole, UK]) was added to 2 ml homogenising buffer. The homogenate was collected in an eppendorf and centrifuged at 14,000g for ten minutes at 4°C. The supernatant was carefully decanted and 10 μl was used to determine the protein levels with a Lowry assay, 10 μl was used for the MAP kinase activity assay and an equal volume of 2X sample buffer[4] was added to the remaining supernatant for Western analysis. This remaining supernatant was then boiled for two to three minutes, before all the aliquots for the three procedures were stored at -20°C.

2.6.1) Lowry Assay

After the cellular protein has been extracted, the protein concentrations present were determined so that equal amounts could be loaded on the SDS gel. To standardise the assay, the following curve was obtained:

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[3] Basic buffer of 20 mM Tris (pH to 7.4) and 2 mM EDTA [Fisher Scientific, Loughborough, UK], containing 10 μg/ml leupeptin (protease inhibitor), 20 μM E64 (calpain inhibitor), 2 μg/ml aprotinin (protease inhibitor), 1 μM pepstatin A (acid protease inhibitor), 50 mM sodium fluoride (ser/thr phosphatase inhibitor), 2.5 mM sodium orthovanadate (tyr phosphatase inhibitor), 62.5 mM of β-glycerophosphate (excess phosphate) [Calbiochem, Nottingham, UK], 0.1 % Triton X-100 (solubilises cells) [Sigma, Poole, UK].

[4] 114 mM Tris, pH 6.8, containing 0.44 % v/v 10 % SDS, 0.22 % v/v glycerol [Sigma, Poole, UK], 0.11 % v/v β-mercaptoethanol and 0.05 % w/v bromophenol blue [Sigma, Poole, UK].

Total protein (μg)	0	20	40	60	80
1 mg/ml BSA (μl)	0	20	40	60	80
Water (μl)	200	180	160	140	120
Total Volume (μl)	200	200	200	200	200

The 10μl assay samples were made up to the same volume by adding 190μl H_2O.

A stock solution of 2 % sodium carbonate, 1 % copper sulphate and 2 % potassium/sodium tartrate [Fisher Scientific, Loughborough, UK] was produced, in a ratio of 100:1:1, and 1 ml of this solution was added to each sample. The samples/standards were then briefly vortexed and incubated at room temperature for ten minutes. 100 μl of Folin reagent [Sigma, Poole, UK], diluted prior to use, 1 ml Folin to 2 ml H_2O, was added to the tubes, then they were vortexed and incubated at room temperature for thirty minutes. 1 ml distilled water was added to each tube and the optical density was measured at a wavelength of 750 nm. This data enabled the determination of the protein concentration of each sample such that equal amounts of protein could be loaded on the Western polyacrylamide gel.

2.7) Electrophoretic Techniques

2.7.1) Northern Analysis

2.7.1.1) Northern Gel Preparation

Total cellular RNA was prepared from VSMC as described in section 4. Equal amounts of RNA (between 15-30 μg/lane) were loaded on an agarose gel (containing 1.2 % w/v agarose [Sigma, Poole, UK], 72 ml DEPC water, 10 ml 10X MOPS (3-[N-moropholino]

propanesulphonic acid [Sigma, Poole, UK])[5], 17.2 ml formaldehyde [Fisher Scientific, Loughborough, UK] and 1.2 µl ethidium bromide [Sigma, Poole, UK]). The RNA samples were made up to 15 µl with DEPC water and then 10 µl gel loading buffer (for each sample prepare a mixture of 1 µl 10X MOPS, 3.5 µl formaldehyde, 4.5 µl DEPC water and 10 µl formamide [Fisher Scientific, Loughborough, UK]) and 3 µl of running dye[6] was added to each sample. The samples were heated at 65°C for fifteen minutes before being quenched on ice, then after a pulse centrifugation the samples were loaded on the gel and separated by electrophoresis (for a 100 ml denaturing gel either 60 V for three hours or 20 V over night) in a submarine tank containing 1X MOPS.

2.7.1.2) Northern Blotting

After being photographed, the gel was soaked in 20X SSC (3M sodium chloride and 0.3M tri-sodium citrate [Fisher Scientific, Loughborough, UK], DEPC treated) for 1 hour. The blotting station was set up by placing a wick of 3MM Whatman paper [Whatman Scientific Ltd, Maidstone, UK] on a glass plate over a tray of 20X SSC. The gel was placed on the wick and the ridges of the wells were removed. The exposed areas of the wick around the gel were covered with Saran wrap to prevent any transfer of 20X SSC around the gel, rather than through the gel itself. The transfer membrane, Hybond N [Amersham, UK] was soaked in 20X SSC, gently laid on the gel and any air bubbles were removed by rolling the membrane flat with a pipette. Three pre-soaked sheets of 3MM Whatman paper were then added, using the pipette to ensure that each was applied without bubbles. Ten sheets of Quickdraw [Sigma, Poole, UK] were placed on this, followed by a 5 cm thick wad of paper hand towels. A glass plate was placed on the hand towels and a weight (approximately 100 g) was added such that the pressure applied was evenly distributed over the whole blot.

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[5] 0.2 M MOPS, 3 M sodium acetate, 0.5 M EDTA, pH 7.

[6] 50 % glycerol, 0.5 M EDTA, 0.05 % bromophenol blue.

Transfer occurred overnight, after which the membrane was washed in 6X SSC and allowed to air dry for 2 minutes. The RNA was then fixed to the membrane by exposure to UV light for 50 seconds, and wrapped in Saran Wrap [Genetic Research Instrumentation Limited, Dunmow, UK] to be stored at 4°C until use.

2.7.1.3) VEGF Northern Probe

To detect the VEGF mRNA, a 204 base pair (bp) fragment was generated from human kidney RNA using two oligonucleotide primers in a polymerase chain reaction (PCR) (37).

The primers were based on the previously described human VEGF cDNA sequence and were as follows:

Forward: 5'- CGCGGATCCAGGAGTACCCTGATATGAG -3'
Reverse: 5'- CCGGAATTCACATTTGTTGTGCTGT -3'

The forward primer has a *BamH1* restriction site built into it and the reverse primer contains an *EcoR1* site so as to facilitate subcloning.

The cDNA for the PCR reaction was generated using 0.1 μg total RNA which was annealed with random hexanucleotides and reverse transcribed for 30 minutes at 42°C using 100 U reverse transcriptase [Gibco BRL, Paisley, UK] in a final volume of 20 μl. The reaction mixture was heat inactivated for 10 minutes at 95°C before the addition of the PCR primers and Taq polymerase [Biotaq, Bioline, UK] to a final PCR reaction volume of 100 μl. The amplification was performed using a DNA thermal cycler for between 30-35 cycles as follows:

- **Denaturation-** 1 minute at 94°C
- **Annealing-** 1 minute at 55°C

- **Extension-** 1 minute at 72°C

The 204 bp cDNA fragment produced was cloned into the polylinker region of pBluescript II SK(+) and the sequence confirmed by Sanger dideoxynucleotide analysis. The cDNA probe produced was found to be the desired fragment, was common to all the known VEGF species and was therefore employed in the Northern analysis.

2.7.1.4) Northern Hybridisation and Washing

Hybridisation was accomplished using the Hybaid Maxi 14 hybridisation oven [Qiagen, Surrey, UK] and bottles. Approximately 200 ml 6x SSC (diluted from 20x stock solution) was poured into an RNase free tray (treated with hydrogen peroxide for 30 minutes prior to use) and the filters for hybridisation were alternatively layered between meshes. These were then carefully rolled such that they would fit in the hybridisation bottle and that rotation of the bottle unrolled the filters and meshes leaving them stuck to the inside if the bottle. The filters were then preheated in the oven at 42°C in about 30 ml of the 6x SSC to prevent the filters drying out. 200µl salmon sperm [Sigma, Poole, UK] was boiled for ten minutes, quenched on ice and added to the pre-hybridisation solution (12.5 ml formamide, 6.25 ml 20X SSPE [7], 2.25 ml DEPC water, 1.25 ml 10 % w/v SDS [ICN, Thame, UK], 1.25 ml 100X Denhardts[8] and 1.5g polyethylene glycol [Sigma, Poole, UK]). The 6X SSC was then discharged from the bottle and replaced with the pre-hybridisation solution. The filters were then incubated for at least four hours at 42°C in the hybridisation oven, to allow the prehybridisation to occur.

The probe was labelled for the hybridisation using the Radprime Kit [Gibco BRL, Paisley, UK]. Prior to labelling, 20 ng of probe (in 11 µl DEPC water) was boiled for ten

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[7] 3 M sodium chloride, 166 M sodium dihydrogen phosphate and 0.74g EDTA [ethylenediaminetetra-acetic acid] per 100 ml distilled water, pH to 7.9 [Fisher Scientific, Loughborough, UK]

[8] 2g type 400 Ficoll, 2g bovine serum albumin and 2g polyvinylpyrrolidone [Sigma, Poole, UK] per 100 ml distilled water

minutes and quenched on ice. To this was added 20 µl Rad Prime buffer, 3 µl AGT mix, 5 µl ^{32}P labelled dCTP [Amersham, UK] and 1 µl Klenow. The labelling reaction was incubated at 37°C for one hour. The labelled probe and another 200 µl salmon sperm aliquot were boiled for ten minutes, quenched on ice and added to the hybridisation solution (as for the pre-hybridisation solution). The pre-hybridisation solution was then poured out of the hybridisation bottle and replaced with the hybridisation solution containing the labelled probe. The hybridisation was then left over night at 42°C.

The filters were then washed in a succession of increasingly stringent wash solutions. Both the filters and the meshes are washed in the first and least stringent wash, which was the 2X solution (2X final concentration of a 20X SSPE stock) with 30 ml 10 % SDS. After twenty minutes washing at 42°C, the filters only were transferred to a 1X SSPE wash for ten minutes at 42°C, and subsequently into a 0.5X SSPE wash for five minutes at 42°C. Sometimes the stringency of the hybridisation required further washing, either for a longer period of time or in a more stringent wash solution, such as a 0.1X SSPE wash.

When sufficiently washed the filters were wrapped in Saran Wrap, and exposed to X-ray film (Kodak X-OMAT AR film [Sigma, Poole, UK]) for between 6-18 hours at -80°C. The autoradiographs were thawed and developed using an X-ray developer (AGFA Curix 60 X-ray Developer [AGFA, Manchester, UK]).

Subtle changes in expression on the autoradiographs could be quantified by analysis using a densitometer (Pharmacia LKB, Image Master DTS [Pharmacia, St Albans, UK]) such that the relative amounts of VEGF mRNA could be compared (see operators manual). The Figures for the levels of expression could be obtained by calculating the percentage difference between the unstretched controls' and the stretched samples' densitometry readings and displayed as a percentage difference. Dividing the VEGF reading by the relevant GAPDH reading performed compensation for mRNA loading variations. GAPDH is a constitiutively-expressed gene, whose expression is not altered

by either CMS or FCS concentrations within the media. Therefore any changes in the levels of VEGF expression can be normalised against GAPDH, such that the effect of CMS on VEGF expression can be analysed.

2.7.2) Southern Analysis

2.7.2.1) Southern Gel Preparation

cDNA was prepared from VSMC as described in section 2.5. Equal volumes of cDNA were loaded on an agarose gel (containing 2.0 % w/v agarose, 125 ml 1X TAE[9] and 1.2 μl ethidium bromide). 1 μl cDNA samples were made up to 7 μl with distilled water and 3 μl of running dye was added to each sample. After a pulse centrifugation the samples were loaded on the gel and separated by electrophoresis (either 60 V for 4 hours or 20 V over night) in a submarine tank containing 1X TAE.

2.7.2.2) Southern Blotting

After being photographed, the gel was washed twice, for fifteen minutes, in denaturation buffer (1.5 M sodium chloride and 0.5 M sodium hydroxide [Fisher Scientific, Loughborough, UK]) at room temperature. This washing procedure was repeated using neutralising buffer (3.0 M sodium chloride and 0.5 M Tris [Fisher Scientific, Loughborough, UK]). This set of washes first denatures the double stranded cDNA and then stabilises it temporarily in the gel. Placing a wick of 3MM Whatman paper on a glass plate over a tray of 20X SSC set up the blotting station. The gel was placed on the wick and the ridges of the wells were removed. The exposed areas of the wick around the gel were covered with Saran wrap to prevent any transfer of 20X SSC around the gel,

[9] 2.0 M Tris, 1 M glacial acetic acid, 50 mM EDTA [Fisher Scientific, Loughborough, UK], pH 7.6.

rather than through the gel itself. The transfer membrane, Hybond N [Amersham, UK] was soaked in 20X SSC, gently laid on the gel and any air bubbles were removed by rolling the membrane flat with a pipette. Three pre-soaked sheets of 3MM Whatman paper were then added, using the pipette to ensure that each was applied without bubbles. Ten sheets of Quickdraw were placed on this, followed by a 5 cm thick wad of paper hand towels. A glass plate was placed on the hand towels and a weight (approximately 100 g) was added such that the pressure applied was evenly distributed over the whole blot.

Transfer occurred overnight, after which the membrane was washed in 2X SSC and allowed to air dry for two minutes. The RNA was then fixed to the membrane by exposure to UV light for fifty seconds, and wrapped in Saran to be stored at 4°C until use.

2.7.2.3) VEGF Southern Probes

The VEGF A isoform probes were designed using the software package Oligo (355), which enables the user to design unique oligo probes for chosen sequences of DNA. The oligo primers were designed to identify the different VEGF A isoforms because the desired sequences were at the exon boundaries of the sites of differential splicing of the gene products. Hence the VEGF A_{121} probe was selected to cross the exon 5-8 junction, VEGF A_{165} was designed to be specific for the exon 5-7 boundary and the full length VEGF A_{189} probe was specific to the exon 6-7 boundary. Once selected for specificity and low levels of cross-reactivity, the primers were synthesised within the university P.N.A.C.L. laboratories.

2.7.2.4) Southern Hybridisation and Washing

The hybridisation and washing protocols for Southern analysis were similar to those of the Northern technique except for the temperature and the hybridisation solutions.

Hybridisation was accomplished using the Hybaid Maxi 14 hybridisation oven [Qiagen, Surrey, UK] and bottles. Approximately 200 ml 6x SSC (diluted from 20x stock solution) was poured into a tray and the filters for hybridisation were alternatively layered between meshes. These were then carefully rolled such that they would fit in the hybridisation bottle and that rotation of the bottle unrolled the filters and meshes leaving them stuck to the inside if the bottle. The filters were then preheated in the oven at 65°C in about 30 ml of the 6x SSC to prevent the filters drying out. 200 µl salmon sperm was boiled for 10 minutes, quenched on ice and added to the pre-hybridisation solution[10]. The 6x SSC was then discharged from the bottle and replaced with the pre-hybridisation solution. The filters were then left to pre-hybridisation for at least four hours at 65°C in the hybridisation oven.

The probe was labelled for the hybridisation using the Radprime Kit. Prior to labelling, 20 ng of probe (in 11 µl DEPC water) was boiled for ten minutes and quenched on ice. To this was added 20 µl Rad Prime buffer, 3 µl AGT mix, 5 µl ^{32}P labelled dCTP and 1 µl Klenow. The labelling reaction was incubated at 37°C for one hour. The labelled probe and another 200 µl salmon sperm aliquot were boiled for ten minutes, quenched on ice and added to the hybridisation solution (as for the pre-hybridisation solution). The pre-hybridisation solution was then poured out of the hybridisation bottle and replaced with the hybridisation solution containing the labelled probe. The hybridisation was then left over night at 65°C.

The probes used for the VEGF PCR fragment detection were labelled employing the TdT (terminal deoxynucleotide transferase) Labelling kit. The oligonucleotide probe was diluted such that a 12 µl aliquot contained 20 ng of probe. Prior to incubation, 4 µl 5X reaction buffer, 1 µl ^{32}P labelled dCTP, 2 µl 10 mM manganese chloride and1 µ TdT enzyme were added to the probe. The labelling reaction was incubated at 37°C for one hour. The labelled probe and another 200 µl salmon sperm aliquot were boiled for ten

[10] 6X SSC [5 ml 20X stock], 5X Denhardts [1.25 ml 100X stock], 0.5 % SDS [1.25 ml 10% stock] and 6.4 % PEG [1.6 g] in 12.4 ml distilled water.

minutes, quenched on ice and added to the hybridisation solution (as for the pre-hybridisation solution). The pre-hybridisation solution was then poured out of the hybridisation bottle and replaced with the hybridisation solution containing the labelled probe. The hybridisation was then left over night at 65°C.

The filters were then washed in a succession of increasingly stringent wash solutions. Both the filters and the meshes were washed in the first and least stringent wash, which was the 2X solution (i.e. 2X final concentration of a 20X SSC stock) with 30 ml 10 % SDS. After twenty minutes washing at 65°C, the filters only were transferred to a 1X SSC wash for ten minutes at 65°C, and subsequently into a 0.5X SSC wash for five minutes at 65°C. The VEGF oligo probes were very tightly bound and required further washing, in a more stringent wash solution, such as a 0.1X SSC wash.

When sufficiently washed the filters were air dried for two minutes, wrapped in Saran Wrap, and exposed to X-ray film for between 2-24 hours at -80°C. The autoradiographs were thawed and developed using an X-ray developer.

2.7.3) Western Analysis

2.7.3.1) Western Gel Preparation

The Western analysis was performed using the Biorad mini gel apparatus. The glass plates were cleaned with ethanol and assembled as directed, then the kit was tested for any leaks with water. The comb was inserted and a mark was made on the front plate 0.5 cm below the teeth so that the fill level of the running gel could be determined. A 10 % polyacrylamide running gel was used (4.0 ml water, 3.3 ml 30 % acrylamide [National Diagnostics, Atlanta, USA], 2.5 ml buffer A [1.5 M Tris, pH 8.8] and 2.5 ml 10 % SDS, with 100 µl 10 % APS [Fisher Scientific, Loughborough, UK] and 4 µl TEMED [Sigma, Poole, UK] and approximately 5 ml gel solution was required for each gel to just above

the mark, to allow for shrinkage. 200 µl IMS was dispensed onto the top of the gel, to ensure that the interface between the running and stacking gel was straight and then the gel was allowed to set for about twenty minutes at room temperature. Once set, the IMS was poured off and the top of the gel was washed with 500 µl 0.1 % SDS. The remaining gel space between the two plates was then filled with a 5 % stacking gel (6.8 ml water, 1.7 ml 30 % acrylamide, 1.25 ml Buffer B [1.0 M Tris, pH 6.8] and 100 µl 10 % SDS, with 100 µl 10 % APS and 10 µl TEMED added prior to use), then the comb was carefully inserted and allowed to set for 15 minutes at room temperature.

