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Cyclic Stretch-Mediated Release of Vascular Endothelial Growth Factor by Vascular Smooth Muscle Cells: A Role in Improved Vascular Graft Patency

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Thesis submitted in fulfilment of the requirements for the degree of MSc (Medicine) in the faculty of Health Sciences, University of Cape Town, South Africa.

Cape Town, August 1999.

Declaration

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Acknowledgements and Dedication

I wish to express my gratitude to:

Prof. Peter Zilla for insightful and invaluable direction.

Prof. Bauer Sumpio for expert advice and a wonderful opportunity.

Dr. Hiroyuki Kito, Dr. Alliric Willis and Emery Chen for patient laboratory assistance.

Dr. Neil Davies for assistance with the preparation of this manuscript.

My wife Tamsin and my parents Robert and Gillian Smith for their loving prayers and encouragement.

This thesis is dedicated A.M.D.G.

Abbreviations

aFGF	Acidic fibroblast growth factor
ATCC	American type culture collection
bFGF	Basic fibroblast growth factor
bp	Base pairs
BSA	Bovine serum albumin
CPM	Counts per minute
DEPC	Diethyl pyrocarbonate
dCTP	Deoxycytidine 5'-triphosphate
DME	Dulbecco's modified Eagle's medium
DPM	Disintegrations per minute
DTT	Dithiothreitol
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ePTFE	expanded polytetrafluoroethylene
FAK	Focal adhesion kinase
FBGC	Foreign body giant cell
FBS	Foetal bovine serum
Flk-1	Foetal liver kinase-1
Flt-1	fms-like tyrosine kinase-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HB-EGF	Heparin-binding-Epidermal growth factor
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HPRT	Hypoxanthine phosphoribosyl transferase
HUVEC	Human umbilical vein endothelial cells
IL-1	Interleukin-1
ITS	Insulin, transferrin, selenium
kDa	KiloDaltons
KDR	Kinase insert domain-containing receptor
NO	Nitric oxide
PBS	Phosphate-buffered saline

PDGF/ -B / -A	Platelet-derived growth factor/ -B chain / -A chain
PECAM-1	Platelet endothelial cell adhesion molecule-1
PET	Polyethylene tetraphthalate
PlGF	Placenta growth factor
PMSF	Phenylmethylsulfonyl fluoride
PU	Polyurethane
RER	Rough endoplasmic reticulum
RT-PCR	Reverse transcription-Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cells
TBS-T	Tris (hydroxymethyl)-aminomethane buffered saline-Tween-20
TGF-β	Transforming growth factor –beta
TNF-α	Tumour necrosis factor-alpha
Tween-20	polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR/ -1 / -2	Vascular endothelial growth factor receptor/ -1 / -2
VPF	Vascular permeability factor
WHO	World health organisation

Abstract

Within the wall of the native artery the endothelial cells (EC) and smooth muscle cells (SMC) are functionally indispensable and interact to a large degree. The vascular EC are known to profoundly effect the proliferative, synthetic and motor functions of the underlying SMC. In a like manner the SMC have been found to contribute to the maintenance and survival of the endothelium by the release of soluble factors. One of the most significant and specific endothelial cell survival factors is vascular endothelial growth factor (VEGF). SMC are the chief vascular source of VEGF and constitutively produce VEGF *in vivo*. The anti-apoptotic action of VEGF for EC occurs via the phosphorylation of the VEGF receptor KDR. Thus, in a healthy state the artery resides in a balanced way with the absence of unrestrained cellular loss or proliferation. However, if the artery has to be replaced due to blockage or damage, and a suitable autologous vessel graft is unavailable, a prosthetic vascular graft may be used.

Increased understanding of the molecular mechanisms involved in vessel function and development has permitted great advances in prosthetic vascular graft design. However, present synthetic grafts remain a last resort in peripheral arterial reconstructions. The key reason for their failure is the absence of host-derived EC which are paramount to synthetic graft patency. Although the fibrin matrix within the graft wall formed during implantation is initially highly favourable to cell integration, healing patterns show the EC and SMC to be excluded from presently available grafts. This may be due to prolonged inflammation, limited porosity and compliance mismatch with the native vessel. Compliance plays a role because cells within an incompliant graft do not experience the appropriate blood flow-related biomechanical forces experienced by cells in the artery. These forces are fluid shear stress on the EC and cyclic stretch on both the EC and SMC due to the rhythmic expansion of the artery wall. Cyclic stretch has been shown to be imperative for normal cell function in both cell types *in vivo*. Therefore, prosthetic vascular grafts should possess a degree of elasticity approaching the natural artery if it is to resemble the function of the natural artery. The development of novel compliant graft materials accompanied by advances in anti-inflammatory treatment and porous graft design may allow EC accompanied by pericytes / SMC to integrate into the graft structure to a greater extent. In this case the challenge will be to retain the functional endothelial layer after endothelial cell invasion without the apoptotic regression normally associated with scar formation.

In the light of studies which show the upregulation of VEGF in contractile cells subjected to cyclic stretch and the profound effects which cyclic stretch has been shown to have on the release of other cytokines by SMC, this study investigates the role which cyclic stretch might play in VEGF expression by SMC in a compliant environment. Furthermore, following observations of receptor phosphorylation in response to cyclic stretch in vascular cells, the effect of cyclic strain on the KDR-mediated endothelial response to locally-released VEGF was also investigated.

Low passage number bovine aortic SMC and EC were plated on collagen-coated elastomer plates and subjected to 10% repetitive strain at 1 Hz. The mRNA expression of VEGF in SMC and the phosphorylation of KDR on EC were determined by northern blotting and western blotting respectively. The biological activity on EC and levels of VEGF secreted into the medium by SMC under cyclic stretch were investigated using a migration assay and ELISA respectively. Cyclic stretch was found to cause a 3.3 (±1.5 $p < 0.005$) fold increase in VEGF mRNA levels over unstretched controls at 4 hours. This biomechanically-induced expression was found to drop slightly by 24 hours and to be approximately equivalent to expression induced by the cytokine bFGF over the same time course. These results correlated with an increase in VEGF levels in media from stretched SMC capable of inducing migration of EC by 1.6 fold although additional EC chemotactic factors appear to be released by stretch. Furthermore, although the levels of KDR remained constant under cyclic stretch, average KDR phosphorylation was found to increase weakly over time due to cyclic stretch. These results show that cyclic stretch affects the VEGF communication between SMC and EC at both the level of VEGF expression by SMC and at the level of VEGF recognition by the KDR receptor on EC. It is possible that through the nitric oxide (NO) pathway, VEGF release may alleviate abnormally high levels of cyclic strain. It is hoped that a better understanding of the role of VEGF communication between stretched SMC and EC will enable the design of a graft in which the level of compliance encourages SMC to maintain a functional endothelium. Following this it is hoped that the low levels of SMC and pericytes invading the graft, pacified by endothelial cell mediation, will not result in intimal hyperplasia but rather play a role in microvessel maintenance and more complete healing.

Contents

Introduction

Opening Remarks

The techniques and knowledge developed in molecular biology over the last 20 years have provided considerable insight into the biology of the vasculature. Breakthroughs as diverse as the construction of rudimentary vessels from cells in culture and strides to halt the progression of cancer have emerged from research involving vascular cells. However, despite great advances in understanding the molecular mechanisms involved in vessel development and function, a successfully lasting replacement of damaged vessels with a synthetic structure has eluded researchers for over four decades in the face of growing need for such technology. During this time, cardiovascular disease has emerged as a leading cause of mortality to the extent that according to the World Health Organisation (WHO) by the year 2020 40% of deaths worldwide will be related to cardiovascular disease. A common surgical solution to cardiovascular diseases – which often manifest themselves in an occlusion or symptomatic narrowing of vessels – is to bypass the blocked or stenosed vessels. Typically, the graft links the inflow portion to the distal part of the occluded artery and acts as a parallel auxiliary supply to the affected area. It is attached by sutures along the junction known as the anastomosis. According to the increasing worldwide trend towards a higher incidence in coronary artery disease, the coronary artery bypass graft operation has become the single most performed operation in the majority of hospitals. Bypass of the coronary arteries may be achieved by diversion of the internal thoracic or gastro-epiploic arteries in the chest cavity or insertion of an autologous vein graft. These grafts commonly use a saphenous vein from the legs. If either of these alternatives were not available, due to damage to the vessels or prior operation, the coronary artery would need to be bypassed by a synthetic vascular prosthesis. However, the poor performance of contemporary synthetic grafts make them obsolete in life-threatening positions such as coronary artery bypass grafts. Peripheral arterial reconstructions, such as those in the leg, are equally important. While in coronary artery bypass grafting the preferred conduit is an autologous artery graft, autologous vein grafts are the conduits of choice for peripheral bypass surgery. However, if no autologous conduit is available, peripheral bypass surgery uses synthetic arterial prostheses as a last resort. The performance of these grafts is, however, poor (Veith et al., 1986) with a 5 year patency of 38% for synthetic bypass grafts versus 68% for autologous vein grafts. One key reason for failure in these grafts is a lack of an endothelium on its surface and thus a lack of normal function. Typically, the pores of contemporary synthetic grafts are initially filled by seeping blood which forms a loose fibrin clot. Ideally, during the course of healing, endothelial and smooth muscle cells would degrade and replace the fibrin matrix in the interstices of the graft and the porous structures would be populated by a healthy, layered cellular structure resembling a native artery (Figure 1). At present, however, the healing responses of prosthetic grafts are characterised by chronic inflammation, often rendering them non-functional by thrombus formation and anastomotic intimal hyperplasia (Davids et al., 1999). Therefore, the goal remains to populate a prosthetic graft with functional vascular cells. This has previously been partly achieved through *in vitro* lining of the synthetic graft surface with cultured, host-derived endothelial cells (Zilla et al., 1999). However, this technique requires sophisticated laboratory infrastructures and is thus limited to major centres. In order to provide all vascular surgeons with a synthetic arterial prosthesis which at least equals the performance of autologous vein grafts, a spontaneously healing graft would be needed. Unfortunately, contemporary prosthetic grafts fail to elicit a spontaneous healing response due to a variety of reasons, amongst them the stiffness of grafts, the lack of porosity and an ongoing chronic inflammatory reaction. In order to understand why vascular cells fail to elicit a spontaneous healing response in presently available synthetic grafts, one needs to understand both the structure and environment of the natural artery and the specifics of the unfavourable environment that contemporary synthetic grafts provide for ingrowing cells. In this study I seek to better understand the influence of the mechanical environment of the vessel on cellular communication in order to allow for both muscular components and vascularity within the design of the synthetic vascular graft.

A. The Cellular Physiology of Native Arteries

1. Cells and Structure

The function of the coronary artery, and other large arteries, is reflected in the structure and cellular components of its wall which consists of three layers: the tunica intima the tunica media and the adventitia (Figure 1).

The tunica intima consists of a flat monolayer of endothelial cells (EC) which provides a smooth interface with the blood flowing through the lumen of the vessel, while allowing diffusion of nutrients and chemical messengers from the blood to the cells in the aortic wall. A smooth luminal lining to the vessel prevents thrombus formation and tight intercellular junctions maintain the integrity of the lining under the high flow conditions found in the arteries (Stevens and Lowe, 1992). These junctions also form a barrier between the blood and cells underlying the endothelial layer (Schnittler, 1998). Communication between the blood and the cells in the vessel wall is thus limited to either diffusion via opened junctions or tightly controlled active transcellular transport of pinocytic vesicles across the EC. Endothelial cells are equipped to function as the interface between the vessel and the blood because of many diverse receptors on their luminal surfaces (Stevens and Lowe, 1992). The high specificity of these receptors allows the EC to respond to changes in levels of metabolites such as glucose (Hempel et al., 1997); ions like Na^+ (Wigham et al., 1994); molecules such as nitric oxide (NO) (Gerol et al., 1998); cytokines like vascular endothelial growth factor (VEGF) (Neufeld et al., 1999); and hormones such as insulin (Milton and Knutson, 1993) within the blood stream. Thus EC are pivotal in communicating signals in the blood to cells lying within the wall of the vessel (Brophy et al., 1997). On the abluminal side of the monolayer the EC secrete and adhere to a specialised extracellular matrix (ECM), the basement membrane, consisting of type IV collagen, laminin, heparan sulphate proteoglycans, entactin, fibronectin and other glycosaminoglycans (Stevens and Lowe, 1992). The basement membrane allows attachment of the EC to the underlying ECM, forms a filtration barrier for substances passing from the blood and controls cellular organisation and differentiation. In large vessels, the basement membrane lies on a distinct layer of collagen and elastin fibres forming the internal elastic lamina, which separates the intima from the underlying second layer, the media (Stevens and Lowe, 1992).

The tunica media is most prominent in larger vessels and has distinct morphology in each of the two types of artery, elastic and muscular. Elastic arteries, of which the aorta is an example, have the thickest media consisting of concentric layers of elastin running circumferentially throughout the media (Stevens and Lowe, 1992). These are interposed with collagen and irregular smooth muscle cells (SMC) with numerous branching processes (Cliff, 1967). The extensive fibrous network of elastin allows the vessel wall to absorb the pressure pulse as the blood leaves the left ventricle of the heart during systole by a rapid and dramatic expansion of the vessel diameter (Stevens and Lowe, 1992). During diastole in the heart, the aortic valve between the aorta and the heart closes, preventing back-flow of blood into the heart before the wall of the aorta returns to its original diameter propelling the blood onward into the circulatory system. This serves to smooth the pulsatile flow in the proximal arteries, maintaining an adequate diastolic pressure and helping to equilibrate the pulsatile output of the heart. This protects the smaller vessels and especially the capillaries from damage by peaks of pressure and high flow. The elastic arteries gradually merge into the muscular arteries. These are distinguished from the elastic arteries by a decrease in elastin in their medias, as the need for elastic recoil diminishes (Stevens and Lowe, 1992). The media of the muscular artery, of which the coronary artery is an example, consists mainly of spindle-shaped SMC arranged circumferentially and perpendicularly to the long axis of the vessel (Mills and Sumpio, 1997).

The vascular SMC are the principal cell type in the media of muscular arteries and are unique to this layer (Stevens and Lowe, 1992). The function of vascular SMC is dynamic and is the result of two interconvertible phenotypic states: a contractile and a synthetic state (Mills and Sumpio, 1997). In the normal adult media, the majority of SMC are contractile. These SMC are highly differentiated and mitotically quiescent with large centrally located nuclei and plentiful tracts of myofibrils in the cytoplasm, which are involved in the contraction of the cells (Mills and Sumpio, 1997). Contraction of these SMC, under the control of the autonomic nervous system as well as through local signalling mediated by EC, allows fine-tuning of the blood flow in the muscular arteries. In addition, SMC respond to agonists that either cause vasoconstriction or vasodilatation (Ross, 1993). Another important trait of vascular smooth muscle is the continued maintenance of tension in the vessel wall after stimulation (Adelstein and Sellers, 1987; Cooke et al., 1987) despite a dissipation of the signalling cascade molecules involved with contraction. This tension is important in the preservation of a consistent level of vascular tone (Brophy et al., 1997). If the structure of the artery wall needs readjustment to cope with changes in flow the vessel SMC may be found in the synthetic state (Mills and Sumpio, 1997). Smooth muscle cells involved in remodelling, repair and development of the artery as well as those found in some pathological conditions, such as atherosclerosis and intimal hyperplasia, have a synthetic phenotype. Cytokines or injury may induce the SMC to adopt this state. In the synthetic state, SMC are proliferative, produce a variety of growth factors / cytokines and have an increased production of ECM molecules such as collagen and sulphated glycosaminoglycans (Mills and Sumpio, 1997). Each of these activities enables synthetic SMC to remodel the vessel wall structure in response to changing structural requirements. The metabolic requirements for such active cells are high and require constant nutrients and oxygen from the blood. Although cells in the wall of smaller vessels obtain their oxygen and nutrients by diffusion from the lumen, the energy requirements for SMC embedded within larger vessel walls are met by small blood vessels, the vasa vasorum, which are branches of vessels originating in the underlying adventitia (Stevens and Lowe, 1992).

The adventitia forms the third and outermost layer of blood vessels. It is separated from the media in large vessels by a condensation of elastin fibres forming the external elastic lamina and is composed largely of collagen fibres interspersed with SMC and fibroblasts (Stevens and Lowe, 1992). These fibres serve to connect the circulatory system to the other organs. An additional function of the adventitia, as mentioned above, is to provide a blood supply for the cells of the media. The mature microvasculature, such as that found in the adventitia of large vessels, consists of EC flanked abluminally by pericytes (D'Amore, 1992a). Pericytes, also known as mural cells, are a morphologically, biochemically, and physiologically heterogeneous group of cells defined *in vivo* by their location within and on the outer side of the basement membrane of microvessels (Shepro and Morel, 1993). As the vascular tree progresses from arteries to arterioles and finally to capillaries, the contractile cell layer adjacent to the endothelium dwindles from a thick media of SMC in the artery to a single pericyte lying adjacent to the EC in the microvasculature (Stevens and Lowe, 1992). Although the pericytes play a more important role in development (Benjamin et al., 1998), there appears to be a functional plasticity and developmental relationship (Hirschi and D' Amore, 1996; Nicosia and Villaschi, 1995) between pericytes and SMC in the vasculature and conversion between the two phenotypes may occur during remodeling (Nicosia and Villaschi, 1995). Co-culture experiments suggest that EC and pericytes interact by means of soluble factors (Hirschi and D' Amore, 1996), integrins and gap junctions (D'Amore, 1992a) and both cell types play a role in vessel assembly, growth control and normal capillary function (Hirschi and D' Amore, 1997; Nehls et al., 1994). For example, pericytes are contractile (Schlondorff, 1987) and contraction in the pericytes allows fine control of blood flow in the capillary and adjusts junctional permeability in EC (Shepro and Morel, 1993). In addition, the pericyte releases basement membrane components (Shepro and Morel, 1993) and cytokines such as transforming growth factor beta (TGF-β) (Antonelli-Orlidge et al., 1989) which maintain the endothelium in a quiescent state (D'Amore and Smith, 1993; Grant and Kleinman, 1997). Such cross-talk between cells in the vasculature is widespread and vital to routine function and adjustment of the vessel.

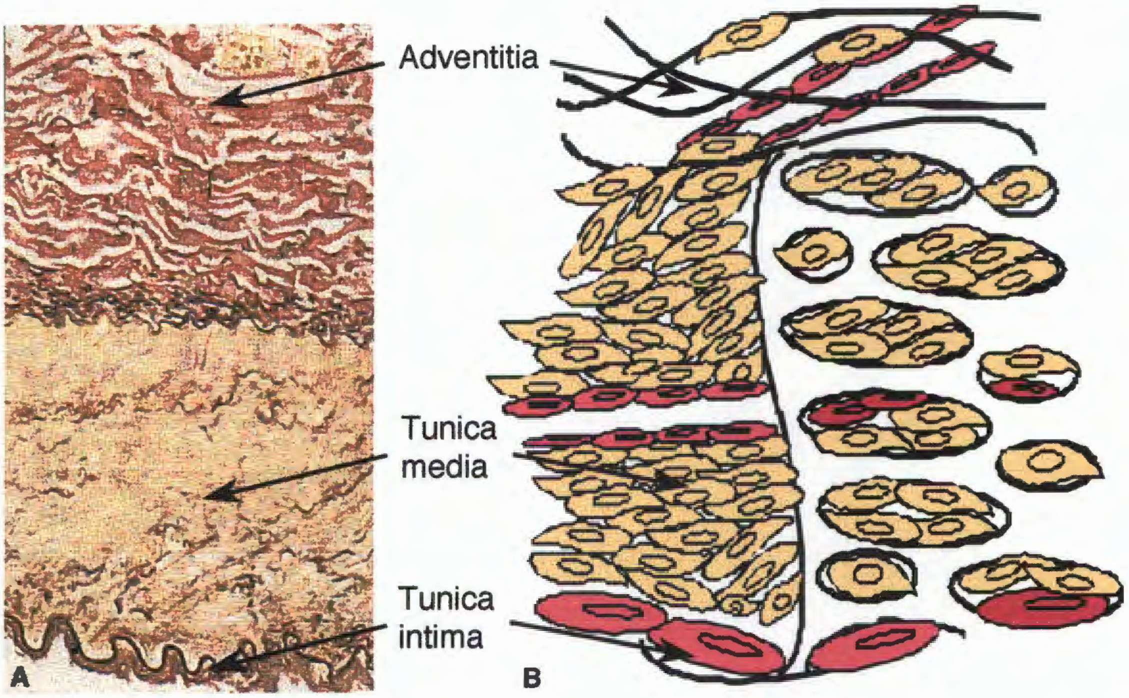

Figure 1. Structure of artery. **A**. Photomicrograph of an elastic artery wall showing the triple layered structure. The endothelial layer of the tunica intima provides an antithrombotic interface with the blood in the lumen and is separated from the tunica media by the undulating, black internal elastic lamina. The smooth muscle cells of the tunica media are stained yellow and are interspersed with black elastic fibres. The black external lamina separating the tunica media and the adventitia can be clearly seen. The collagen-rich adventitia is stained red in this preparation (Stevens and Lowe, 1992). **B**. Schematic diagram of ideal healing. The pink endothelial cells and yellow smooth muscle cells from native microvessels at left would populate the porous graft structure at right without overt proliferation to create a functional prosthesis.

2. Stimuli Effecting Vascular Wall Cells

a. Cytokines

Although the layered vessel may appear to remain quiescent over many years, the cellular environment of the arterial wall is far from insentient. Changing conditions of flow within the vessel require constant adaptation of the vessel structure (Nicosia and Villaschi, 1999) even in the absence of dramatic short-term vascular remodelling such as angiogenesis. This is brought about by abundant interactions between the two main cell types present: SMC, EC and their ECM.

EC and SMC in the wall of the artery undergo a large number of interactions. Although gap junctions between adjacent SMC and EC may allow the transfer of ions and small molecules (Lash et al., 1990), the major mode of communication between the cell types is through the release of cytokines. Endothelial cells and SMC both contribute to the cytokine cross talk in the coronary artery wall making the *in vivo* milieu at once interdependent and complex (Brophy et al., 1997; Mills and Sumpio, 1997). For simplicity, a brief review of the contributions of endothelial cell cytokine production on SMC and smooth muscle cell cytokine production on EC is considered.

As mentioned above, EC are responsive to levels of diverse blood components especially cytokines. Such stimulation may induce the subsequent endothelial cell production of cytokines / growth factors including platelet-derived growth factor (PDGF); basic fibroblast growth factor (bFGF); transforming growth factor-beta (TGF-β) interleukin-1 (IL-1) (Ross, 1993) and vasoactive substances such as nitric oxide (NO) (Li et al., 1995, Wennmalm, 1994; Busse, 1995) and endothelin (Estrada et al., 1995; Luscher, 1990; Yamagishi et al., 1993). Many of these molecules are capable of affecting proliferation and contractile response in vascular SMC. Thus, it would appear that the presence of an intact endothelium also mediates signals from the blood to the SMC in a paracrine fashion (Brophy et al., 1997). In this way, the maintenance of an intact endothelial layer may maintain the SMC in the contractile phenotype (Mills and Sumpio, 1997) and act as an inherent brake on smooth muscle cell proliferation (Couffinhal et al., 1997; Doornekamp et al., 1996). This is supported by the finding that smooth muscle cell proliferative, synthetic and contractile responses to many agents on their luminal surface depend on whether the endothelial layer is intact or damaged (Loscalzo, 1995; Peiro et al., 1995; Shimokawa and Takeshita, 1995). Damage to the endothelial layer, by toxins in the blood (Painter, 1991) or surgical manipulation, (Quist and LoGerfo, 1992) can result in phenotypic modulation of SMC to a synthetic and proliferative state (Mills and Everson, 1995; Painter, 1991) and participate in the pathogenesis of all the major vascular disorders. The upset of the normal balanced situation between the cells may lead to unrestrained cellular proliferation *in vivo* such as underlying the pathological proliferation of SMC forming hyperplasia of the intima and vessel occlusion (Allen et al., 1994; Chervu, 1990).

Cytokines or hypoxia may also switch the phenotypic state of the SMC from the quiescent contractile state to the synthetic state (Mills and Sumpio, 1997). As mentioned above, SMC in the synthetic state can express a variety of growth factors / cytokines (Ross, 1993) including PDGF (Holt et al., 1994); bFGF; TGF-β; IL-1; vascular endothelial growth factor (VEGF) and heparin-binding epidermal growth factor (HB-EGF) (Abramovitch et al., 1998) and thus contribute to the cytokine cross talk in the vessel wall (Mills and Sumpio, 1997). The secretion of many of these factors, such as PDGF-B, acts in both an autocrine manner to affect smooth muscle cell proliferation and in a paracrine fashion on the neighbouring endothelium (Ross, 1993). Other factors such as VEGF are specific for the EC and result in selective migration and proliferation of these cells (Ferrara and Davis, 1997). Thus, the complex local cytokine environment in the artery will to a large extent define any adjustment of the vessel wall. In addition to the short-term release of cytokines associated with fine tuning the vessel structure, cytokines may also contribute to the structure of the vessel in a more long-term fashion. For example, in addition to its role in proliferation and migration, VEGF produced by the SMC has also been proposed to have a role in the continued maintenance and repair of the mature endothelium (Couffinhal et al., 1997) as covered below. As the SMC are the chief source of VEGF in the vessel wall (Williams et al., 1995), it is reasonable to assume that damage to SMC in the media would affect this upkeep. Due to the interdependence of the two cell types, the maintenance of an intact endothelium will in turn act as an inherent brake on smooth muscle cell proliferation (Couffinhal et al., 1997) as mentioned above. Although cytokines have a specific and potent affect on EC and smooth muscle cell activities in the arterial wall this is not the only contributing factor. The ECM released by these cells also influences their activities in the vessel wall.

b. Extracellular Matrix

Firstly, the composition of the underlying ECM has been shown to increase or attenuate the responses to cytokines by the EC and SMC (Powell et al., 1996) and hence may contextualise the cellular responses to cytokine stimuli. Secondly, ECM components have been shown to have a variety of effects on the remodelling (Liu et al., 1990; Nehls and Herrmann, 1996) and other phenotypic characteristics of EC and SMC. For example, EC are held in a quiescent state *in vivo* by the presence of collagen IV and laminin in their basement membranes (Grant and Kleinman, 1997) and they may be protected from apoptosis by vitronectin (Isik et al., 1998). In a similar fashion, a heparin-like glycosaminoglycan in the basal lamina of SMC maintains the cells in the contractile phenotype (Campbell et al., 1988) *in vitro*. It would be reasonable to assume that disruption of ECM contacts, such as those associated with injury to the vessel, would result in modulations of the phenotype. Thus, it seems that the ECM influences the cellular response in a less dramatic but more lasting way than cytokines.

c. Biomechanical Forces

In addition to humoral and matrix stimuli, another sustained influence on endothelial cell and smooth muscle cell physiology in the vessel wall is the biomechanical environment (Ziegler and Nerem, 1994; Gimbrone, 1997). This has been found to have profound effects on vascular cell physiology and a consideration of the forces involved in the design of a synthetic vascular graft may be key to the design of a more natural cellular responses in these prostheses. The flow of blood in the vessel results in two major haemodynamic forces acting on the EC and SMC (Figure 2). Namely: shear stress from the flow of blood and cyclic stress from the blood pressure.