When set the comb was gently removed and any unpolymerised acrylamide was washed away with upper buffer (10X upper electrode buffer: 0.2 M Tris, 2.0 M glycine [Sigma, Poole, UK] and 0.2 % w/v SDS). The gels were clipped together to form the upper reservoir and filled with 1X upper buffer. A 5 cm depth of 1X lower buffer (10X lower electrode buffer: 0.5 M Tris, pH 8.3) was poured in the electrophoresis tank and the upper reservoir was placed into the electrophoresis tank. Any bubbles present at the base of the gels were removed with a Pasteur pipette. Prior to loading, ensure the lower buffer covers the lower electrode and that the upper buffer reservoir is full. The samples were boiled for 5 minutes before loading, the markers (Sigma High M.W. Standards [Sigma, Poole, UK]) were boiled for 30 seconds prior to loading. Electrophoresis was carried out using constant amps, 30 mA until the samples passed into the running gel, when it was increased to 32-34 mA.

2.7.3.2) Western Blotting

When the gel had run, i.e. the dye had reached the bottom of the polyacrylamide, the plates were dismantled, the stacking gel removed and the bottom left corner of the gel was cut off as a maker for orientation, before it was soaked in blotting buffer[11] whilst the blotting equipment was assembled. The nitro-cellulose membrane (0.45µm Protein Nitro-

[11] 75 mM glycine [Sigma, Poole, UK], 50 mM Tris, 0.05 % w/v SDS and 20 % v/v methanol.

cellulose [Schleicher & Schnell -distributed by Anderman and Co.]), eight sheets of 3 mm Whatman paper and two wool pads were soaked simultaneously in blotting buffer. Whilst submerged in blotting buffer, the blot was assembled as follows, one wool pad and four Whatman papers, carefully rolling out air bubbles with a pipette each time, then the gel, the membrane and finally the remaining four Whatman papers and the last wool pad. The blotting cassette was carefully closed and locked, then inserted into a tank half full with blotting buffer, with the gel on the negative side (black) of the membrane. A magnetic stirrer and cooling coil were added to the blotting tank before the tank was then filled to the top with blotting buffer. Transfer was achieved using a constant voltage of 100 V (0.75 amps, limit 1.98) for one hour.

After one hour, the blot was dismantled, and the membrane was stained with a 2 % ponsceau solution [Sigma, Poole, UK]. This indicated whether the blot had been successful and allowed the markers to be removed. The membrane was de-stained with distilled water so that it could be probed for either inactive unphoshorylated MAP kinase (checks for consistent loading) or activate phosphorylated MAP kinase [New England Biolabs, Hitchen, UK].

2.7.3.3) MAPK Detection

To detect the relative abundance of inactive unphoshorylated MAP kinase (MAPK) and activated phosphorylated MAP kinase (phospho-MAPK), the membranes' non-specific binding sites are blocked with a 10 % Marvel milk powder solution in PBS-T[12]. The MAPK membranes were blocked over night at 4°C, whilst the phospho-MAPK membranes required blocking for 2 hours at room temperature (all room temperature incubations were performed on a slow rocking platform). After blocking the membranes underwent a standard washing regimen of five 5 minute washes in PBS-T (these were

[12] 83 mM di-sodium hydrogen orthophosphate, 20 mM sodium di-hydrogen orthophosphate, 100 mM sodium chloride [Fisher Scientific, Loughborough, UK] and 0.1 % v/v Tween-20 [Sigma, Poole, UK], pH 7.5.

performed on a fast rocking platform). The primary antibody for both assays was used in a 1/500 dilution in Marvel/PBS-T. This was applied in a humid air tight box, where 500 μl of the diluted antibody was dispensed on to a glass plate and the filter was gently laid on the antibody solution (M. Boarder, personal communication). The MAPK membranes were incubated for 2 hours at room temperature, whilst the phospho-MAPK membranes required an over night incubation at 4°C. Following this the membranes were washed as mentioned above. The secondary antibody (anti-rabbit HRP labelled [Amersham, UK]) was added to both the membranes as a 1/5000 dilution in Marvel/PBS-T and incubated at room temperature for 1 hour, after which the membranes were washed as before. The protein/antibody complexes were detected using the Amersham ECL detection system. The ECL regents A and B were mixed in equal volumes such that 500 μl could be evenly applied to each 20 cm^2 blot. After 1 minute the excess was blotted off and the membranes covered with Saran wrap, and the filter was then exposed to X-ray film at room temperature for between 5-30 minutes before developing.

2.8) MAP Kinase Activity Assay

A MAP kinase assay was established to directly measure MAPK activity. The VSMC were cultured and stimulated by CMS and the intracellular proteins extracted (as previously described in sections 2.2 and 2.6 respectively). The total amount of protein per sample was calculated using the Lowry assay, so that equal amounts of protein could be assayed for MAP kinase activity.

The principle of the assay is the detection of MAPK phosphorylatory activity. To do this an aliquot of VSMC protein extract was incubated with a synthetic ERK substrate, myelin basic protein (sequence APRTPGGRR [P.N.A.C.L., Leicester University, UK]). The phosphorylation of MAPK substrate in the presence of radio-labelled ATP (^{32}P ATP [Amersham, UK]) over twenty minutes was taken to represent MAPK phosphorylatory activity. To confirm that the phosphorylatory activity being measured was due to the action of MAPK, the assay was repeated in the presence of a MAP2K (MEK) inhibitor

which eliminates MAPK (ERK) activity. Therefore the MAPK substrate, which is phosphorylated in the presence of the inhibitor, is caused by MAPK-independent phosphorylation, and provides an indication as to the level of the background phosphorylation induced by other kinases.

Days beyond activity date	% Activity	^{32}P ATP/µl	Cold ATP/µl
0	100	30	110
1	95.3	31.47	108.53
2	90.8	33.03	106.97
3	86.5	34.68	105.32
4	82.4	36.42	103.58
5	78.5	38.22	101.78
6	74.8	40.11	99.89
7	71.2	42.12	97.88
8	67.9	44.19	95.81
9	64.7	46.38	93.62
10	61.6	48.69	91.31
11	58.7	51.12	88.88
12	55.9	53.67	86.33
13	53.3	56.28	83.72
14	50.7	59.16	80.84
15	48.3	62.1	77.9
16	46.1	65.07	74.93
17	43.9	68.34	71.66
18	41.8	71.76	68.24
19	39.8	75.36	64.64
20	37.9	79.17	60.83
21	36.1	83.1	56.9

Table 1- A table to indicate the dilution factors required so as to compensate for the radioactive decay of the source isotope to maintain consistency between chronologically independent assays

Firstly, a solution of ^{32}P ATP/ATP was made, see table above, and then a stock of 'cold' 250 µM ATP was used to dilute the 'hot' ATP to the experimental optimum of 50 µM.

The reaction mixture was then made up of 5 μl 125 mM magnesium chloride, 5 μl 5 mM substrate, 5 μl ^{32}P ATP/cold ATP solution and 10 μl sample. The two controls were made by substituting the firstly the sample with water, to detect baseline phosphorylation, and then the substrate with a BSA solution, to detect non-specific phosphorylation levels.

The reaction mixtures were made whilst the eppendorfs were on ice and then centrifuged (pulsed at 13000 r.p.m.) before being incubated at 30°C for twenty minutes. The reaction was stopped by adding 20 μl 20% w/v TCA [Sigma, Poole, UK], then centrifuged (pulsed at 13000 r.p.m.) and spotted onto 2 cm^2 P81 phospho-cellulose paper [Whatman Scientific Ltd, Maidstone, UK]. When dry the excess label was washed off with a 75 mM phosphoric acid [Fisher Scientific, Loughborough, UK] solution using two 1 minute washes, one 5 minute wash and one 1 hour wash, after which the phosphoric acid was removed with a 5 minute wash in water. The Whatman papers were then placed into scintillation vials with 10 ml Emulsifier safe [Packard, Pangbourne, UK], vortexed and counted, using a ^{32}P program, on a Packard 2200 CA Tri-Carb Liquid Scintillation Analyser [Packard, Pangbourne, UK].

3) RESULTS

3.1) VSMC Expression of VEGF mRNA/peptide

3.1.1) The Effect of Serum on VEGF mRNA Expression

3.1.1.1) Aim

The aim of these initial experiments was to ascertain the viability of the cultured VSMC so as to ensure the cells were metabolically active and able to respond to known stimuli in the expected manner. The first part of the hypothesis is concerned with the production of VEGF by the cultured VSMC in response to cyclical mechanical strain. However, before the effect of chronic strain can be determined, the baseline responses had to be analysed. The addition of foetal calf serum (FCS) to VSMC has been shown to increase the production of VEGF mRNA, and that the magnitude of response can be increased with increasing serum concentration (37). Thus, challenging cultured human VSMC with FCS will enable the mRNA extraction and electrophoresis techniques to be optimised, and stimulate a suitable concentration of VEGF mRNA to ensure accurate determination of molecular weight.

During the development of the control conditions, modified Hams F12 media (see Methods, section 2.1) containing 1 % FSC was used to determine the baseline expression of VEGF, as per the publication by Williams *et al* (37). However, the VEGF mRNA proved to be a rare transcript and was not detectable in media with such low serum concentrations and on the small populations of VSMC in the stretch plate wells. In order to rectify this situation, the RNA from two plates was pooled in the same preparation such that the concentration was doubled in each sample, however, the VEGF mRNA was still barely detectable. Further experimentation demonstrated that high levels of VEGF

mRNA were produced when two plates were harvested together in high FCS concentrations (20 % in the media), suggesting that the techniques were operational but still sub-optimal. This led to assaying the control cells in 5 % FCS which enabled VEGF mRNA to be detected in static cultures. Furthermore it was demonstrated that this level was not maximal when incubated in 5 % FCS media because increasing the FCS concentration in the overlying media could vastly elevate the level of VEGF mRNA expression (see Figure 11). The optimal time for exposure to the serum was taken as 3 hours, as this was the accepted time for maximal gene expression (37).

The cost of each stretch plate, allowing for shipping and import tax, was approximately ten pounds. Also, each time point assayed required the mRNA of two plates to be pooled to achieve a detectable signal, which meant that each sample cost twenty pounds in culture plates alone before other consumables and tissue culture materials were included. As such, these high costs prohibited high numbers of repeat assays. During the completion of the research, most experiments were repeated a minimum of three times, unless comparative work in the laboratory supported the data and allowed an experiment to be performed twice. Similarly, the number of control (rested) stretch plates was restricted as it was difficult to justify the costs for two expensive plates per time point to undergo static incubation. Therefore the control plates were either the extreme conditions, i.e. 5 % or 20 % FCS, or alternate time points of a stretch regimen, i.e. if the stretch was at one minute intervals from 1 to 7 minutes, the controls would be at 1, 3, 5 and 7 minutes of static culture.

The VSMC underwent static culture in modified Hams F12 media containing either 5 % or 20 % FCS. Previous investigation determined that 5 % FCS was a good negative control because it did not stimulate VEGF expression, whereas the media containing the 20 % FCS was used a positive control to upregulate the VEGF mRNA.

3.1.1.2) Results

The VEGF mRNA expression data presented in the following chapters compares the level of the gene of interest against that of the widely utilised 'housekeeping' gene GAPDH. The expression of GAPDH is used to assess changes in VEGF expression in response to cyclical mechanical strain, because it is constitiutively expressed and can thus be used as a reference point for basal nuclear mRNA expression. The level of expression is presented as a ratio of the VEGF densitometry reading divided by the GAPDH densitometry figures. As both data is obtained from the same Northern blot, this compensates for any variation in mRNA levels between the lanes.

The VSMC when challenged with 5 % and 20 % FSC media for three hours produced VEGF mRNA in a dose dependent manner. As the concentration of FCS increased in the media, the expression of VEGF mRNA expressed increased accordingly (see Figure eleven). Increasing the concentration of FCS however, had no effect on the expression of GAPDH, which is consistent with previous investigations (37).

3.1.1.3) Discussion

Serum, in this case foetal calf serum, is widely used as an inducer of cellular proliferation during *in vitro* culture (356). Furthermore, the expression of VEGF mRNA by human VSMC has been demonstrated to be temporally related to the growth phase of cells and proportional to the potency of the stimulus (37). Consistent with the previous investigation, the data demonstrates that serum produces a minimum two-fold increase in VEGF mRNA expression. The diagram below presents two such investigations, each showing similar increases in VEGF expression when VSMC are challenged with 20 % FCS. When the FCS growth stimuli was removed (1 % FCS serum), the cells remained in a quiescent state and therefore did not express any detectable VEGF message.

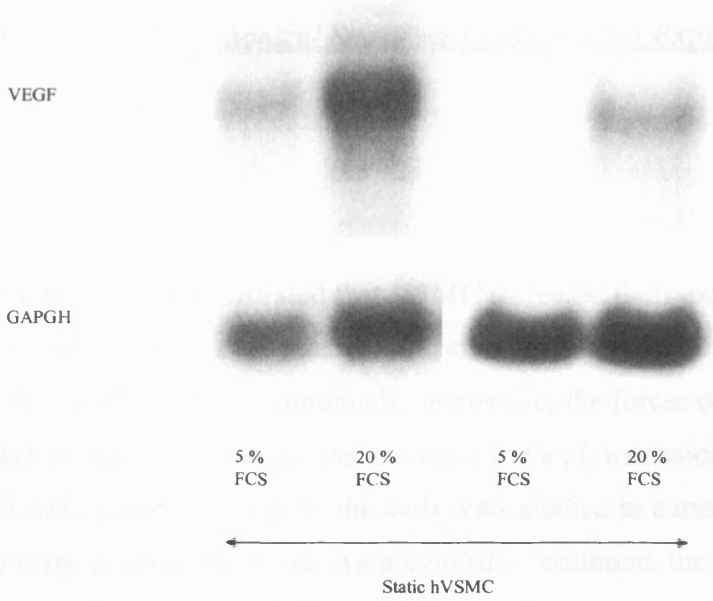

Figure 11- Autoradiograph showing the effect of FCS challenge on VEGF mRNA expression by VSMC.

These positive and negative regulatory experiments provide the necessary control samples for further analyses and verifies that the cells were viable and metabolically competent. Furthermore, this not only confirms that VSMC synthesise VEGF mRNA when exposed to high concentrations of serum, but also establishes the fact that the Northern technique, the probes and the initial time point of three hours, to be used in subsequent experiments were performing optimally.

3.1.2) The Effect of Cyclical Mechanical Strain on VEGF mRNA Expression

3.1.2.1) Aim

The previous section of data demonstrated that VSMC increased their expression of VEGF mRNA in response to increasing serum concentrations in the overlying media. In their natural environment, VSMC are continually exposed to the forces of cyclical stretch because the passage of blood along the vessel causes a wave of distension in the cells. To recreate a more realistic milieu for culture, the cells were studied in a mechanically active state so as to mimic the *in vivo* conditions. As previously mentioned, the cyclical mechanical strain was applied using the Flexcell apparatus.

The experiments were designed along simple time courses so as to determine the optimum length of exposure to cyclical mechanical strain to achieve the greatest expression of VEGF mRNA. Once the optimal time for exposure had been determined, a more detailed investigation of the effect of cyclical mechanical strain on VEGF expression was undertaken.

Two time courses were used to during this series of experiments, primarily due to the expense and time required to harvest sufficient mRNA for each time point. The first course analysed the effect of exposure to cyclical mechanical strain for fifteen, thirty and sixty minutes and two, three and six hours. This was to determine the rapidity of onset of the effect of strain on VEGF expression. The second time course examined the effect of long term exposure to strain where the mRNA was harvested after three, six, twelve and twenty-four hours. As determined in the previous section, the static controls were incubated in 5 % FCS (negative) and 20 % FCS (positive). The cells were analysed in Hams F12 media containing 5 % FCS and exposed to strain equivalent to the physiological blood pressure of 120/80 mmHg, at a rate of 60 cycles per minute. The Flexercell equipment was calibrated such that 0.133 kPa of vacuum applied produced the same physical deformation to the base of the plate as 1 mmHg blood pressure would

administer to the vessel wall (see Flexercell Strain Unit Operator's Manual [Flexcell, MacKeesport, USA]). Therefore, to achieve a degree of strain equivalent to 120/80 mmHg, the vacuum was set to15.96 kPa to represent the systolic pressure and 10.64 kPa to represent the diastolic pressure.

3.1.2.2) Results

The initial experiments shown below investigated the effect of cyclical mechanical strain on VEGF expression over a six-hour period. The VEGF/GAPDH ratios for the static 5 % and 20 % FCS controls were 0.27 and 1.27 respectively. During the first hour of cyclical mechanical strain the ratio decreased to 0.05 at 15 minutes, 0.03 at 30 minutes and 0.14 at 1 hour. This level then increased to 0.48 at two hours and 0.533 after three hours. The ratio then diminished to 0.21 at six hours.

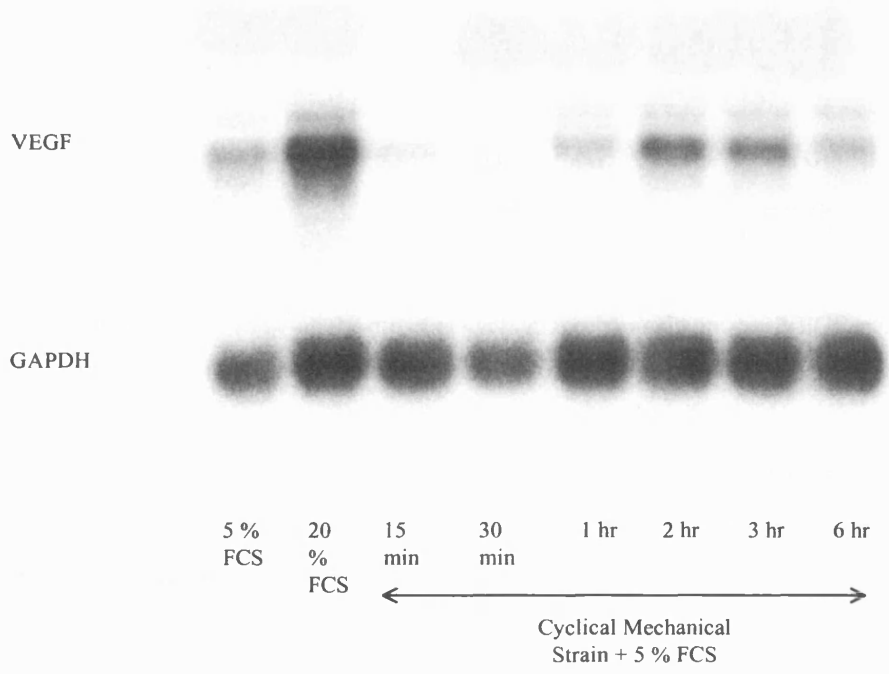

Figure 12- Autoradiograph showing the effect of cyclical mechanical strain on VEGF expression by hVSMC over a six-hour incubation period. The second lane shows the response to FCS as a control stimulus.