- ## Shear Stress

Firstly, the flow of the blood across the luminal surface of the vessel produces a tractive force known as shear stress (Dewey, 1984). The magnitude of shear stress oscillates with the cycles of systole and diastole in the heart. Only those EC lining the wall are subjected to this force and they respond by elongating and aligning with the direction of flow (Malek et al., 1993; Zhao et al., 1995). This reduces the tractive force on the cells and prevents detachment of the endothelial layer by extremes of shear (Ballermann and Ott, 1995; Franke et al., 1984). At the ultrastructural level, the re-orientation of intracellular actin and myosin filaments accompanies this response (Franke et al., 1984; Ives et al., 1986). However, it is not known if the parallel alignment of EC (Dewey, 1984) is an active response by the cell to reduce the shear force experienced or a passive adaptation due to the flow. Shear stress also induces a spectrum of functional responses in the EC (Nerem et al., 1993) including regulation of angiogenesis (Ando and Kamiya, 1993); modulation of the production of vasoactive substances (Nerem et al., 1993) like NO (Uematsu et al., 1995) and endothelin (Malek and Izumo, 1992) as well as the alteration of signal transduction pathways (Ando and Kamiya, 1993; Papadaki and Eskin, 1997; Ando and Kamiya,1996). Co-culture studies have demonstrated that many of the effects of shear on the EC are translated to the SMC by the release of soluble factors (Cucina et al., 1998; Nackman et al., 1998; Traub and Berk, 1998). Shear stress may thus play a role in the normal communication between the cells mentioned above. Damage to the endothelial cell layer disrupts this regulatory interaction and subjects the SMC to shear stress, both of which may lead to uncontrolled proliferation in the SMC (Kraiss et al., 1991; Sterpetti et al., 1993). It was hypothesised that shear stress may be used to precondition endothelialised vascular grafts prior to implant, following the observation that EC exposed to shear stress adhere more tightly to a surface (Ballermann and Ott, 1995). In the light of this observation, it was hoped that this would result in improved retention of the endothelial layer *in situ*. In addition, the expansion of the elastic artery to accommodate the pulse of blood keeps the blood velocity and thus the level of shear stress from peaking to elevated levels (Fung and Liu, 1993). Due to the relationship between flow and expansion (Fung and Liu, 1993), if the wall is able to expand to 10% of its diameter during systolic pulse, the pulsatile increase in blood velocity will lead to an increase in shear force 25% lower than experiences by EC in an inelastic vessel which does not expand with each pulse. Thus, it may be equally important for a synthetic arterial graft to possess some of the elastic properties of the artery if it is to mimic the physiological levels of shears stress experienced in the artery. This material-dependent property is known as graft compliance.

• Cyclic Stress

The above mentioned expansion stems from the pressure waveform on the artery wall created by the increased volume of blood in the aorta during systole and results in the second force acting on the EC and SMC. Although experiments to determine the effect of pulsatile pressure on SMC (Watase et al., 1997) have shown to cause an increase in proliferation and cell alignment, direct pressure may have limited effects on EC and SMC. This is explained by the fact that cells consist mostly of incompressible water. Thus, physiological levels of pressure exerted on cells cultured on a rigid substrate should have little effect on cell morphology but will be equilibrated across the cell (Ives et al., 1986). However, in the elastic artery, pressure creates an oscillating variation in the vessel circumference. This fluctuation results in a cyclic stretch on the cells of the vessel wall in the circumferential direction (Sumpio, 1989) (Figure 2) which affects the cell-cell junctions, cell membrane and cytoplasm of both EC and SMC *in vivo*.

Effects of Cyclic Stress on Endothelial Cells

Because it is situated on the internal surface of the vessel, the luminal endothelium is subject to the lowest cyclic stretch *in vivo*. However, a number of experiments in culture have shown this cyclic stretch to have important influences on EC (Sumpio et al., 1988a).

The translation of a mechanical signal, such as pulsatile stretch, into cellular events is a complex and poorly understood process and the precise means by which EC detect and respond to cyclic stretch is not known. Possible stretch receptor candidates include the integrins $\alpha_5\beta_1$ and $\alpha_2\beta_1$ (Yano et al., 1997) which physically link the ECM substratum and the cell (Shattil and Ginsberg, 1997) via condensations of cytoskeletal proteins known as focal adhesion plaques (Albelda and Buck, 1990). This hypothesis is supported by the observation that integrins are redistributed at the periphery of the cell during cyclic stretch (Yano et al., 1997). Concurrent cytoskeletal remodelling aligns the EC perpendicularly to the strain. Thus, although EC receive their primary orientation cues from shear stress, as mentioned above, the two forces work in harmony to orientate the EC in the vessel wall (Ives et al., 1986; Zhao et al., 1995). Further mechanosensor candidates include two cytoskeletal proteins within the focal adhesion plaque, paxillin and focal adhesion kinase (FAK), and platelet endothelial cell adhesion molecule-1 (PECAM-1). PECAM is a receptor which is concentrated at the region of cell apposition between adjacent EC (DeLisser et al., 1993) which has been shown to be activated by phosphorylation in response to shear stress (Osawa et al., 1997). The demonstration of activation of the PDGF receptor-α in SMC by cyclic stretch (Hu et al., 1998) raises the possibility of ligand-free physical-force-induced activation of a variety of cytokine receptors on other cell types including EC although the effect which such activation has on ligand binding is unknown. Finally, the highly-expressed VEGF receptor kinase insert domain-containing receptor (KDR) (Quinn et al., 1993), which is discussed in detail below, plays a role in the detection of shear stress by EC (Chen et al., 1999) and it is interesting to postulate whether it is also important as a stretch receptor.

Cyclic strain is also found to affect cellular signalling intermediates downstream of the initial mechanoreceptor (Letsou et al., 1990; Brophy et al., 1993; Segurola et al., 1997; Sumpio and Banes, 1988a; Yano et al., 1996b). Although much is left to elucidate about the role of cyclic stretch in the pathways which involve these molecules, modulation of their function may bring about many of the altered cellular responses seen in EC under cyclic stretch. These include changes in gene expression (Iba and Sumpio, 1992; Sumpio et al., 1988a); proliferation (Sumpio et al., 1987); matrix secretion (Sumpio, 1989) and the release of factors capable of affecting smooth muscle cell contractile function (Awolesi et al., 1995; Macarthur et al., 1994; Popp et al., 1998; Sumpio and Widen, 1990).

Effects of Cyclic Stress on Smooth Muscle Cells

In addition to any influences from the adjacent EC, SMC are affected directly by cyclic stretch in a distinct way to EC (Mills et al., 1993). Stretch is the contributory factor for orientation in SMC as compared to EC, which prefer fluid flow as a stimulus for alignment. SMC are seen to align perpendicularly to the direction of cyclic strain *in vitro* (Sumpio and Banes, 1988b). This may be due to breakdown of cell attachments or a cellular response to migrate and rotate in order to minimise the strain on the cell. The aligning effect of stretch noted *in vitro* is mirrored *in vivo* where histological observations show a spiral orientation of the SMC parallel to the longitudinal axis of the vessel (Peters et al., 1983; Stevens and Lowe, 1992). The cells may align spirally rather than circumferentially due to a secondary alignment response to the minor cyclic stresses experienced along the length of the vessel (Figure 2) (Deng et al., 1994). The aligning response in SMC is paralleled by changes in cell phenotype (Birukov et al., 1995; Reusch et al., 1996).

Cyclic mechanical stimulation *in vitro* has dual effects on phenotypic characteristics of SMC depending on the regimen of stretching. In different models cyclic stretch induces the production of ECM (Sumpio et al., 1988b) and growth factors such as PDGF-B (Wilson et al., 1993), both characteristic of a synthetic phenotype, and induces increased expression of the contraction regulating protein h-caldesmon (Birukov et al., 1995), considered a marker of the differentiated contractile smooth muscle state (Glukhova et al., 1988). Overall cyclic stretch appears to induce the maintenance of the differentiated contractile phenotype based on myosin isoforms expressed (Reusch et al., 1996). However, the varied response by SMC under stretch (Reusch et al., 1996) may be due to distinct sub-populations of SMC (Lindner et al., 1995; Villaschi et al., 1994) or due to the heterogeneous strain profile produced by the strain apparatus used (Gilbert et al., 1994).

Alternatively, growth and differentiation in SMC may not be mutually exclusive (Owens, 1996). *In vivo* differences in mechanical stimulation of arterial vascular SMC may maintain the vascular SMC in the contractile phenotype or a predominantly biosynthetic mode (Sottiurai et al., 1983). At the ultrastructural level, characteristic features of contractile smooth muscle cell morphology such as myofilaments became less distinct (Sottiurai et al., 1983) and rough endoplasmic reticulum (RER) profiles were more abundant in cyclically stretched cells (Sottiurai et al., 1983). This observation is consonant with an increase in ECM synthesis (Leung et al., 1976).

Collagen production has been found previously in SMC in static culture (Burke et al., 1977) and within a vascular graft (Mesh et al., 1995) but synthesis of collagen, noncollagenous ECM protein (Sumpio et al., 1988b) and other ECM components (Leung et al., 1976) by SMC has been shown to increase under cyclic stretch. These observations may represent an adaptive response to increased cyclic stretch by the SMC to strengthen the artery by releasing ECM proteins (Leung et al., 1976) although they also parallel the composition changes of stiffened artery walls associated with increased medial tensile stress (Leung et al., 1976) such as associated with intimal hyperplasia (Shrestha et al., 1992). However, other studies have shown a decrease (Kulik and Alvarado, 1993) or an increase (Grande et al., 1989) in collagen production depending on the nature of stretch applied (Sottiurai et al., 1983). Furthermore, *in vivo* production of ECM by SMC under cyclic stretch may be curbed by ECM components in the artery wall (Akita et al., 1993; Sakata et al., 1990; Campbell et al., 1988), the adjacent EC (Powell et al., 1997) and the reduction of ECM production accompanying the promotion of some of the characteristics of the contractile state in SMC by cyclic stretch (Reusch et al., 1996). Thus the precise effect of cyclic stretch on ECM production has yet to be determined. In addition to ECM production, cells under cyclic stretch form numerous attachments to their moving ECM substrate and each other (Leung et al., 1976).

Attachments with the nascent ECM are important in alignment of SMC in response to stretch. These attachment sites are also responsible for transmission of mechanical force signals to the cells (Wilson et al., 1995) and studies in which cells were stretched on different substrates have shown them to be very specific (Ingber, 1991). As was the case with EC, SMC have been shown to sense mechanical strain through interactions of integrin receptors in the plasma membrane with matrix sites fixed to the moving substrate (Wilson et al., 1995). However, different mechanoreceptors may communicate the varied responses of SMC to cyclic stretch such as proliferation and differentiation (Reusch et al., 1996) and the mechanisms by which forces are transmitted across the cell surface and transduced into a cytoskeletal response are largely unknown. A number of intracellular signalling mechanisms are found to take part during the smooth muscle cell response to cyclic stretch (Kulik et al., 1991; Mills et al., 1990; Wiersbitzky et al., 1994; Davis et al., 1994; Davis et al., 1992; Bialecki et al., 1992).

In addition to direct stimulation by cyclic stretch, SMC may by stimulated by cytokines released autocrinely under cyclic stretch. This is supported by the observation in some cell stretching experiments that cells were initially slower to proliferate in lower concentrations of foetal bovine serum (FBS) suggesting a switch to autocrine growth stimulation by low levels of growth factors (Birukov et al., 1995). The production of cytokines, despite the observation of some differentiating effects of cyclic stretch on SMC (Reusch et al., 1996), which should diminish the production of cytokines (Mills and Sumpio, 1997), once again demonstrates the dual effects of cyclic stretch on smooth muscle cell phenotype. One candidate cytokine for the role of growth stimulation is the potent smooth muscle cell mitogen PDGF, which has been shown to be released by SMC (Ross, 1993). Cyclic strain appears to induce the production and secretion of PDGF-BB and PDGF-AA, which act in an autocrine manner (Wilson et al., 1993). In addition, cyclic strain has been shown to cause activation of the PDGF receptor-α on vascular SMC (Hu et al., 1998). At present it is not certain whether stretch modulates the activity of PDGF on SMC by affecting the production of the cytokine (Birukov et al., 1995), the response of the receptor (Hu et al., 1998) or both. Although a potential autocrine mediator of smooth muscle cell proliferation under stretch (Brogi et al., 1994; Ross, 1993; Klagsbrun and Edelman, 1989), bFGF appears not be involved in the proliferative response of SMC to stretching (Wilson et al., 1993). TGF-β is a further cytokine secreted by SMC (Ross, 1993) that is capable of enhancing both smooth muscle cell proliferation and migration (Bell and Madri, 1989). In the heart, myocardial stretch is associated with rapid induction of VEGF gene expression in the related cardiocyte under the mediation of TGF-β produced by the cardiocyte (Li et al., 1997a; Seko et al., 1999). However, whether TGF-β is released during cyclic stretch by SMC and whether this has any effect on smooth muscle cell proliferation has yet to be determined. VEGF itself has also been shown to be released under cyclic stretch by the contractile cell lying adjacent to the endothelium in the kidney, the mesangial cell (Gruden et al., 1997). The mesangial cell is the pericyte of the kidney (Schlondorff, 1987; Stevens and Lowe, 1992) and is very similar to the vascular SMC in terms of embryonic origin, position in the vasculature and function. Although VEGF may have dramatic effects on the underlying endothelial cell physiology, as covered below, VEGF has not been shown to have any dramatic effect on smooth muscle cell behaviour. Despite evidence of basal levels of VEGF expression by vascular SMC *in vitro* (Ferrara et al., 1991) and *in vivo* (Couffinhal et al., 1997), to date no work has looked into the release of VEGF by vascular SMC under cyclic stretch.

The role of the cytokines released due to cyclic stretch on smooth muscle cell proliferation may be of secondary importance to the origin of the SMC. Smooth muscle cell proliferation under cyclic stretch has been found to be increased, unchanged or decreased in various studies (Birukov et al., 1995; Sumpio, 1989). Discrepancies may result from experimental differences (Sumpio and Banes, 1988b; Yang et al., 1993) or variation in the effectiveness of pulsatile stretch as a proliferative stimulus in SMC from different blood vessels. For example, stretching proved inefficient in stimulating proliferation in cultured SMC obtained from the human mammary artery (Predel et al., 1992) but induced proliferative responses in cultured human coronary SMC (Birukov et al., 1995). In a similar way, SMC from the relatively inelastic saphenous vein are seen to proliferate under cyclic stretch while those of the more elastic aorta were not (Dethlefsen et al., 1996). This may explain the hyperproliferative response, which may be found in these cells after implant of saphenous vein in the arterial circulation, and the lack of this response in bypasses using the internal mammary artery. It therefore appears that the proliferative response to cyclic stretch is dependent on the source of the SMC. Thus, if one is aiming at the spontaneous growth of SMC into porous synthetic grafts, the source of SMC populating the graft may be key to its success.

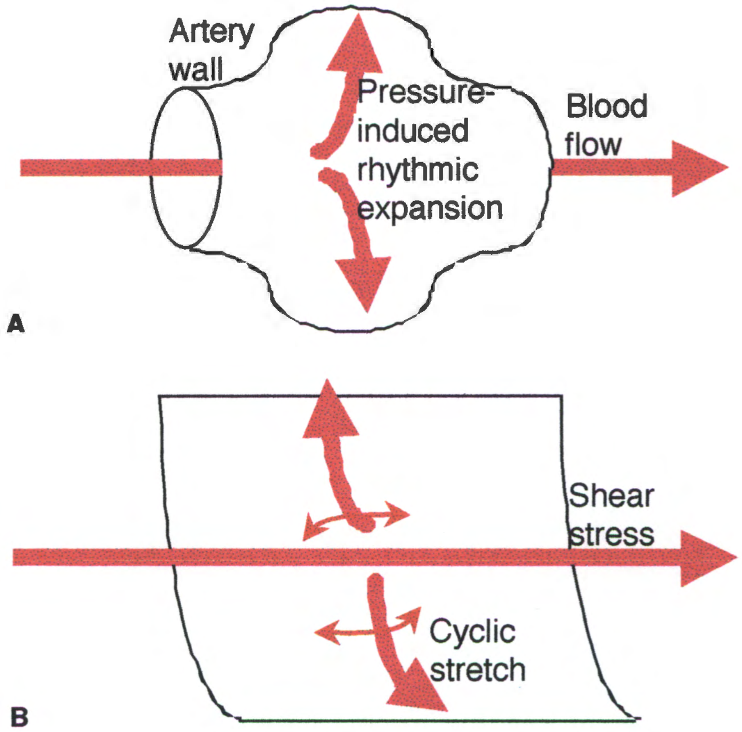

Figure 2. Haemodynamic forces acting on the cells of the artery wall (after Ives et al., 1986). **A**. Schematic diagram displaying effects of blood flow on the artery **B** Schematic diagram showing how the forces are experienced by cells on a flat surface. Blood flow across the lumen in **A** results in a shear force shown in **B** which effects the physiology and alignment of the endothelial cells lining the lumen. The rhythmic expansion of the artery wall shown in **A** can be visualised as a linear cyclic stretch in **B**. Although the major direction of stretch (thick curved arrows) is perpendicular to the long axis of the artery, a minor component of stretch (thin curved arrows) acts parallel to the long axis of the artery. Both EC and SMC have been shown to align due to cyclic stretch and this force influences many other functions in both cell types.

B. Pathophysiology of Prosthetic Graft Healing

1. Healing Response in Present Grafts

As can be seen above, both EC and SMC have properties vital to the proper function of blood vessels. In the same way, their presence in a graft is imperative to its proper healing and function. Based on the normal structure and function of the artery, the goal in the complete healing of prosthetic grafts remains three-fold: Firstly, the formation of an antithrombotic endothelial layer / neointima on the luminal surface which is maintained over time. Secondly, well-healed interstices containing both a muscular component and functional vasa vasora. The SMC strengthen the wall of the graft and provide the contractile and controlling function found in the artery. Finally, these cellular components should be resident in a material whose surface characteristics, structure and mechanical properties are optimal to both the physical demands on the graft and mature healing of the cellular components. However, currently available synthetic arterial grafts fail to heal (Davids et al., 1999). Neither surface healing in the form of endothelialisation nor complete wall healing through thorough population with SMC occurs, even after decades of implantation. On the contrary, these grafts are rather typified by a long-term inflammatory response and generally acellular structures (Davids et al., 1999). A brief synopsis of the cellular response and healing patterns observed in these materials is presented. Presently two materials are widely used in small diameter vascular prostheses: expanded polytetrafluoroethylene and polyethylene terephthalate.

The first of these is expanded polytetrafluoroethylene (ePTFE), which is used commercially in a form resulting from the stretching of the extruded polymer. This results in a characteristic structure of nodes with fine fibres bridging internodal spaces of between 30μm and 60μm (Figure 3A). The initial interstitial fibrin matrix formed after implant of this material becomes ordered over time into a chronically inflamed triple layered biological structure (Davids et al., 1999). Firstly, the blood interface portion of the graft is often populated by macrophages and lies adjacent to a distinct and largely acellular central protein matrix layer (Joos and Sandra, 1990). The final outer adventitial layer of the graft is also rich in macrophages and later becomes populated with foreign body giant cells (FBGC) characteristic of unresolved healing (Davids et al., 1999).

An alternative substance for graft manufacture is polyethylene terephthalate (PET). This polyester is manufactured for grafts as a yarn commercially known as Dacron (Figure 3B). This yarn may be woven or knit, with varying warp and weave into a tubular graft or fibres may be pressed into a felt resulting in a more random arrangement of fibres. The cellular response to Dacron implants is characterised by macrophages, a prevailing absence of SMC and, in the more porous woven or felt structures, the presence of large FBGC-associated endothelial sinuses which fail to penetrate the persistent luminal fibrin matrix lining the blood surface (Davids et al., 1999; Herring et al., 1984). If used as a small diameter bypass graft, this poor healing pattern leads to a disappointing patency (Berger et al., 1972).

The poor cellular response to these two graft materials may in part also be due to their structural and physical properties. At present, the non-healing response in these grafts, which is generally accepted to be characterised by the prolonged presence of FBGC and macrophages, may curtail the appropriate ingrowth of EC and SMC (Davids et al., 1999). The adhesion of macrophages to the graft wall is mediated by the absorption of blood proteins onto the graft structure, which is in turn dependent on the chemical groups available on the polymer surface (Davids et al., 1999). As new surface treatments for polymers arise and anti-inflammatory therapies improve, it is hoped that the degree of chronic inflammation will diminish. In this case, the primary challenge to graft healing will be the achievement of a sustained ingrowth of EC and SMC. However, the pore sizes in contemporary ePTFE and PET grafts are ill defined and too narrow to allow the ingrowth of EC transmurally (Golden et al., 1990b). Even in high porosity ePTFE (internodal distance 100µm), EC in the graft wall seem to originate primarily from the adjacent vessel wall and transmural ingrowth may be restricted by the network of fine internodal fibres (Tsuchida et al., 1997) (Figure 3A). In addition, grafts of these materials are inelastic and this rigidity may present the cells with an environment foreign to that found in the natural compliant artery (Ziegler and Nerem, 1994; Gimbrone, 1997; Brossollet, 1992). The replacement of a relatively inelastic wound fibrin ECM following implant with more elastic cellular components during cellular ingrowth (Greisler et al., 1992) means that in the end, the mechanical properties of the polymer are the limiting factor for the ability of the healing graft to undergo cyclic deformation *in situ*. Although compliance may not act alone (Uchida et al., 1989; Uchida et al., 1993), it has an important effect on vascular cell physiology (Zenni et al., 1993). If the walls of the graft do not expand to accommodate the pulse of blood, the flow rate past the EC will oscillate to abnormally high levels. This will have an effect on the alignment (Ives et al., 1986) and function of these cells, as shown above, and may be destructive to the endothelium. Similarly, the SMC in the walls of these grafts can be assumed to not be subject to sufficient cyclic deformation due to the non-expansion of the wall. This force has also been shown to have an effect on the alignment and physiology of the SMC, as discussed above (Sumpio and Banes, 1988b). It is possible that altered production of cytokines such as PDGF-A and -B and TGF-β in intimal EC (Sapienza et al., 1998) and SMC (Golden et al., 1990a) within some present inelastic prosthetic grafts, which is thought to be responsible for proliferation associated with intimal hyperplasia, may stem from an abnormal mechanical environment. Although this adverse development will only affect the central graft portion once it is populated by SMC, it may seriously enhance the luminal narrowing of the anastomosis (Shrestha et al., 1992). This hyperplastic intimal reaction (Bryan and Angelini, 1994) is the second most important reason for the failure of synthetic vascular grafts.

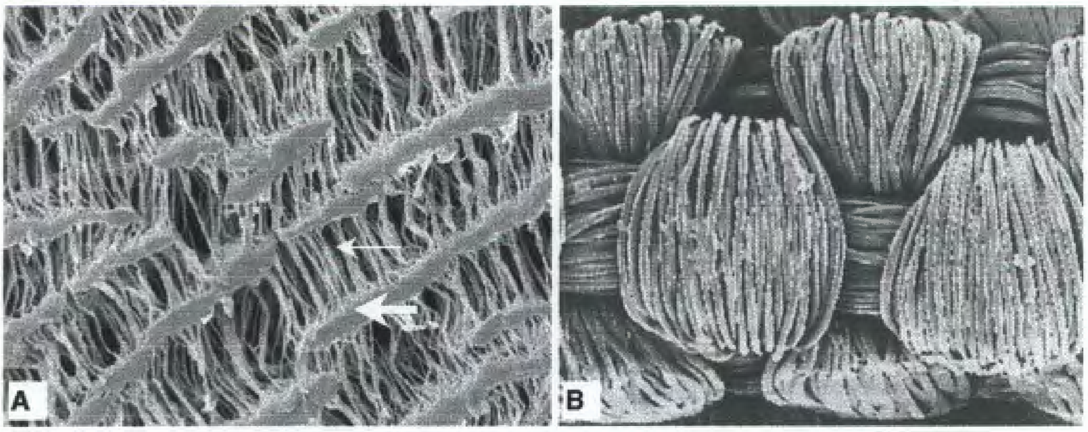

Figure 3. Scanning electronmicrographs of polymers used in prosthetic vascular grafts. **A**. Expanded polytetrafluoroethylene (ePTFE) (Courtesy of D. Bezuidenhout, Medtronic Institute for Biomedical Research, University of Cape Town Medical School, Cape Town, South Africa). Note the characteristic structure of nodes (thick arrow) separated by fine fibres (thin arrow). Although the internodal distances (45 – 60 μm) are large enough to allow cellular infiltration into the graft structure, successful integration is minimised by the internodal fibres. **B**. Polyethylene terephthalate (PET) (Dacron) (Guidoin et al., 1987). Dacron fibres (10 μm) joined into approximately 300 μm bundles and woven together to form the wall of a tubular graft. Once again the structure is highly impenetrable to cells and highly inelastic.

2. Facilitated Healing through Vascular Cell Transplantation

As can be seen from healing patterns in presently available grafts, EC don't spontaneously cover the graft surface further than one centimeter from the anastomosis (Poole et al., 1996). In a first step to actively achieve this goal, autologous endothelial cell transplantation has been shown to significantly improve graft performance (Zilla et al., 1999). The clinically most successful approach was *in vitro* endothelialisation prior to implant (Zilla and et al., 1987). This involves culture of EC harvested from the patient followed by protein coating of the prosthesis and rotation of a suspension of these cells onto the graft surface (Meinhart et al., 1997). Alternatively, other studies have used venous (Noishiki et al., 1992) and microvascular EC from fat (Jarrell et al., 1986; Schmidt et al., 1988) to seed synthetic vascular grafts of Dacron (Matsuda and Miwa, 1995) and ePTFE (Stansby et al., 1994).

The role of SMC in graft healing has been largely overlooked in cell transplantation because of their pathological role in intimal hyperplasia (Bryan and Angelini, 1994; Painter, 1991). This uncontrolled proliferative response to a number of factors has been a leading cause in graft failure and many strategies have therefore aimed at smooth muscle cell exclusion (Pitsch et al., 1996; Powell et al., 1996). Nevertheless, seeding with SMC alone (Oene et al., 1987) and accompanying endothelial cell seeding (Noishiki et al., 1993; Kanda et al., 1993a) has been carried out in some studies and SMC in these grafts have been shown to mature over time and demonstrate the contractile characteristics of SMC of the native artery (Noishiki et al., 1993; Baitella et al., 1993; Kanda et al., 1993b; Kanda et al., 1995). Although their long-term function *in vivo* has still to be tested, some particularly ambitious studies report formation of an entire functional artery from cultured EC and SMC (L'Heureux et al., 1998; Niklason et al., 1999) subjected to appropriate biomechanical forces. It therefore appears that, rather than excluding SMC, a balance between the EC and SMC is important for functional healing in the graft wall.

This is in concordance with the situation in the artery wall, in which SMC and EC reside in a balanced and interdependent way without overt proliferation.

Although grafts with cellular constituents have been found to have better long-term prospects in animal models and clinical use, the endothelial lining of synthetic grafts involves technically demanding and costly cell culture facilities. Furthermore, adequate, healthy cells are not always available for seeding in surgical situations and therefore the design of a graft in which the spontaneous transmural ingrowth of the EC occurs, would be preferable.

3. Components for a Spontaneously Healing Graft

SMC and EC populating the pores of a vascular graft originate from tissue adjacent to the graft and are enticed to enter the graft by stimuli specific to each cell. A brief consideration of the mechanisms of recruitment of each of these cells is presented.

EC populating the graft wall have been shown to originate from a variety of different sources in different models, ranging from transanastomotic ingrowth from the natural artery (Clowes et al., 1986a), circulating endothelial cell stem cells (Hollier et al., 1986; Scott et al., 1994; Shi et al., 1998) or transmural ingrowth from microvessels of the adventitial tissue (Clowes et al., 1986b; Wu et al., 1996). As transanastomotic ingrowth is limited in humans (Sauvage et al., 1971; Szilagyi et al., 1965) and the importance of blood-borne endothelial precursors in the human model is controversial and uncertain, the most promising source of luminal EC is from transmural ingrowth (Wu et al., 1996). The importance of this source of EC is demonstrated by the way that endothelial cell infiltration depends on the tissue surrounding the vessel in canine experiments (Sterpetti et al., 1992). As the capillary sprout from the adventitia faces the abluminal surface of the graft, it is confronted with a network of fibrin within the pores and a mildly hypoxic environment which is initially highly promigratory (Davids et al., 1999; Liu et al., 1990). Both in terms of peptide sequences in the matrix (Nicosia and Bonanno, 1991) and cytokines such as PDGF (Green et al., 1997) and VEGF (Weatherford et al., 1996) embedded during the formation of the clot (Devine and Carter, 1995), fibrin provides an ideal structural scaffold for neovascularisation and EC have been shown to infiltrate fibrin matrices such as those in the interstices of the graft wall *in vitro* (Hiraoka et al., 1998; Nehls and Herrmann, 1996). Although EC have been found to migrate on both loose and dense fibrin matrices, the formation of differentiated capillaries needs matrices with a lower fibre density (Nehls and Herrmann, 1996). Over time, due to the continued presence of clotting factors, platelets and macrophages in the blood, the fibrin matrix on the blood surface of the graft becomes progressively denser and more saturated with ingrowth inhibitory cytokines, making it more impenetrable to EC migrating towards the lumen, thus creating a window of time available for such invasion (Davids et al., 1999). Endothelial cells invade the graft by degrading and replacing the fibrin matrix in the interstices of the implanted graft by a process known as angiogenesis (Liu et al., 1990).

a. Angiogenesis

Blood vessels develop by two processes: vasculogenesis and angiogenesis (Sage, 1997). Vasculogenesis is the embryonic formation of the larger vessels from primordial EC in the mesoderm (Beck and D'Amore, 1997). Angiogenesis is the growth of new vessels from pre-existing ones (Folkman and Shing, 1992). Angiogenesis is fundamental to development, reproduction, wound repair in response to injury and augmenting existing vessels in response to tissue hypoxia (Ferrara and Davis, 1997; Pepper, 1996a). It is also found in a number of pathological conditions including arthritis, diabetic proliferative retinopathy, haemangioma and the recruitment of capillaries by solid tumours (Ferrara and Davis, 1997). Much of the knowledge of the process of angiogenesis stems from studies involving these conditions and the cellular mechanics are well characterised (Figure 4). In a spontaneously healing graft angiogenesis is important for the development of capillaries from vessels in the adventitia to infiltrate the graft by transmural ingrowth. These capillaries will hopefully provide the luminal surface with EC and establish the vasa vasorum within the vessel wall (Davids et al., 1999).

To bring about such dramatic remodelling requires activation of the quiescent EC in the adventitial vessels (Folkman and Shing, 1992). The microvasculature is typically quiescent, with a very low cellular turnover (D'Amore, 1992b; Folkman and Shing, 1992). It is held in this state by specific inhibitory molecules in the basement membrane such as collagen IV (Grant and Kleinman, 1997) and cytokines such as TGF-β (Antonelli-Orlidge et al., 1989) and angiopoietin-1 (Darland and D'Amore, 1999; Goede et al., 1998) released by adjacent pericytes. Due to this maintenance, the presence of pro-angiogenic molecules does not necessarily result in angiogenesis. Rather, a balanced concentration-dependent situation exists between positive and negative regulatory factors *in vivo,* which allows fine control of the process (Pepper, 1997a). A large variety of cytokines, peptide fragments, signalling molecules, vasoactive peptides and ions have been shown to activate EC to angiogenesis (Bussolino et al., 1997). Activation involves the conversion from a quiescent phenotype to an invasive and proliferative morphology. A dramatic alteration of cell physiology and upregulation of gene expression in the activated EC accompany this switch (Vernon and Sage, 1995). Many of the genes expressed are involved in the disruption of cell adhesion and migration of the EC such as proteases (Mignatti and Rifkin, 1996) which act locally to degrade the basement membrane of the parent vessel underlying the endothelial layer. EC then migrate, as a capillary sprout, through the basement membrane into the surrounding matrix towards the angiogenic stimulus (Sage, 1997). The cells at the front of this sprout continue to release surface-bound proteolytic enzymes as they progress through the interstitial ECM (Pepper et al., 1996b). Elongation of the capillary ingrowth is accompanied by cell proliferation in the portion nearest to the migratory front (Sage, 1997). In the region immediately posterior to this a lumen is formed - by the expansion of the intercellular space to form vacuoles (Meyer et al., 1997) - as the migration continues (Sage, 1997). Two contiguous tubular sprouts fuse to form a new functional capillary loop (Sage, 1997). As the nascent tube of EC forms, EC release factors to attract pericytes to accompany them (Benjamin et al., 1998). In fact, EC are seldom found free of pericytes and cultured EC in the absence of pericytes have been shown to release endothelin-1, which promotes the proliferation of pericytes (Yamagishi et al., 1993) and PDGF-BB, which may recruit and induce differentiation of pericytes / SMC (Hirschi et al., 1999). As mentioned above, once beckoned, the pericyte plays an active role in endothelial cell maintenance and regulation of the capillary function. After fusion of two endothelial sprouts to form a new capillary, pericytes migrate to the apex of the new capillary loop and halt angiogenesis in a contact dependent manner (Schott and Morrow, 1993). This may involve the release of TGF-β which inhibits endothelial cell proliferation and maintains the differentiated state (Antonelli-Orlidge et al., 1989). Vessel maturation is completed by secretion of a novel basement membrane (Sage, 1997). The components of the basement membrane and endogenous negative regulators of angiogenesis return the new vessel to a quiescent state.

The angiogenic progression of capillaries arising from outside of the adventitia and entering the wall of the graft is affected by stimuli from the fibrin matrix, inflammatory cells and angiogenic cytokines which are released by these cells and embedded in the matrix during clotting.

• Matrix

The maintaining function which collagen IV and laminin in the basement membrane have on endothelial cell physiology (Grant and Kleinman, 1997) demonstrates the potent effect which proteins in the ECM have on EC which are dependent on adhesion for the completion of the cell cycle (Stromblad and Cheresh, 1996; Watanabe and Dvorak, 1997) ECM constituents, or their degraded fragments, may affect each stage of angiogenesis (Sage, 1997) including proliferation and guidance of migration. EC undergoing angiogenesis in an implanted prosthetic vascular graft use proteins like thrombospondin-1 (Adams, 1997; Sage and Bornstein, 1991) which originate from the blood and macrophages (Sunderkotter et al., 1994) and are bound in the fibrin matrix within the pores of the graft. As mentioned above, fibrin provides an ideal scaffold for EC undergoing transmural angiogenesis and although the fibrin matrix within the pores of the graft is largely acellular, EC may come into contact with inflammatory cells associated with the graft.

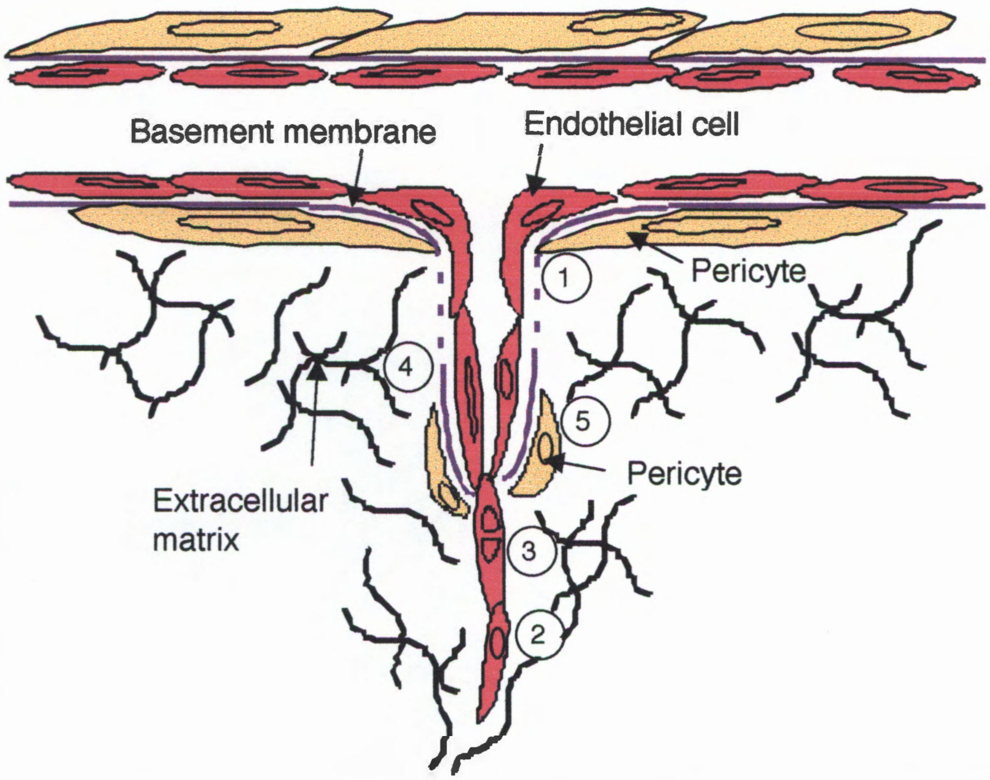

Figure 4. Schematic representation of the process of angiogenesis (after Sage, 1997). **1.** Basement membrane degradation followed by **2.** Directed endothelial cell migration with associated extracellular matrix degradation. **3.** Endothelial proliferation. **4.** Lumen formation **5.** Pericyte recruitment, basement membrane synthesis and neocapillary stabilisation.

• Macrophages

Macrophages are ubiquitous in normal and especially inflamed tissues (Sunderkotter et al., 1994). After implant of a prosthetic vascular graft macrophages degrade and enter the fibrin within the graft pores from the luminal blood and from capillaries in the adventitia in a similar way to normal wound healing (Leibovich and Wiseman, 1988). Macrophages are activated by hypoxia, such as found in wounds and in proximity to tumors, chemical stimuli or by cytokines in the matrix or released by other cells (Folkman and Shing, 1992). Activated macrophages can produce a plethora of proteases, and growth factors including bFGF; PDGF; VEGF; TGF-β; thrombospondin-1 (Cavaillon, 1994; Sunderkotter et al., 1991) and tumour necrosis factor -alpha (TNF-α) (Leibovich et al., 1987). This diversity (Takemura and Werb, 1984) and the additional release of angiogenic cytokines (Sunderkotter et al., 1991) - incorporated during clotting - by fibrinolysis associated with macrophage migration allows activated macrophages to influence each phase of the angiogenic process. In this way, the angiogenic capabilities of macrophages are comprehensive and unsurpassed by any other cell and their initial presence within the graft may stimulate capillary ingrowth such as is found in wound healing (Hunt et al., 1984; Leibovich and Wiseman, 1988). However, on encountering the material surface of the graft macrophages tend to adhere to the graft or fuse with other macrophages to form FBGC in a material-dependant fashion (Davids et al., 1999). The inability of these cells to remove the inflammatory stimulus of the graft results in their continued presence with the concomitant production of partly antiangiogenic cytokines which, as described before, may halt endothelial cell ingrowth and attenuate the recruitment of pericytes, leading to disappointing cellular ingrowth (Davids et al., 1999).

• Cytokines

During the course of angiogenesis, EC encounter a variety of cytokines derived from the blood, platelets, neighbouring cells and ECM-bound reserves. Cytokines involved in the process of angiogenesis may be classified as either direct or indirect (Folkman and Shing, 1992). Direct cytokines, such as VEGF and bFGF, can trigger migration and / or proliferation of EC. Indirect cytokines, such as TGF-β (Harmey et al., 1998) and TNF-α (Frater et al., 1987), cannot directly promote migration and / or proliferation of EC but have an effect on the angiogenic process which is modulated through other cells or cytokines (Sunderkotter et al., 1991). The angiogenic cytokines can also be classified into those capable of driving the whole process of angiogenesis such as VEGF (Thomas, 1996) and those capable of promoting only discrete sections of the angiogenic process. For example, TNF-α only induces the production of proteases in EC (Niedbala, 1993).

The effects of these individual factors on the EC are contextualised *in vivo* by interactions between the cytokines (Rifkin et al., 1990); ECM (Powell et al., 1996); cytokine receptors (Mandriota et al., 1996) and other cells. Thus the effect of individual cytokines on the process of angiogenesis is difficult to dissect. Despite this complexity, a brief overview of some of the key cytokines involved in angiogenesis is helpful to understanding the process more fully (Schott and Morrow, 1993).

Basic fibroblast growth factor (bFGF) and the related acidic fibroblast growth factor (aFGF) are ubiquitous cytokines found in many tissues (Schott and Morrow, 1993). Basic FGF production is induced in EC, SMC and nonvascular cells by a variety of stimuli such as hypoxia and other cytokines (Brogi et al., 1994). Basic FGF and aFGF are released into the ECM by the cells and bind to heparan sulphate proteoglycans in the basement membrane (Baird and Ling, 1987). Such binding increases the stability of the cytokines and forms a reservoir of the factors that may be released by degradation of the basement membrane during invasion of migrating EC regulating angiogenesis in a paracrine fashion (Klagsbrun, 1992). Basic FGF has been shown to induce mitogenesis in multiple cell types including SMC and EC (Slavin, 1995). This effect coupled with a promotion of protease production (Presta et al., 1986), migration and tube formation (Montesano et al., 1986) in EC makes it a potent direct inducer of angiogenesis. Furthermore, the upregulation of production of the angiogenic cytokine VEGF in SMC (Stavri et al., 1995a) and the co-ordinate upregulation of the VEGF receptor KDR expression in EC (Pepper and Mandriota, 1998) are brought about by bFGF. These interactions are capable of multiplying the initial angiogenic signal.

Another angiogenic growth factor released from the matrix during endothelial sprout invasion is TGF-β (Cox and Maurer, 1997; Rifkin et al., 1990). In contrast to the role of bFGF, TGF-β inhibits proteolysis (Rifkin et al., 1990); halts endothelial cell proliferation (Baird and Durkin, 1986; Frater et al., 1986); downregulates expression of receptors for other angiogenic cytokine (Mandriota and Pepper, 1997) and stimulates ECM production (Pepper, 1997b) *in vitro*. These anti-angiogenic properties of TGF-β may serve to balance the angiogenic effect of bFGF (Saksela et al., 1987). Following angiogenesis, pericytes in contact with EC have been shown to release TGF-β which has been implicated in maintaining the quiescent differentiated state in EC (RayChaudhury and D'Amore, 1991) without inhibiting pericyte proliferation (D'Amore and Smith, 1993). Preliminary evidence indicates that some of the components of the ECM released by EC in response to TGF-β may play a part in this anti-proliferative effect of TGF-β (RayChaudhury and D'Amore, 1991). However, despite these diverse inhibitory effects on angiogenesis *in vitro,* TGF-β is angiogenic *in vivo* (Madri et al., 1988). This may be due to the modulation of the *in vivo* angiogenic response by other cell types such as SMC (Brogi et al., 1994), macrophages and monocytes (Cavaillon, 1994). For example, TGF-β is chemotactic for macrophages and may induce the production of direct angiogenic molecules such as VEGF in these cells (Harmey et al., 1998). A direct pro-angiogenic effect on EC by TGF-β is that of tube formation. TGF-β has been shown to induce formation of a branching tubular structure in EC *in vitro* (Madri et al., 1988) and its functions in capillary remodelling are possibly more consistent with the completion of angiogenesis and the establishment of mature neocapillaries.

Another cytokine shown to play a role in endothelial cell tube formation during angiogenesis is PDGF-B (Battegay et al., 1994). PDGF is composed of homologous polypeptide chains, termed A and B, that are expressed as mitogenically active homo- or hetero-dimers with different roles and cellular specificities (Schott and Morrow, 1993). Of these, PDGF-BB is the most potent inducer of mitosis for EC (Marx et al., 1994). Production may be induced in a variety of cells including SMC and EC in response to injury (Ross et al., 1990) or cytokines, such as TGF-β (Starksen et al., 1987) and TNF-α

(Li et al., 1997b). In addition to its role in tube formation and mitogenic activity for EC *in vitro* (Marx et al., 1994), its effect on EC *in vivo* may be mediated through the release of VEGF and bFGF by vascular SMC (Brogi et al., 1994). Following angiogenesis, the endothelial cell sprout may also direct the recruitment and differentiation of pericytes / SMC by the mediation of PDGF-BB (Hirschi et al., 1999). Thus PDGF-BB plays a part in the recruitment of both components of the capillary (D'Amore, 1992a).

In contrast to the multi-cellular responses elicited by the above cytokines, the response to VEGF is potently and specifically mitogenic for micro- and macro-vascular EC (Shibuya, 1995). It may exist as one of four different molecular species: $VEGF_{121}$; $VEGF_{165}$; $VEGF_{189}$ and $VEGF_{206}$, which are distinguished by the number of amino acids in each molecule. These isoforms arise from alternative splicing of a single gene and have different physical properties (Ferrara and Davis, 1997). $VEGF_{121}$ is a free soluble form; $VEGF_{165}$ is found both free and bound to the cell surface and ECM; $VEGF_{189}$ and $VEGF_{206}$ are completely sequestered by the ECM (Ferrara and Davis, 1997). The differences in ECM binding by the isoforms are due to variations in heparin affinity and directly affect their bioavailability (Park et al., 1993). The heparin-bound $VEGF_{189}$ and $VEGF_{206}$ isoforms in the basement membrane have limited access to and thus little effect on the EC (Ferrara and Davis, 1997) and the non-heparin binding $VEGF_{121}$ isoform is considerably less mitogenic than the $VEGF_{165}$ species (Ferrara and Davis, 1997). This may be due to mediation of VEGF-receptor binding by the interaction of $VEGF_{165}$ with heparan sulphate on the cell surface which may orientate and localise the cytokine to its receptor and thus contribute to effective signal transduction and stimulation of EC (Dougher et al., 1997; Tessler et al., 1994). $VEGF_{165}$ is the predominant molecule released by a variety of normal and transformed cells (Ferrara and Davis, 1997). As the majority of the research into this cytokine has dealt with this major isoform (hereafter referred to as VEGF), this work will focus on its role in angiogenesis.

VEGF is produced throughout the body by a variety of normal embryonic or adult tissues and abnormal or tumour cells which require the recruitment of capillaries (Shibuya, 1995). The chief source of VEGF in the vasculature is the SMC / pericytes (Couffinhal et al., 1997; Ferrara et al., 1991; Williams et al., 1995) although temporally activated macrophages may be responsible for VEGF production (Harmey et al., 1998) and recent reports indicate the possibility of autocrine production of VEGF by stimulated EC (Namiki et al., 1995; Wang et al., 1999). VEGF production in SMC / pericytes (Ferrara et al., 1991), as with many cells in the body (Ferrara and Davis, 1997), can be induced by hypoxia (Brogi et al., 1996; Shweiki et al., 1992), a variety of hormones (Shibuya, 1995) and notably by other angiogenic cytokines (Williams, 1997) including the above mentioned bFGF (Stavri et al., 1995a), PDGF-BB (Stavri et al., 1995b; Williams et al., 1995) and TGF-ß (Brogi et al., 1994). The centrality of VEGF in the smooth muscle cell-mediated interactions of other angiogenic cytokines with EC emphasises its importance in the process of angiogenesis. The specificity of VEGF activity for EC in the absence of a smooth muscle cell response (Ferrara and Davis, 1997) also makes it stand out from the other angiogenic cytokines.

The specificity of VEGF for EC is defined by the presence of the two predominantly endothelial cell specific VEGF receptors (Thomas, 1996): fms-like tyrosine kinase-1 (Flt-1) and kinase insert domain-containing receptor (KDR). (Although Flt-1 has recently been identified to induce protease release in SMC (Wang and Keiser, 1998) and to mediate the mitogenic activity of VEGF for pericytes (Takagi et al., 1996; Yamagishi et al., 1999), both of which may contribute to angiogenesis, the activity of VEGF receptors in other cells is rare (Neufeld et al., 1999; Shibuya, 1995).) Both of the receptors are tyrosine kinases and respond to binding of VEGF by dimerising and autophosphorylating (Hanahan, 1997; Heldin, 1995; Mustonen and Alitalo, 1995). Although present on the mature endothelium, Flt-1 (also known as VEGF receptor-1 (VEGFR-1)) is unable to drive proliferation and chemotaxis alone and appears to play more of a role in developmental vasculogenesis (Kroll and Waltenberger, 1997; Weatherford et al., 1996). The function of Flt-1 in the adult vasculature remains unclear (Waltenberger et al., 1996) but may be associated with ongoing vascular repair and maintenance of permeability (Peters et al., 1993). By contrast, KDR (also know as foetal liver kinase-1 (Flk-1) and VEGF receptor-2 (VEGFR-2)) can transduce signals for mitogenicity, chemotaxis and cytoskeletal organisation (Neufeld et al., 1999). The difference in cellular response to the activation of each of the receptors is reflected in their distinct signal transduction pathways following phosphorylation (Waltenberger et al., 1994). While little is known of the signal transduction events following Flt-1 activation, in the case of KDR, the diverse, and in some cases unique (Doanes et al., 1999), signal transduction cascades triggered by phosphorylation correlate with the diversity of endothelial cell functions associated with this receptor (Kroll and Waltenberger, 1997).

The level of the VEGF receptors on the endothelial cell surface is regulated by many of the same factors affecting the expression of VEGF in other cells (Shibuya, 1995). This parallel regulation of the ligand and its receptor may tighten the control of angiogenesis by co-ordinating the release of VEGF with the expression of its receptor. Cytokines such as bFGF increase both the levels of KDR on the endothelial cell surface (Pepper and Mandriota, 1998) and VEGF secreted by adjacent SMC (Stavri et al., 1995a). Similarly, hypoxia, shown to induce production of VEGF in many cell types (Brogi et al., 1996; Shweiki et al., 1992) - and recently in EC (Liu et al., 1995) - has been demonstrated to upregulate the expression of the KDR receptor on EC in some studies (Brogi et al., 1996; Waltenberger et al., 1996) although it is unaffected in others (Gerber et al., 1997). This regulation of receptor expression may enable VEGF produced by hypoxic EC to act in a paracrine fashion during angiogenesis.

The role of VEGF in angiogenesis is not limited to proliferation. VEGF also induces migration by its potent and endothelial cell-specific chemotactic nature (Abramovitch et al., 1998; Neufeld et al., 1999; Senger et al., 1996; Visweswaran et al., 1997). In parallel to the induction of endothelial cell migration, VEGF can also induce the production of proteases in microvascular (Lamoreaux et al., 1998; Mandriota and Pepper, 1997) and lymphatic (Pepper et al., 1994) EC. These proteases are involved in the migration of the EC during angiogenesis (Mignatti and Rifkin, 1996) in conjunction with VEGF-upregulated adhesion molecules (Senger, 1996; Senger et al., 1996). Early research focussed on the ability of VEGF to increase the permeability of the endothelial layer (hence the synonym vascular permeability factor (VPF) (Senger et al., 1983)). An increase in vascular permeability, mediated by a loosening of intercellular connections or the induction of fenestrations (Shibuya, 1995), may be a crucial step in angiogenesis (Dvorak et al., 1995). It has been proposed that plasma escaping through the permeabilised endothelial layer may form an extravascular fibrin gel ideal for migration of EC (Senger et al., 1993). In addition, VEGF can induce the formation of tubular structures *in vitro* (Goto et al., 1993).

A further crucial function of VEGF is that of endothelial cell survival and maintenance. During vasculogenesis *in vivo*, VEGF (Alon et al., 1995; Gerber et al., 1999) and both of its receptors (Fong et al., 1995; Millauer et al., 1993; Shalaby et al., 1995) have been shown to be essential for vessel maturation. However, the widespread presence of VEGF in postnatal vessels after development (Couffinhal et al., 1997) has led to investigations into a "housekeeping" function for VEGF. Quiescent EC expressing both the KDR (Quinn et al., 1993) and Flt-1 (Peters et al., 1993) receptors may be exposed to this constitutive VEGF stimulus without the induction of angiogenesis (Couffinhal et al., 1997). The expression of these receptors in mature vessels suggests a role in ongoing repair and "housekeeping" (Jakeman et al., 1992). In a similar fashion, although it may not be the only survival factor necessary for EC from all tissues (Etoh et al., 1989; Karsan et al., 1997), VEGF has been found to improve endothelial cell survival on polystyrene culture dishes (Watanabe and Dvorak, 1997) and to be imperative for synovial endothelial cell survival *in vitro* (Yamane et al., 1994). A number of studies have elucidated the nature of the signalling pathways involved in maintenance and repair of the luminal endothelium by VEGF (Gerber et al., 1998; Nor et al., 1999). The survival function of VEGF seems to be mediated by the KDR receptor rather than Flt-1 (Gerber et al., 1998; Meeson et al., 1999). This is in contrast with the proposed roles of Flt-1 as the dominant receptor in maintenance of the quiescent endothelium (Peters et al., 1993) and KDR as the dynamic mediator of angiogenesis (Millauer et al., 1993; Quinn et al., 1993). VEGF seems to promote cell survival by halting apoptosis (Nor et al., 1999) experimentally-induced in EC by anchorage disruption (Watanabe and Dvorak, 1997) or cytokines (Spyridopoulos et al., 1997) or in haematopoietic cells by radiation (Katoh et al., 1995). In addition to its function in survival, VEGF may also play a long-term role in maintenance of correct permeability of the endothelium (Shibuya, 1995). In adult wound healing, EC invading the fibrin matrix (Liu et al., 1990) may regress by apoptosis as the local levels of angiogenic cytokines diminish. This leaves a collagenous and acellular scar. Thus, the continued maintenance of the EC appears to be as important to healing of the vascular graft as the recruitment of EC during angiogenesis (Desmouliere et al., 1997; Kischer, 1992).