These data demonstrate that the optimal time to study the effect of cyclical mechanical strain on VEGF expression was three hours. To confirm that this time point was the optimal expression, a longer time course was performed to study the expression levels of VEGF up to 24 hours. The data supports the earlier finding that three hours is the optimal exposure and shows that although reduced, the increased VEGF expression persists beyond the 24 hour period of strain. The ratios were 0.03 and 0.34 for the 5 % and 20 % controls respectively and 0.46, 0.27, 0.13 and 0.05 for the three, six, twelve and twenty-four hour strains respectively.

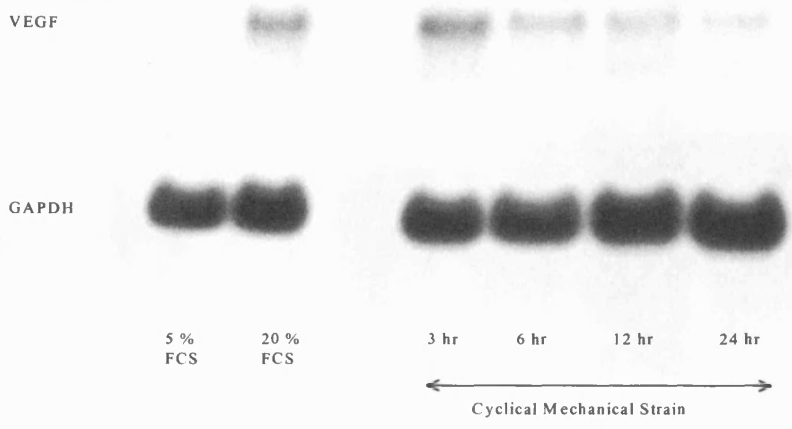

Figure 13- Autoradiograph showing the effect of cyclical mechanical strain on VEGF expression by hVSMC over a twenty-four hour incubation period.

These time courses indicate that, as with the higher concentrations of FCS, the optimal time for exposure to strain to assess steady state VEGF mRNA expression analysis is 3 hours. As highlighted earlier in this section, the high cost of each experiment was a limiting factor during the completion of this work. As earlier work in our laboratory had demonstrated that both FCS and PDGF maximally induced VEGF expression at three hours, no further time courses were required. The results presented in the previous section show that FCS induces VEGF mRNA in a dose dependent manner. In continuation of this theme, sets of plates were exposed to same degree of strain (120/80 mmHg), in increasing concentrations of FCS. Data from these experiments would

indicate whether or not the strain induced VEGF mRNA expression was maximal or could the level be further potentiated by additional stimuli.

The Northern analysis indicates that not only does strain induce the expression of VEGF mRNA, but that it acts to potentiate the effect of increasing FCS concentration such that the effect of both stretch and FCS gives a greater increase than either components alone. Those cells stretched in Hams F12 containing 1 % FCS increased their VEGF/GAPDH ratio to 0.20 compared to 0.06 of the 1 % static culture. These data followed this pattern through the increasing concentrations of serum indicating that strain can induce VEGF expression beyond that of serum initiation alone. The 5 % stretch was 1.42 compared to 1.20 for the rest cells and the 15 % stretch was 2.83 compared to 1.87 for the static cells.

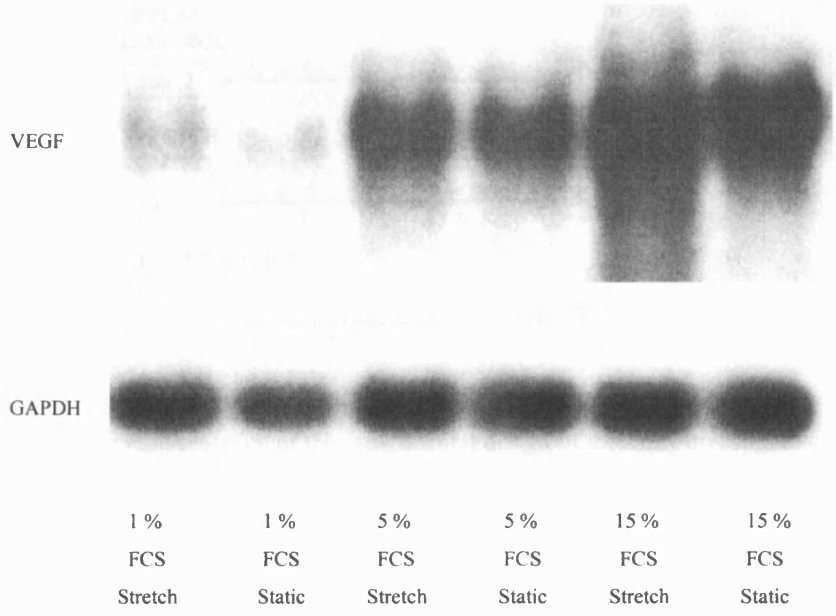

Figure 14- Autoradiograph demonstrating the additive effect of cyclical mechanical strain and FCS concentration.

The data thus far indicates that cyclical mechanical strain induces VEGF mRNA expression, however, the response returns towards its baseline level of expression during continued strain over twenty-four hours. This is similar to the effect observed with FCS

alone. The last experiment in this section sought to determine if the return to baseline expression was because the VSMC had adjusted their response and adapted to the new, mechanically active environment, or simply been exhausted of functional capacity. As the addition of a second stimulus, in this case strain added to the known stimulus of serum, caused a greater VEGF response than either alone, it can be proposed that the cells have not been exhausted of their functional capacity. Thus, the next experiment was designed to assess the effect of the two known stimuli (15 % FCS and exposure to strain) after the VSMC had undergone twenty-four hours of strain. These data would confirm if the cells had adapted to a stimulus to down regulate VEGF expression.

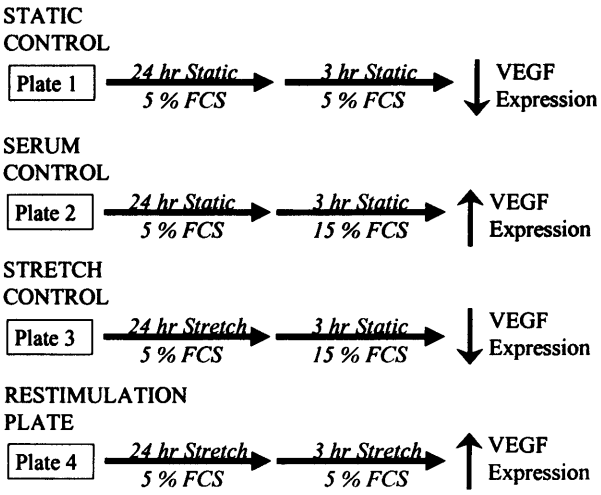

Figure 15- A schematic representation of the experimental design of the assay investigating the effect of re-challenging stretched human VSMC to a second stimulus, demonstrating the relative level of VEGF mRNA expression observed.

The cells were conditioned for twenty-four hours in 5 % FCS by either static or mechanically strained incubation, before exposure to a three-hour test pulse. This ensured that the initial VEGF stretch response had been maximally expressed and returned to baseline. The cells were then exposed to combinations of either rest or strain and low or high serum concentrations. The two control plates that were static for twenty-four hours show the normal response when then exposed to 5 % and 15 % FCS, with ratios of 0.27

and 0.88 respectively. The sample in the third well was stretched at 120/80 mmHg for twenty-four hours and then exposed to 15 % FCS in a static environment for three hours.

The VEGF expression ratio in the static 15 % FCS pulse was 0.14, suggesting that the VEGF response was blunted after twenty-four hours exposure to cyclical mechanical strain. However, the cells exposed to the lower serum concentration in the mechanically active environment were able to elicit a VEGF ratio of 0.76, demonstrating that the gene could be further induced by strain a second time.

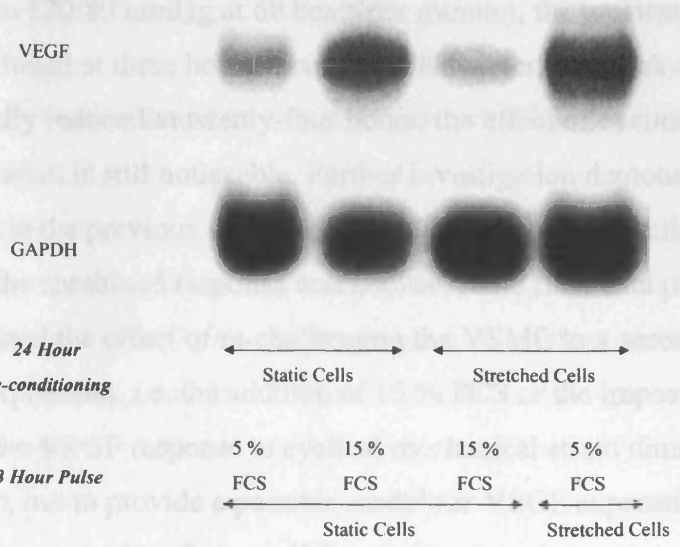

Figure 16- Autoradiograph demonstrating the effect of long term exposure to strain and re-stimulation by serum and cyclical mechanical strain on VEGF mRNA expression at 3 hours by human VSMC.

3.1.2.3) Discussion

Cyclical mechanical strain has in published data been shown to induce a diverse array of cellular events in different cells. Cells in the arterial wall exhibit alignment responses when exposed to cyclical mechanical strain (357). Bovine EC, VSMC and fibroblasts

align themselves perpendicular to direction of the strain, regardless of the cellular species, and this response increases as the amplitude and frequency of stretch increases (25).

The results in this section focus on the effect of repeated mechanical strain on the expression of the potent mitogen VEGF. A positive inducement of VEGF with strain could indicate a novel mechanism by which cyclical mechanical strain could induce hyperpermeability of the overlying endothelium.

When exposed to a regimen of maximal cellular elongation of 24 % at a frequency of 1 Hz (equivalent to 120/80 mmHg at 60 beats per minute), the greatest VEGF mRNA expression was found at three hours incubation. However, this peak diminishes and although markedly reduced at twenty-four hours, the effect of cyclical mechanical strain on VEGF expression is still noticeable. Further investigation demonstrated that the serum response shown in the previous section could be potentiated by cyclical mechanical strain, and that the combined response was additive. The final data presented in this section investigated the effect of re-challenging the VSMC to a second stimulus known to induce VEGF expression, i.e. the addition of 15 % FCS or the imposition of strain in 5 % FCS. Thus far, the VEGF response to cyclical mechanical strain diminishes after twenty-four hour stretch, but to provide a possible model for VEGF expression *in vivo*, further investigation was required to discover if the strain induced expression could be repeated in cells previously stimulated with strain. The data demonstrates that the expression of VEGF could be re-induced after the cells had been stretched for twenty-four hours (24 % maximal elongation). It was found that the control cells that were kept in static incubation for twenty-four hours could be stimulated to synthesise VEGF mRNA with high serum concentrations. However, the cells were previously exposed to twenty-four hours of cyclical mechanical strain and the addition of 15 % FCS media did not induce the expected increase in VEGF mRNA. Furthermore, the level of VEGF harvested from the cells was less than the static, low serum control cells. The cells that were stretched for twenty-four hours and then pulsed again for three hours in low serum media elicited a greater VEGF mRNA response than the high serum static cells. These data suggest that

the mechanisms for synthesis and release of VEGF via strain and serum are independent, and that the VEGF response to strain is repeatable whereas the serum response is not.

A later investigation by Seko *et al* supported these findings by demonstrating that VEGF is induced by cyclical mechanical strain in rat retinal pigment epithelial cells (358). This indicates a potential mechanism for strain inducing increased levels of VEGF resulting in an increased risk of retinal neovascularisation.

Early studies in avian skeletal muscle demonstrated that repetitive mechanical stimulation induced cell proliferation. This response was shown, in part, to be regulated by the prostaglandins E2 and F2 alpha, as inhibition of these with indomethacin reduced the cell growth (359). The application of mechanical strain caused in increase in prostaglandin levels of between 50-100 %, and during continued application of strain, the levels returned to baseline (359). These data were supported by a report investigating the effect of mechanical load on rat myocytes, which stimulates the production of immediate early (IE) genes and hypertrophy (360). The study identified a single stretch activated ion channel, which when blocked with gadolinium, did not have any effect on the IE genes or hypertrophy. Furthermore, disruption of microtubules with colchicine, inhibition of actin microfilaments by cytochalasin D or arresting contractile activity by tetrodotoxin did not affect the stretch-induced IE gene expression or hypertrophy. These results suggest that stretch-activated ion channels, microtubules, microfilaments, and contractile activity are not the mechanotransducers of strain induced growth, rather that cell stretch may cause a release of a growth factor(s), which in turn initiate a cascade of growth responses of cardiac myocytes (360).

Cyclical mechanical strain at 1 Hz on neonatal rat VSMC caused cellular proliferation via the autocrine action of PDGF (28) and twenty-four hours exposure to strain caused a 3.5-fold increased in PDGF mRNA (63). Following studies indicated that this reaction was mediated by extracellular matrix (collagen) interactions with the cells during strain and it could be potentiated with angiotensin II (59), (62), (361). This information suggests that there are a host of responses stimulated by cyclical mechanical strain to induce growth in

VSMC, and this complicated diversity is necessary to facilitate the actions of the many antagonistic circulating factors involved in regulating cell growth.

The data presented in this section clearly demonstrates that cyclical mechanical strain can increase expression of VEGF mRNA by up to 6-fold and that the optimal time of exposure is three hours. The optimal incubation time for VEGF expression is the same for strain and FCS induction, as was demonstrated by Williams *et al* who achieved a 6.6-fold increase in VEGF mRNA after three hours. Similarly, after twenty-four hours exposure to serum, the VEGF signal had diminished to baseline expression (37). Different stimuli have shown different increases in VEGF mRNA, in that hypoxia appears to by a highly potent provocation of expression. When Levy and co-workers subjected PC12 cells to a hypoxic environment, VEGF mRNA expression increased by a factor of twelve compared to baseline levels (362). Moreover, Stavri *et al* demonstrated a 30-fold increase in VEGF expression after 12-24 hours exposure to hypoxic conditions (328). Whilst investigating a potential mechanism for endothelial dysfunction by angiotensin II, Williams *et al* discovered that the expression of VEGF was induced in a concentration and time dependent manner by angiotensin II (363). This increase in VEGF could be inhibited by the addition of the specific AII inhibitor losartan (363). These data were confirmed by Gruden *et al*, who observed that angiotensin II could induce a 1.5 to1.7-fold increase in VEGF expression in human mesangial cells (364). A recent report published by Seko and co-workers investigated the effect of cyclical mechanical strain on cultured rat ventricular cardiac myocytes (365). Interestingly, the report observed maximal VEGF message after one hour of strain, which is significantly sooner than the three hours recorded in our laboratory (37).

3.1.3) The VEGF mRNA Isoform Expression by VSMC

3.1.3.1) Aim

The previous results sections have demonstrated that VSMC synthesise VEGF in response to cyclical mechanical strain. However, as discussed in the introduction, there are a variety of VEGF isoforms produced by VSMC, therefore it is necessary to identify which of the species are expressed in the stretched and static cells. This introduces the possibility that the species synthesised could be differentially affected by the degree of strain imparted on the cells.

The results thus far clearly indicate that VEGF A is expressed by human VSMC when exposed to cyclical mechanical strain or suitable FCS concentrations. It was unknown however, which of the different VEGF A isoforms the human VSMC had the potential to produce. Therefore, an RT PCR was designed to establish which of the VEGF A isoforms were actually expressed by human VSMC.

Duplicate sets of plates were prepared for exposure to cyclical mechanical strain or for the control static culture. Two concentrations of FCS were used, either 5 % or 15 % FCS, because it has been demonstrated earlier in this thesis that in low FCS concentrations, VEGF expression is reduced. Therefore, the higher FCS concentration may induce detectable levels of a rare species, so as to assess the breadth of expression by the cells. After three hours, the mRNA was harvested and then reverse transcribed into double stranded message. This was then amplified by PCR using primers specific to VEGF-A. The resultant DNA products were electrophoresed and transferred to a membrane and probed using Southern techniques to confirm the authenticity of the bands. At the time these experiments were performed, the VEGF A_{145} species had not been fully characterised. Therefore, a probe was not generated due to the lack of available published information.

3.1.3.2) Results

After PCR amplification, the samples were electrophoresed on a 2 % agarose gel. The high concentration of agarose was needed to ensure the separation of the low molecular weight bands that were to be generated from the amplification.

The gel was run and when separation was achieved, it was photographed and the DNA was transferred to a membrane for Southern blotting.

The photograph of the gel shows clearly that the PCR reaction has generated four bands, the weight of which spans from approximately 570 bp to 790 bp. The predicted molecular weight for the VEGF isoforms from the PCR primers is 589 bp for VEGF A_{121}, 659 bp for VEGF A_{165} and 734 bp for VEGF A_{189}. The molecular weight for the VEGF A_{206} isoform was not calculated from the sequence in the PCR section, because it does not contain the 17 amino acid insert between exons six and seven. However, as three bases code for each amino acid, it can be postulated that the extra fragment would be approximately 51 bp heavier than the VEGF A_{189} fragment, at a molecular weight of 785 bp. This suggests that all four VEGF A isoforms are produced by human VSMC.

To confirm the identity of the fragments, probes were generated for the VEGF A isoforms (as described in the Methods section). The VEGF A_{121} probe was chosen to span the exon five/eight junction, as this sequence is specific to this isoform.

Similarly the exon five/seven junction was chosen for VEGF A_{165} and the exon six/seven junction for VEGF A_{189}. The final probe for VEGF A_{206} was deigned to span the junction of the unique insert section. The probes were in excess of twenty-five bases in length and the middle of the sequence was the splice junction, so as to increase the specificity and reduce the levels of cross reactivity.

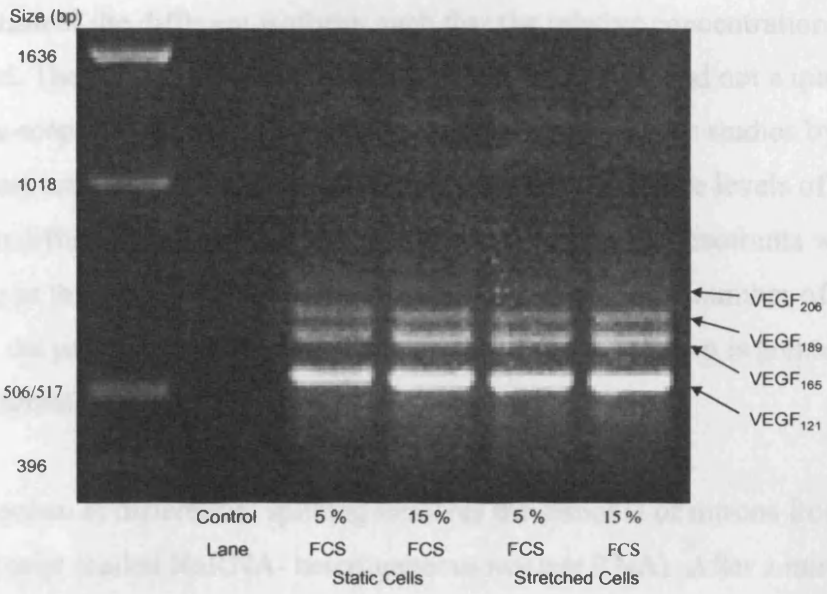

Figure 17- Gel photograph identifying the VEGF A isoforms synthesised by VSMC when exposed to cyclical mechanical strain.

When analysed separately with these probes, the band on the autoradiograph produced by the probe for VEGF A_{121} had migrated 116 mm, the same distance from the wells as the band on the photograph, confirming that the smallest band on the gel is VEGF A_{121}. The same results were found using the other two probes, thus confirming the identity of the middle two bands as VEGF A_{165} and VEGF A_{189} respectively. The probe for the VEGF A_{206} isoform bound to the autoradiograph 105 mm from the wells in the gel; a distance that corresponded to the distance migrated by the largest PCR fragment thus confirming its identity as the largest VEGF species.

3.1.3.3) Discussion

The data presented in this section clearly demonstrates that the four major species of the VEGF A gene are expressed in human VSMC. When the distances between the single bands and the top of the autoradiograph (the wells of the gel) were measured, each corresponded with the distance the relevant band had migrated on the gel. Whilst authenticating the identity of the bands, the technique unfortunately does not allow for

quantification of the different isoforms such that the relative concentrations could not be determined. The design of the technique as a qualitative PCR and not a quantitative PCR limited the scope of information available for discussion. Earlier studies by other investigators used *in situ* hybridisation techniques to quantify the levels of VEGF mRNA detected in different tissue types. The quantification of the four isoforms would prove interesting as this assay produces a proportionately higher copy number of the shorter species as the possibility of these being transcribed in the reaction is greater than the longer isoforms.

The mechanism of differential splicing involves the removal of introns from the primary RNA transcript (called HnRNA- heterogeneous nuclear RNA). After a number of intermediate splicing species, during which the remaining introns and specific exons are removed, the final mRNA species is produced. The mechanism by which the specific VEGF A isoforms are regulated and expressed still has yet to be described.

Previous investigation in rat tissues had discovered that VEGF A_{206} was not detected in any of the vascularised tissues analysed (286), suggesting that this information was new and possibly species specific. Bacic *et al* also noted the smaller VEGF A_{189} species accounted for over half of the VEGF found in the heart and lung, but was the least expressed in the brain, kidney and spleen. VEGF A_{165} was the predominant species expressed in the brain and kidney, however VEGF A_{121} was most abundant in the kidney and lung (286). A study of ovine corpora lutea demonstrated that one third of the VEGF expressed in these cells was the VEGF A_{121} isoform (366). This suggests that every tissue/cell type in the body does not express all of the species and that certain conditions may induce a preferential isoform. This has been found to be the case with VEGF A_{145}, which appears to only be produced by normal tissues and carcinomas of the female reproductive system (201), (216). The data in this section demonstrates for the first time the capability of human VSMC to generate the four main VEGF species. It will be important in future work to develop a quantitative PCR technique to detect whether there is differential expression of the various VEGF A isoforms in response to different stimuli, such as strain, FCS concentration, hypoxia and growth factors such as angiotensin II.

An interesting area for further investigation would be the development of a quantitative PCR that would allow the analysis of the levels of isoform expression at different degrees of strain. This would identify potential roles and preferential conditions of expression for the different species of VEGF.

3.1.4) The Effect of Cyclical Mechanical Strain on VEGF Peptide Expression

3.1.4.1) Aim

The data presented thus far has demonstrated that cyclical mechanical strain induces expression of VEGF mRNA in human VSMC, and that the cells are capable of synthesising the main four identified species. The next part of the hypothesis was to investigate the whether the RNA was translated into a detectable peptide.

Initial experiments were performed at 3-6 hours, however the VEGF A_{165} peptide could not be detected. Therefore, the VSMC were exposed to cyclical mechanical strain for a greater period of time to ensure that the mRNA had sufficient time to be translated and the product modified into the mature secreted peptide.

The overlying media was initially sampled from both stretched and static VSMC cultures at three, six, twelve, twenty-four, forty-eight and seventy-two hours. The ELISA assay was then performed immediately or the samples were frozen until required. Once the optimal time for peptide expression had been determined, all subsequent analysis was performed for that duration of strain.

3.1.4.2) Results

The initial experiments were performed by assaying the overlying media of cells incubated in 1 % FCS. These samples contained no measurable levels of VEGF A_{165} peptide, i.e. less than 5 pg/ml. Those cells incubated in higher concentrations of 10 % or 15 % FCS had very pronounced increases in peptide expression. To increase the concentration of secreted peptide per millilitre of media, the volume of the overlying media was halved from 2 ml to 1 ml, and as such, the levels of VEGF A_{165} peptide were then detectable in those cells incubated in 1 % FCS. Previous work within the laboratory

had demonstrated that FCS per se does not contain significant amounts of VEGF peptide (H Patel, personal communication).

To ensure that the cells were still viable after starving to quiescence for forty-eight hours, several duplicate experiments were performed to check that the VSMC did not perish during the subsequent seventy-two hour pulse time. The results obtained from these trial stretches demonstrated that the cells were metabolically viable and producing VEGF peptide. Visual examination confirmed that the cells were healthy in appearance and not lifting away from the plate.

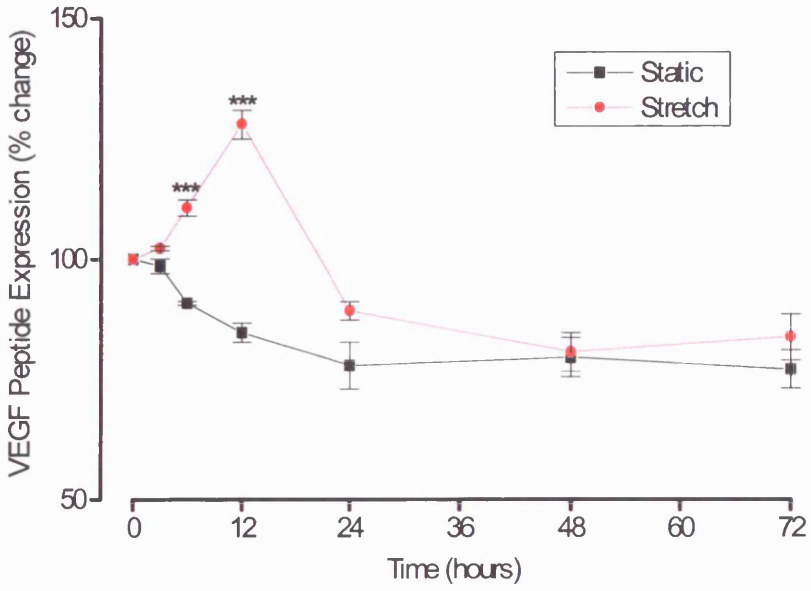

Figure 18- A graph to show the effect of cyclical mechanical strain on VEGF A_{165} peptide expression in hVSMC over a seventy two hour period, *** denotes $p < 0.001$ (one-way ANOVA).

The time courses demonstrated that the expression of the peptide reached maximum levels after 12 hours exposure to cyclical mechanical strain (128 % of the control static incubation, $p < 0.001$) and confirms that the optimal exposure period to cyclical mechanical strain for VEGF peptide expression is twelve hours.

Time (hours)	Duplicate 1		Duplicate 2		Duplicate 3	
	Stretched Cells	Static Cells	Stretched Cells	Static Cells	Stretched Cells	Static Cells
0	121	123	148	152	137	133
3	125	121	151	154	139	128
6	130	112	165	139	155	120
12	148	101	196	135	177	111
24	104	84	132	128	127	108
48	98	88	109	125	120	113
72	109	96	110	106	119	111

Table 3- A table showing the duplicate experiments of VEGF A_{165} peptide expression (pg/ml) assayed by ELISA in hVSMC over a seventy-two hour period of either active or static incubation.

Time (hrs)	Stretched Cells	SEM	Static Cells	SEM	P Value
0	100	0	100	0	1
3	102	1	99	1	0.082
6	111	2	91	0	<0.001
12	128	3	85	2	<0.001
24	89	2	78	5	0.096
48	81	4	80	4	0.849
72	84	5	77	4	0.345

Table 4- A table showing the average peptide production from hVSMC, expressed as percentage change of control (t0 for each experimental group), showing the standard error of the mean (SEM) and the statistical significance as determined by one-way ANOVA.

When the triplicate assays were analysed by one-way ANOVA, the only time points to show a statistically significant difference in VEGF peptide expression between stretched and static cells were the six and twelve hour incubations. Although both time points were significant ($p < 0.001$), the twelve hour time point repeatedly gave a better differentiation. This, therefore, was the optimal incubation time used for the subsequent assays.

The next experiment was designed to determine whether the differences in VEGF peptide production (static versus stretch) observed in the presence of 1 % FCS where accentuated when the concentration of FCS in the media was increased. This was an important experiment because the previous study examining the interaction between strain and 15 % FCS suggested an additive effect on VEGF mRNA expression when the two stimuli were combined. Thus human VSMC were stretched for twelve hours in the presence of 1 %, 5 % and 15 % FCS and the VEGF A peptide was measured.

FCS in Media (%)	Stretched Cells		Static Cells	
	Total VEGF (pg/ml)	Production per hour (pg)	Total VEGF (pg/ml)	Production per hour (pg)
1	688	57.37	537	44.75
5	1286	107.18	1225	102.12
15	2078	173.15	2088	174.01

Table 5- A table showing typical data obtained when VEGF A_{165} peptide production is determined after twelve hours incubation in either an active or static environment, in 1, 5 and 15 % FCS media.

The assay results shown in Table 5 indicate that 1 % FCS provides the optimal media for determining the effect of cyclical mechanical strain on VEGF expression, because the 1 % FCS concentration does not effect VEGF production. The effect of strain on these cells causes a 28 % increase in VEGF A_{165} protein expression compared to the rested cultures. The increasing concentrations of serum in the media mask the differences in the other cells, with the 5 % media demonstrating a non-significant 4 % increase in peptide expression and the 15 % media showing 0.5 % less VEGF peptide in the stretched cells.

These data provided the final link for the final study, allowing for the definitive assay to be performed. Compiling this data, it was determined that the optimal conditions for investigating the effect of cyclical mechanical strain on VEGF peptide expression were forty eight hours starvation in 1 % FCS with no media change, straight into the 12 hour stretch/static culture period.

FCS in	Set One		Set Two		Set Three	
Media (%)	Stretched Cells	Static Cells	Stretched Cells	Static Cells	Stretched Cells	Static Cells
1	688	537	644	425	521	450
5	1286	1225	745	644	691	714
15	2078	2088	1201	1147	1205	1155

Table 6- A table showing duplicate data obtained when VEGF A_{165} peptide production is determined after twelve hours incubation in either an active or static environment, in 1, 5 and 15 % FCS media (all values shown as pg/ml).

FCS in	Set One		Set Two		Set Three		P Value
Media (%)	Stretched Cells	Static Cells	Stretched Cells	Static Cells	Stretched Cells	Static Cells	
1	128	100	152	100	116	100	0.038
5	239	228	175	156	153	159	0.821
15	387	389	282	270	268	258	0.902

Table 7- A table showing duplicate data obtained when VEGF A_{165} peptide production is determined after twelve hours incubation in either an active or static environment displayed as percentage change in expression compared to control (1 % FCS static incubation). Statistical analysis performed using one-way ANOVA.

The duplicate data above corroborates the data, demonstrating that 1 % FCS media is the optimal concentration, with the averages of the data showing a statistically significant increase of 32 % in VEGF peptide expression with cyclical mechanical strain compared to the static controls ($p < 0.038$). Whereas the 5 % and 15 % media cells showed 8 % and 6 % increases in peptide expression respectively when compared to their serum controlled static cells. The data shows that cyclical mechanical strain increases VEGF peptide production in 1% FCS. However, when the serum concentration was increased, there was no additive effect of strain with 5 % or 15 % FCS.

3.1.4.3) Discussion

The data presented in this section clearly demonstrates for the first time that cyclical mechanical strain increases VEGF A_{165} peptide expression by human VSMC. The average increase is 32 % compared to the static incubations in 1 % FCS media ($p < 0.001$). This is consistent with the earlier sections of the result chapters, which have shown that strain increases VEGF A_{165} mRNA expression and now it has been demonstrated that the increase in mRNA is translated into increased VEGF peptide production, that is subsequently secreted by the cell. This is important as it could allow the strain-induced VEGF to act as a paracrine hormone, influencing the overlying endothelial cells.

The analysis of VEGF mRNA had to be performed using media containing 5 % FCS media whereas the peptide analysis was performed using 1 % FCS media. This difference in the levels if detection of the VEGF signal is due to the sensitivity of the assays, in that the immunological techniques employed in the ELISA provide a far more sensitive response than the Northern analysis.

Previous work into the area of VEGF peptide expression is limited with the work concentrating on the levels during specific pathologies, such as rheumatoid arthritis (367) and leiomyomata (368). Both of these pathologies are associated with increased vessel permeability and proliferation, which are characteristic actions of VEGF. Interestingly, the level of peptide was increased in both conditions and the expression of the receptors VEGFR1 and VEGFR2 by the adjacent EC was increased (367). The data in this thesis is the first demonstration that human VSMC produce and secrete the VEGF A peptide, which could then act locally on the endothelium. Although the data suggests a paracrine mode of action for VEGF *in vitro*, the experiment to support this hypothesis *in vivo* has still to be performed.

3.1.5) The Effect of Increasing Degrees of Cyclical Mechanical Strain on VEGF Expression

3.1.5.1) Aim

Having previously determined that cyclical mechanical strain induced the expression of VEGF mRNA and peptide, the next step was to simulate a physiological scenario. The studies so far in this thesis have compared static culture against cell stretch. It was now important to determine whether the strain-induced VEGF mRNA/peptide response was proportionate to the magnitude of strain applied to the human VSMC. The strain applied to the human VSMC *in vitro* was varied so as to mimic strain associated with different levels of blood pressure.

The optimal time of three hours exposure to strain had been established so this time point was used for these studies. The degree of strain applied to the cells was set at normal physiological blood pressure equivalent to 120/80 mmHg (an overall increase in length of 24 %), an intermediate hypertensive pressure of 160/100 mmHg (increase of 27 %) and an extreme hypertensive pressure of 200/120 mmHg (increase of 30 %).

3.1.5.2a) Results- Effect of Varying Levels of Strain on VEGF A mRNA Expression

The initial experiment to investigate the effect of increasing the degree of strain applied to the VSMC simply compared the level of VEGF expression in those cells stretched to mimic a normal blood pressure (120/80 mmHg) compared to human VSMC stretched to a greater degree to mimic a higher blood pressure (200/120 mmHg). Strain was applied for three hours to ensure a maximal VEGF mRNA response. The densitometry data demonstrated that the human VSMC exposed to the higher degree of strain increased their VEGF mRNA expression to a greater degree than those cells exposed to mechanical

strain mimicking a normal blood pressure. The VEGF/GAPDH ratios were 0.27 and 0.58 for the normotensive and hypertensive strains respectively.

The data in Figure 19 is a scan of the autoradiograph in which the initial strain response was repeated and investigated in more depth. It can be seen that the cells exposed to normal blood pressure conditions have increased their VEGF expression compared to the 5 % FCS control cells (0.03 and 0.09 respectively). The graph below illustrates that VSMC exposed to 20 % FCS in static culture increase VEGF/GAPDG ratio to 0.21. Increasing VEGF expression was observed in the 160/100 mmHg cells, where after three hours cyclical mechanical strain the ratio rose to 0.34. At 200/120 mmHg, the expression ratio was maximal at 0.45. Note that the VEGF mRNA response to higher levels of strain is greater than that induced by 20 % FCS. This response was specific to VEGF mRNA expression, as there was no strain-dependent change in GAPDH expression.

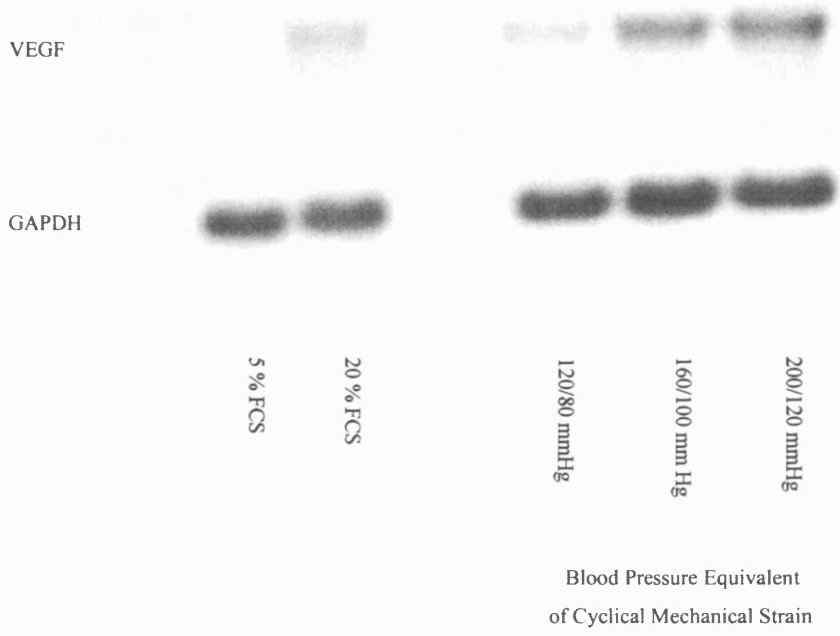

Figure 19- Autoradiograph showing the effect of increasing degrees of cyclical mechanical strain on VEGF mRNA production.

This data demonstrates that cyclical mechanical strain induces a strain-dependent increase in VEGF mRNA expression by human VSMC after three hours exposure to strain. The response is 'strain-dose' dependent in that higher levels of strain induce a greater level of VEGF mRNA expression.

3.1.5.2b) Results- Effect of Varying Levels of Strain on VEGF A Peptide Production

Previous sections of the results chapter demonstrated that cyclical mechanical strain increased the level of VEGF mRNA expressed, which in turn, lead to an increase in the level of VEGF peptide secreted into the overlying media. The logical step to take after showing that the level of VEGF mRNA produced increases with increasing degrees of strain was to investigate the effect of elevated levels of strain on VEGF A peptide production.

The VSMC were exposed to the same regimen of strain that was used to asses the effect of cyclical mechanical strain on VEGF mRNA expression, which was 24 % maximal elongation (equivalent to 120/80 mmHg), 27 % maximal elongation (160/100 mmHg) and 30 % maximal elongation (200/120 mmHg). As determined in the earlier studies, the optimal time period to detect a strain induced increase in VEGF peptide was 12 hours. VEGF A peptide secretion into the media overlying the human VSMC was assessed in the presence of three different concentrations of FCS and at three different levels of strain to define whether there was an interaction between these two stimuli. As previous data has shown that strain increases VEGF peptide as compared to static culture, for these experiments, the control levels were provided by the cells exposed to normal levels of strain (120/80 mmHg).