Due to the specificity of the endothelial cell response in the absence of overt smooth muscle cell proliferation, a number of groups have proposed strategies to use the VEGF-KDR ligand-receptor signalling system to improve healing of vascular grafts. One *in vitro* strategy employed VEGF and heparin embedded in fibrin glue to selectively increase endothelial cell proliferation while avoiding smooth muscle cell growth (Weatherford et al., 1996). It is hypothesised that this approach will increase patency of the graft *in vivo* by encouraging the development of an antithrombotic endothelium in the absence of unrestrained smooth muscle cell proliferation associated with intimal hyperplasia. Masuda et al (1997) used VEGF alone attached to the graft to improve the transmural and transanastomotic endothelialisation of vascular grafts. As discussed above, the presence of an antithrombotic endothelium has been shown to improve graft patency (Zilla et al., 1999). A further study used transfection of vascular SMC with the VEGF gene *in vitro* in the hope that increased production of VEGF by such SMC seeded on the graft will promote the growth of an endogenous endothelium *in vivo* (Visweswaran et al., 1997). If these or similar strategies are successful in promoting the endothelialisation of the graft the challenge remains to populate the wall of the graft with a functional muscular component.

b. Musculogenesis

Following the ingrowth of capillaries from the adventitia, a number of scenarios exist for the accompanying migration of SMC into the graft wall. Despite isolated evidence of SMC originating from the blood (Feigl et al., 1985; Kouchi et al., 1998) and differentiated myofibroblasts (Okahara et al., 1995; Greisler et al., 1992) these scenarios seem unlikely to provide the bulk of the SMC and the most likely source for proposed contractile cells in the pores of a graft is by attraction via cytokines released by the EC, the fibrin matrix and macrophages.

As mentioned above, angiogenic EC migrating into a matrix are usually accompanied by pericytes (D'Amore, 1992a). Pericytes have been shown to be precursors for SMC and may be recruited and induced to mature under the mediation of PDGF-BB and other factors released by the EC (Darland and D'Amore, 1999; Swinscoe and Carlson, 1992). Recent work has also shown the pericytes to be subject to the mitogenic effects of VEGF in parallel to the endothelial cell response (Yamagishi et al., 1999) allowing the simultaneous migration of the whole capillary in response to this cytokine. Ingrowing SMC may also originate from the media of the adjacent vessel at the anastomosis (Clowes et al., 1986a; Okahara et al., 1995). There appears to be some phenotypic plasticity between the related SMC and pericytes and a sub-population of SMC in the vessel wall may be recruited as pericytes by EC during angiogenesis (Nicosia and Villaschi, 1995). These findings have been supported *in vivo* (Zacharias et al., 1988), where EC have been shown to promote infiltration and proliferation of SMC in a number of histological observations (Allen et al., 1994).

Chemotactic cytokines for SMC / pericytes may also be incorporated during the formation of the fibrin matrix within the interstices of a prosthetic graft and thus induce smooth muscle cell invasion (D'Amore, 1992b) into the wall of the structure. During proteolytic migration, the SMC or pericyte may utilise the sequences within the fibrin matrix (Yee et al., 1998) and protein components (Naito et al., 1992; Patel et al., 1997) as a scaffold in a similar way to migratory EC. However, despite these potential sources of SMC it is the observation that they are conspicuous by their absence in most implanted vascular grafts (Davids et al., 1999).

4. The Possible Role of Compliance in a Spontaneously Healing Prosthetic Vascular Graft

Developments in cell seeding have been paralleled by improvements in the materials involved in vascular grafts. One amongst many materials which shows promise in small diameter vascular prostheses is biostable polyurethane (PU) (Coury, 1999). This is a thermoplastic polymer into which pores of a larger size than those found in ePTFE and PET can be fashioned by the addition of a soluble filler to the polymer while it is still in solution (Deon Bezuidenhout, University of Cape Town Medical School, Cape Town, South Africa, personal communication). The filler is then dissolved out of the structure leaving a network of interconnecting pores with sizes up to the 60-100μm diameter range. This pore size is sufficient for the ingrowth of whole capillaries; bundles of SMC; the secretion of an elastin- and collagen-rich matrix and the rearrangement of EC and SMC involved in healing. It is hoped that this will facilitate the integration of the cellular components of the graft wall including SMC (Hess et al., 1983) and vasa vasorum. In addition to larger pore sizes, a chemically modified surface of the PU may reduce the absorption of blood proteins and thus lower the adhesion of macrophages to the graft (Coury, 1999). It is hoped that this will in turn attenuate the prolonged inflammatory response seen in present grafts which precludes successful integration. In the absence of inflammation the main goal for mature healing in the graft is that of maintenance of the cellular components without regression or hyperproliferation. In this case, the biomechanical properties of the graft may significantly contribute in a more sustained way to the long-term cellular responses. PU is also distinguished from ePTFE and Dacron as a graft material by its physical properties (Coury, 1999). Some porous PUs are elastomeric and as such may better approximate the mechanical environment found in the natural artery. As mentioned above, such matching of compliance has been shown to improve healing (van der Lei et al., 1987) and has been shown to reduce hyperproliferation of SMC (Miwa et al., 1993). Although little is known about the long-term healing patterns in compliant grafts, the material qualities of thermoplastic elastomers set them aside from the other polymers currently used in vascular prostheses.

Considering the manifold effects of cyclic deformation on vascular SMC, the presence of SMC within the interstices of a compliant graft brings with it some intriguing possibilities. Smooth muscle cells have been shown to produce ECM, alter proliferation and produce growth factors in response to cyclic stretch *in vitro* (Mills et al., 1993; Reusch et al., 1996; Sumpio and Banes, 1988b). If these responses are mirrored *in situ*, the possibility exists that, in addition to a structural and functional role, SMC stretched within the graft wall might play a major part in maintaining EC and altering their activity by release of ECM and cytokines as observed above.

EC have been shown to be paramount with regards to the patency of synthetic grafts. Although the fibrin matrix within the graft wall is initially highly favourable to endothelial cell migration, EC fail to achieve full thickness transmural ingrowth into most of the contemporary vascular prostheses, most likely due to prolonged inflammation, limited porosity and the build-up of a hostile ingrowth matrix. However, as soon as these obstacles are overcome, by advances in anti-inflammatory treatment and graft design, the challenge will not be so much to induce endothelial cell invasion as to retain the functional endothelial layer. In light of the intense interrelation between EC and SMC in the vessel wall and the maintaining role of VEGF in the vasculature, SMC appear to be important players in endothelial cell maintenance. The low levels of SMC and pericytes potentially invading a graft are not likely to become a bulk of SMC resembling the natural vessel wall Therefore, it is unlikely that SMC - pacified by endothelial cell mediation – will become the villains of intimal hyperplasia. On the contrary, it is more likely that they will complete a physiological healing pattern of synthetic vascular grafts by avoiding a stiff collagen matrix and facilitating the maintenance of functional capillaries and endothelial coverage through their secretion of VEGF.

In typical wound healing, decreasing tissue levels of VEGF lead to a purposeful regression of the abundant microvessels of granulation tissue. As the oxygen supply to a wound matrix improves due to the new vessels, the production of VEGF decreases (Benjamin et al., 1998). Due to the survival role which VEGF has on EC this decrease in

VEGF concentrations results in cell apoptosis (Benjamin et al., 1998). In addition, macrophages may mediate the apoptosis of capillary loops by deprivation of VEGF in the plasma (Meeson et al., 1999). These mechanisms result in a trimming of the vascular tree based on oxygen demand prior to pericyte recruitment. Pericytes, recruited in parallel by VEGF (Yamagishi et al., 1999) or by PDGF-BB (D'Amore, 1992a) released by the EC, establish contact with the endothelial cell tube. Establishment of pericyte contact with the EC results in an abrogation of the survival dependence on VEGF and the establishment of a quiescent phenotype in the EC (Benjamin et al., 1999). This is consistent with the observation of lower endothelial cell turnover in vascular beds in which the pericyte: endothelial cell ratio approaches 1:1 such as the brain microvasculature (Stevens and Lowe, 1992) and the observed vulnerability of EC supplying tumours, which lack pericytes, to apoptosis in the absence of high concentrations of VEGF (Benjamin et al., 1999). The maintenance of the endothelial cell by the pericyte may be mediated by the contact-dependent production of collagen IV into the basement membrane and TGF-β, which maintains the EC in a quiescent state, lowers the expression of the VEGF receptor KDR (Mandriota et al., 1996) and stimulates the production of further basement membrane components responsible for inducing quiescence in EC (Darland and D'Amore, 1999; RayChaudhury and D'Amore, 1991). Pericytes also release the related angiopoietin-1 and angiopoietin-2 in a paracrine fashion (Darland and D'Amore, 1999). Angiopoietin-1 has been show to be important for endothelial cell survival during development and to reduce apoptosis and increase stability of mature capillaries (Kwak et al., 1999; Papapetropoulos et al., 1999) in conjunction with VEGF (Asahara et al., 1998). While VEGF may be important in early capillary formation, the maintaining role of angiopoietin-1 seems to be limited to later stabilisation of the capillary (Peters, 1998). In contrast, angiopoietin-2 destabilises capillaries and appears to be involved in migration of EC during angiogenesis (Asahara et al., 1998). In this way a balance between destabilising factors and inhibitors is established which results in a capillary which is not dependant on high concentrations of cytokines for survival (Darland and D'Amore, 1999; Hanahan, 1997). Finally, pericytes have been demonstrated to produce angiopoietin and VEGF as a survival factors in stable capillaries in the corpus luteum (Goede et al., 1998; Reynolds and Redmer, 1998) and in response to many of the same stimuli inducing

VEGF production in the SMC (Kim et al., 1998). It appears therefore that, although the dependence on VEGF for endothelial cell survival decreases with the arrival of pericytes in the mature capillary (Benjamin et al., 1998), low levels of VEGF are still produced by the pericyte in a similar way to that observed by SMC *in vivo* (Couffinhal et al., 1997; Ferrara et al, 1991). Given the similarities in function and response between the two cell types (Nicosia and Villaschi, 1995), the production of VEGF by mesangial pericytes (Gruden et al., 1997) and other contractile vascular cells (Seko et al, 1999) under stretch and the profound effects of cyclic stretch on SMC including cytokine production (Mills et al., 1993), it seemed worthwhile investigating the role which cyclic stretch might play in VEGF expression in SMC potentially populating a compliant graft. To date no other study has addressed this question.

I hypothesised that SMC under physiological levels of cyclic stretch will express and release VEGF. The first aim of this work was to determine the extent and time course of VEGF expression by SMC due to these levels of cyclic stretch using an *in vitro* model. In addition, if any VEGF was expressed by SMC under cyclic stretch it would be useful to determine whether it was released by the cells at sufficient levels into the cultured media to affect endothelial cell physiology. Following this, I wished to determine whether cyclic stretch induced VEGF expression in other mammalian species and if cyclic stretch could influence the expression of other cytokines such as PDGF, bFGF and TGF-β.

Although VEGF released by stretched SMC may be capable of inducing a response in EC it is additionally possible that VEGF receptors on stretched EC may be affected by cyclic stretch. Studies showing the phosphorylation of cytokine receptors under cyclic stretch in the absence of their respective ligands (Hu et al., 1998; Osawa et al., 1997) raise the possibility that similar activation of the VEGF receptors on EC by stretch alone may occur *in vivo*. This may alter the endothelial cell response to locally-released VEGF from stretched SMC. Of the two constitutively expressed VEGF receptors on EC, KDR appears to play the predominant role in mediating endothelial cell survival due to VEGF. In addition, KDR has also been recently demonstrated to be phosphorylated by shear stress and thus act as a mechanoreceptor for this force (Chen et al., 1999). Therefore, a further aim of this work was to determine the effect of cyclic stretch on KDR expression and phosphorylation on EC. I aimed to outline the timecourse and magnitude of any response compared to the response from VEGF stimulation. This information should give an indication of how cyclic stretch will effect EC response to VEGF released by SMC under cyclic stretch. It is hoped that a better understanding of the role of VEGF communication between stretched SMC and EC will enable the design of a graft in which the level of compliance encourages the maintenance of a functional endothelium and more complete healing.

In order to investigate this relationship *in vitro* EC and SMC must be cultured under cyclic stretch. In light of accumulating evidence that cells residing in a dynamic system *in vivo* may be affected by the stresses to which they are subjected, a number of culture systems have been developed to grow cells under stretched conditions. Their use has led to considerable insight into the effects of cyclic stretch on both EC and SMC (covered above).

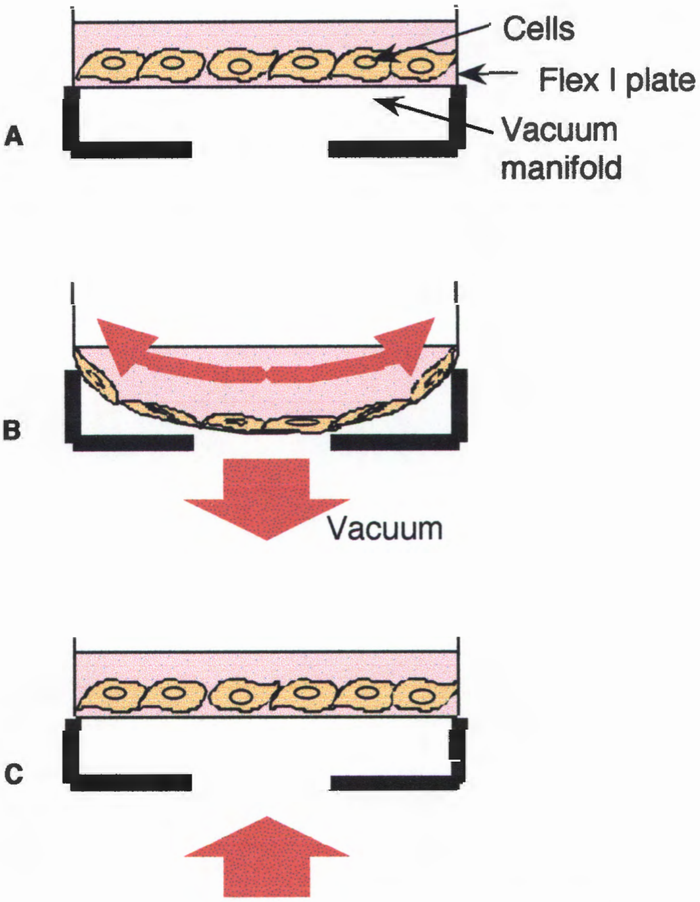

Figure 5. Schematic diagram of *in vitro* application of cyclic stretch to cells (after Sumpio et al., 1987). **A**. Cells seeded in Flex I plate placed in vacuum manifold in neutral position. **B**. Vacuum is applied to base of Flex I plate resulting in deformation. Cells adherent to the elastomeric base of the plate are subjected to the same elongation as the base (curved arrows). **C**. Vacuum is released and Flex I plate resumes neutral position. Cells were cycled at 10% deformation and 1 Hz (0.5 s deformation alternating with 0.5 s in the neutral position).

In order for cells to be stretched *in vitro* they must be grown on a deformable surface. These substrates are either rectangular (uniaxial) or circular (biaxial) in design and range from plastic culture dishes (Banes et al., 1985) to silicone rubber membranes (Ives et al., 1986) to elastin membranes (Leung et al., 1976). Rectangular surfaces are stretched mechanically while circular systems rely on vacuum deformation. The chief difficulties encountered are the maintenance of sterility and consistent levels of stretch and largely for these reasons the vacuum system has emerged as the model of choice. An early study using a circular system was carried out using vacuum pressure to deform aortic tissues from swine and dogs (Thibault and Fry, 1983). Subsequently, vacuum pressure was used to deform rigid culture dishes (Banes et al., 1985) and more recently circular dishes with elastomeric bottoms have been used (Harris et al., 1990) (Figure 5). Despite the fact that plating efficiency and the growth rate of some cell types on these flexible bottomed plates was decreased relative to those cultured on polystyrene tissue culture surfaces, the level of stretch can most easily be controlled in this system (Gilbert et al., 1994). Although studies using cyclic stretch are in no way a complete simulation of the complex *in vivo* environment due to the study of isolated biomechanical forces and cell types, the use of such cell culture systems may allow a more physiological study of systems under pulsatile stretching (Langille, 1993). Using similar systems, cyclic stretch has been show to exert important effects on phenotypic expression and growth of a number of cell types *in vitro* including vascular cells (Sumpio, 1989); fibroblasts (Banes et al., 1985); bone (Hasegawa et al., 1985) and epithelial cells (Brunnette, 1984). These studies have shown that physical deformation is perceived differently by different cell types and is not a generalised cell response (Sumpio and Banes, 1988b). Of all cells in the body normally subjected to mechanical deformation EC and SMC within the arterial wall are prototype. In these cells the forces experienced are relevant to the regulation of cell morphology and function. Following this, I endeavour to use a commercially available vacuum-based cyclic stretch apparatus to investigate the effect of cyclic stretch on VEGF communication between vascular SMC and EC.

Methods and Materials

1. Cell Culture

Bovine Endothelial Cells

Thoracic aortas of calves were removed aseptically and placed in phosphate buffered saline (PBS) with 200 U/ml penicillin and 200 μg/ml streptomycin and transferred on ice to the tissue culture facility. After removal of side vessels, fat and the adventitia to eliminate other cell types, the vessels were opened in a laminar flow hood and bovine aortic EC were obtained by the standard laboratory technique of gently scraping the internal surface of the aorta (Sumpio et al., 1987). Cells were maintained in Dulbecco's modified Eagles (DME) high glucose medium/Ham's F-12 1:1 (with 50 mM HEPES buffer, pH 7.2 and 10mM L-glutamine and $NaHCO_3$) supplemented with 10% vol/vol heat-inactivated foetal bovine serum (FBS), 5 μg/ml deoxycytidine/thymidine, antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and antimycotic (amphotericin B 250 ng/ml). The cells were incubated at 37°C in a humidified 5% CO_2 incubator until confluent. Cells were seeded at low density and in many cases cultures were clonal. EC were identified by their typical closely opposed polygonal "cobblestoned" morphology of confluent monolayers seen at 100x magnification under phase contrast microscopy and maintenance of density-dependent inhibition after serial passage. Cells isolated in this way have previously been shown to be free of other cell types (Sumpio et al., 1987). At confluency, EC were subcultured using 0.01% trypsin for detachment. Passages 2-4 were used in subsequent experiments. Cells were seeded at densities corresponding to confluency (6x 10^4 cells/cm^2), as determined by using a Coulter cell counter. They were subsequently grown in specialised Flex I plates for the application of cyclic stretch and quiescence induced by using 0% FBS and 1x insulin, transferrin, selenium (ITS) supplement (final concentrations: insulin 10mg/L, transferrin 5.5 mg/L and selenium 5.0 μg/L) (Libby and O'Brien, 1983) for 24 hrs prior to experiment. The EC must be induced to quiesce to approximate the *in vivo* condition where they are non-proliferative and nonmigratory (Couffinhal et al., 1997). If the cells are in a proliferative phenotype during the experiment then results may be due to increases in cell number rather than the stimulus being tested. In addition the use of serum-free medium ensures that the EC are not stimulated to by growth factors in the serum. Media with 0% FBS with 1x ITS was found to be effective in inducing quiescence of SMC with maintenance of high cell numbers (see below) and when the same conditions were applied to endothelial cell culture they were found to be as effective.

Bovine Smooth Muscle Cells

SMC were isolated using standard laboratory technique (Sumpio and Banes, 1988b; Ross, 1971). After complete removal of the endothelial layer by mechanical scraping, two-millimeter sections of the aortic wall were removed and placed in a 25 ml culture flask with medium consisting of DME (with 25 mM HEPES buffer, pH 7.2 and 2 mM L-glutamine and 44 mM $NaHCO_3$) supplemented with 10% heat-inactivated FBS, antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and an antimycotic agent (amphotericin B 250 ng/ml). The sections were then incubated at 37°C in a humidified 5% CO_2 incubator until smooth muscle cell outgrowth was detected. Uniform populations of SMC were identified by their characteristic morphology and undulating “hill and valley” confluent monolayer in culture as seen at 100x magnification under phase contrast microscopy (Sumpio and Banes, 1988b). Cultures isolated in this way were previously determined to be free of contaminating fibroblasts and EC by staining with an anti-α-actin antibody, which stains for the smooth muscle cell-specific protein α-actin (Powell et al., 1997). Cells were allowed to grow to confluency before subculture using 0.01% trypsin. Passages 2-4 were used in subsequent experiments. Cells were also seeded at confluency ($5x\ 10^4$ cells/cm^2) in specialised Flex I plates for the application of cyclic stretch and quiescence induced by culture in 0% FBS + 1x ITS (final concentrations: insulin 10mg/L, transferrin 5.5 mg/L and selenium 5.0 μg/L) (Libby and O'Brien, 1983) for 24 hrs prior to experiment. It was necessary to induce quiescence in SMC in 0% FBS to establish a baseline for detection of VEGF as serum has been shown to upregulate VEGF mRNA expression in SMC (Williams et al., 1995). This is possibly due to the presence of growth factors in commercial FBS. Furthermore, the SMC must be induced to quiesce to approximate the *in vivo* condition where they are non-synthetic and non-proliferative. If the cells are in a synthetic / proliferative phenotype during the experiment then results may be due to non-specific cytokine expression or increases in cell number rather than the stimulus being tested (Namiki et al., 1995). Equal numbers of SMC were seeded in media with 0.5% FBS or 0% FBS with or without ITS to determine the conditions best suited to induce quiescence but allowing maximal cell adherence. After 24 hours the plates were examined under phase contrast microscopy and washed 2x with PBS to remove floating cells. Adherent SMC were then resuspended by incubation with 0.01% trypsin and counted using a Coulter counter.

Rat Smooth Muscle Cells

Passage 1 rat aortic SMC were purchased from the American type culture collection (ATCC) and cultured with DME (with 25 mM HEPES buffer, pH 7.2 and 4 mM L-glutamine, 0.1 mM sodium pyruvate and 18 mM $NaHCO_3$) supplemented with 10% vol/vol heat-inactivated FBS, antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and antimycotic (amphotericin B 250 ng/ml). These cells were allowed to grow to confluency before subculture using 0.01% trypsin. Passages 2-4 were used in subsequent experiments. Cells were seeded at confluency (5x 10^4 cells/cm^2), as determined using a cell counter, in specialised Flex I plates for the application of cyclic stretch and quiescence induced using the same conditions and supplements used for bovine SMC.

2. *In Vitro* Application of Cyclic Strain to Cultured Cells

Apparatus

The stress unit (described in detail by Banes et al., 1985) consists of a vacuum manifold with recessed ports connected by vacuum lines to a regulator solenoid valve that is controlled by a computer with a timer program. The timer controls the duration and frequency of the applied stress. Cells were cultured on collagen-coated 25 mm 6-well culture plates with a flexible elastomer bottom, Flex I plate, that could be deformed to a known percentage elongation by controlling the level of vacuum (Figure 5). The collagen coating may improve plating efficiency of EC and other cell types on the elastomeric surface (Sumpio et al., 1987) by providing a substrate to which the cells can readily attach (Watanabe and Dvorak, 1997). Since the cells were found by microscopic examination to remain attached to the surface of the dish they presumably experienced the same force that was applied to the plate bottom (Sumpio et al., 1987). Although the mechanical stress applied to adherent cells varies with the position of the cells in the culture plate due to the nature of stretch application, with a gradient of increasing stress from the centre of the dish to the periphery, an average strain can be calculated (Gilbert et al., 1994).

Stretch conditions

For these experiments the membrane bottoms were subjected to 150 mm Hg (20 kPa) of vacuum, which produces an average strain of 10% on attached cells, at a rate of 60 cycles/min (1Hz) (0.5 s deformation alternating with 0.5 s in the neutral position (Figure 5)). Ten percent stretch is used as it is within the range of percentage deformation experienced by the walls of arteries (Awolesi et al., 1995; Lee and Wilson, 1986) and allows comparison with other studies and 1Hz used as it is the approximate physiological rate of stretch. Unstretched cells grown on identical collagen-coated Flex I plates were used as negative controls. Cells in stretched and control plates were checked at 100x magnification under phase contrast microscopy after induction of quiescence to confirm that the cells maintained the integrity and confluence of the monolayer. In addition, each plate was viewed after the experiment to determine whether the cells had remained adherent to the plates during the experiment. Plates in which the majority of the cells were not adherent after induction of quiescence or after the experiment were discarded. Fluid agitation and vibratory oscillations encountered by cells due to the application of cyclic stretch, which may have artifactual effects on cells during the course of the experiment (Leung et al., 1976), are found to be minimised by the use of this vacuum deformation system (Birukov et al., 1995; Davis et al., 1994). The use of this system also allows comparison of the results with the findings of other groups using the same system.

EC were subjected to cyclic strain for time intervals up to 2 hours in accordance with expected time courses of receptor phosphorylation recorded in the literature (Hu et al., 1998). Smooth muscle cells were subjected to up to 24 hrs of stretch following the expected time course of cytokine expression (Gruden et al., 1997). The use of quiescent cells and short time points (Wilson et al., 1993)should minimise bias due to cell proliferation during the experiment.

3. RNA Extraction and Northern Blot Analysis

Experimental Set-up

Quiescent bovine and rat SMC were subjected to cyclic stretch to determine the effect of cyclic stretch on VEGF expression. Control and experimental cells were seeded identically on 4 Flex I plates. One plate remained unstretched and served as a negative control for any effects of culture of SMC under cyclic stretch while another plate was subjected to cyclic stretch as described above. Bovine bFGF was added to a further plate of SMC at the published value of 10 ng/ml as a positive control for expression of VEGF (Stavri et al., 1995a). (In addition, the concentration dependence of bFGF as a positive control for induction of VEGF mRNA was determined to check the suitability of the published concentration of 10ng/ml for further experiments.) TNF-α is not known to stimulate VEGF production in SMC and thus was added at 10 ng/ml to the final plate of SMC to control for any response of the SMC to the process of addition of cytokines.

Time Courses

Cells were stimulated by cyclic stretch and bFGF for 4 hours following the demonstration in a 24 hour time course of VEGF mRNA expression that the maximal induction of VEGF mRNA due to both of these stimuli occurred at 4 hours.

RNA Extraction

Smooth muscle cell RNA was extracted by the guanidium isothiocyanate method using TRIZOL as per the manufacturers instructions. Briefly, after washing the cells twice with PBS, 1 ml of TRIZOL per 10 cm^2 of culture plate surface area was added to the cells and the lysate homogenised through a pipette before sitting for 5 minutes at room temperature. Chloroform (0.2 ml per ml of TRIZOL used in sample preparation) was added, the solution shaken vigorously and left at room temperature for 15 minutes. It was then centrifuged at 12,000 g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol per ml of TRIZOL used in sample preparation added. This was mixed, allowed to stand at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed and the pellet washed in at least 1 ml of 75% ethanol per 1 ml of TRIZOL used in sample preparation before centrifuging at 12,000 g for 5 minutes at 4°C. The pellet was dried using a pipette to the point of removing all drops of ethanol and resuspended in an appropriate volume of DEPC-treated water. For the concentrations of RNA required for this study, the RNA from one 30 ml six-well Flex I plate was resuspended in 15 μl of DEPC-treated water.