The initial assay (Table 8) confirmed that as the media concentration increased the concentration of VEGF peptide increased. Those cells assayed in the 1 % FCS media increased the VEGF peptide concentration to 119 % and 147 % that of control (normal strain) in the 160/100 mmHg and 200/120 mmHg plates respectively. The VSMC

stretched in the 5 % media demonstrated increases of 128 % and 142 % that of control in the 160/100 mmHg and 200/120 mmHg plates respectively, and the 15 % media showed smaller increases of 111 % and 121 % respectively.

FCS in Media (%)	120/80 mmHg (24 % elongation)	160/100 mmHg (27 % elongation)	200/120 mmHg (30 % elongation)
1	420	499	616
5	826	1058	1169
15	1507	1671	1824

Table 8- A table showing a representative VEGF production after twelve hours incubation in an active environment, in 1, 5 and 15 % FCS media (all values shown as pg/ml).

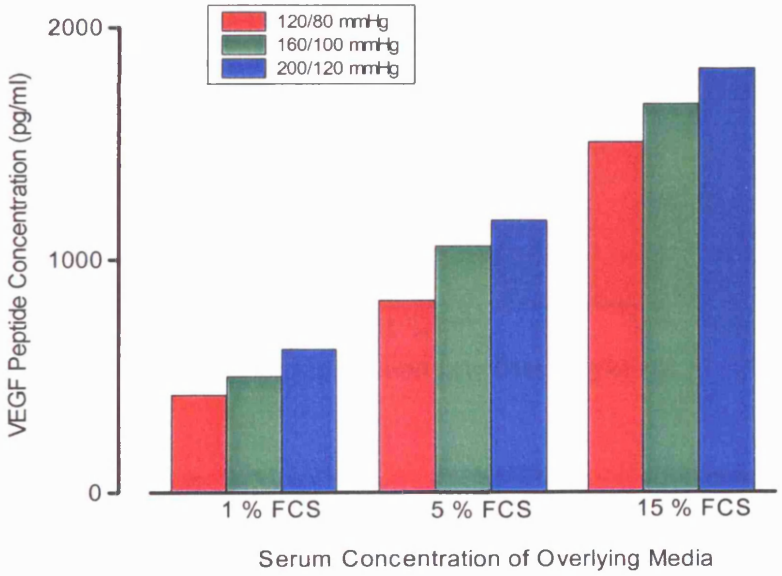

Figure 20- A graph demonstrating the effect of increasing levels of strain and increasing concentrations of serum on VEGF peptide production by human VSMC.

When repeated, the data indicated that as with the VEGF mRNA results, the greatest increase in peptide was found when the degree of strain was increased from 24 % maximal elongation to 27 % maximal elongation. The experiments had to be repeated on

numerous occasions as a substandard batch of plates meant that some of the elastomer bases were not sealed to the plates properly causing malfunction when the vacuum was applied. During the longer periods of strain, malfunction would render the equipment unable to apply the desired vacuum to produce the experimental stretch conditions. This would mean that during certain experiments, such as a twelve-hour overnight stretch, the experiment would have to be discarded, as the exact time of malfunction could not be determined.

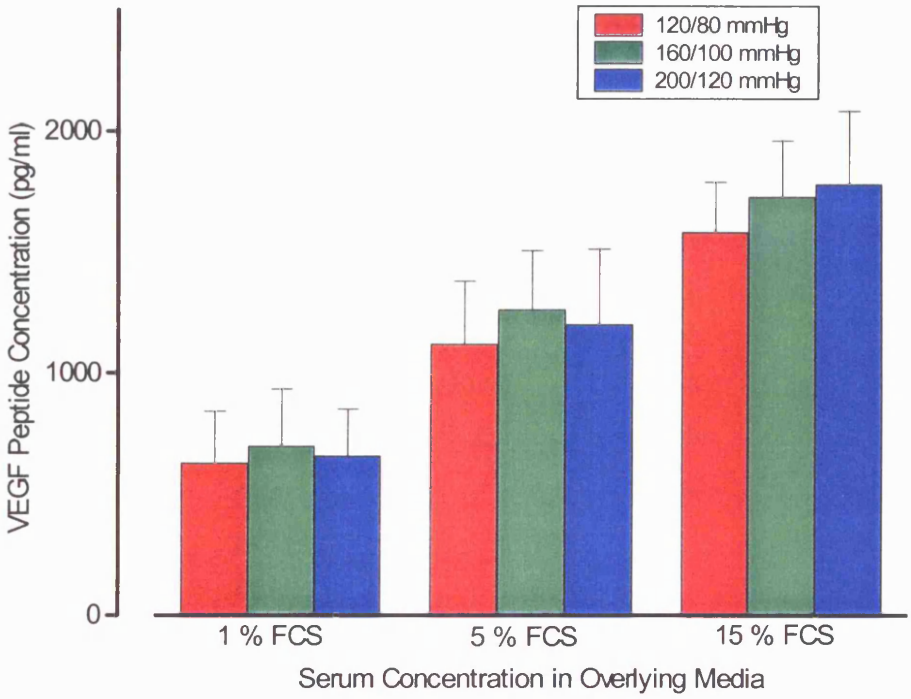

Figure 21- A graph demonstrating the average effect of increasing levels of strain and increasing concentrations of serum on VEGF peptide production by human VSMC.

The data was compiled (Figure 26) and analysed using the Friedman two-way analysis of variance. This was so that the difference between the two lower degrees of strain could be investigated. As can be seen from the data (Table 9) the increase in peptide production when the VSMC were stretched to 27 % maximal elongation was statistically significant when compared to the peptide production of the cells stretched to 24 % of their original length.

FCS Concentration (%)	VEGF A_{165} Peptide Production (pg/ml)		
	120/80 mmHg	160/100 mmHg	200/120 mmHg
1	628	698	657
5	1121	1264	1202
15	1585	1732	1784
1	329	390	284
1	303	404	#
5	672	1063	770
5	715	990	#
15	1270	1209	1312
15	1143	1526	#
1	1140	1110	1426
1	1933	2220	1308
5	2108	2714	1812
5	2463	1842	2792
15	2499	2324	2585
15	2485	3055	3100
1	223	311	196
1	227	324	196
5	571	739	576
5	661	606	527
15	1041	1196	1048
15	1191	1195	973

Table 9- A table showing the duplicate data for the levels of VEGF peptide expression by human VSMC at increasing degrees of strain (the # symbol denotes plates that were destroyed by the higher level of strain and no sample could be taken).

The data used to perform the statistical analysis was obtained from nine separate experiments that are tabulated below (Table 9). Assuming chi-square distribution with two degrees of freedom, the increase in VEGF peptide production when the VSMC are stretched to a greater elongation of 27 % (equivalent to 160/100 mmHg) is significant ($p= 0.037$).

3.1.5.3) Discussion

The data presented above clearly demonstrates a dose dependent response of VEGF mRNA expression to an increasing magnitude of cyclical mechanical strain. This is also shown in the resultant peptide expression, which also shows an increase in response to increasing degrees of strain. This may be of particular relevance when considering the increase in permeability observed in hypertensive vessels as this suggests a possible mechanism whereby the magnitude of strain experienced by the vascular wall could correlate with a proportionate increase in VEGF production by human VSMC. As VEGF is a potent permeabilising agent, its increase in expression during strain may well cause an increase in vessel permeability allowing cellular invasion and the development of such vascular pathologies as atherosclerosis.

The second point of interest within the data is the reduction in peptide response at the highest degree of strain (30 % maximal elongation). The greatest peptide expression was observed in the 160/100 mmHg plates (27 % elongation) when cultured in 1 % and 5 % FCS media. A possible reason for this decline in expression in those cells exposed to the greatest degree of cyclical mechanical strain is that the apparatus is not designed to operate optimally at this degree of strain. Thus, the degree of strain applied could vary, especially during the longer twelve-hour strain period, causing a variation that is amplified during incubation. Further investigation of the stretch effect at this degree of strain requires more reliable systems. A major problem was the elastomer bases often became detached at 30 % elongation and the calibration of the machine at this level was not guaranteed. Conversely, there was a study published by Seko *et al*, which investigated the effect of cyclical mechanical strain on VEGF peptide expression in rat cardiac myocytes. The data demonstrates that strain does induce a 1.7 fold increase in VEGF peptide expression using the two strain regimens, but does not show any difference between the two degrees of strain (365). The two regimens used were 15 % elongation at 1 Hz and 10 % elongation at 2 Hz, which are very mild degrees of strain.

Furthermore, the protocol investigates the effect of two variables simultaneously, i.e. the frequency of strain and changes in degree of strain. To better qualify their data, Seko *et al* would have to investigate the effect if these changes individually as well as in combination. The detection of dose dependent VEGF expression in this thesis could be because the degrees of strain applied to human VSMC were physiologically relevant for the human vasculature.

Previous data in the thesis demonstrates that cells exposed to cyclical mechanical strain for twenty-four hours can still be stimulated to express VEGF, which indicates a direct physiological mechanism by which over stimulation causes a down regulation of the gene. Moreover, the dose dependent response demonstrated by the cells in these experiments suggests that the human VSMC have a sophisticated mechanism for the detection of strain because it is capable of producing a proportionate response to the magnitude of strain applied.

Previous work centred on pre-eclampsia during pregnancy where it was determined that VEGF levels rose during the condition (369). Interestingly, the level of VEGF in the pre-eclamptic patients was not elevated prior to the clinical disease. This is in keeping with the data above, as an increase in cyclical mechanical strain exerted on the vasculature of the pre-eclamptic patients would induce a greater degree of strain on the smooth muscle cells increasing the level of expression of the gene.

A study performed by Sharp *et al* investigated the potential link between microvascular disease and elevated blood pressure in diabetic patients. The level of VEGF detected in the blood by PCR was directly correlated to the patients' blood pressure. As the level of all measures of blood pressure increased (diastolic, systolic and mean) the level of VEGF detected in the blood increased significantly (370). These data supports that presented in this thesis by presenting a human model *in vivo* that demonstrates the strain response of VEGF expression in the vasculature.

3.2) Intracellular Signalling Pathways in VSMC in Response to Cyclical Mechanical Strain

3.2.1) The Effect of Cyclical Mechanical Strain on Phosphorylation of MAP Kinase

3.2.1.1) Aim

The data presented above indicates that cyclical mechanical strain induces an increase in expression of VEGF mRNA in human VSMC. Subsequent analysis demonstrated that this was associated with an increase in secreted VEGF peptide in the media overlying the cells. The previous studies also show that human VSMC produce VEGF in proportion to the magnitude of mechanical strain applied to the cells. The second area of interest in the thesis was the potential mechanism by which the cyclical mechanical strain initiated these intracellular events. As discussed in the introduction, the MAP kinase cascade was identified as a likely candidate for the transduction of the extracellular deformation into an intracellular event.

The first objective was to ascertain whether or not MAP kinase phosphorylation was stimulated by cyclical mechanical strain in human cells. At the time this study was initiated, this had not been defined. The cells were exposed to the same regimen that was used for the VEGF analysis, however the period of exposure was reduced to minutes as the activation of MAPK is very rapid. The positive control used in the experiments was ATP stimulation, because this has been well documented to activate the MAPK cascade in VSM cells.

The antibody used for the detection of MAPK was specifically designed to only recognise the phosphorylated form of the signalling peptide, as opposed to total MAPK. The phosphorylated MAPK is indicative of the activated MAPK and thus the level of

phosphorylated MAPK defines the magnitude of MAPK activation in response to the extracellular stimulus (cyclical mechanical strain).

3.2.1.2) Results

The first experiments were designed to optimise the protein isolation from the stretch plates. Initially four samples were obtained from each stretch plate, the first was the resultant mixture after three wells were harvested together by pooling the carried over 50 µl homogenising buffer from well to well. The remaining three wells were harvested individually with 50 µl homogenising buffer to prepare the final three samples. The Western analysis obtained from these samples produced very faint bands for these four samples, the strongest being the three-well pool. So as to maximise the protein harvested from each stretch plate, all six wells were then harvested with 50 µl homogenising buffer being transferred from well to well. Also the plates were allowed a longer thaw time after treatment with liquid nitrogen, so as to reduce the risk of diluting the sample with condensation from the plate.

The next step after optimising the concentration of protein harvested from the plates was to design a suitable time course of strain to observe the effect on phosphorylated MAPK levels. The human VSMC were plated out and exposed to strain for between one to seven minutes strain, with the static controls being incubated for one, three, five and seven minutes. Figure 22 shows an autoradiograph obtained from the Western analysis. The protein concentration within the samples was assayed and the samples diluted such that the same amount of protein was loaded into each well and the molecular weight of the resultant bands produced confirmed their identities as p44 and p42.

The data in Figure 22 clearly demonstrates that the levels of phosphorylated MAPK increase with longer exposures to cyclical mechanical strain. To enable numerical comparisons to be made, the control baseline (100 %) was determined from the two

minute strain data because the levels of p42 and p44 were too low in the static incubations for the densitometer to detect and quantify.

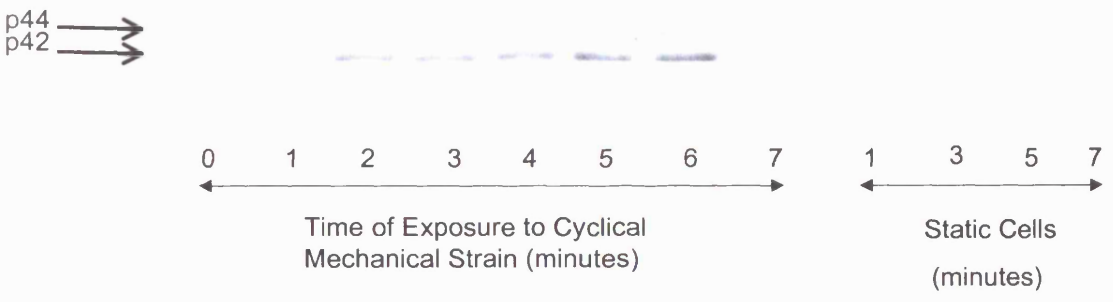

Figure 22- Autoradiograph to demonstrate the effect of cyclical mechanical strain on levels of phosphorylated MAPK (p44and p42) in human VSMC.

The phosphorylated MAPK peaks after approximately five to six minute's exposure to strain (both the p42 and p44 are about 300 % that of control) and by seven minutes the response has returned to baseline. Thus the activation of MAPK by cyclical mechanical strain is rapid, substantial and short lived.

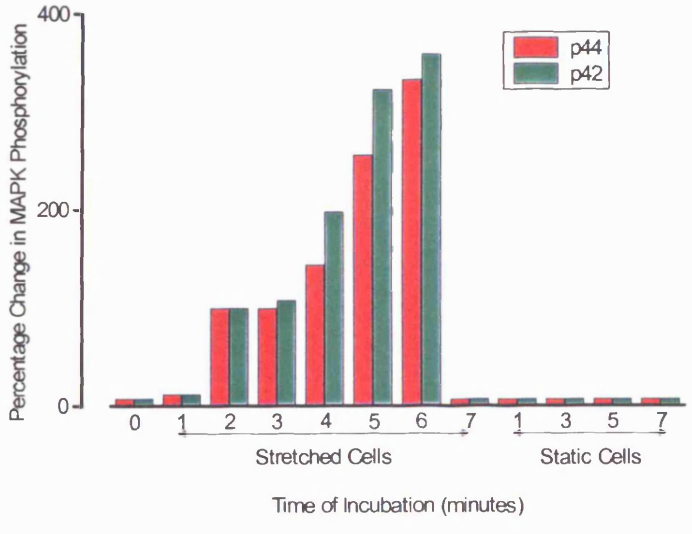

Figure 23- A graph to demonstrate the effect of cyclical mechanical strain on levels of phosphorylated MAPK (p44and p42) in human VSMC as determined by densitometry.

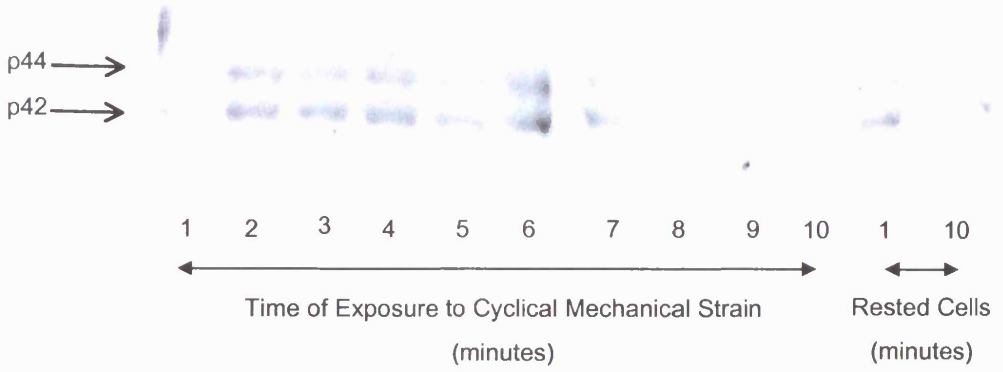

Figure 24- Autoradiograph to demonstrate the effect of cyclical mechanical strain on MAPK phosphorylation over time.

Further experiments, shown in Figures 24 and 25, confirmed that the maximal phosphorylation of MAPK was achieved after four to six minute's exposure to cyclical mechanical strain. The p44 peptide phosphorylation was elevated to 286 % and 214 %, at four and five minutes respectively, compared to the baseline at one minute static incubation.

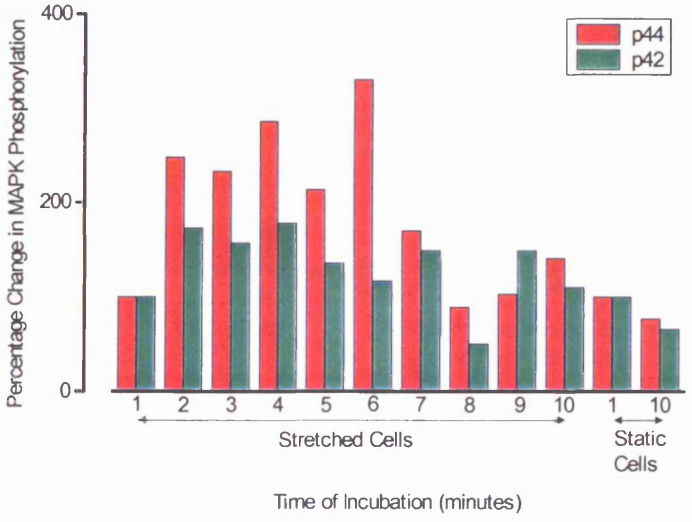

Figure 25- A graph to demonstrate the effect of cyclical mechanical strain on levels of phosphorylated MAPK (p44and p42) in human VSMC as determined by densitometry.