Gel Electrophoresis

RNA (10-20 μg, as determined by ultraviolet (UV) spectrophotometry) was mixed with 3x volume of formamide sample loading buffer (appendix 1) per volume of RNA solution and incubated for 15 min at 65°C. Following this, 1.5 μl of 1 mg/ml ethidium bromide was added to each sample and the RNA was electrophoresed through a 1% agarose/2% formaldehyde gel. The purity and integrity of the mRNA was checked by UV spectrophotometry and by the appearance of the RNA after gel electrophoresis. If RNA degradation was visualised as a smeared sample in gel electrophoresis or if protein contamination was detected as a ratio of absorbencies at 260 nm / 280 nm of less than 1, the experiment was discarded. The gel was photographed under UV, cut to an appropriate size and rinsed once for 15 minutes in ddH_2O and twice in 20x SSC with an inversion of the gel before the last rinse.

Northern Blotting and Membrane Fixation

Hybond-N nylon membrane was cut to fit the gel and soaked in 10x SSC prior to transfer. The gel was placed on a paper wick, the ends of which lay in 500 ml of 10x SSC. The membrane was placed on the gel, all bubbles removed and overlaid with blotting paper, towelling and a weight. Drawing of the 10x SSC by the blotting paper without passing through the gel was prevented by surrounding the gel/membrane interface with plastic wrap. Transfer was carried out overnight.

After RNA transfer, the gel and membrane were viewed on a UV lightbox to check for completeness of transfer by visualisation of the 18S and 28S rRNA bands, the membrane was photographed and the 18S and 28S rRNA bands marked on the membrane. The RNA was then fixed to the membrane by 3 exposures of UV crosslinking at 254 nm for 120, 150 and 150 seconds respectively before the membrane was sealed in a plastic hybridisation bag and stored at 4°C until probing.

Prehybridisation and Probe Preparation

Before probing, the membrane was briefly wet in 2x SSC in a sealable plastic hybridisation bag before 1 ml/25 cm^2 of membrane of hybridisation solution was sealed in the bag and the membrane preincubated at 42°C for 2 hours. During this time the radiolabelled murine probe for VEGF and human probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared. There is a very high sequence homology between the murine and bovine VEGF cDNA and the human and bovine GAPDH which allow the cross-species use of probes. GAPDH was chosen as a ubiquitously expressed gene against which to compare changes in VEGF expression due to the maintenance of steady levels of GAPDH expression under conditions of cyclic stretch and cytokine stimulation of bovine vascular cells in culture (Awolesi et al., 1995; Williams et al., 1995). The constancy of the GAPDH signal for different samples was also used as a check for the efficiency of transfer and equality of RNA loading.

To 50 ng of probe DNA (as determined by UV spectrophotometry at 260 nm) 45 µl of TE was added, boiled for 2 minutes to eliminate secondary structure, placed on ice and pulse centrifuged at 12,000 g to collect the condensate from the roof of the tube. This volume was placed with the pellet in the Ready-To-Go kit and thoroughly mixed. A 5 µl aliquot of α-^{32}P dCTP was added to the random prime reaction and mixed and the tube incubated at 37°C for 15 minutes. During this incubation the storage buffer in a G-25 Sephadex column was drained by centrifugation at 1100 g for 2 minutes. The DNA solution was then pulse-centrifuged at 12,000 g and the entire volume placed in the centre of the Sephadex column. The column is placed in a collection tube and spun at 1100 g for 4 minutes to pass the DNA through the column and remove any unincorporated α-^{32}P dCTP nucleotides. The degree of incorporation of the α-^{32}P dCTP into the eluant radiolabelled probe is then determined by measuring the disintegrations per minute (DPM) emitted by 1 µl of the probe in a scintillation counter.

Hybridisation, Washing and Autoradiography

A volume corresponding to either 1x 10^6 DPM of the probe for VEGF mRNA or 0.5x 10^6 DPM of the probe for the more prevalent GAPDH mRNA per ml of hybridisation solution used in the prehybridisation stage was boiled for 2 minutes with 0.5 µl of ssDNA and 20 µl of TE. The solution was then placed immediately on ice and pulse-centrifuged at 12,000 g before addition to the prehybridisation solution. The membrane was hybridised with the probe overnight at 42°C.

After hybridisation, the membrane was removed from the hybridisation bag and washed twice in 100 ml of 3x SSC/0.1% SDS at 55°C with shaking for 15 minutes. Progress and efficiency of washing was checked by scanning the blot with a Geiger-Muller counter. The washes were selected to result in a signal of up to 4000 counts per minute (CPM). The membrane was drained of the wash solution, sealed in a new hybridisation bag and placed between two sheets of autoradiographic film. On the outside of these films intensifying screens were placed to amplify the signal. Autoradiography was carried out at -80°C for between 1 and 4 days. The length of exposure was determined by the specific probe, count by Geiger-Muller counter and progress of exposure as monitored by developing of one of the films. Developing was carried out in an automatic developer. The optical density of hybridisation signals on developed films were analysed using a scanning densitometer for quantification of steady state mRNA levels. The sizes of the hybridising bands for VEGF and GAPDH were confirmed, by comparison with the marked positions of the 28S and 18S rRNA bands, at the published sizes of 4.2 kilobase (kb) (Williams et al., 1995) and 1.2 kb (Awolesi et al., 1995) respectively.

Stripping

Membranes probed for VEGF were stripped by washing in 100 ml of boiling 0.1% SDS before subsequent hybridisation for GAPDH. After the membrane was placed in the solution, it was allowed to cool to room temperature with shaking and the efficiency of the stripping checked by scanning the blot with a Geiger-Muller counter. If necessary, the stripping was repeated until a count below 500 CPM was obtained. After stripping, the membrane was placed directly into an appropriate volume of hybridisation solution at 42°C for hybridisation.

Species-Specificity Pilot Study

The species specificity of the smooth muscle cell response to cyclic stretch was determined using a pilot study which duplicated the above work in every aspect except using rat aortic SMC in place of bovine aortic SMC.

4. Conditioned Medium and Migration Assay

Experimental Set-up

To investigate the release by SMC of soluble chemotactic factors for endothelial cell migration, assays were carried out using conditioned media from SMC on EC made quiescent as above (0% FBS and 1x ITS for 24 hours). EC were exposed to the following variables of smooth muscle cell media: conditioned media from SMC stretched for 4 hours; media from unstretched SMC, which served as the negative control for any effects of cyclic stretch on the release of factors by the SMC; the conditioned media from SMC to which bFGF at 10 ng/ml had been added for 4 hours served as the positive control for VEGF conditioned media; unconditioned smooth muscle cell medium (0% FBS and 1x ITS), which was used to control for any cellular effects on the medium during the 24 hours of induction of quiescence and the time course of the experiment. Migration values were all standardised against the values obtained using unconditioned smooth muscle cell media. Although the use of 0% FBS in the media may result in increased random chemokinesis as compared to the use of 10% FBS (Waltenberger et al., 1994), this should be corrected for by standardising against the negative controls.

Smooth muscle cell were seeded and quiescence induced for 24 hours using the same conditions as used previously (0% FBS and 1x ITS for 24 hours) before being treated with bFGF at 10 ng/ml, stretched or unstimulated for 4 hours. Cells were examined under phase contrast microscopy following induction of quiescence and after each treatment to ensure adherence and if the cells were damaged the plates were discarded. After the treatment, media were collected on ice, spun down at 12,000g at 4°C to clear any cellular debris and stored at -80°C until use in the migration assay. Clarity and pH of the media were also checked to screen for contamination or pH alteration stemming from smooth muscle cell exposure.

Antibody Abrogation

In order to determine the role of VEGF in the chemotactic response of EC to smooth muscle cell conditioned media, the above set of experiments were duplicated with anti-human VEGF antibody at 50 µg/ml added to the media of the second set of experiments for 20 minutes at room temperature in order to neutralise any VEGF in the media. Due to the conservation of the VEGF structure between species, it is reasonable to assume that the high concentrations of the polyclonal anti-human VEGF antibody will cross-react with any bovine VEGF released by the SMC.

Endothelial Cell Migration Assay

For the migration assay, a blind-welled chemotaxis chamber was used which consists of a bottom plate containing wells and a matching top plate separated by a polycarbonate membrane with 8 µm pores (Figure 6). This pore size allows the exchange of molecules and migrating EC between the two chambers. The interface between the plates is sealed by a gasket which overlays the filter. Before placement of the filter, gasket and top plate and sealing of the apparatus, 30 µl of unconditioned media or media from unstimulated control SMC, stretched SMC and bFGF-stimulated SMC were placed into the bottom plate. Following this, the filter and gasket are laid on the bottom wells, sealing the apparatus and 50 µl of the quiescent endothelial cell culture at 1x 10^6 cells/ml were placed into the wells in the top plate. The chamber was placed in a cell culture incubator for 4 hours to allow the EC to migrate through the filter. Four hours is short enough to minimise the effects of proliferation due to factors in the smooth muscle cell conditioned medium while long enough to allow for the upregulation of the molecular mechanisms behind migration (Senger et al., 1996) and has been used in other endothelial cell migration studies (Ikeda et al., 1995; Rosen et al., 1990). At the end of this time, the filter was removed and any surface cells scraped off the top surface, on which the EC were seeded using a cotton swab, followed by fixing in 70% ethanol at -20°C for 20 minutes before staining with haemotoxylin overnight. In this way, any cells passing through the membrane at the time of fixation were bound and could be counted by microscopy. The filters were destained in ddH_2O, placed on a microscope slide and overlaid with glycerol and a coverslip before counting for quantification of endothelial cell migration. Migratory activity for each well was recorded as the average count of 5 random fields at 400x magnification for each experimental value. Each experiment was repeated 6 separate times.

ELISA

VEGF Enzyme-linked Immunosorbant Assay (ELISA) was carried out using a polyclonal antibody raised in rabbits against the N-terminal portion of the mouse VEGF protein. This anti-VEGF polyclonal antibody has been found to be permissively reactive across-species, reacting with both rat and mouse protein (Claffey et al., 1996). It was hoped that this lack of species specificity and the conserved nature of the portion of the VEGF protein against which the antibody is designed will allow the detection and quantification of the bovine VEGF in conditioned media samples from SMC. Although the mRNA expression levels of VEGF may fluctuate with time, the level of VEGF in the media is expected to increase over time without degradation as found in other studies (Katoh et al., 1995; Visweswaran et al., 1997), allowing the analysis of the cumulative VEGF production after 24 hours. It was hoped that by 24 hours the levels of VEGF would be sufficient to allow quantitation of VEGF secretion by the SMC. Media from SMC treated with bFGF at 10 ng/ml for 24 hours served as the positive control while media from unstimulated SMC served as a negative control. Smooth muscle cells were stretched for 24 hours. Media samples were collected on ice from experimental and control cells and any cellular material removed by centrifugation at 12,000 g for 5 minutes at 4°C before storage at -80°C until use in the ELISA. The ELISA was set up as follows (Yeo et al., 1992): A microtitre well was coated with an antibody raised in rabbit against the C-terminal of mouse VEGF, washed thoroughly with TBS-T and blocked with haemoglobin solution. After another wash, the media dilutions were placed in the well and incubated at 20°C for 2 hours with shaking. After further washing, the anti-VEGF N-terminal antibody was added and the solution further incubated for 2 hours at 20°C and washed. The anti-VEGF N-terminal antibody was labelled with Eu^{3+}, which dissociates on addition of a detection reagent to form a highly fluorescent chelate that can be detected by an ELISA reader and compared to the results obtained with a series of human VEGF standards. In each experiment the samples were tested in triplicate and the values averaged.

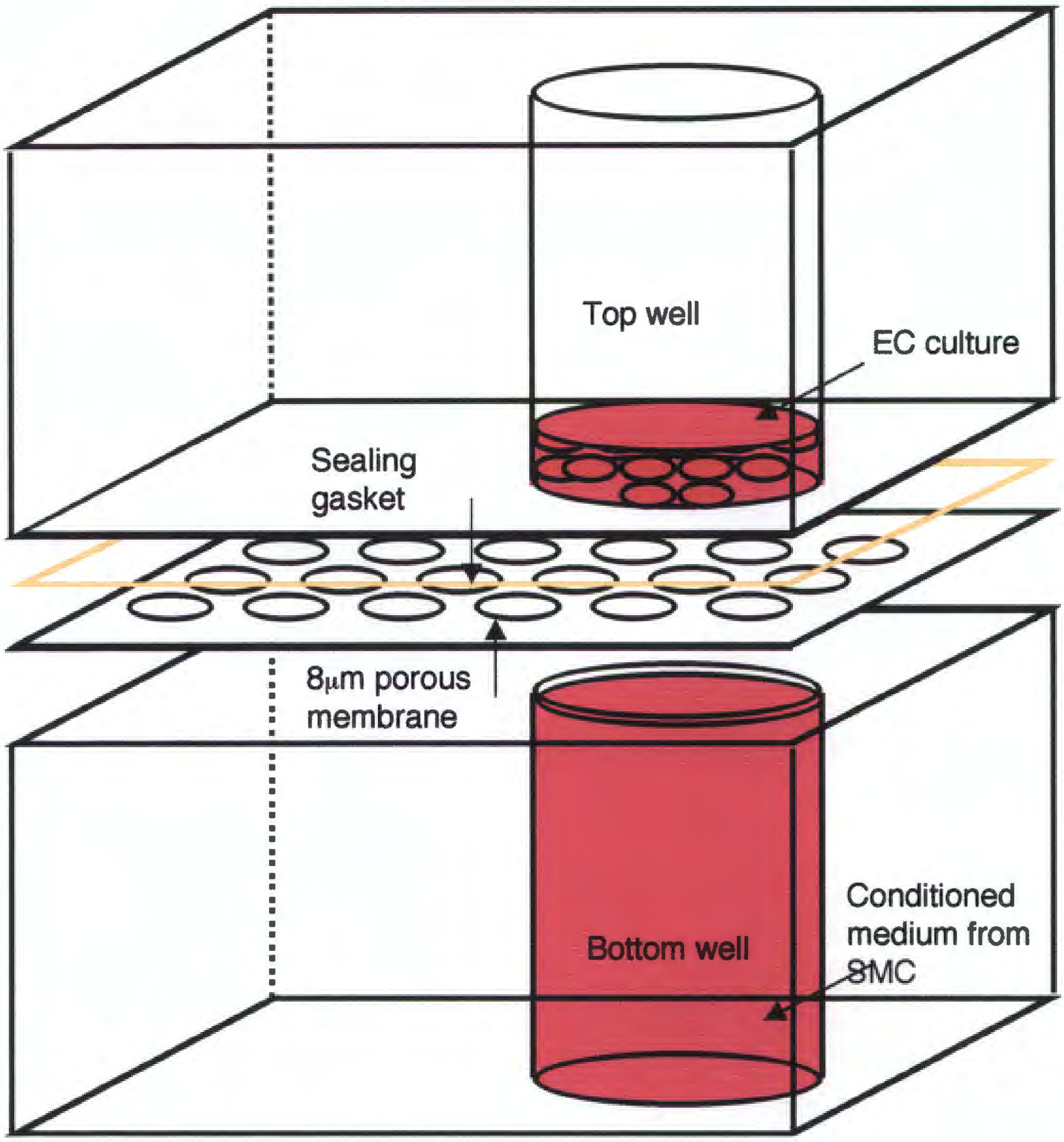

Figure 6. Schematic diagram of the chemotaxis chamber used in the migration assay. When the apparatus is sealed, EC seeded in the top well which are induced to migrate towards the SMC conditioned medium in the bottom well are trapped in the 8μm porous membrane and counted after staining. The count gives an indication of the levels of chemotactic factors in the conditioned media.

5. Immunoprecipitation and Western Blot Analysis

Experimental Set-up

To test whether cyclic stretch was able to bring about KDR phosphorylation in the absence of the VEGF ligand, quiescent EC were subjected to periods of cyclic stretch. Quiescent cells were used to approximate the non-proliferative cell state *in vivo.* Unstretched EC, with and without the addition of human recombinant VEGF at 50 ng/ml for 20 minutes (Waltenberger et al., 1994; Nor et al., 1999; Gerber et al., 1998), served as positive and negative controls for the phosphorylation of KDR respectively.

Time Courses

The time course of phosphorylation of the VEGF receptor KDR (Flk-1) was found by examining protein isolated from identically seeded cells at time intervals following the start of cyclic stretch. A preliminary 3 hour time course of KDR phosphorylation, demonstrated that the maximal 3.4 fold induction of KDR phosphorylation that occurred after 2 hours (Figure 13 C - Experiment 1) was followed by a slight decrease to 3.0 fold at 3 hours (timepoint not shown in Figure 13 C). Thus in subsequent repeats cells were stimulated by cyclic stretch for up to 2 hours. Similarly, protein isolated after 10 minutes of cyclic stretch in another preliminary experiment exhibited only 1.4 fold phosphorylation compared to protein isolated from unstretched EC (timepoint not shown in Figure 13 C). This was very similar to the value of 1.0 fold phosphorylation found at 30 minutes in this experiment (Figure 13 C - Experiment 4) therefore in subsequent experiments samples were taken at 30 minute intervals.

Protein Isolation

To isolate protein from each sample, EC were washed twice in cold PBS before harvesting by scraping in 100 µl of EBC lysis buffer supplemented with 1 mM PMSF, 1 mM $NaVO_3$, 10 µg/ml aprotinin and 10 µg/ml leupeptin. Lysates were centrifuged at

12,000 g for 1 minute to remove cellular debris and the supernatant transferred to a fresh tube. Samples were stored at -80°C until quantification. Protein quantification was performed using the Bradford assay and 2 µls of protein were added to 998 µl of ddH_2O and mixed well before addition of 1 ml of the assay reagent. The change in colour was compared to a range of bovine serum albumin (BSA) protein standards using a spectrophotometer at 595 nm. Between 300 and 500 µg of each protein sample was diluted to a total volume of 500 µl in EBC buffer for subsequent immunoprecipitation. If less than 300 µg of protein was available the experiment was discontinued.

Immunoprecipitation

4 µg of anti-KDR (anti-Flk-1) antibody was added to each sample, mixed thoroughly and rotated at 4°C overnight. Due to the cross-species reactivity of the anti-KDR antibody, it is reasonable to assume that the high concentrations of the polyclonal anti-mouse KDR antibody will cross-react with any bovine Flk-1 expressed by the EC. During this time the protein A-Sepharose beads were prepared. For each sample, 40 µl of a 50% protein A-Sepharose bead slurry in 70% ethanol was washed 3x in EBC buffer to remove the storage ethanol and resuspended as a 50% slurry in EBC buffer. A 20 µl aliquot of this solution was added to each sample, mixed thoroughly and rotated at 4°C for 2 hours.

The beads were then pelleted by centrifugation at 12,000 g at 4°C for 1 minute and the pellet washed 3x in NET-N supplemented with 1 mM PMSF, 1 mM $NaVO_3$, 10 µg/ml aprotinin and 10 µg/ml leupeptin. The pellet was resuspended in 20 µl of protein sample buffer, boiled for 5 minute, pulse centrifuged at 12,000 g and 4°C and stored on ice until electrophoresis.

Gel Electrophoresis

Samples were electrophoresed on a 5% non-reducing SDS polyacrylamide gel with a high molecular weight protein marker before overnight electrophoretic transfer to a nitrocellulose membrane at 4°C. The gel was stained with a Coomassie-based dye to ensure complete transfer and equivalent loading in gel lanes while the membrane was blocked in 10% fat-free milk in tris-buffered saline-Tween-20 (TBS-T) for 1 hour at 4°C with shaking to prevent non-specific binding of the antibody. Transfer efficiency was also determined by observation of the transfer of the prestained molecular weight marker from the gel to the membrane and if transfer was poor, the blot was discarded. Similarly, if the protein samples ran in a streak during gel electrophoresis, indicative of protein degradation, rather than as discrete bands, the samples were discarded. The membrane was then probed with the anti-tyrosine phosphorylation antibody (PY-20) at 1:2500 dilution in 10% fat-free milk in TBS-T for 1 hour to detect tyrosine phosphorylation levels of Flk-1. After this time the membrane was washed in TBS-T at 4°C for 20 minutes followed by two washes for 5 minutes to remove any unbound antibody. The PY-20 antibody is conjugated to a horseradish peroxidase enzyme, which produces a light signal detectable by film after exposure to enhanced chemiluminescence (ECL) reagents.

Visualisation

ECL reagents were prepared as per the manufacturers instructions and washed over the membrane for one minute before the membrane was drained, blotted dry and placed between two sheets of plastic wrap. ECL film was exposed to the wrapped membrane for up to 10 minutes although the majority of results could be seen after 10-30 seconds of exposure. The 210 kiloDalton (kDa) immunoreactive band corresponding to the KDR receptor protein was determined by comparison with the molecular weight marker and by subsequent exposure to an anti-KDR antibody (see below – Stripping and Reprobing). The optical densities of immunoreactive signals on developed films were analysed using a scanning densitometer for quantification of phosphorylation levels.

Stripping and Reprobing

To probe for KDR, membranes were stripped in a high salt buffer with 7% β-mercaptoethanol at 65°C for 30 minutes before washing as previously and blocking in

10% fat-free milk in TBS-T for 1 hour at 4°C with shaking. The membrane was then labelled with the anti-KDR antibody as primary antibody at 1:2500 dilution in 10% milk in TBS-T for 1 hour. After this time the membrane was washed in TBS-T at 4°C as before. The membrane was then labelled with the secondary antibody, an anti-rabbit IgG peroxidase conjugate, at 1:2000 in 10% milk at 4°C for 1 hour. Washes were performed as before to remove any unbound secondary antibody before detection of bound antibody was performed using the ECL protocol as above. The size of bands detected on the film was found by comparison with the molecular weight marker and used to identify the 210 kDa protein as KDR. The optical densities of immunoreactive signals on developed films were once again analysed using a scanning densitometer for quantification of KDR levels. These values were used to correct for loading differences between samples and to ensure that KDR expression remained constant throughout the experiment. The KDR receptor is highly expressed in quiescent EC (Gerber et al., 1999) and no positive control for the presence of the receptor is therefore necessary. Blots in which a 210 kDa band was not found after probing with both PY-20 and anti-KDR were discarded.

6. RT-PCR

Reverse Transcription

To examine the feasibility of using reverse transcriptase-polymerase chain reaction (RT-PCR) to measure expression of the cytokines VEGF, PDGF-B, TGF-β and bFGF in SMC stretched for 4-24 hours, total RNA from stretched SMC, as isolated above (RNA Extraction and Northern Blotting – RNA Extraction), was reverse transcribed into DNA for PCR as follows:

2 μg of RNA were mixed with 0.5 μg of Oligo-dT primer and DEPC-treated water to a total volume of 12 μl in a thin-walled polymerase chain reaction (PCR) tube. The mixture was placed at 68°C for 10 minutes in order to remove any secondary structure in the RNA before being placed on ice. Appropriate volumes of the following reagents were added in a total reaction volume of 20 μl to reach final concentrations of: 1x reverse transcriptase

buffer; 10 mM dithiothreitol (DTT); 0.5 mM each of dATP, dCTP, dGTP and dTTP; 0.25 U/µl of RNAse inhibitor and finally 2.5 U/µl of reverse transcriptase (RT). Negative controls to establish the RNA as the sole source of any DNA present in the reaction mixture were set up in parallel except the RT was replaced by an appropriate volume of DEPC-treated water. The solutions were thoroughly mixed and heated to 37°C for 10 minutes and 42°C for 50 minutes before the reaction was stopped by heating to 94°C for 2 minutes (to denature the RT and RNA) and placed on ice. The reaction was further halted and the concentration of nascent DNA adjusted for PCR by the addition of 20 µl of DEPC-treated water to the reaction mixture.

Polymerase Chain Reaction

2 µl of the RT reaction were amplified in a fresh thin-walled PCR tube in 1x PCR buffer, using final concentrations of 2.5 U *Taq* DNA polymerase; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 2 mM of sense and antisense primers (Table 1) and DEPC-treated water to total reaction volume of 50 µl. Cycle temperatures and numbers were adjusted for the specific thermal cycler and *Taq* DNA polymerase used in this study until a distinct PCR DNA product band for each cytokine, of the published molecular weight as confirmed by comparison with a common DNA molecular weight ladder, was obtained on gel electrophoresis (see below – Gel Electrophoresis). The primers used for VEGF are able to amplify all of the isoforms of this cytokine, allowing the determination of which isoforms are being expressed. Primers for β-actin and hypoxanthine phosphoribosyl transferase (HPRT) mRNA expression were also used to determine the feasibility of using these genes as controls. The β-actin gene has been shown to maintain constant expression under a variety of stimuli making it a suitable control (Ninomiya et al., 1992). HPRT was included as its expression has been specifically shown to remain unaltered during VEGF upregulation (Iizuka et al., 1994) and its robust response and high molecular weight in comparison to β-actin allow it to be run on the same percentage gels as the cytokines. The reaction was performed in a PCR Express thermal cycler. Cycle parameters were generally as follows: Samples were taken from ice to a preheated block at 94°C for a 1 minute melting step followed by a 1 minute annealing step at the temperature indicated in

table 1, followed by a 1 minute extension step at 72°C except in the case of HPRT which was elongated at 65°C due to the primer design. The melting, annealing and elongation steps were cycled 30 times before a final extension at the appropriate extension temperature for 10 minutes. This final step allowed any products in which extension was not complete during the cycling to reach their full length.

Gel Electrophoresis

Products were electrophoresed with a 100 base pair (bp) DNA ladder on a 2% agarose gel containing 1 µl/ml ethidium bromide and photographed on an UV transilluminator. Negative controls from the RT step, as mentioned above, were run in parallel to each sample.

7. Statistical Analysis

Results are expressed as mean (± standard deviation) for the indicated number of independent observations (*n*). Densitometric values obtained for VEGF mRNA and KDR phosphorylation signals were normalised over standards for GAPDH and KDR levels respectively to correct for loading differences in studies involving gel electrophoresis. This adjustment was therefore not necessary for the results obtained from the migration assay or ELISA procedure. Following this adjustment, values in all studies were normalised to the individual unstretched control for each experiment and data were expressed as fold of the unstretched control. Statistical comparison of the differences between means of control and experimental expression of VEGF mRNA as determined by northern blotting and protein levels as determined by ELISA were made using paired one-tailed Student's *t*-tests. Comparison of the differences between experimental means of KDR phosphorylation levels and the control mean were made using a one-tailed Student's *t*-tests. Comparison between migration results and their respective antibody-abrogated controls was made with a 2-sample *t*-test with distinct variances. The degree of statistical significance of the differences of the means is reflected by their *p* values, which are indicated in the text.

Sequence **(Reference)**	**Sense Primer**	**Antisense Primer**	**Anneal Temp.**
VEGF (Nicol et al., 1997)	ATG CGG ATC AAA CCT CAC C	ATC TGG TTC CCG AAA CCC TG	57°C - 60°C
PDGF-B (Holt et al., 1994)	CCG CAC CAA CGC CAA CTT CC	TTT GGC TCG CTG CTC CTG GG	62°C
TGF-β (Dahiya et al., 1996)	GCC CTG GAC ACC AAC TAT TGC T	AGG CTC CAA ATG TAG GGG CAG G	64°C
bFGF (Sheu et al., 1996)	GCA AAA ACG GGG GCT TCT TCC TGC G	CCA GTT CGT TTC AGT GCC ACA TAC CAA	65°C
β-actin (Ninomiya et al., 1992)	ATC ACC ATT GGC AAT GAG CG	TTG AAG GTA GTT TCG TGG AT	65°C
HPRT (Iizuka et al., 1994)	AAT TAT GGA CAG GAC TGA ACG TC	CGT GGG GTC CTT TTC ACC AGC AAG	56°C

Table 1. Primers and annealing temperatures used in RT-PCR. Cycle temperatures and numbers were adjusted for the specific thermal cycler and *Taq* DNA polymerase used in this study until a distinct band for each gene, of the published molecular weight, was obtained. The optimum annealing temperature found is listed.