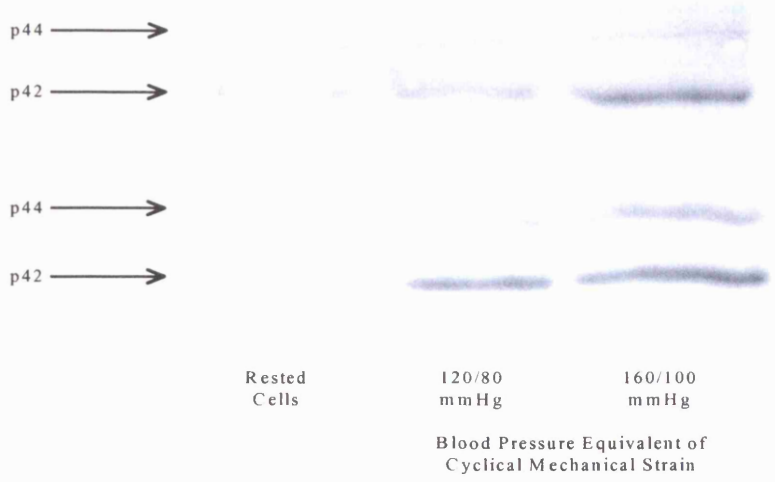

Figure 26- Two autoradiographs demonstrating the effect of increasing degrees of cyclical mechanical strain on MAPK phosphorylation.

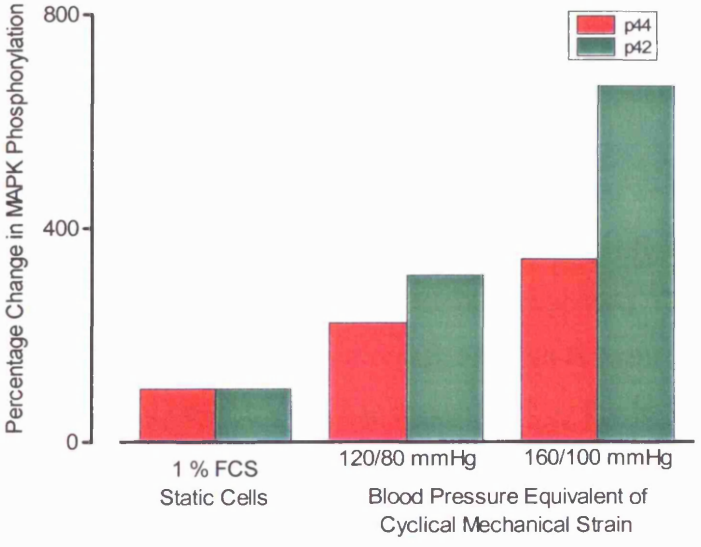

Figure 27- A graph demonstrating the percentage change in MAPK phosphorylation when VSMC are exposed to increasing levels of cyclical mechanical strain.

The strain-induced activation of VEGF mRNA and protein expression was strain-dose dependent. The next series of experiments set out to determine whether the level of MAPK activation was proportionate to the magnitude of mechanical strain applied to the human VSMC. The data obtained examining VEGF expression indicated that stretching to mimic a blood pressure of 160/100 mmHg produced the optimal expression and therefore, for these experiments, the higher degree strain of 200/120 mmHg was not examined.

The human VSMC were stretched at equivalent pressures of 120/80 mmHg and 160/100 mmHg for five minutes, and the cell preparations analysed using Western blot. The level of phosphorylated p44 and p42 in the 120/80 mmHg cells rose to 224 % and 312 % of the static control cells respectively. The phosphorylation peaked at 160/100 mmHg where the levels were 343% for p44 and 668 % for p42 (Figures 26 and 27). The second investigation in Figure 26 confirmed that the 160/100 mmHg gave the maximal phosphorylation of p44 and p42 with levels of 364 % and 362 % respectively, over that of the control.

3.2.1.3) Discussion

Exposing human VSMC to cyclical mechanical strain induces significant phosphorylation of p44 and p42 MAPK after two minutes exposure. This increased phosphorylation continued until six minutes, thereafter the level reduced back to near baseline. Moreover, the activation of MAPK (as determined by the level of phosphorylated MAPK) was proportionate to the magnitude of strain. For both of these assays, the data could only be obtained twice, due to the complexity and cost of the procedure.

Yamazaki *et al* published a report that confirmed the activation of the MAPK cascade in rat cardiac myocytes in response to cell stretch (371). Furthermore, they demonstrated that the upstream activators of MAPK, i.e. MAPKKK (Raf-1 and MEKK) proteins were

also activated after 1 minute of cyclical mechanical strain and were maximally phosphorylated at 2 minutes stretch. The MAPKK (MEK) peptides were phosphorylated after 1-2 minutes stretch and maximally so after 5 minutes strain. The MAPK and p90rsk proteins were activated and maximally phosphorylated at 8 and 10 minutes respectively (371) (372). The paper concluded that cyclical mechanical strain activated the MAPK cascade in the order of MAPKKK and MEKK, MAPKK (MEK), the ERKs and p90rsk. Subsequently it was illustrated that cyclical mechanical strain increased the expression of angiotensin II, which potentiated cardiac myocyte hypertrophy. Using the type I AII receptor antagonist CV-11974, studies both *in vitro* and *in vivo*, demonstrated a reduction in MAPK activity and hypertension-induced cardiac hypertrophy respectively (372). In addition, CV-11974 caused regression of cardiac hypertrophy *in vivo* thus acting in a cardioprotective manner.

In my experiments, the level of activation of p44 and p42 differs between experiments. Zhou and colleagues demonstrated that the different isoforms reacted differently when stimulated with insulin. Rat skeletal muscles were injected with either insulin or saline after a twenty-four hour fast, and the levels of phosphorylated MAPK assessed after exercise. Their data demonstrated sequential activation of two MAP kinases in muscles, the isoform which activates/deactivates more rapidly was p44, while the more slowly responding isoform was p42 (373). The physiological relevance of this is not known, although it may well play a role in the diversity of signalling mechanisms and specificity of actions of the MAPK family.

Moreover, the application of increasing degrees of strain causes an increase in MAPK phosphorylation, which is in keeping with the dose dependent increase in VEGF mRNA and peptide expression when exposed to strain. A recent publication by Ingram *et al* confirmed this occurrence, when they demonstrated that 29 % maximal elongation could increase p44/42 phosphorylation in mesangial cells approximately 8-fold, whereas the 20 % elongation cells did not phosphorylate detectable levels of the MAP kinase proteins (15). Interestingly in this study, the time of exposure to cyclical mechanical strain required to achieve maximal MAPK phosphorylation was one hour, which was

substantially longer than other investigators. Whether this is a phenomenon of mesangial cells compared to a more rapidly acting muscle cells remains to be determined.

The data in this section not only indicates that the level of phosphorylated MAPK increases when human VSMC are exposed to strain, but also that the level of phosphorylation of the signalling molecule is directly proportionate to the degree of strain applied. Within a few minutes exposure, the level of phosphorylated MAPK in the cells stretched to 24 % maximal elongation (120/80 mmHg equivalent) increased two-fold. However, the cells stretched to 27 % maximal elongation had increased levels of activated MAPK in excess of three times that of control cells. This supports the earlier suggestion of a sophisticated cell that can respond according to the level of strain applied to it. Furthermore, it suggests that the proliferative response of VSMC is likely to be a direct effect of strain, rather than a secreted factor such as PDGF, because of the rapidity of the signalling response.

It seems likely that the strain-induced activation of MAPK may be involved in the signal transduction of the strain-induced increase in VEGF mRNA. Liang *et al* have shown that cyclical mechanical strain increased the expression of brain natriuretic peptide mRNA in rat cardiac myocytes via a MAPK/JNK dependent pathway (374).

3.2.2) The Effect of Cyclical Mechanical Strain on MAP Kinase Activity

3.2.2.1) Aim

When exposed to cyclical mechanical strain, the level of phosphorylated MAP kinase increased in VSMC, although the actual activity of the kinase needed to be determined. The Western analysis in the previous section indicated the increase in phosphorylated protein in the cells, although further analysis was necessary to determine if the actual activity of MAPK was increased.

The cell lysates were obtained for the activity assay using the same technique as for the Western electrophoresis. The amount of cellular protein from each plate was standardised enabling the same concentration from each preparation to be analysed in the assay. The assay itself monitored the radioactive phospho-labelling of a known MAPK substrate, myelin basic protein (MBP). Thus, the higher the level of labelled MBP, the greater the phosphorylating activity of the cell lysate.

3.2.2.2) Results

The initial experiments following the published assay protocol appeared to give spurious and misleading data, which provided little support for the results obtained by Western analysis.

The initial studies yielded very high counts (DPM) which showed no differentiation between static of stretched cells and were very erratic with no form or pattern. To ensure that the assay was performing at its optimal conditions, the incubation times, temperature and sample dilutions were confirmed by two experiments (Figures 28 and 29). There were two parts of the protocol that may have caused the problems. The first was the incubation time and temperature for the initial reaction. The second was the washing

regimen, which may have been too stringent for the label. For the experiments in Figures 28 and 29, the samples were obtained from VSMC after ten minutes incubation with 10^{-6} M ATP, a known stimuli of MAPK phosphorylation. Performing the assay with phosphorylated MAPK rich samples also confirms the efficacy of the assay and MAPK isolation technique. The first assay was designed to ensure that the reaction end point had not been achieved within the twenty minute incubation at 30^{0}C. Ideally the reaction should still be progressing when it is halted, such that the rate of reaction is maximal but not exhausted. This would distinguish between relative phosphorylated MAPK concentrations in the samples, as those with greater levels of active MAPK would have phosphorylated more substrate. To determine the dynamics of the reaction, the samples underwent the same stimulus to obtain ten samples containing the same level of activated MAPK. The reaction was started and the samples stopped at 0, 5, 10, 15, 20, 25, 30, 45, 60 and 120 minutes.

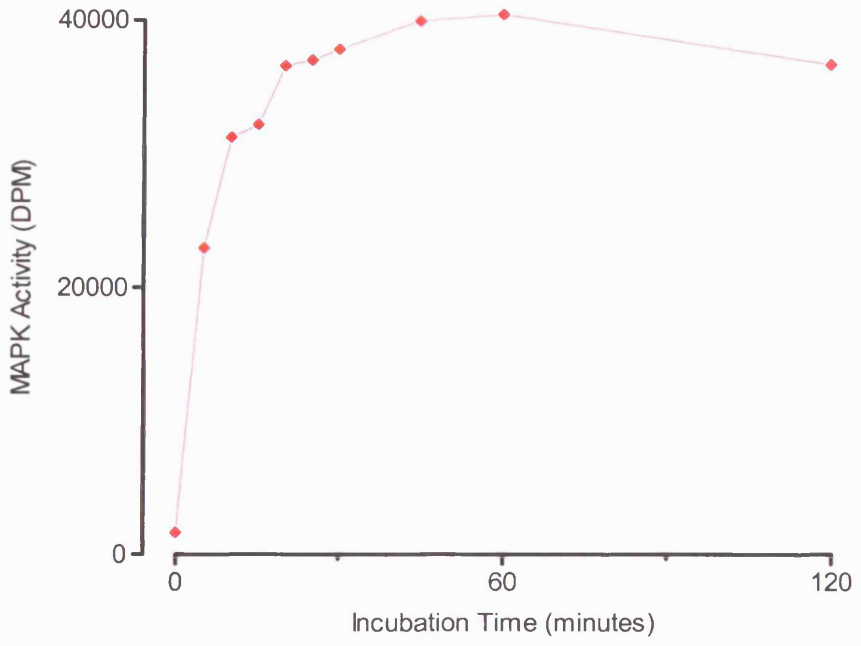

Figure 28- A graph to show the time course designed to determine the optimal length of incubation for the MAPK activity assay.

As can be seen from Figure 28, the rate of reaction was maximal at twenty minutes when the documented assay protocol was halted. Therefore, if the reaction has not exhausted itself at this time point, the problem with the assay was not the length of the reaction incubation.

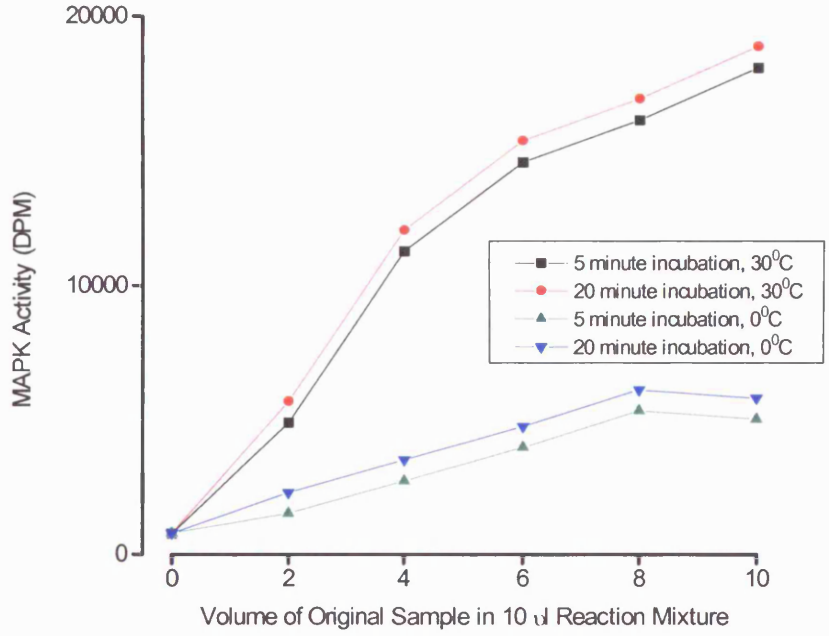

Figure 29- A graph to show the differentiation between five and twenty minutes incubation at both 30 °C and 0 °C for increasing titrations of ATP induced MAPK phosphorylation.

The second experiment was designed to investigate two potential problems within the assay. The first problem may have been the dilution of the sample. If the concentration of the isolated MAPK was too high, the reaction would be overwhelmed and any differences in MAPK levels would be masked. The second series of experiments compared the activity after 5 and 20 minutes incubation at 30^0C, because the fastest rate of reaction was seen after 5 minutes incubation, and this might have provided sample differentiation of activity when compared to the standard twenty minute incubation. As can be seen from Figure 29, the activity recorded in the five minute incubation and the twenty minute incubation is very similar at each temperature, indicating that the temperature of the reaction incubation is rate limiting. Furthermore, as the concentration of the sample

increases in all four reactions, so does the activity, however, the major factor would appear to be the length of time the reaction is allowed to progress.

The assay was repeated using the samples at the highest protein concentration possible. All the samples were diluted to the same concentration as the weakest protein concentration sample, such that each 10 µl sample used in the assay would contain the same amount of intracellular proteins. Again the assay showed no differentiation between the samples. A further experiment was designed to ensure the background signal was minimised, in which the filter papers were washed over increasing time periods to ensure that all the non-specific radiation was removed.

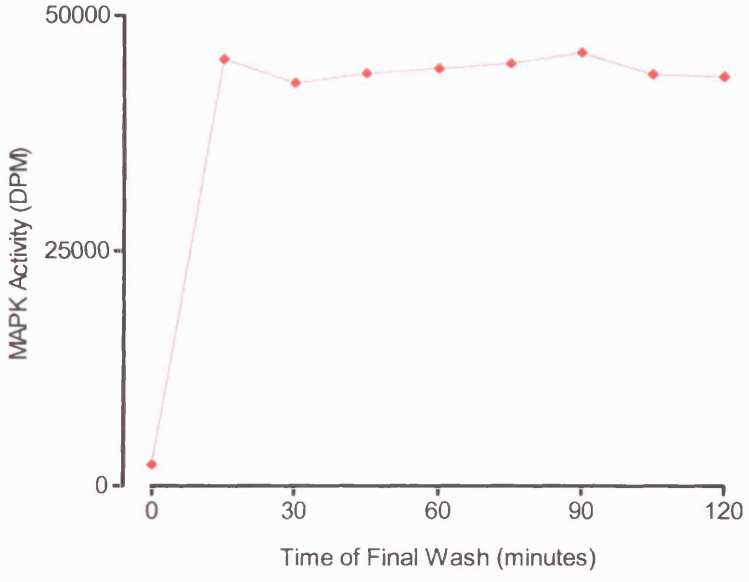

Figure 30- A graph to show the activity after increasing washing times (where zero indicates the BSA control levels).

The ATP stimulated samples were allowed to react at 30 °C for twenty minutes prior to being terminated by the addition of 20 % TCA. The reactions were then dispensed onto the filter paper and allowed to air dry. After which they were washed in phosphoric acid twice for one minute and then once for five minutes. The samples were then washed for two hours with one filter being removed every fifteen minutes so as to determine the

optimal washing time that would reduce the background radiation. The experiment above suggests that the recorded counts are those incorporated and that the standard washing protocol of one hour final wash is sufficient to remove the unbound label.

The assays were then performed using the protocol (as stated in the Methods section) which was optimised by the above data.

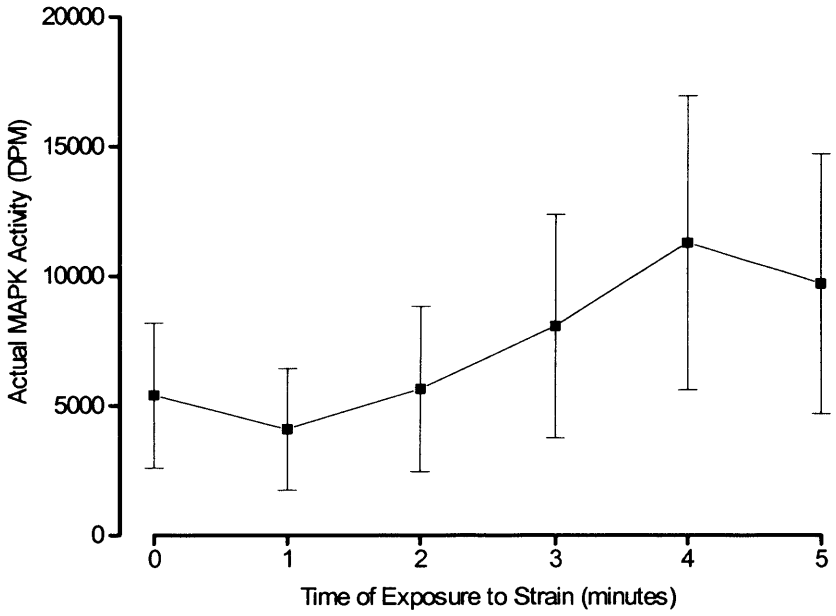

Figure 31- A graph showing the time course of MAPK activity in VSMC exposed to cyclical mechanical strain (data not standardised).