Acknowledgements

Murine probe for VEGF from Dr. Koji Maemura, Cardiovascular Biology Laboratory, Harvard School of Public Health, Boston, Massachusetts, USA. Human probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Dr. Bauer Sumpio, Department of Vascular Surgery, Yale University School of Medicine, New Haven, Connecticut, USA. VEGF ELISA carried out by Dr. Kevin Claffey, Beth Israel Deaconess Medical Centre, Boston, Massachusetts, USA. Photomicrographs of SMC under cyclic stretch taken by Dr. Ira Mills, Department of Vascular Surgery, Yale University School of Medicine, New Haven, Connecticut, USA. Electronmicrographs of vascular graft materials taken by Deon Bezuidenhout, Medtronic Institute for Biomedical Research, University of Cape Town Medical School, Cape Town, South Africa. Assistance with statistical analysis from Prof. Tim Dunne, University of Cape Town, Cape Town, South Africa.

8. Materials

Tissue culture

Rat aortic smooth muscle cells (product number CRL-1444) were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA. Tissue culture media Dulbecco's modified Eagle's medium (DME) (product number D-5648) for SMC, deoxycytidine (product number D-0776), thymidine (product number T-1895), bovine bFGF (product number F-3133), HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) buffer (product number H3375), sodium pyruvate (product number P-4526) and insulin, transferrin, selenium (ITS) supplement (product number I-3146) were obtained from Sigma chemical company St. Louis, Missouri, USA. Tissue culture media Dulbecco's modified Eagle's medium (DME)/F12 (product number 32500-019) for EC, antibiotic/antimycotic (product number 15240-039), trypsin-EDTA (product number 45300-019), magnesium and calcium free phosphate buffered saline (PBS) (product number 14190-201) were obtained from Gibco BRL Grand Island, New York, USA. Foetal bovine serum (FBS) from Hyclone Logan, Utah, USA. Flexercell Stress Unit and Flex I collagen-coated culture plates (product number P-1001C) were obtained from Flexcell International Corporation, Hillsborough, North Carolina, USA. Cell counter was obtained from Beckman Coulter Incorporated. Fullerton, California, USA.

RNA extraction and northern blotting

TRIZOL (product number 15596-026) was obtained from Gibco BRL Grand Island, New York, USA. Formaldehyde (product number F1635), formamide (product number F9035), single stranded salmon sperm DNA (ssDNA) (product number D-7656), Denhardts solution (product number D2532), Diethyl Pyrocarbonate (DEPC) (product number D5758) and Dithiothreitol (DTT) (product number D5545) were obtained from Sigma chemical company St. Louis, Missouri, USA. Hybond-N nylon membrane for nucleic acids (product number RPN303N) and Ready-To-Go DNA random oligonucleotide primed nucleotide labelling beads deoxycytidine 5'-triphosphate (dCTP) (product number 27-924001) were obtained from Amersham Pharmacia Biotech Piscataway, New Jersey, USA. BioMax MS autoradiographic film for northern blotting (product number 8377616), BioMax MS intensifying screens (product number 8827438) and X-OMAT automatic developer were obtained from Kodak, Rochester, New York, USA. Gel photographic film (product number T-667) and camera (product number DS-34) were obtained from Polaroid Cambridge, Massachusetts, USA. Scanning Densitometer was manufactured by Molecular Dynamics, Sunnyvale, California, USA. Quickspin Sephadex G-25 fine Column (product number 1273922) was obtained from Boehringer Mannheim now Roche Molecular Biochemicals Indianapolis, Indiana, USA. Easytides Deoxycytidine 5' triphosphate (α-^{32}P dCTP) (product number NEG513S) was obtained from NEN Lifesciences Boston Massachusetts, USA. Pancake Geiger-Muller survey meter (Product number 3-90) was obtained from Ludlum Measurements Incorporated. Sweetwater, Texas, USA. Scintillation counter and spectrophotometer were manufactured by Beckman Coulter Incorporated. Fullerton, California, USA.

Migration assay

Anti-human VEGF antibody (product number AF-293-NA) was obtained from R&D Systems Inc. Minneapolis, Minnesota, USA. Reusable chemotaxis chamber (48 well) (product number AC48) and Poretics Polycarbonate 8 μm membrane (product number PFB8) were obtained from Neuro Probe Incorporated. Gaithersburg, Maryland, USA.

Immunoprecipitation and western blotting

Anti-Flk-1 (Anti-KDR) antibody (SC-504) was obtained from Santa Cruz Biotechnologies, Santa Cruz, California, USA. Anti-tyrosine phosphorylation antibody (PY-20) (product number P11625) was obtained from Transduction Laboratories Inc. Lexington, Kentucky, USA. Human VEGF (293-VE) was obtained from R&D Systems Inc. Minneapolis, Minnesota, USA. Hybond-ECL nitrocellulose membrane for proteins (product number RPN2020D), protein A Sepharose CL-4B (product number 17-0963-03) for immunoprecipitation, ECL chemiluninescence detection reagents (product number

RPN2109), anti-rabbit immunoglobin G (IgG) peroxidase conjugate (product number NA934) and film (product number RPN2103H) were obtained from Amersham Pharmacia Biotech Piscataway, New Jersey, USA. Bradford protein quantification assay (product number 5000002) and high range molecular weight marker (product number 1610309) were obtained from Bio Rad Laboratories, Hercules, California, USA. Phenylmethylsulphonyl fluoride (PMSF) (product number P7626) was obtained from Sigma chemical company. St. Louis, Missouri, USA. GelCode Coomassie-based dye was obtained from Pierce chemical company. Rockford, Illinois, U.S.A. Scanning densitometer was manufactured by Molecular Dynamics, Sunnyvale, California, USA. Spectrophotometer was manufactured by Beckman Coulter Incorporated. Fullerton, California, USA.

RT-PCR

Primers for PCR for VEGF (Nicol et al., 1997), PDGF-B (Holt et al., 1994), TGF-β (Dahiya et al., 1996), bFGF (Sheu et al., 1996), β-actin (Ninomiya et al., 1992) and Hypoxanthine phosphoribosyl transferase (HPRT) (Iizuka et al., 1994) were made by Department of Medical Biochemistry University of Cape Town Medical School, Cape Town, South Africa. DNA ladder (100 bp) (product number 15628-019), Oligo-dT reverse transcription primer (product number 18418-012) were obtained from Gibco BRL Grand Island, New York, USA. Gel photographic film (product number T-667) and camera (product number DS-34) were obtained from Polaroid Cambridge, Massachusetts, USA. dATP, dCTP, dGTP, dTTP mix (product number 1277049), RNAse inhibitor (product number 799017) and *Taq* DNA polymerase with *Taq* buffer (product number 1146173) were obtained from Boehringer Mannheim now Roche Molecular Biochemicals Indianapolis, Indiana, USA. M-MuLV Reverse transcriptase and reverse transcriptase buffer (product number 253S) were obtained from New England Biotechnology. Beverly Massachusetts, USA. PCR thermal cycler PCR Express (product number HBPX) was manufactured by Hybaid Franklin, Massachusetts, USA.

9. Appendix of solutions

RNA solutions:

(All made up with autoclaved ddH_2O)

DEPC-treated water
0.1% DEPC
stir overnight
Autoclave

MOPS
1 M MOPS
pH to 7.0
Autoclave and store in dark

10x MOPSB
200 mM MOPS (pH 7.0)
50 mM NaOAc (pH 7.0)
10 mM EDTA
store in dark

RNA gel
made up in DEPC-treated H_2O
1% agarose
1x MOPSB
2% formaldehyde
Make up fresh

RNA electrolysis buffer
1xMOPB
Make up fresh

4x RNA sample buffer
made up in DEPC-treated H_2O
1.43x MOPSB
7% formaldehyde
0.6x formamide
Make up fresh

Hybridisation Solution
5x Denhardts Solution
5x SSPE
0.1% SDS
0.1 mg/ml ssDNA
50% formamide
Store at 4°C

20x SSPE
3 M NaCl
25 mM EDTA
0.2 M NaH_2PO_4

SDS
10% SDS
pH to 7.2
Autoclave briefly

ssDNA
1 mg/ml
denatured at 65°C for 30 min
and 42°C for 120 min
Store at 4°C

20x SSC
3 M NaCl
0.3 M NaCitrate
pH to 7.0
Autoclave

DNA Solutions:

TE
10 mM Tris
1 mM EDTA
pH 8.0

TAE
40 mM Tris.Acetate
2 mM $Na_2EDTA.2H_2O$

DNA gel
made up in 1x TAE
1% agarose

Protein Solutions:

4x Separating gel
1.5 M Tris
0.4% SDS
pH to 8.8
Filter and store at 4°C

4x Stacking gel
0.5 M Tris
0.4% SDS
pH to 6.8
Filter and store at 4°C

2x Sample buffer
0.125 M Tris.Cl pH 6.8
2% SDS
20% glycerol
2% β-mercaptoethanol
0.001% bromophenol blue

5x Protein Electrophoresis buffer
125 mM Tris
1 M glycine
5% SDS
pH to 8.8
Store at 4°C

Transfer buffer
25 mM Tris
0.19 M glycine
20% methanol
Store at 4°C, use 3x

10x TBS-T
0.2 M Tris
1.37 M NaCl
0.01% Tween-20
pH to 7.6,
Store at 4°C

EBC lysis buffer
50 mM Tris
120 mM NaCl
0.5% NP40
pH to 7.4
add PMSF to 1 mM, $NaVO_3$ to 1 mM, aprotinin to 10 µg/ml and leupeptin to 10 µg/ml before use.

NET-N
20 mM Tris
100 mM NaCl
1 mM EDTA
0.5% NP40
add PMSF to 1 mM, $NaVO_3$ to 1 mM, aprotinin to 10 µg/ml and leupeptin to 10 µg/ml before use

Results

1. Optimisation of Conditions for Induction of Quiescence and Application of Cyclic Strain

Smooth muscle cells were grown with 0.5% FBS and 0%FBS with and without 1x ITS supplement to determine the optimum conditions for induction of quiescence with maximal cell adherence. Adherence of SMC grown under the above low serum contitions was compared to cells grown with 10% FBS as positive control using a cell counter. As shown in Figure 7, the best conditions for induction of quiescence with high cell adherence in bovine SMC were found to be with media containing 0% FBS with 1x ITS supplement. Cells grown under these conditions showed 94% adherence compared to only 84% adherence by SMC cultured in FBS-free media without ITS. Examination of cultures under microscopy prior to resuspension of the cells concurred with the cell count data. The same conditions used for bovine SMC were found to be excellent for induction of quiescence with the maintenance of adhesion in both bovine EC and rat SMC.

In each of the experiments performed, morphological examination of cultured cells under phase contrast microscopy before the experimental treatment showed that EC and SMC maintained the confluence and integrity of the monolayer after conditions used to induce quiescence. Plates in which cells had bunched or the monolayer was detached were discarded. The same examination after the experiment demonstrated that the cells had remained adherent to the culture plates throughout the experiment. This demonstrated that the treatments used were not destructive to the cells and that, where applicable, the cells were subject to the forces applied to the culture plates. If a disruption of adherence was noted, the plate was discarded. In addition, SMC which were subjected to cyclic strain were noted to orientate perpendicularly to the radial direction of cyclic strain (Figure 8) after longer time periods of cyclic stretch as noted in previous studies (Sumpio and Banes, 1988) although this was accompanied by some cell loss. This indicates that the 10 % level of stretch used was capable of influencing the physiology of adherent SMC and EC.

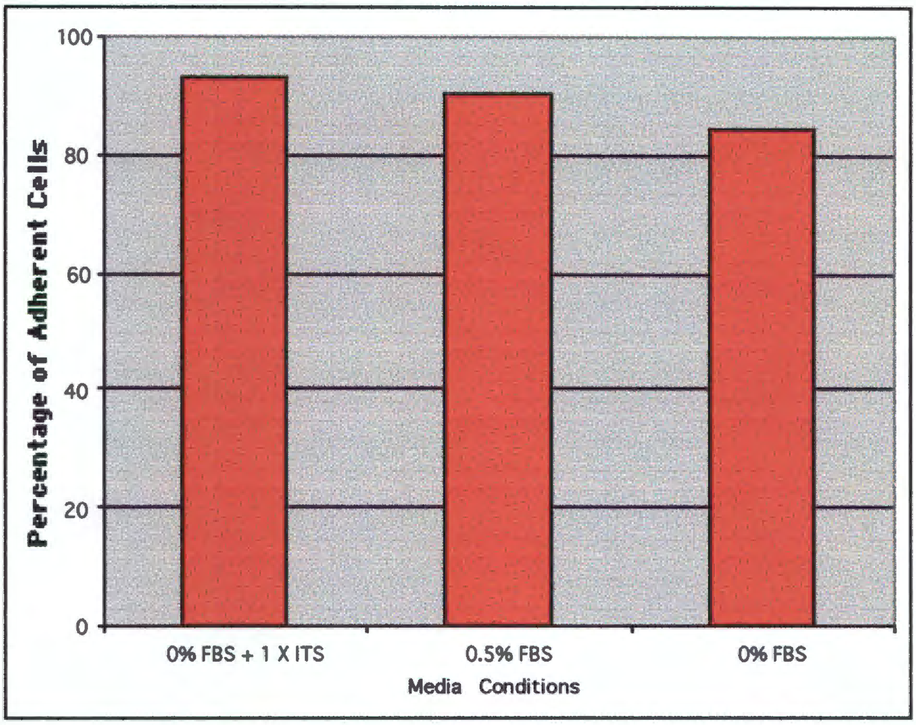

Figure 7. Optimisation of culture conditions for induction of quiescence. Bovine SMC were seeded at confluency on Flex I plates in media with 0.5% FBS or 0% FBS with or without ITS to determine the conditions which best induced quiescence but allowed maximal cell adherence. After 24 hours the plates were examined under phase contrast microscopy and washed 2x with PBS to remove floating cells. Adherent SMC were then resuspended by incubation with 0.01% trypsin and counted using a Coulter counter. Results are expressed as a percentage of adherent cells grown in 10% FBS. These results, which were corroborated by microscopy, show that SMC may be induced to adopt the quiescent phenotype with 0% FBS and 1x ITS with minimal loss of cells. The same conditions were found to be suitable for induction of quiescence in EC.

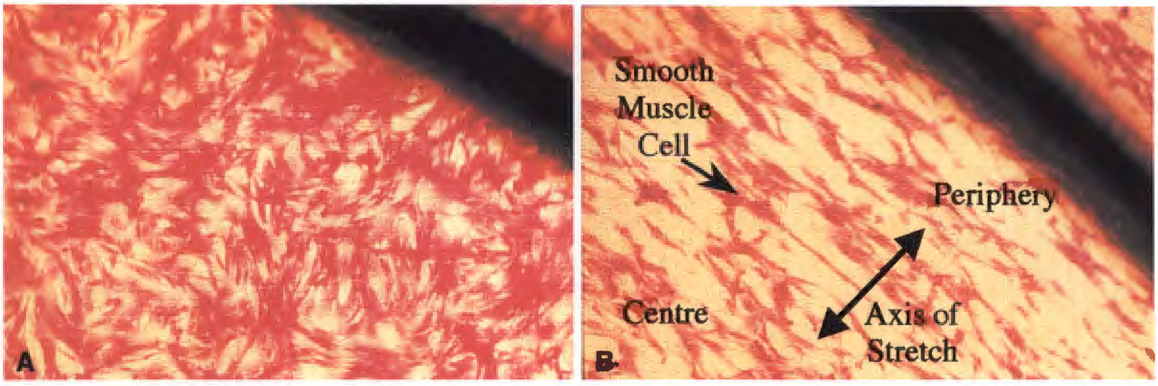

Figure 8. Photomicrographs taken at 100x magnification indicating morphological effects of cyclic stretch on SMC (Courtesy of I. Mills, Yale University School of Medicine, New Haven, USA) **A**: Unstretched SMC with a random alignment. **B**: SMC align perpendicularly to axis of stretch after 24 hours of 10%cyclic stretch. Axis of stretch, the centre and periphery of the dish and a smooth muscle cell are indicated in panel B. Cells exposed to shorter time points exhibited less dramatic alignment in proportion to the period of stretch experienced. This figure is representative of neither the degree of alignment nor the confluency of the SMC on the Flex I plates after 4 hours. As shown in panel B, a limited loss of adherence may be seen after 24 hours of cyclic stretch. However, this was not observed for 4 hours of cyclic stretch.

2. VEGF Expression in Smooth Muscle Cells under Cyclic Stretch

Optimisation of Controls

VEGF expression in bovine aortic SMC under cyclic stretch was determined by northern blotting. Control and experimental cells were seeded identically on Flex I plates with bFGF at 10 ng/ml added as a positive control for VEGF expression while unstretched cells served as the negative control for the effects of cyclic stretch. VEGF mRNA expression induced by bFGF at 10 ng/ml and 50 ng/ml was statistically indistinguishable ($p > 0.05$) after 3 hours (Figure 9). Furthermore, after 6 hours bFGF was found to bring about the same induction of VEGF mRNA expression at 10 ng/ml and 50 ng/ml (data not shown). Thus, bFGF at a concentration of 10 ng/ml was considered sufficient to induce VEGF mRNA expression in SMC for the 4 hour time period of the experiments and at a suitable level for a positive control. Addition of TNF-α, which is not known to induce VEGF expression in SMC, did not result in a significant alteration of VEGF mRNA expression from the levels observed in unstretched controls, thus demonstrating that the response to bFGF was specific and not due to the process of cytokine addition.

Optimisation of RNA Extraction, Northern Blotting and Autoradiography

RNA isolation from each 30 cm^2 Flexcell I plate yielded at least 10 ng of RNA of adequate integrity and purity for northern blotting as determined by UV spectroscopy and gel electrophoresis. Neither RNA degradation nor protein contamination was detected in any of the experiments considered (see Methods and Materials – RNA Extraction and Northern Blot Analysis - Gel Electrophoresis). In each experiment, the transfer of the mRNA to nylon membrane by northern blotting was found to be complete by comparison of the gel and membrane under UV radiation. Only membranes which displayed the 18S and 28S rRNA bands and whose corresponding gels did not contain visible amounts of RNA after transfer were subsequently probed. Probing the membrane for VEGF mRNA displayed a 4.2 kilobase (kb) band corresponding to the published molecular weight of VEGF mRNA and probing the same blot for GAPDH displayed a band corresponding to the published size of GAPDH mRNA at 1.2 kb for each experiment (Figures 9, 10 and 11). Comparison of the densitometric values obtained after scanning the GADPH bands indicated that mRNA loading and northern blot transfer was equal for all samples in each experiment.

VEGF mRNA Expression

In each experiment a weak VEGF mRNA signal was noted in samples from cells which were treated with neither bFGF nor cyclic stretch, indicating a low basal level of VEGF expression by these cells. As these cells were in the quiescent phenotype, this expression is due to neither serum factors nor switching by the SMC to the synthetic phenotype but rather a phenomenon of differentiated cells which has been observed previously *in vivo* (Couffinhal et al., 1997).

A time course measuring VEGF mRNA expression under cyclic stretch after various time intervals showed the upregulation of VEGF mRNA expression to be maximal, at greater than 3 fold of the expression in unstretched controls, after 4 hours. By 24 hours the expression of VEGF mRNA due to cyclic stretch had dropped slightly to 90 % of the expression from cyclic stretch at 4 hours (Figure 10). The induction of VEGF mRNA expression in the positive control - by addition of bFGF at 10 ng/ml - was also found to peak at around 4 hours and to drop to 80% of this peak value by 24 hours (Figure 10). These results show that the increase in VEGF mRNA expression shown at 4 hours is maintained, with a slight decrease, until 24 hours. Therefore, further experiments were carried out over a 4 hour time period.

Seven independent experiments, conducted with different preparations of SMC, in which application of cyclic stretch, RNA extraction, transfer and probing met stringency criteria were included in the study and their results are summarised in Figure 11. These experiments demonstrated an increase in VEGF mRNA expression to 3.3 fold (± 1.5) of unstretched controls after 4 hours. This was of a similar magnitude to the VEGF mRNA expression induced by bFGF at 10 ng/ml for 4 hours (Figure 11).

Species-Specificity Pilot Study

In order that the species specificity of this response could be investigated, a pilot study was undertaken in which rat aortic SMC were treated with the same regimen of cyclic stretch and positive control of bFGF at 10 ng/ml as the bovine cells. Rat SMC were found to exhibit averages of 5 fold (n = 1) and 2.7 fold (n = 2) increases in VEGF mRNA expression over unstretched controls to these stimuli respectively, indicating that the smooth muscle cell response to cyclic stretch in terms of VEGF mRNA expression may not be confined to the bovine model.

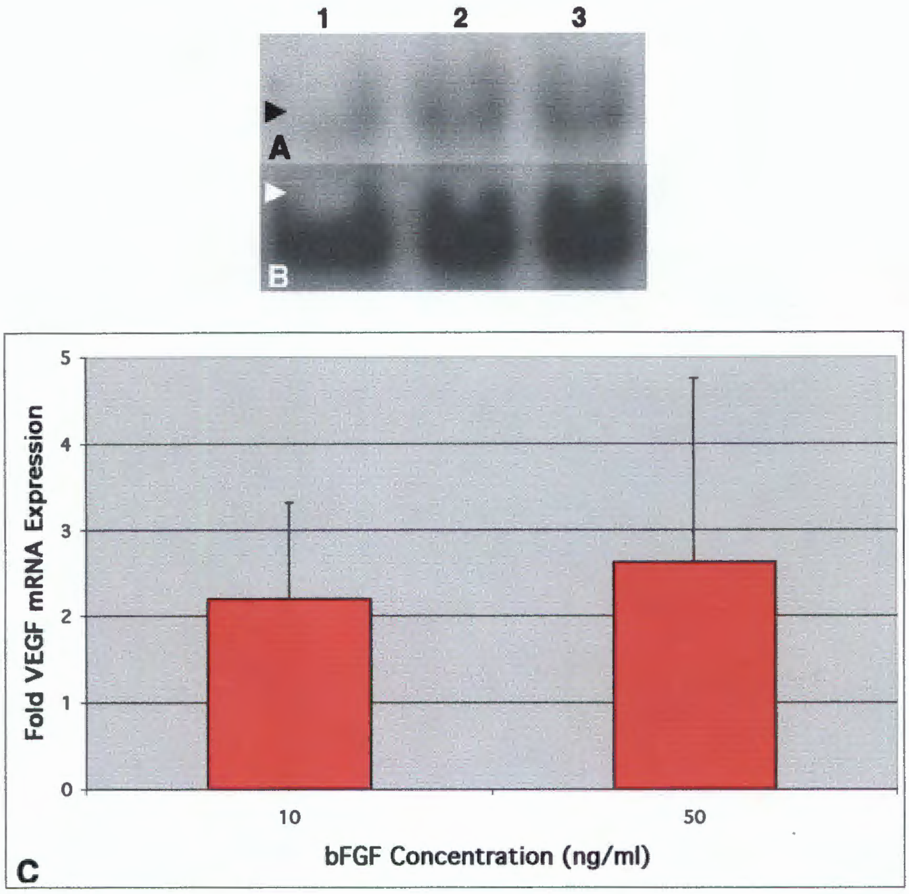

Figure 9. Northern blot showing VEGF mRNA expression in response to different concentrations of bFGF. The figure is a typical autoradiographic result of 5 experiments. **A**: VEGF. Total RNA of SMC probed for VEGF mRNA. The arrow represents the location of the 28S rRNA band. A band corresponding to the VEGF mRNA can be seen at low constitutive levels in untreated SMC in lane 1. Lane 2 and 3 contain mRNA from SMC stimulated for 3 hours with bFGF at 10 ng/ml and 50 ng/ml respectively. **B**: GAPDH. The same blot stripped and probed for GAPDH mRNA. The consistency of the band density in all lanes indicates equal mRNA loading and transfer as well as uniform probe hybridisation. The arrow represents the location of the 18S rRNA band. **C**: Densitometric analysis of the levels of VEGF mRNA expression corrected against GAPDH mRNA expression levels and expressed as a fold of untreated controls. Mean increases in VEGF mRNA expression due to bFGF at 10 and 50 ng/ml were indistinguishable at the 95% confidence interval (bars represent standard deviation, n = 5).

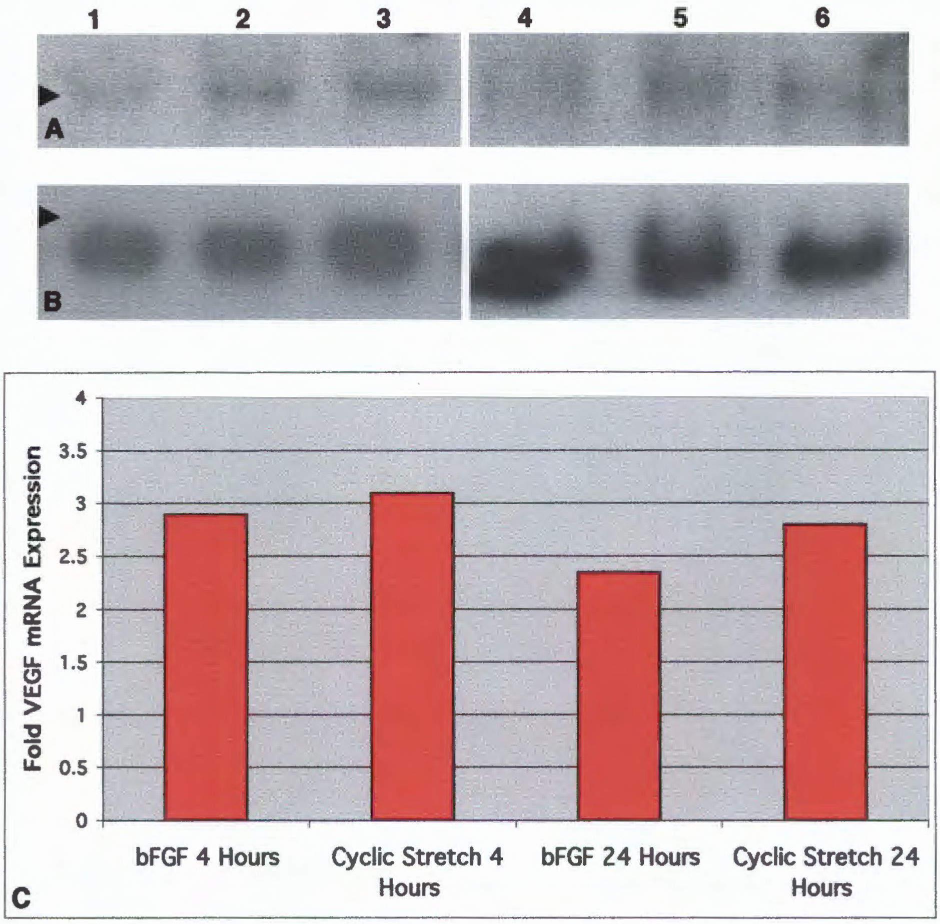

Figure 10. Northern blot showing change in VEGF mRNA expression due to both bFGF and cyclic stretch stimulation at 4 and 24 hours. **A**: VEGF. Total RNA of SMC probed for VEGF mRNA. The arrow represents the location of the 28S rRNA band. Comparison of the migration of the VEGF mRNA bands seen in lanes 1-6 with the migration of the 28S rRNA band indicates the size of the VEGF mRNA to be 4.2 kb. A band corresponding to the VEGF mRNA can be seen at low constitutive levels at 4 and 24 hours in unstretched SMC in lanes 1 and 4 respectively. Lanes 2 and 3 contain mRNA from SMC stimulated with bFGF at 10 ng/ml for 4 hours and 4 hours of 10% cyclic stretch respectively and each indicate an increase in VEGF mRNA expression over the unstretched control. Lanes 5 and 6 contain mRNA from SMC stimulated with bFGF at 10 ng/ml for 24 hours and 24 hours of 10% cyclic stretch respectively and show a slight decrease in VEGF mRNA expression relative to the expression at 4 hours. **B**: GAPDH. The same blot stripped and probed for GAPDH mRNA. The arrow represents the location of the 18S rRNA band. Comparison of the migration of the GAPDH mRNA bands seen in lanes 1-3 with the migration of the 18S band indicate the size of the GAPDH mRNA to be 1.2 kb. The figure represents a typical autoradiographic result. **C**: Densitometric analysis of the levels of VEGF mRNA expression corrected against GAPDH mRNA expression levels and expressed as a fold of unstretched controls. VEGF mRNA expression due to both bFGF and cyclic stretch can be seen to decrease slightly between 4 and 24 hours.