A simple time course was performed in triplicate to analyse the effect of cyclical mechanical strain on MAPK activation in the VSMC (Figure 31). The average baseline count obtained from unstretched cells was 6965 DPM. During the first minute of strain, the amount of phosphorylated MAPK fell, giving an average count of 5215 DPM. The level recovered after two minutes strain (average 7178 DPM). After three minutes strain, the average level increased to 11053 DPM, reaching its peak at four minutes strain, providing a mean of 15116 DPM. At the five minute time point, the level of activated MAPK had tailed off, giving a reading of 13021 DPM. To provide a clearer interpretation

of this data, the readings were standardised by calculating the relative percentage change in phosphorylation, using the unstretched sample (t0) as 100%.

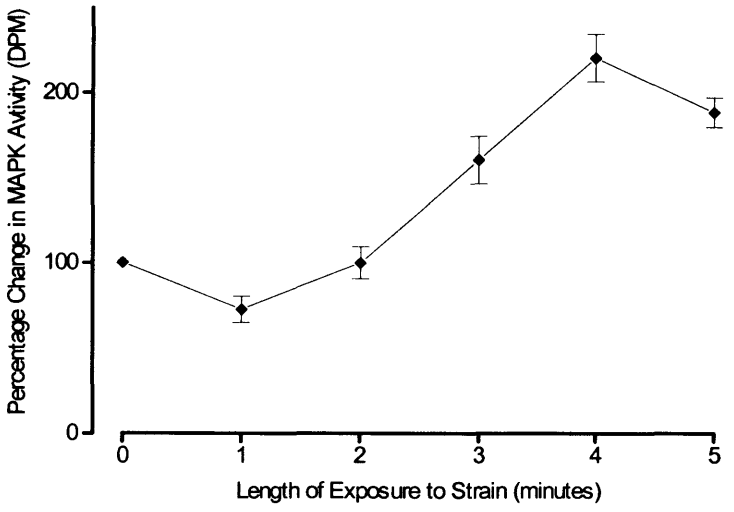

Figure 32- A graph showing the standardised time course of MAPK activity in VSMC exposed to cyclical mechanical strain (n=3).

Time (min)	Duplicate 1 (DPM)	Duplicate 2 (DPM)	Duplicate 3 (DPM)	Percentage Change	SEM	Statistical Significance
0	6839	9357	4789	100.0	0.0	-
1	4148	8129	3368	72.6	7.7	>0.05
2	5914	11055	4566	100.0	9.4	NS
3	9441	14801	8918	160.8	14.0	>0.01
4	15795	18112	11442	221.1	14.0	>0.01
5	12397	16784	9882	189.0	8.7	>0.01

Table 10- A table showing the triplicate time course data obtained from the MAPK activity assay, with the standardised percentage change from control (t0) and the standard error of the mean. The statistical significance compared to the control is also listed.

The data presented in Figure 32 shows the standardised DPM readings for the three experiments, which have then been averaged, including a SEM calculation. The graph

clearly indicates that after a slight dip in phosphorylation levels after one minute's exposure to cyclical mechanical strain, the level increases. This data is supportive of the cruder average of the total counts, by suggesting that the maximal strain time for MAPK activation is four minutes.

3.2.2.3) Discussion

The development of a precise and accurate assay for measuring MAPK activity required intensive examination of all stages of the procedure. After investigation, the final procedure was developed that demonstrated that maximal activity (two-fold increase) was detected after four minutes exposure to cyclical mechanical strain. These data are consistent with that obtained from the Western analysis, which demonstrated that the maximal levels of phosphorylated MAPK were detected by electrophoretic techniques at four to five minute's exposure to cyclical mechanical strain.

This experiment was designed to confirm that the increase in phosphorylated MAPK seen in the Western analysis was associated with an increase in MAPK activity. The MAPK activity was not assayed in time-matched static cultures as the Western analysis had previously demonstrated that there was not an increase in phosphorylated MAPK levels in static human VSMC cultures. Therefore it was deemed an expensive use of plates to perform activity assays on static control cultures that did not depict significant MAPK activity.

This scale of increase in MAPK activity has been demonstrated in other studies. The three-fold increase in MAPK activity observed when human polymorphonuclear neutrophils are stimulated with the human haematopoietic hormone granulocyte-macrophage colony-stimulating factor, can be negated using the tyrosine kinase inhibitor genistein (375). A study performed by Yamazaki *et al* supported this data by demonstrating that mechanical strain induced MAPK activity in cultured neonatal rat cardiac myocytes maximally after ten minutes exposure (376). This level of activity returned to baseline after thirty minutes of strain. Begum and co-workers observed a two-

fold increase in MAPK activity when insulin-stimulated mitogenesis was induced in rat VSMC. Furthermore, this activity was shown to be dose-dependent, with peak activation between five and ten minutes after exposure to 100 nM insulin (377).

More recently in human mesangial cells, exposure to high degrees of cyclical mechanical strain (29 % elongation) has demonstrated an eight-fold increase in MAPK phosphorylation after exposure to one hours strain. Interestingly, of the other kinase families, the stress-activated protein kinase/Jun terminal kinase (SAPK/JNK) pathway was not altered by strain. Furthermore the p38/HOG pathway, which was not detectable prior to exposure to strain, demonstrated a marked increase within thirty minutes incubation that was sustained for at least two hours (15).

3.2.3) The Effect of PD 98059 Inhibition in VSMC Signalling Pathways

3.2.3.1) Aim

To define whether the strain-induced activation of MAPK was involved in the induction of VEGF production, 2'-Amino-3'-methoxyflavone (PD 98059) a selective inhibitor of MAPKK (MEK) phosphorylation of MAPK was used. The inhibitor halts the MAPK cascade at the MEK 1 peptide by binding to the inactive peptide preventing phosphorylation by upstream activators (378). This binding therefore prevents further phosphorylation of the downstream MAPK substrates. Analysis has demonstrated that PD98059 does not effect the activity of at least eighteen other serine/threonine protein kinases (378).

The addition of PD 98059 to both human and rat cultured puerperal uterine cells abolished oxytocin induced MAPK activation in a dose dependent manner (379). Similar experiments have demonstrated that MAPK activation by other stimuli, such as hydrogen peroxide and PDGF, can be blocked using PD 98059 indicating that different stimuli utilise the same cytosolic phase of the cascade (380), (381).

This compound was added dissolved in the culture media to allow access to the cells prior to exposure to cyclical mechanical strain. Due to the toxicity of the PD98059 to the cells, the addition of the inhibitor was immediately prior to the commencement of strain. The compound was added to inhibit MAPK to determine whether the strain-induced activation of MAPK could be reduced, and ultimately to determine if the strain-induced expression of VEGF mRNA was MAPK dependent.

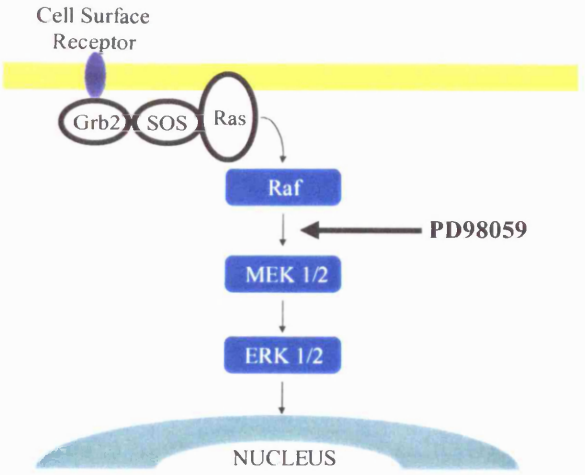

Figure 33- A schematic representation of the mode of action of the MAPK inhibitor PD98059.

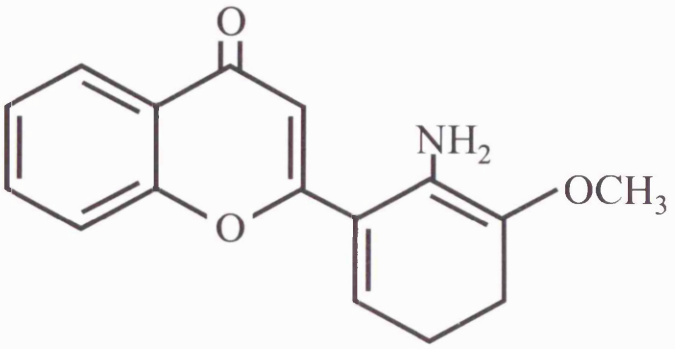

Figure 34- The molecular structure of the MAPK inhibitor PD98059.

3.2.3.2) Results

The initial experiment design used the IC_{50} for the product as stated in the Calbiochem data sheets. The results demonstrated that co-incubation of stretch human VSMC with 2 μM PD 98059 reduced the baseline production of phosphorylated MAPK (Figure 35).

Data presented in the previous section demonstrated that four minutes exposure to cyclical mechanical strain was required to elicit optimal MAPK phosphorylation.

The data presented in Figure 35 shows a simple time course of MAPK activity levels after one, two, four and six minutes incubation. The control levels were obtained from a static incubation and the positive controls were from stretched cells. At the optimum incubation for stretch activated MAPK activity, the addition of PD98059 to stretched human VSMC reduced the activity from 39 % above control in the normal stretched cells to approximately 21 % increase in stretched cells co-cultured with the MEK inhibitor, i.e. the inhibitor reduced the strain-induced MAPK phosphorylation by 50 %.

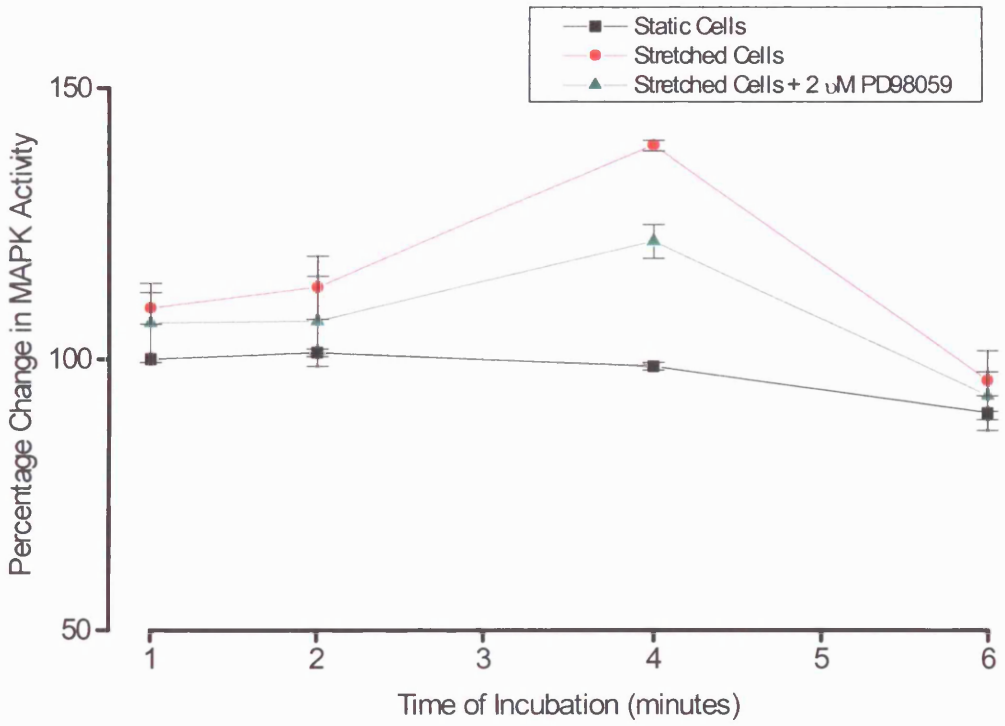

Figure 35- A graph to show the effect of PD 98059 inhibition of MEK on MAPK activity in strained VSMC, using static cells as baseline controls.

This experiment was poorly reproducible in my early studies. I later discovered the reproducibility of this assay was difficult due to the short life of the PD 98059 when

reconstituted in DMSO. It was found that after 48 hours storage at –20 °C the inhibitor activity had all but ceased and thus, had to be replaced.

Time (min)	Duplicate 1			Duplicate 2		
	Static Cells	Stretched Cells	Stretched + PD98059	Static Cells	Stretched Cells	Stretched + PD98059
1	5612	5973	5577	7117	7993	8113
2	5717	6024	5542	7156	8467	8207
4	5499	7769	6659	7078	9996	8885
6	4876	5072	4985	6640	7228	6955

Table 11- A table showing the effect of PD 98059 inhibition (2μM) of MEK on MAPK activity in static and strained VSMC.

Time (min)	Static Cells	SEM	Stretched Cells	SEM	Stretched + PD98059	SEM
1	100.0	0.0	109.4	2.9	106.7	7.3
2	101.2	0.7	113.2	5.8	107.0	8.3
4	98.7	0.7	139.4	1.0	121.7	3.1
6	90.1	3.2	96.0	5.6	93.3	4.4

Table 12- A table showing the standardised data of PD 98059 inhibition (2μM) of MEK on MAPK activity in strained VSMC.

3.2.3.3) Discussion

The addition of the MEK inhibitor PD98059 at the manufacturers stated IC_{50} reduced the detectable MAPK activity in stretched VSMC by approximately half, compared to the stretched control. Having previously demonstrated that MAPK is responsive to strain in a dose dependent manner, the logical step is now to investigate if the level of VEGF mRNA can be reduced by the addition of this inhibitor. This data would then support the

final part of the hypothesis by providing a potential signaling mechanism for the expression of strain induced VEGF expression.

At the time of these investigations, PD98059 was the only selective MEK inhibitor available, and its high level toxicity made it difficult to work with because of low reproducibility. The importance of the MAPK cascade in maintaining cell function has been highlighted by a number of recent reports. Miyamoto *et al* presented data that demonstrated that the MAPK cascade was vital in sustaining the bFGF autocrine signaling loop in rat aortic smooth muscle cells. When PD 98059 inhibited the cascade *in vivo*, the lack of FGF promoted apoptosis (382). Conversely, reductions in levels of phosphorylated MAPK have indicated potential mechanisms for decreasing the development of certain pathologies. The inhibition of PDGF activation of the ERK peptides reduced the migration of vascular smooth muscle by 32 % (383). Furthermore a study by Brown *et al* investigated the effect of PD 98059 on homocysteine action on cultured vascular smooth muscle cells isolated from chick embryos (384). High levels of homocysteine have been linked to the development of atherosclerosis and are thought to be a key factor in atherogenesis as it has been shown to promote proliferation and activate the transcription factor c-fos via the ERK2 isoform. The addition of PD 98059 was shown to reduce the levels of homocysteine activated MAPK (384). Taken together, these data suggest that MAPK is one important link in regulation of cell function, with potential implications for cardiovascular development, remodeling and disease. Interestingly, the inhibition of smooth muscle cell migration induced by PDGF could only be reduced by a third when the MAPK cascade was blocked (383), suggesting that although important in its role, the MAPK cascade is not sufficient for the full response.

3.2.4) The Effect of PD98059 Inhibition on VSMC Expression of VEGF mRNA

3.2.4.1) Aim

Thus far the data has shown that cyclical mechanical strain causes an increase in VEGF mRNA expression and peptide synthesis and furthermore, both can be upregulated by increasing the degree of strain applied to the VSMC. A potential signaling pathway was identified and investigated and it was seen that strain increased the level of activated members of the cascade (MAPK), also in a dose dependent manner, and that the level of strain induced phosphorylated MAPK could be reduced with a cascade specific inhibitor. Therefore, to test the final part of the hypothesis, the remaining experiments were designed to see if the signaling cascade investigated was involved in the expression of VEGF.

The VSMC were exposed to cyclical mechanical strain for three hours, the optimal time of exposure for VEGF mRNA expression, whilst being co-incubated with 2 μM PD 98059, a known inhibitor of MAPK activity. If the MAPK cascade was involved in the transduction of the strain induced increase in VEGF expression, then inhibiting the pathway would result in a reduction in VEGF mRNA. The cells were then harvested and the mRNA extracted and electrophoresed as described previously and the levels of mRNA were analysed.

3.2.4.2) Results

The initial experiment design used the IC_{50} for the product as stated in the Calbiochem data sheets.

The results obtained demonstrated that co-incubation of stretched VSMC with 2 μM PD98059 reduced the strain induced VEGF/GAPDH ratio to about one third that of cells

stretched in normal 5 % FCS media (0.21 and 0.78 respectively). Furthermore, the level of VEGF mRNA was reduced in the static culture with PD 98059, by approximately 50 % compared to the rested cells in 5 % FCS media, with ratios of 0.15 and 0.25 respectively.

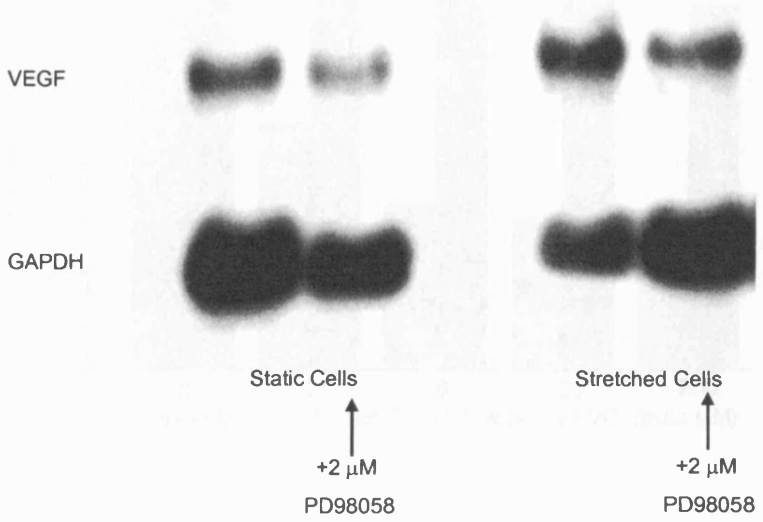

Figure 36- Autoradiograph demonstrating the effect of MEK inhibition on cyclical mechanical strain induced VEGF mRNA expression

This data suggests that the addition of the MEK inhibitor reduced the mechanical strain-induced expression of VEGF mRNA, however a second experiment was required to fully qualify the effect of higher concentrations of the peptide. The next sets of plates were incubated in an increasing concentration gradient, with the media containing 2, 6, 20 or 200 μM PD98059 (the control plates had media with no added PD98059). The plates had the media added to them, containing the increasing concentrations of PD98059, and then they were exposed to cyclical mechanical strain for three hours, which has previously been demonstrated as being the optimal incubation period for VEGF mRNA expression.

The densitometry analysis (Figure 37) showed that as the concentration of PD98059 in the overlying media increased the level of VEGF mRNA expressed decreased. The ratio

of VEGF to GAPDH in the plates was 0.58, 0.29, 0.22, 0.22 and 0.21 for the 2, 6, 20 and 200 μM PD98059 plates respectively.