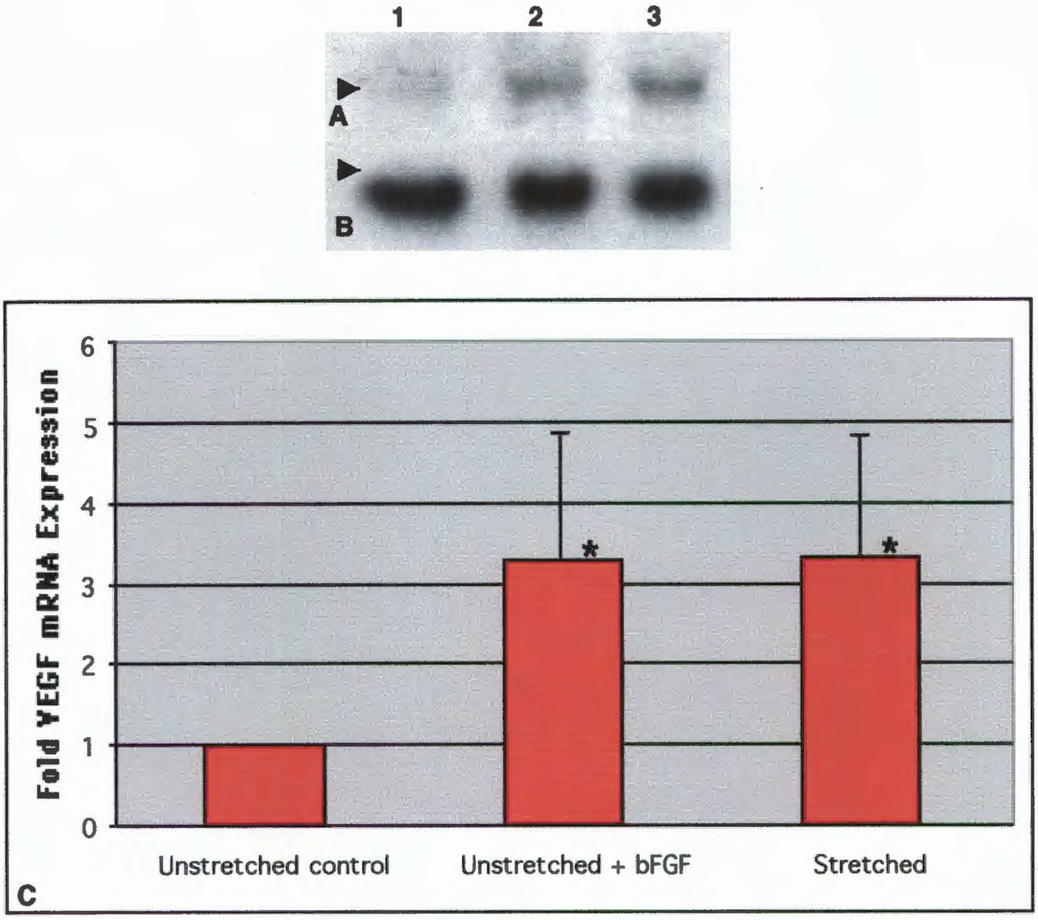

Figure 11. Northern blot showing an increase in VEGF mRNA expression due to both bFGF and cyclic stretch stimulation. The figure is a representative autoradiographic result for all 7 experiments. **A**: VEGF. Total RNA of SMC probed for VEGF mRNA. The arrow represents the location of the 28S rRNA band. Comparison of the migration of the VEGF mRNA bands seen in lanes 1-3 with the migration of the 28S rRNA band indicates the size of the VEGF mRNA to be 4.2 kb. A band corresponding to the VEGF mRNA can be seen at low constitutive levels in unstretched SMC in lane 1. Lane 2 and 3 contain mRNA from SMC stimulated with bFGF at 10 ng/ml for 4 hours and 4 hours of 10% cyclic stretch respectively and each indicate a dramatic increase in VEGF mRNA expression. **B**: GAPDH. The same blot stripped and probed for GAPDH mRNA. The consistency of the band density in all lanes indicates equal mRNA loading and transfer as well as uniform probe hybridisation. The arrow represents the location of the 18S rRNA band. Comparison of the migration of the GAPDH mRNA bands seen in lanes 1-3 with the migration of the 18S band indicate the size of the GAPDH mRNA to be 1.2 kb. The figure represents a typical autoradiographic result. **C**: Densitometric analysis of the levels of VEGF mRNA expression corrected against GAPDH mRNA expression levels and expressed as a fold of unstretched controls. Both bFGF and cyclic stretch stimulated cells upregulated VEGF mRNA expression in SMC to a statistically significant degree over unstimulated controls at the 99% confidence interval (bars represent standard deviation, * indicates $p < 0.005$, $n = 7$).

3. Activity of VEGF Released by Smooth Muscle Cells

Optimisation of Controls

VEGF has been demonstrated to have effects on diverse aspects of endothelial cell physiology including migration and survival. To determine the biological activity on EC of VEGF released by SMC, the culture media from quiescent SMC - both unstretched and stretched for 4 hours - were analysed for their ability to induce migration in quiescent EC in an *in vitro* chemotaxis assay. Unconditioned smooth muscle cell media was used as a negative control while the media from SMC exposed to bFGF at 10 ng/ml for 4 hours was chosen as a positive control. The pH and clarity of media collected from all SMC was found to be similar to that of unconditioned media indicating that smooth muscle cell conditioning had not led to pH changes or contamination.

Both at assembly of the migration chamber (Figure 6) and after the execution of the assay each well was inspected for air-bubbles obstructing the interface of the EC and the test media. If a bubble was found in a well of any of the variables in a given experiment the entire experiment was discarded. On these criteria, 6 independent experimental repeats were included in this study and migration analysed by counting fixed and stained filters.

Antibody Abrogation

In addition to the above controls, anti-VEGF antibody was added to a duplicate repeat of conditioned media from each condition to neutralise any VEGF in the media. Comparison of endothelial cell migration induced by the conditioned media in the presence and absence of the anti-VEGF antibody will establish the proportion of the endothelial cell migration that is due to VEGF. This is important as it demonstrates whether VEGF plays a part in the endothelial migration due to smooth muscle cell conditioned media and also quantifies the contribution of VEGF to the migratory response, establishing a baseline for comparing the results. As shown in Figure 12, the increases in endothelial cell migration due to conditioned media from stretched, bFGF-stimulated and unstretched SMC were not completely inhibited by the addition of the anti-VEGF antibody. This indicates the possible involvement of other factors in the migratory response of EC to media. However, the statistically significant ($p < 0.05$) decrease in the migration increases following addition of anti-VEGF in all samples indicates that VEGF may be a chief factor involved.

In one of the 6 experimental repeats mentioned above the migration values induced by anti-VEGF antibody treated media from bFGF- and stretch-stimulated SMC were respectively 4.1 and 6.4 fold higher than that of the average migration induced by the same antibody-treated samples in the other experiments. This result was possibly due to failure of the antibody and the repeat was considered aberrant and not considered. The results of the remaining 5 experiments are considered below and shown in Figure 12.

Endothelial Cell Migration

All media conditioned by SMC were shown to induce migration of EC (Figure 12). Conditioning of media by unstimulated SMC resulted in an 8.3 (± 2.4) fold increase in endothelial cell migration compared to unconditioned media. This dropped to 3.2 (± 1.3) fold with the addition of anti-VEGF antibody, indicating that the majority of the endothelial cell migration response to media from unstimulated SMC resulted from the presence of VEGF in the media. Conditioned media from SMC exposed to bFGF at 10 ng/ml to for 4 hours resulted in endothelial migration to 8.2 (± 3.8) fold of unconditioned media (5.5 (± 3.0) fold with anti-VEGF). These data show that bFGF did not induce release of endothelial cell chemotactic molecules by the SMC into the conditioned medium to a greater extent than unstretched controls alone. In addition, comparison of results with and without anti-VEGF antibody indicate that VEGF contributed only 40% to the endothelial migration response to conditioned media from SMC treated with bFGF. Cyclic stretch for 4 hours was, by contrast, found to stimulate the production of soluble factors by the SMC which increased endothelial cell migration to 14.6 (± 2.8) fold of the level of unconditioned media. This value was diminished to 6.7 (± 3.0) fold with anti-

VEGF antibody, indicating that VEGF contributed to 55% of the endothelial migratory response. The relevance of the results is covered in the discussion section of this work.

ELISA

In order to determine the levels of VEGF released by SMC under cyclic stretch, a pilot ELISA study ($n = 3$) was carried out over 24 hours on the conditioned culture media of SMC. The media from bFGF-stimulated SMC served as a positive control and results were compared to human VEGF standards to quantify the VEGF concentration (Table 2).

In this way bFGF and cyclic stretch were found respectively to induce average secretion of 8.7 ng/ml and 7.1 ng/ml of VEGF into the media over 24 hours. By comparison, cumulative levels of VEGF secretion by unstimulated SMC during the course of induction of quiescence and the experiment were approximately 6.0 ng/ml. This value is high in comparison to the levels secreted by stimulated cells. Nevertheless, VEGF levels in the media were corrected against control media from unstimulated SMC to determine the relative expression due to bFGF and cyclic stretch. In the bFGF positive control the levels of VEGF corresponded to a statistically significant ($p < 0.05$) 1.5 (± 0.2) fold average increase of VEGF over unstimulated cells. However, in media from SMC stretched a statistically insignificant ($p > 0.05$) increase in average VEGF levels of 1.2 (± 0.2) fold of the levels of unstimulated controls was found (Table 2). These apparently contradictory results and the possible mechanisms behind them are discussed in the discussion section of this work.

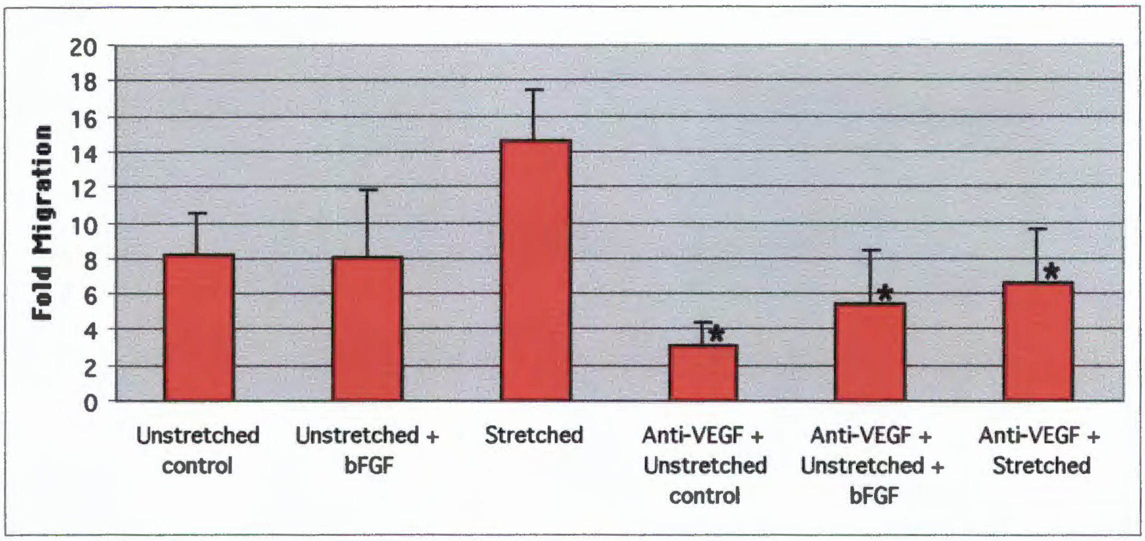

Figure 12. Graph showing endothelial cell migration in response to media from SMC. Results are standardised against the EC migratory response to unconditioned smooth muscle cell media and expressed as a fold of this response (bars represent standard deviation, $n = 5$). Endothelial cells migration was increased in response to media from unstretched SMC, bFGF treated SMC and SMC exposed to cyclic stretch. The high chemotactic response to media from unstretched control cells may be due to the release of chemotactic factors such as VEGF and bFGF by these cells over time during induction of quiescence. This is supported by the inability of media from bFGF-stimulated SMC to increase the VEGF-mediated migratory response of EC over the response due to media from unstretched control SMC. The relevance of these findings is addressed in the discussion section of this work. The statistically significant reduction in migration (* indicates $p < 0.05$) due to the addition of a VEGF-neutralising antibody in all cases indicates that although not alone, VEGF may be a chief mediator of the chemotactic response of EC to the conditioned media of unstretched and stretched SMC. It also demonstrates that VEGF is released by the SMC in a biologically active form at sufficient levels to elicit a response by the EC. An increase in VEGF secretion into the media of SMC due to cyclic stretch is consonant with the increase in VEGF expression due to the cyclic stretch noted at the mRNA level above.

Media Sample	**Unstimulated**	**10ng/ml bFGF 24 Hrs**	**10% Cyclic stretch 24 Hrs**
VEGF concentration (ng/ml)	6.0	8.7	7.1
Fold VEGF Production (*n*=3)	1	1.5 (± 0.2) *	1.2 (± 0.2)

Table 2. VEGF protein levels in smooth muscle cell conditioned media as determined by ELISA. VEGF protein was found in all smooth muscle conditioned media. The level of VEGF secretion in unstimulated SMC correlates with the constitutive expression in unstimulated SMC demonstrated in this work by northern blot and previously shown *in vivo* (Couffinhal et al., 1997). However, the high concentration found in these cells after 24 hours may mask the release of VEGF due to bFGF and stretch during this time. Thus, although the concentration of VEGF in the media is increased by both the addition of bFGF and cyclic stretch, only the increase due to bFGF is statistically relevant at the 95% confidence interval (* Denotes $p < 0.05$).

4. Effect of Cyclic Stretch on KDR Phosphorylation in Endothelial Cells

Optimisation of Controls

Although VEGF released by SMC under cyclic stretch *in vivo* may influence the adjacent EC, a number of studies suggest that it is possible for VEGF receptors on EC to be phosphorylated by cyclic stretch in the absence of VEGF from SMC or EC. If KDR (Flk-1) is activated purely as a mechanoreceptor by cyclic stretch then this may affect the endothelial response to locally-released VEGF. To further investigate whether KDR is a mechanoreceptor quiescent EC were stretched on Flex I plates, proteins extracted and KDR phosphorylation determined by immunoprecipitation for KDR and immunoblotting with an anti-tyrosine phosphorylation antibody PY-20. Quiescent cells were used as they mimic the non-secretory, non-proliferative phenotype found *in vivo* and cells in this phenotype are not expected to release VEGF (Namiki et al., 1995; Couffinhal et al., 1997).

VEGF at the published value of 50 ng/ml (Waltenberger et al., 1994; Nor et al., 1999; Gerber et al., 1998) was added to unstretched EC as a positive control for KDR phosphorylation while unstretched cells served as the negative control for the effects of cyclic stretch. VEGF at 50 ng/ml was found to increase KDR phosphorylation to an average maximum of 12.3 fold of unstretched controls by 20 minutes of exposure. This concentration and time was thus considered appropriate for a positive control for KDR phosphorylation. Blots which showed no phosphorylation of KDR in response to VEGF were discarded.

Optimisation of Protein Isolation, Immunoprecipitation and Gel Electrophoresis

Protein isolation from each plate yielded 300 – 500 μg of protein for immunoprecipitation as determined by the Bradford assay. If less than 300 μg of protein was available the experiment was discontinued. Similarly, if the protein samples ran in a streak during gel electrophoresis, indicative of protein degradation, rather than as discrete bands, the samples were discarded. Following electrophoresis, the protein transfer to the nitrocellulose membrane was found to be thorough in all experiments by staining the gel and observation of the transfer of the prestained molecular weight marker.

Visualisation

Bands obtained in immunoblotting were compared with the prestained molecular weight marker and found to be at the published value of 210 kDa for KDR (Brogi et al., 1996). The identity of KDR was further confirmed by stripping the same blot and probing with an antibody specific for KDR. Although a band of the same size and shape was present when immunoblotting with KDR, as confirmed by comparison with the molecular weight marker, there were unfortunately a high number of background bands after stripping and reprobing of the blot (Figure 13 B). These extra bands may be due to inter-species cross-reactions by the antibody or high non-specific binding after stripping. However, the constancy of band densities for KDR over time indicated that the levels of KDR expression remained constant under cyclic stretch and did not contribute to changes in levels of KDR phosphorylation (Figure 13 B). Only blots which showed visible bands at 210 kDa after probing with both PY-20 and the anti-KDR antibody were analysed by densitometry.

KDR Phosphorylation

Time courses for phosphorylation of KDR determined that values of phosphorylation before 30 minutes were similar to the values at 30 minutes (1.4 fold of unstretched controls at 10 minutes) and were found to plateau at approximately 3 fold of that of unstretched cells after 180 minutes. Therefore, further experiments were carried out between 30 and 120 minutes. Four independent experiments (assigned 1 – 4), conducted with distinct preparations of EC, met the stringency criteria in terms of application of cyclic stretch, response to VEGF, isolation of sufficient undenatured protein, efficient transfer and identification of a signal for KDR following immunoblotting and their results are summarised in Figure 13. Experiment 1 displayed an initial decrease in phosphorylation compared to unstretched controls (fold phosphorylation < 1 fold of unstretched controls) to 90 minutes followed by an increase in phosphorylation to greater than 3 fold of unstretched values at 120 minutes (Figure 13 C). Experiment 2 followed a similar trend to experiment 1 although phosphorylation levels at 30 and 60 minutes were both higher (greater than 3.5 fold of unstretched controls) than those found at the same time intervals in experiment 1. Although these results are included in the study they appear to be anomalous when compared to the low phosphorylation values at 30 and 60 minutes obtained in the other experiments and the values from experiment 2 lead to the large standard deviation in the mean of all the experiments at 30 and 60 minutes (Figure 13 C). Experiments 3 and 4 followed the same initial decrease followed by an overall increase in phosphorylation over time as seen in experiment 1 although the response at 90 and 120 minutes in experiment 4 was lower than that seen in the other experiments (Figure 13 C). This may be due to an unobserved difference in the cells following induction of quiescence, which resulted in a muted or delayed response. This result appears to deviate from the robust increase in phosphorylation observed at 90 and 120 minutes in the other experiments and the values from experiment 4 mutes the mean increase in phosphorylation for all the experiments. Furthermore, this result diminishes the statistical significance of the differences of the mean responses at 90 and 120 minutes from unstretched controls (120 minutes relevant at the 95% confidence interval ($p < 0.05$) and 90 minutes relevant at the 93% confidence interval ($p < 0.07$) without values from experiment 4). As demonstrated by the mean values for the 4 experiments (Figure 13 C), cyclic stretch appears to induce an initial statistically irrelevant decrease in receptor phosphorylation followed by a modest increase in KDR phosphorylation to 2.5 fold of the unstretched control which is only statistically significant at the 94% confidence interval ($p < 0.06$ $n = 4$). Both the biological relevance of the trends observed and whether the mechanisms behind them are direct or indirect are addressed in the discussion section of this work.

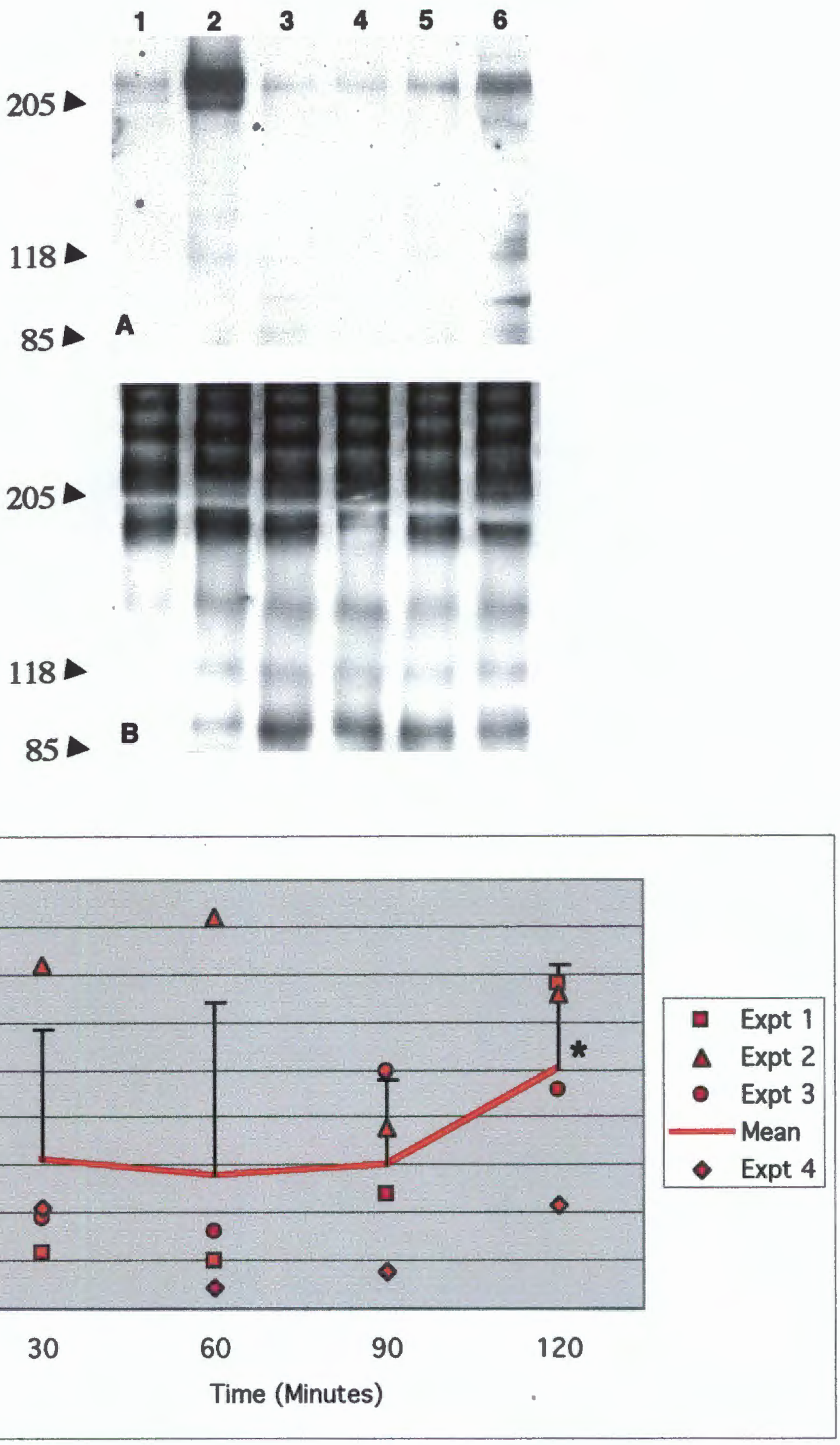

Figure 13. Effect of cyclic stretch on KDR phosphorylation in EC. **A**: Immunoblot with PY-20 for KDR (Flk-1) phosphorylation. Arrows indicate the position and size of the molecular weight marker standards. The 209 kDa band corresponding to KDR is just above the 205 kDa molecular weight marker. Bands for KDR were identified by their molecular weight of approximately 210 kDa as determined by comparison with the molecular weight marker and by subsequent probing with anti-KDR antibody. Lanes: 1.

Cell lysate of unstretched negative control; 2. Cell lysate of EC stimulated with VEGF at 50 ng/ml for 20 minutes demonstrates a 10-fold increase in KDR phosphorylation as compared with the unstretched control; 3 to 6. contain cell lysates of EC subjected to 30, 60 , 90 and 120 minute of cyclic stretch respectively. The figure represents a typical result. Phosphorylation can be seen to decrease slightly relative to the unstretched control at 30 and 60 minutes but to increase dramatically at 90 and 120 minutes to reach 3 fold of unstretched values. **B**: Immunoblot with anti-KDR antibody. The same blot was stripped and probed with anti-KDR antibody. Although there is a high background after stripping, for reasons covered above, the constancy of band density in all lanes of the blot demonstrates that protein loading is equal for all samples. Thus, the increase in KDR phosphorylation in stretched EC observed in A occurs without an increase in KDR levels. **C**: Densitometric analysis of the levels of KDR phosphorylation over time for 4 experiments expressed as fold of KDR phosphorylation in unstretched controls and corrected against KDR protein levels. Results for each experiment are elaborated on in the text. Despite the outlying values at 30 and 60 minutes in experiment 2 (▲) and the muted response at all time points in experiment 4 (◆), the overall trend, as portrayed by the mean value for the four experiments (red line), is an increase in KDR phosphorylation in response to cyclic stretch over time to a 2.5 fold response at 120 minutes. This response is statistically relevant compared to unstretched controls at the 94% confidence interval (vertical bars represent standard deviation, * indicates $p < 0.06$, $n = 4$).

5. RT-PCR Determined Cytokine Expression in Smooth Muscle Cells under Cyclic Stretch

Optimisation of RT-PCR and Controls

Contractile cells such as SMC are known to produce a variety of cytokines under conditions of cyclic stretch. Accordingly, a pilot study to determine the feasibility of using RT-PCR to determine the expression of VEGF, PDGF-B, TGF-β and bFGF mRNA in SMC under cyclic stretch for 4-24 hours was carried out as described above (Methods and Materials - RT-PCR) using published primers. The results of gel electrophoresis of PCR products, which are summarised in Figure 14, indicate a distinct DNA product band for VEGF, TGF-β and bFGF at the published molecular weight (as confirmed by comparison with a common DNA molecular weight ladder). This demonstrates that the primers and conditions listed above (Methods and Materials – Table 1) are suitable for RT-PCR analysis of expression of these cytokines in SMC under cyclic stretch. In all cases, no DNA band was obtained in controls in which RT was excluded, indicating that PCR products were not due to unspecific binding of the primers (Figure 14 A, lane 2). This indicates the expression of VEGF, bFGF and TGF-β mRNA by SMC after 4-24 hours of cyclic stretch.