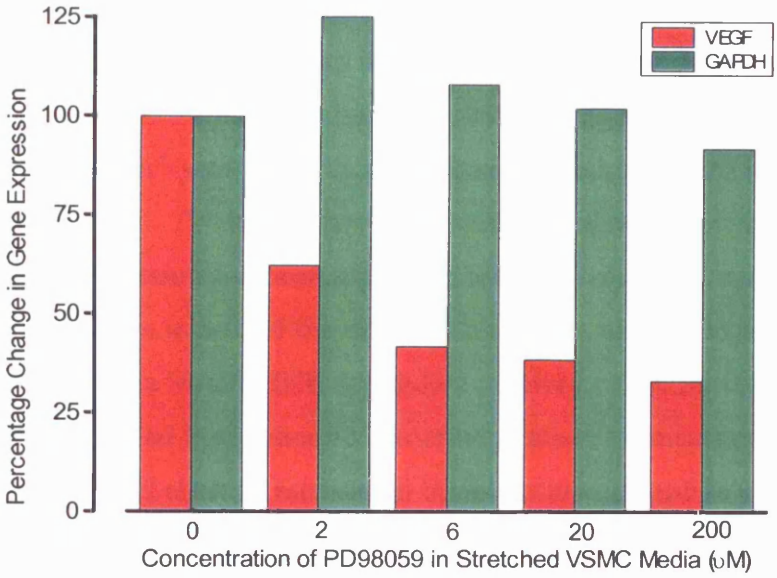

Figure 37- A graph demonstrating the effect of increasing concentrations of PD98059 on VEGF and GAPDH mRNA expression by stretched human VSMC.

3.2.4.3) Discussion

Addition of the MEK inhibitor PD98059 resulted in a reduction in the expression of VEGF mRNA induced when VSMC were exposed to cyclical mechanical strain. Initial experiments using the IC_{50} of 2 μl PD98059 reduced the level of VEGF expression by approximately 50 %. The inhibition appeared not to be linear as the concentration of the inhibitor added was increased. Although the expression of VEGF decreased slightly as the concentration of the MEK inhibitor increased, it was not proportionate to the amount of inhibitor added. The maximal inhibition was achieved at a concentration 6 μM in the overlying culture media. Due to the difficulty in maintaining a stable solution of PD 98059, this experiment was only completed twice. The time consuming methodology made it difficult to identify the sub-optimal step in the assay. A study investigating the

activation c-fos by cadmium, also observed that whilst this response was reduced by the addition of PD98059, it was not completely removed (385).

This data appears to be consistent with the initial hypothesis which stated that strain induced VEGF expression was regulated via the MAPK pathway. However, the lack of total inhibition at the higher concentrations of PD98059 suggests that the MAPK cascade may not be the only route by which cyclical mechanical strain is transduced by the cell into an intracellular event. The hypothesis that kinase cascades are involved in the regulation of gene expression has been supported by Liang *et al* demonstrating that cyclical mechanical strain increased the expression of brain natriuretic peptide mRNA in rat cardiac myocytes via a MAPK/JNK dependent pathway (374). Furthermore, they found this to be potentiated in a paracrine/autocrine manner, because conditioned media from stretched cells could elicit an increase in brain natriuretic peptide promoter activity in static cells (386). This suggests an important autocrine/paracrine role in mediating strain-dependent activation of cardiac-specific gene expression.

The initial studies in this arena investigated the effects of strain on EC, such as Wang and co-workers, who demonstrated that cyclical mechanical strain increased endothelin-1 expression in human umbilical vein endothelium. This was thought to be regulated by protein kinase C, as inhibition of this protein completely abolished the endothelin-1 response (387), (388). However, as mentioned earlier in the thesis, shear stress is expected to play a more physiologically relevant role in the mechanical stimulation of EC, as it is the force with which they are in direct contact (2). An example of such was submitted by Cowan *et al* who demonstrated that cultured EC subjected to laminar shear stress of 15 dynes/cm2, increased the gap junction protein connexin 43 mRNA levels approximately four fold at one hour exposure. This remained elevated for the duration of shear force (56).

4) CONCLUSION

The hypothesis that formed the basis for this study was designed to investigate the effect of cyclical mechanical strain on VEGF expression in human VSMC. The main points of investigation were:

1- Human vascular smooth muscle cells synthesise VEGF mRNA and that the expression of VEGF mRNA is increased in response to cyclical mechanical strain *in vitro*.
2- The increase in VEGF mRNA is associated with an increase in VEGF peptide production.
3- The level of VEGF mRNA and peptide produced by human VSMC in culture is responsive to cyclical mechanical strain applied in a 'dose dependent manner'.
4- The mitogen activated protein kinase cascade in human vascular smooth muscle is activated in response to cyclical mechanical strain.
5- The strain-induced expression of VEGF is dependent on strain-induced MAPK activation.

During the closing discussion, each section of the hypothesis will be addressed in turn.

4.1) Human VSMC Response to Strain

The initial experiments provided the necessary positive/negative control markers by demonstrating that the human VSMC were viable in culture, and could produce VEGF mRNA when challenged with a known stimulus (FCS). Further investigation demonstrated that exposing human VSMC to cyclical mechanical strain caused a maximal six-fold increase of VEGF mRNA expression that peaked at three hours, but then diminished to baseline expression after twenty-four hours strain. During the completion of this thesis, this data was supported by a publication submitted by Muratore

et al who exposed a mixed culture of murine pulmonary cells to cyclical strain (389). Expression of VEGF mRNA increased to a peak at 2-4 hours, but also reduced to baseline expression after twenty-four hours strain (389).

Furthermore, my studies demonstrate that the cyclical mechanical strain induction of VEGF mRNA was synergistic with increasing concentrations of FCS in the media, such that the exposure to strain at the highest serum concentrations could increase the level of expression by an extra 50 % compared to high serum alone. The strain-induced expression was also found to be repeatable in previously strained cells. Cells that were stretched for twenty-four hours and then exposed again to strain for three hours in low serum media elicited a greater VEGF mRNA response than the pre-strained, high serum static cells, suggesting independent mechanisms for synthesis of VEGF in human VSMC.

PCR analysis if the VEGF message produced by the cells demonstrated that all four of the major VEGF A isoforms were expressed after exposure to strain. Investigation in rat tissues had demonstrated that VEGF A_{206} was not expressed in any vascularised tissues, and the VEGF A_{189} species accounted for over half of the VEGF found in the heart and lung (286). The data demonstrates for the first time the capability of human VSMC to generate the four main VEGF species.

4.2) Human VSMC Produce VEGF Peptide in Response to Strain

The increases in VEGF peptide detected after exposure to cyclical mechanical strain were subtler than those observed for the mRNA. The average increase of VEGF A_{165} peptide was 116 % compared to the static incubations in 1 % FCS media. The differences in the levels could be attributed to shortcomings in the ELISA assay, in that the sensitivity may have not been optimal or the detection of one single species may not illuminate the true pattern of expression of the VEGF proteins. Also, the reduced proportionate increase in VEGF peptide production as compared to mRNA expression may indicate that the message synthesis and peptide secretion mechanisms operate via independent pathways.

Although the level of mRNA increases with the application strain, the release of the active peptide may be regulated by other circulating factors. However, it is consistent with the earlier data by confirming that strain increased VEGF mRNA expression is translated into increased VEGF peptide production.

Taken together, the data above demonstrates that cyclical mechanical strain increases the level of VEGF mRNA produced by human VSMC in culture and that this message positively correlates to VEGF A_{165} peptide concentrations in the overlying media.

4.3) Human VSMC VEGF Production in Response to Increasing Degrees of Strain

The data presented in this thesis clearly demonstrates a dose dependent response of VEGF expression to the degree of cyclical mechanical strain applied to the cells. When maximally strained to 24 % of their length (equivalent to 120/80 mmHg) the levels of VEGF mRNA increase nearly three fold compared the control cells. If the degree of maximal strain is increased to 27 and 30 % of their length (equivalent to 160/100 and 200/120 mmHg respectively) the increase in expression is in excess of four times that of the controls. This is also shown in the resultant peptide expression, which also shows an increase in response to increasing degrees of strain, with the greatest expression observed when the cells were strained to 27 % of their length. This is of particular relevance when considering the increase in permeability observed in hypertensive vessels as this suggests a possible mechanism for this phenomenon. As VEGF is a potent permeabilising agent, its increase in expression during strain may well cause the increase in vessel permeability associated with hypertension, allowing cellular invasion and the development of such vascular pathologies as atherosclerosis.

Supporting the theory that VEGF production *in vivo* could be, in part, regulated by strain is the fact that cells exposed to cyclical mechanical strain for twenty-four hours can still be stimulated to express VEGF when re-challenged with the strain stimulus. Taken

together with the dose dependent response demonstrated by the cells in these experiments, the data suggests that the human VSMC have a sophisticated mechanism for the synthesis of VEGF, which is capable of producing a proportionate response to the magnitude of strain applied.

4.4) Cyclical Mechanical Strain and the MAPK Cascade

The MAPK cascade was identified as a potential signalling pathway by virtue of their involvement with gene regulation initiated by extracellular matrix/integrin activation. Exposure to cyclical mechanical strain increased the levels of phosphorylated MAPK within two minutes, which peaked at four-five minutes. This pattern was repeated when the actual activity of the peptide was analysed from cell lysates. After 24 % maximal elongation, the phosphorylated MAPK levels doubled compared to the control cells and at 27 % maximal elongation, the levels of phosphorylated MAPK tripled, indicating that the level of induction was also dependent upon the degree of strain applied to the human VSMC.

Further investigation of the strain response, analysing the effect of a MAPK pathway inhibitor (PD98059) demonstrated that the induction of phosphorylated MAPK could be reduced when strained cells were co-incubated with the inhibitor. Addition of the MEK inhibitor at the manufacturers stated IC_{50} reduced the detectable MAPK activity in stretched VSMC by approximately half as compared to the stretched control. Having previously demonstrated that MAPK is responsive to strain in a dose dependent manner, the logical step was to investigate the effect of MAPK inhibition on VEGF mRNA expression.

The data from the chapters in the above section confirm the possibility that the MAPK cascade may be involved in the regulation of strain induced VEGF expression. Both VEGF and phosphorylated MAPK are increased by exposure to cyclical mechanical strain in a dose dependent manner and furthermore, the levels of induced phosphorylated

MAPK are diminished when incubated with a MEK inhibitor, suggesting the necessity for the complete pathway in the stretch response.

4.5) MAPK Inhibition and VEGF mRNA Expression

Addition of the MEK inhibitor PD98059 resulted in a reduction in the expression of VEGF mRNA induced when VSMC were exposed to cyclical mechanical strain. Initial experiments using the IC_{50} of 2 μl PD98059 reduced the level of VEGF expression by approximately 50 %. The inhibition appeared not to be linear as the concentration of the inhibitor added was increased. The expression of VEGF in the plates was maximally inhibited at a PD98059 concentration of 6 μM and further increases to 20 and 200 μM PD98059 gave no significant reductions of expression. Although the expression of VEGF decreased as the concentration of the MEK inhibitor increased, it was not proportionate to the amount of inhibitor added, indicating the potential involvement of further signalling pathways in the strain induced expression of VEGF in human VSMC.

4.6) Mechanotransduction in Human VSMC

The data presented in this thesis presents a novel pattern of VEGF expression in human VSMC and suggests a conceivable mechanism by which the cells transduce the cellular deformation into VEGF mRNA/peptide expression. The diagram below summarises the interactions discussed in the hypothesis.

Cyclical mechanical strain causes deformation of the cell surface, inducing activation of integrin receptors bound to the extracellular matrix. This in turn phosphorylates FAK, which initiates the MAPK cascade, resulting in activated MAPKAPK entering the nucleus and causing increased expression of VEGF mRNA. The resultant mRNA is translated into VEGF peptide that is the either stored in the cell or released. Once outside

the cell, the VEGF peptide can act as a paracrine or autocrine factor, causing an increase in vascular permeability or stimulating angiogenesis.

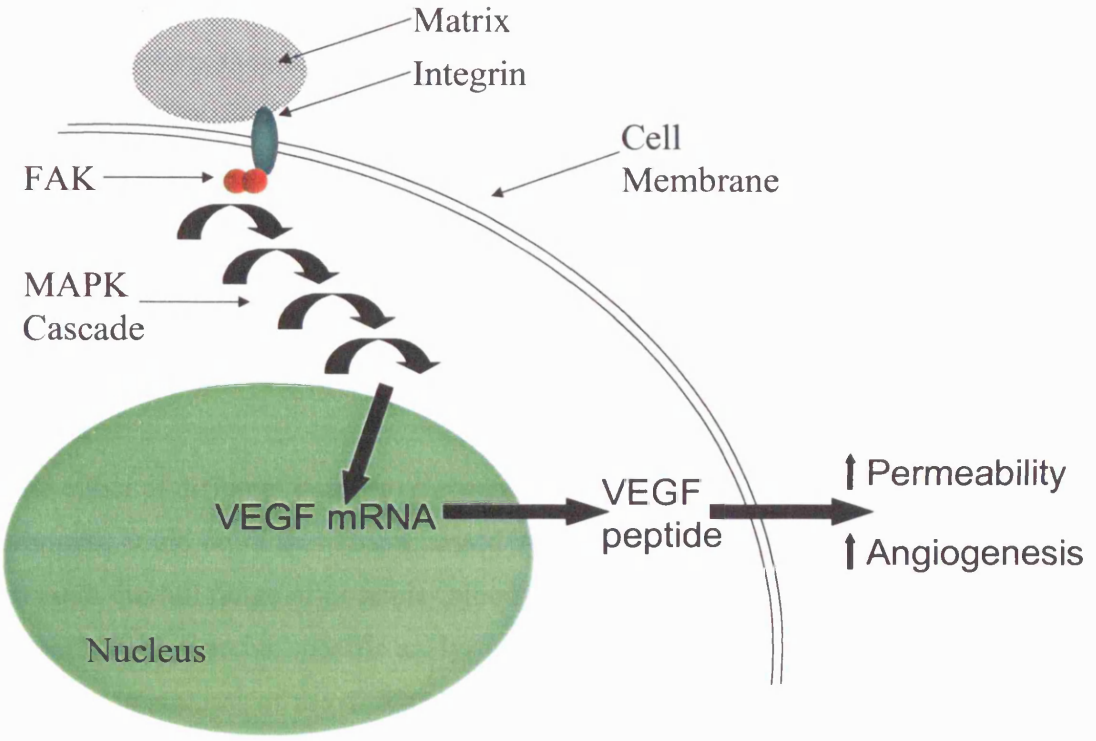

Figure 38- A schematic representation of the potential mechanism by which human VSMC transduce cyclical mechanical strain into VEGF peptide expression.

4.7) Future Work

Many interesting possibilities have been highlighted in this thesis which would warrant further investigation so as to determine a clearer picture as to the complete strain-induced activation of VEGF mRNA via the MAPK cascade. An investigation into the effect of combined pulsatile mechanical and shear stress on whole vessels could provide experimental conditions closer to those found *in vivo*, however, the complexity of such systems delays their development.

The data in this thesis demonstrates that human VSMC can produce the four main isoforms of VEGF A when exposed to cyclical mechanical strain. However, extensive published data demonstrates that the levels of expressed species in smooth muscle are not equal, therefore, the mechanism that regulates differential expression of these species remains unclear. Strain may induce different isoforms as the magnitude of strain increases, preferring different species for different conditions. The development of quantitative mRNA assays would allow the investigation of the effect of strain on the different isoforms. This thesis did not investigate the effect of strain on the other VEGF isoforms (VEGF B and VEGF D), although there may be strain regulation of expression involved with these vascular peptides.

The effect of different degrees of strain was demonstrated in this thesis, however, this response could be further characterised by a detailed analysis of VEGF expression through the full range of possible 'blood pressures', ranging from hypotensive to hypertensive. Species specific analysis would again provide a vital insight to the expression patterns of the different isoforms and provide a possible developmental importance for the multiple species produced by human VSMC. Also, pulse pressure, i.e. the difference between systolic and diastolic pressure, could be varied to determine its role on the response, independent of absolute increases in systolic or diastolic pressures.

Within the MAPK cascade, only the p44/p42 peptides were studied due to time constraints. However, an understanding of the effects of strain on the upstream regulators of the cascade, such as FAK, Ras or Raf, would give a clearer picture as to regulation of the strain-induced response in human VSMC. The MAPK peptides, p44/p42, also demonstrated a dose dependent response to the degree of strain applied to the cells and further investigation could analyse the effect of increasing strain of other members of the MAPK cascade. At the time of these experiments, the MEK inhibitor was the only peptide available for use in the assays. Using the newer, more effective inhibitors accessible for inhibiting the MAPK cascade would provide further interesting data as to the strain effect on the cascade. The current MAPK inhibitor employed for this research did not provide a complete block of the MAPK intracellular signal peptide, thus making

analysis difficult. There are now a number of MAPK inhibitors available that are reconstituted with sterile distilled water and inhibit different stages of the cascade, such as the Raf-1, A-Raf and B-Raf peptides. The PD98059 is reconstituted in DMSO, which was found to be toxic to the cells. Therefore, investigating different stages of the pathway with less toxic inhibitors would enable superior analysis of the role of this cascade in the VEGF/strain hypothesis. There are specific human anti-MAPK IgG antibodies that could facilitate lateral analysis of other cascades, such as the SAPK pathway, providing other potential routes for mechanotransduction of the stretch stimulus. Different intracellular signalling cascades could be employed during different strain regimens, i.e. hypotensive conditions.

4.8) Clinical Relevance

The clinical importance of the regulation of VEGF expression hypothesis demonstrated in this thesis has yet to be examined. The effect of VEGF knock out was demonstrated by Carmeliet, who described the rapid and lethal deterioration of developing embryos in both heterozygous and homozygous deficient animals (289). The necessity for VEGF continues throughout life, as it is vital for maintenance if the endothelium (297), inducing ovarian angiogenesis for reproduction (285) and wound healing (294). Given this information, it is easy to see how disruption of the normal control of expression of VEGF could induce the variety of pathologies linked to the peptide. This would support the hypothesis that as the level of blood pressure increases VEGF production by VSMC, the peptide could then cause an increase in endothelial permeability, allowing cellular and protein extravasation, leading to the fatty streaks associated with the early stages of atherosclerosis. As the plaque develops the increased VEGF production would in turn cause the increase in vascularisation associated with the plaque site. The vasculoproliferative disorders, such as tumour development and diabetic retinopathy, are associated with significant increases in VEGF expression. Furthermore, elevated levels of VEGF have been associated with the development of pathologies involving increased permeability, such as endometriosis and preeclampsia.

The important observation in this thesis was the increase in VEGF expression when the level of strain was changed to mimic normotensive and hypertensive states. This provides a potential link to certain pathologies seen in patients with elevated blood pressure. The development of atherosclerotic plaques is prevalent in hypertension where two major vascular changes are increased endothelial permeability and cellular migration. These two changes are induced by VEGF, and the increased blood pressure of the hypertensive patient could lead to increased VEGF expression resulting in the initiation of the atherosclerotic plaque and instability of the plaque.

This is compelling evidence that mechanical factors may play a role in plaque initiation and instability. The present thesis highlights one mechanism whereby strain could induce the production of a potent cytokine in the vascular wall.

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