Cytokine Expression in Smooth Muscle Cells under Cyclic Stretch

Following gel electrophoresis, VEGF PCR DNA products were visualised as two bands (Figure 14 A, lane 3), determined to be 312 bp and 180 bp in size, which represent the VEGF_{165} and VEGF_{121} isoforms respectively. This expression confirms the expression of VEGF mRNA by SMC under cyclic stretch found in northern blots and mentioned above (Results - VEGF Expression in Smooth Muscle Cells under Cyclic Stretch – VEGF mRNA Expression). The band corresponding to the PCR DNA product of amplification with primers to bFGF was found to be of the published value of 300 bp (Figure 14 B, lane 2). TGF-β PCR DNA products were similarly detected at 161 bp (Figure 14 C, lane

2). PDGF-B PCR DNA products were not detected despite exhaustive manipulation of conditions for reasons which were not due to experimental failure but possibly cellular considerations covered in the discussion section of this work. In addition, 100 bp PCR DNA products following RT-PCR carried out to detect β-actin mRNA from under varying conditions of cyclic stretch were found to be constant (Figure 14 D, lanes 2-6). This ideally suits its use as a control against which to standardise the expression of cytokine mRNA under cyclic stretch. Finally, HPRT PCR DNA product was found to be expressed at its published value of 378 bp, allowing it to be used as an alternative standardising control (Figure 14 E, lane 2). These results demonstrate that VEGF, bFGF and TGF-β mRNA are expressed by SMC under cyclic stretch and RT-PCR can be used to determine the expression of these cytokines and appropriate controls by SMC under cyclic stretch.

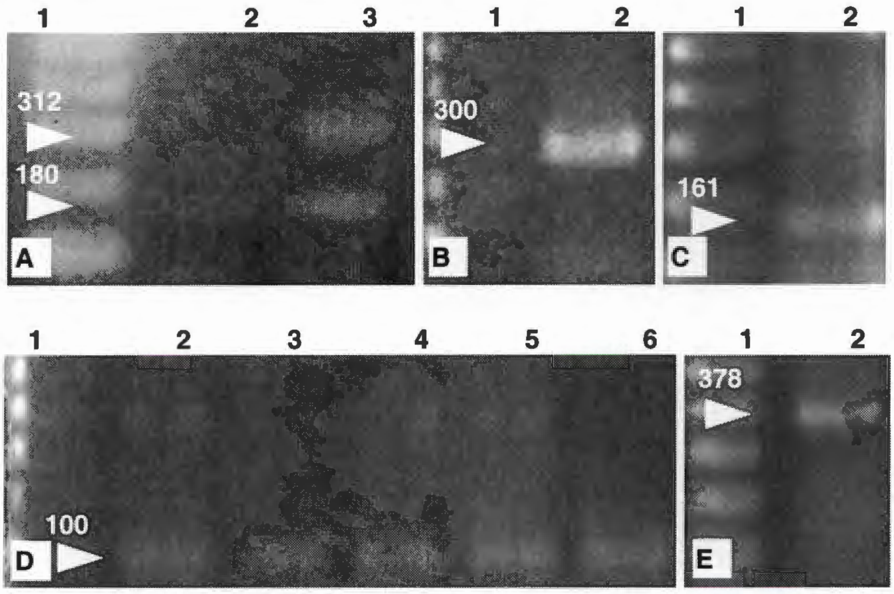

Figure 14. DNA gels of products of RT-PCR performed on RNA from SMC under cyclic stretch. Gels are representative of results obtained. Band sizes, as confirmed by comparison with DNA ladders in lane 1 in each gel, are indicated in base pairs (bp) by arrows. **A**: VEGF. Lanes: 1 100 bp DNA ladder; 2 no RT negative control (not shown for other cytokines); 3 PCR DNA product from SMC subjected to 4 hours of cyclic stretch showing two bands at 180 bp and 312 bp corresponding to mRNA for the VEGF$_{121}$ and VEGF$_{165}$ isoforms respectively. **B**: bFGF. Lanes: 1 100 bp DNA ladder; 2 PCR DNA product from SMC subjected to 4 hours of cyclic stretch showing a band at 300 bp corresponding to the bFGF mRNA. **C**: TGF-β. Lanes: 1 100 bp DNA ladder; 2 PCR DNA product from SMC subjected to 4 hours of cyclic stretch showing a band at 161 bp corresponding to the TGF-β mRNA. **D**: β-actin. Lanes: 1 100 bp DNA ladder; 2 and 3 PCR DNA product from unstretched control SMC primed in the RT reaction by random primer and oligo-nucleotide primer respectively; 4-6 PCR DNA product from SMC subjected to 1, 24 and 8 hours of cyclic stretch respectively. **E**: HPRT. Lanes: 1 100 bp DNA ladder; 2 PCR DNA product from SMC subjected to 4 hours of cyclic stretch showing a band at 378 bp corresponding to the HPRT mRNA.

Discussion

1. Experiments conducted and Induction of Quiescence

This study investigates the role which cyclic stretch might play in VEGF expression by SMC in a compliant environment. The time course and magnitude of VEGF mRNA expression due to cyclic stretch were measured by northern blotting. In addition, the levels of VEGF secreted into the medium by SMC under cyclic stretch and the biological activity on EC of secreted VEGF were investigated using an ELISA and migration assay respectively.

Furthermore, following observations of KDR receptor phosphorylation in response to shear stress (Chen et al., 1999), the effect of cyclic strain on KDR phosphorylation was investigated by immunoprecipitation and western blotting of stretched EC to determine whether KDR acts as a mechanoreceptor for cyclic stretch. This may affect KDR-mediated endothelial response to locally-released VEGF.

For these experiments low passage number bovine aortic SMC and EC were plated on collagen-coated elastomer plates and subjected to 10% repetitive strain at 1 Hz. In order to best approximate the *in vivo* situation, where the majority of the SMC are found in the quiescent / contractile phenotype, cells used in these experiments were induced to quiesce by culturing them in 0% FBS prior to the experimental procedure. The removal of serum has the added benefit of creating a common experimental baseline as commercially available FBS contains an undefined mixture of hormones, nutrients and other components, which may lead to SMC stimulation and experimental variation. This is especially important when using bovine cells and FBS. Serum is unphysiological for SMC, which are never exposed to serum *in vivo*, however, serum starvation in culture is also an unphysiological environment for SMC and leads to increased proteolysis (Libby and O'Brien, 1984) because specific factors in serum are necessary to maintain SMC viability in culture. Therefore, the supplementation of smooth muscle media with some of these factors in ITS supplement (Libby and O'Brien, 1983) allows a cellular phenotype which approaches that found in the artery without a large loss in viability (Figure 7). In the experiments in this study this means that SMC under cyclic stretch are not stimulated by factors in the media and are in the contractile phenotype. The very limited expression of growth factors and non-proliferative characteristics of SMC in this phenotype ensure that any VEGF expression arises from the stimulus being tested and neither from activated smooth muscle cell secretion nor an increase in cell numbers. Furthermore, the absence of extraneous factors in the media allows the easy detection of the released VEGF by ELISA and endothelial migration assay.

In like manner, the maintenance of EC in the quiescent phenotype by the use of serum-free culture both ensures that EC are physiologically close to the state found *in vivo* and that they are not subject to stimulation by other factors found in serum. However, as with SMC, ITS supplements are required for survival as unsupplemented serum-free culture of EC has been shown to lead to apoptosis (Gerber et al., 1998). In this study the maintenance of EC in the quiescent phenotype ensures that endothelial migration in the migration assay is due to factors in the smooth muscle cell-conditioned serum and that phosphorylation of the KDR receptor in the cyclic stretch experiments does not occur due to factors released by active EC. Furthermore, non-proliferative quiescent cells will not bias results as there will be a minimal increase in cell numbers during the experiment.

2. VEGF Expression in Smooth Muscle Cells under Cyclic Stretch

Time Course of VEGF Expression

The experimental conditions were based on the work of Gruden et al (1997) and Seko et al (1999), which previously demonstrated the upregulation of VEGF in other contractile cell types under cyclic stretch. Quiescent SMC were exposed to various periods of cyclic stretch followed by northern blot analysis to determine the expression of VEGF mRNA by these cells. Unstretched SMC served as a negative control while bFGF was added as a positive control for VEGF expression. VEGF mRNA expression in aortic SMC was found to increase due to cyclic stretch to greater than 3 fold of unstretched controls at 4 hours (Figures 10 and 11) and decrease slightly thereafter to 90% of this value by 24 hours. This time course of VEGF mRNA expression in SMC under cyclic stretch is consistent with the VEGF mRNA expression profiles over time which were shown in other studies to be induced in SMC by serum (Williams et al., 1995), cytokines (Brogi et al., 1994; Pedram et al., 1997), or other known VEGF inducers (Namiki et al., 1995). The time course of VEGF expression found in SMC is also similar to the time course of VEGF expression induced by cyclic stretch in contractile mesangial cells (Gruden et al., 1997). The similarity in VEGF expression due to cyclic stretch between SMC and mesangial cells, which are the smooth muscle cell-like pericyte adjacent to the endothelium in the kidney, further underlines their functional and structural similarities (Schlondorff, 1987).

The VEGF expression response of SMC to the bFGF positive control in this work followed a similar pattern of expression to that obtained due to cyclic stretch. This pattern was also consistent with previous findings in other work (Stavri et al., 1995a) although the addition of 10 ng/ml of bFGF has previously been shown to increase VEGF mRNA in a similar time course although more rapidly and robustly in rabbit aortic SMC (Stavri et al., 1995a). The consistent response in bovine cells to bFGF at 10 ng/ml throughout our experiments indicates that this difference in levels of VEGF mRNA expression may be specific to the species or experimental set-up used. Preliminary experiments with rat SMC showed similar patterns of VEGF mRNA expression due to bFGF at 10 ng/ml and cyclic stretch indicating that the production of VEGF in response to bFGF at 10 ng/ml and cyclic stretch may be a common response for both bovine and rat SMC.

Possible Mechanisms behind VEGF Expression

The increase in VEGF mRNA expression due to cyclic stretch noted in this study may be due to the direct action of biomechanical forces on SMC such as noted in studies using

SMC and other contractile cells (Gruden et al., 1997; Wilson et al., 1993; Li et al., 1997a; Seko et al., 1999) and EC subjected to shear stress (Cucina et al., 1998). Alternatively, given the large number of cytokines which have been shown to induce VEGF expression in SMC (Brogi et al., 1994; Stavri et al., 1995a; Stavri et al., 1995b), it is possible that this VEGF expression may be a secondary result under the autocrine mediation of other cytokines such as bFGF, PDGF-B and TGF-β (Seko et al., 1999). However, such a mechanism would simply shift the basis of the response into the next link of the chain, meaning that stretch induced upregulation of these cytokines leads to the upregulation of VEGF.

It is worthwhile noting that the VEGF mRNA expression responses by SMC to bFGF and cyclic stretch in this study are similar in both magnitude and time course (Figures 10 and 11). Furthermore, preliminary RT-PCR analysis showed that bFGF mRNA may also be expressed by SMC under cyclic stretch (Figure 14), although whether it was expressed prior to 4 hours was not investigated. As bFGF has previously been shown to induce the rapid expression of VEGF (Stavri et al., 1995a), these findings leave the question open as to whether bFGF is indirectly the trigger for the VEGF mRNA upregulation by SMC in response to cyclic stretch in the present study. Although the 4 hour time course of expression of VEGF under cyclic stretch demonstrated here appears to be too short for novel bFGF transcription and translation followed by induction of VEGF expression, cyclic stretch may induce release of bFGF stores from intact cells or from the basement membrane produced *in vitro* (Stiemer et al., 1993). This mechanism has been proposed for the release of TGF-β by stretched myocardial cells (Li et al., 1997a). Another possible mechanism of release for bFGF is from damaged cells (Stavri et al., 1995a) although no visible damage to the monolayer was seen after application of cyclic stretch for 4 hours. It is unfortunate that without the complete controls the results from RT-PCR for bFGF do not demonstrate that bFGF expression results from cyclic stretch. However, other studies have shown bFGF to be released from SMC stretched at higher than physiological levels (Cheng et al., 1997). Basic FGF has been shown to be released by SMC after only 15 minutes of cyclic stretch at 14% (Cheng et al., 1997) and low levels of bFGF (10 ng/ml) have previously been shown to induce dramatic VEGF expression by 1 hour (Stavri et al., 1995a). Therefore an autocrine mechanism for upregulation of VEGF mRNA by cyclic stretch involving bFGF would fit within the 4 hour time course noted for stretch-induced VEGF mRNA expression in this study.

Furthermore, according to the time course for stretch- and bFGF-induced expression of VEGF mRNA in the present study, VEGF mRNA upregulation in response to both stimuli had diminished by 24 hours. Therefore, it is possible that, if bFGF is involved in mediation of the increased expression of VEGF by SMC, this effect diminishes as the time of exposure approaches 24 hours. This hypothesis agrees with another study that observed that bFGF was not involved in the smooth muscle cell response to cyclic stretch after 48 hours, although shorter time periods were not considered in their study (Wilson et al., 1993). However, VEGF expression has very recently been demonstrated in SMC in the absence of bFGF expression (Feng et al., 1999) and in response to levels of cyclic stretch (4%) which do not result in significant bFGF release (Cheng et al., 1997) indicating that VEGF expression due to cyclic stretch may occur independently of bFGF expression at low levels of stretch. In addition, the distinct patterns of VEGF protein secretion in stretch- and bFGF-stimulated SMC after 4 hours, - demonstrated by endothelial cell migration studies (Figure 12) - indicate that, although the magnitude of VEGF mRNA upregulation by the two stimuli in this study may be similar at 4 hours, the mechanisms underlying the upregulation may be distinct. More work is required to determine whether bFGF plays a significant role in the induction of VEGF expression at the physiologically relevant 10% cyclic stretch.

PDGF, is an alternative candidate for mediating the VEGF mRNA upregulation in response to cyclic stretch in SMC. A marked upregulation in release of PDGF-A and PDGF-B by SMC under cyclic stretch has been previously demonstrated (Wilson et al., 1993) and 10 ng/ml of PDGF-BB has been shown by other groups to induce VEGF mRNA expression by 3 hours (Brogi et al., 1994; Williams et al., 1995). Therefore, an autocrine mechanism for the stretch-induced expression of VEGF mRNA expression involving the release of PDGF-B could occur within the 4 hour increase in VEGF mRNA expression found in the present study. However, the preliminary RT-PCR data did not show PDGF-B mRNA expression in SMC under cyclic stretch for 4 hours despite exhaustive manipulation of conditions used. However, without appropriate controls it is difficult to interpret this finding. Cross species hybridisation for this assay is probable due to high sequence homology (Herren et al., 1993; Lindner et al., 1995) so the lack of expression may be due to the predominant expression of the PDGF-A isoform by the cells due to differences in smooth muscle cell origin (Malek et al., 1993) or because the time of stretch is shorter than the 24 hours of cyclic stretch previously shown to cause PDGF-B mRNA expression (Wilson et al., 1993). It is possible that PDGF-B may not be expressed by SMC under cyclic stretch after 4 hours and may therefore play no role in the expression of VEGF seen at this time. It is hoped that with the appropriate controls, quantitative RT-PCR can determine the role, if any of PDGF-B in autocrine control of VEGF expression. Another possible mechanism for VEGF expression due to cyclic stretch is via mediation of the PDGF receptor-α. The activation of the PDGF receptor-α by cyclic stretch, which has been shown by another group to occur before 30 minutes in the absence of either PDGF ligand (Hu et al., 1998), may initiate smooth muscle cell responses, such as increased VEGF expression, normally initiated by the activation of the receptor by PDGF-B and may lead to the induction of VEGF mRNA by SMC under cyclic stretch in this study.

VEGF mRNA has also been shown by other groups to be rapidly upregulated in response to cyclic stretch in contractile cardiac myocytes under the mediation of TGF-β (Li et al., 1997a; Seko et al., 1999) and it is possible that a similar mechanism occurs with SMC, where as little as 1 ng/ml of TGF-β has been shown to induce the expression of VEGF after 3 hours (Brogi et al., 1994). Given the rapid rise in TGF-β levels following cyclic stretch it has been hypothesised to be released from cellular stores by cyclic stretch rather than produced by the induction of novel TGF-β transcription (Li et al., 1997a). In this study, preliminary analysis using RT-PCR showed that TGF-β mRNA was expressed by SMC under 4 hours of cyclic stretch (Figure 14) although this data does not directly demonstrate that TGF-β expression is due to stretch. However, VEGF expression under cyclic stretch has been recently shown to occur in the absence of TGF-β in mesangial cells (Gruden et al., 1999), indicating that TGF-β is not central to the expression of

VEGF in all contractile cells under cyclic stretch. It is hoped that quantitative RT-PCR can show whether TGF-β plays a role in VEGF upregulation due to cyclic stretch in aortic SMC.

Levels and Activity of VEGF Released

Smooth muscle cells exposed to cyclic stretch for 4 hours were shown to upregulate the expression of VEGF mRNA by 3.3 (± 1.5 $p < 0.005$) fold of unstretched controls (Figure 11). Although the magnitude of this response is lower than the response previously found to occur in response to serum or hypoxia in other studies (Brogi et al., 1996; Stavri et al., 1995b; Williams et al., 1995), it is similar to the response induced by TGF-β (Brogi et al., 1994), PDGF-B (Stavri et al., 1995b) and endothelin (Pedram et al., 1997). Furthermore, the similarities between SMC and mesangial cells with respect to time course of stretch-induced VEGF expression noted above are found with respect to the magnitude of VEGF mRNA expression (Gruden et al., 1997). Once again, the similarity in magnitude of responses between SMC and mesangial cells is consistent with their functional and structural similarities (Schlondorff, 1987). Although the isoforms of VEGF expressed by stretched SMC in my work could not be determined by northern blotting, they were later shown by pilot RT-PCR experiments to be $VEGF_{121}$ and $VEGF_{165}$ (Figure 14). These isoforms are freely soluble and have been shown to be capable of inducing endothelial cell migration (Ferrara and Davis, 1997). Levels of released VEGF shown to elicit proliferative and migratory responses in EC in other studies have been shown to result from 2 - 3 fold induction in VEGF mRNA in SMC (Ferrara et al., 1991; Pedram et al., 1997). Furthermore, levels of VEGF previously shown to influence endothelial cell migration and proliferation, in a dose dependent manner (Waltenberger et al., 1994) have also been previously shown to be sufficient to reduce endothelial cell apoptosis and thereby increase endothelial cell survival (Gerber et al., 1998; Nor et al., 1999). However, the link between VEGF mRNA expression and an effect on endothelial physiology cannot be inferred from other work. Thus, the 3.3 fold upregulation of VEGF mRNA in SMC under cyclic stretch found in this work, needed to be directly shown to be capable of inducing sufficient VEGF production to effect endothelial cell behaviour and thus survival.

In order to determine whether the increase in VEGF expression by SMC under cyclic stretch found at the mRNA level resulted in the secretion of VEGF at biologically significant levels, conditioned media of SMC under cyclic stretch were used in an endothelial cell migration assay. Smooth muscle cells subjected to cyclic stretch for 4 hours released soluble factors into their media, which increased endothelial cell migration by greater than 14 fold of unconditioned media. However, after correction against the relatively high secretion of endothelial cell chemotactic factors by unstretched SMC (inducing approximately 8 fold the endothelial cell migration of unconditioned media), the dramatic release of endothelial cell migratory factors by stretched SMC only represents a 1.8 fold increase in secretion of endothelial cell migratory factors brought about by subjecting them to cyclic stretch. Furthermore, as can be seen in Figure 12, the addition of anti-VEGF antibodies does not completely attenuate the endothelial cell migration response to media from unstretched and stretched SMC inferring that other factors contributing to endothelial cell migration may be released by both unstretched and stretched SMC in this study. Addition of anti-VEGF antibodies showed that the relative contributions of other factors to endothelial cell migration in media from stretched and unstretched SMC were both found to be approximately 40% of the total migration. Basic FGF has been shown to be released by SMC due to cyclic stretch (Cheng et al., 1997) and is likely to contribute to endothelial migration in this experiment. However, as also shown in the data and confirmed by Yoshida et al (1996), VEGF is twice as powerful at inducing endothelial migration and the two cytokines are not synergistic. This is confirmed by the preliminary RT-PCR data from the present study which demonstrates the expression of the endothelial cell chemotactic factor bFGF by SMC under cyclic stretch (Figure 14) although cyclic stretch is not directly demonstrated to lead to bFGF expression. Taken together these findings lends weight to the theory presented above that VEGF production in SMC under cyclic stretch may be accompanied by or mediated by the production of other factors. Calculating the approximately 60% contributions of

VEGF alone to the total induced endothelial cell migration, stretch is shown to induce a 1.6 fold increase in VEGF-mediated endothelial cell migration after 4 hours.

The levels of endothelial cell migration induced by the stretched smooth muscle cell conditioned media in my study are consistent with migration values previously reported in response to VEGF (Pedram et al., 1997; Senger et al., 1996) and other cytokines (Ikeda et al., 1995; Rosen et al., 1990) in other studies over the 4 hour time interval. Furthermore, the endothelial cell migratory response due to media from stretched SMC in this work correlates with the expression of VEGF mRNA in a similar way to that found by other groups. Greater than 3 fold VEGF mRNA expression in SMC due to application of endothelin has previously been shown to result in greater than 3 fold secretion of VEGF protein by the SMC which was capable of inducing 1.8 fold migration in EC over 4 hours (Pedram et al., 1997). Similarly, PDGF–B stimulation of VEGF mRNA in EC was followed by an approximately 3 fold secretion of VEGF into the media which induced an approximately 2 fold increase in VEGF-mediated endothelial cell migration (Wang et al., 1999). The increases in VEGF-induced endothelial cell migration over 4 hours in the present study are furthermore found to correspond to the levels of VEGF found in the media.

Following the demonstration of VEGF mRNA expression by SMC under cyclic stretch and the release of levels of VEGF into the medium which caused migration of EC, attempts were made to quantify the levels of VEGF secreted. Surprisingly, the ELISA pilot study to determine the cumulative levels of VEGF secreted into the media after 24 hours of stretch found only a modest 1.2 fold increase in VEGF. Although this small increase is not representative of the increase in VEGF mRNA expression after 4 hours, this low level of secretion is consistent with some findings by other groups. Seko et al showed greater than 3 fold upregulation in VEGF mRNA by cardiac myocytes to lead to only a 1.7 fold increase in VEGF protein over 24 hours (Seko et al., 1999). However, the 1.2 fold increase in VEGF expression due to cyclic stretch in the present study is statistically indistinguishable from unstretched controls ($p > 0.05$) indicating that cyclic stretch may have no effect on the release of VEGF after 24 hours. In light of the 3.3 fold increase in VEGF mRNA shown at 4 hours due to cyclic stretch this is surprising.

The divergence between mRNA expression at 4 hours and protein secretion at 24 hours may arise from long-term basal but constitutive expression of VEGF by the SMC during 24 hours of induction of quiescence and 24 hours of experiment on the collagen-coated Flex I plates. Although the 0% FBS in the culture medium should minimise VEGF expression, a low level of VEGF expression was observed in unstretched cells (Figures 9,10 and 11) and expression of VEGF by SMC seeded on collagen has been reported previously (Nicosia et al., 1997). In the present study, the secretion of high levels of approximately 6 ng/ml of VEGF into the medium by unstimulated SMC during 24 hours of induction of quiescence and the 24 hour course of the experiment may swamp the VEGF secretion response to stretch-stimulation. This may account for the low fold increase in protein secreted by stretched cells as compared to unstretched cells. VEGF has little effect on SMC and therefore would not be detected and down-regulated in the absence of mediating EC. The gradual release of cumulatively high levels of VEGF by unstimulated SMC in culture would also explain the high (approximately 8 fold) VEGF-mediated migration response to media from unstretched SMC found in this study and mentioned above. Levels of VEGF at 6 - 7 ng/ml are consistent with reported levels previously shown to be necessary to bring about endothelial cell migration (Senger et al., 1996) at an approximate 1.3 fold increase in over 6 hours (Waltenberger et al., 1994). Furthermore, a study which related the 2 – 4 ng/ml (or 2 – 4 ng of VEGF / 10^6 SMC) produced by VEGF-transfected SMC after 48 hours to the induced endothelial cell migration demonstrated a greater than 3 fold increase in migration after 72 hours (Visweswaran et al., 1997). If the measured VEGF of the supernatant in the present work are related to cell number, the approximately 6 ng/ml of VEGF found in unstretched SMC was equivalent to 17 ng / 10^6 SMC. While, the differences in VEGF expression and induced migration between my study and the one involving VEGF-transfected SMC may be due to experimental differences in time courses of both expression and migration and different migration assays, it may also be due to high VEGF secretion over time by SMC coated on collagen. By comparison with the findings of others, it appears that the levels of VEGF secretion are very high in unstretched controls given the consistently low VEGF mRNA expression found over the 24 hour time period in preliminary time course experiments. Thus, the gradual release of high levels of VEGF during induction of quiescence and the experiment by unstretched and stretched SMC seeded on collagen appears to be the most likely reason for the high levels of VEGF and the lack of a distinctive VEGF secretory response to cyclic stretch at 24 hours.

However, an alternative reason for the low fold increase in VEGF secretion in stretched cells compared to unstretched cells at 24 hours in this study may be a decrease in VEGF secretion over time after the production at 4 hours found by the endothelial cell migration assay. Other studies using mesangial cells under cyclic stretch have shown a profile of VEGF protein secretion that lags behind the mRNA expression (Gruden et al., 1997). They showed an initial 2.4 fold peak increase in VEGF mRNA expression after 6 hours which drops to basal levels by 12 hours and was only subsequently followed by a delayed 3.1 fold increase in secreted VEGF protein levels after 12 hours which returned to basal levels by 24 hours. In the present study - while the modest values of VEGF protein in the media at 24 hours from stretch-stimulated SMC are similar to previously reported values - they do not approach the robust 3 fold increase seen at the mRNA level in response to cyclic stretch when considered as a fold of unstretched controls. Rather, it may be the case that a short-lived increase in levels of secreted VEGF that has decreased by 24 hours has occurred similar to the VEGF expression profile of mesangial cells in response to cyclic stretch. A more thorough ELISA determination of levels of VEGF protein secretion over shorter time periods needs to be carried out to further characterise the secretion of VEGF resulting from the increase in VEGF mRNA expression after 4 hours of stretch. This may indicate whether the small fold increase in VEGF secretion in stretched cells over unstretched cells is due to high levels of secretion by unstretched cells or to an increase in VEGF expression by SMC under cyclic stretch which has started to wane by 24 hours.

In vivo other mechanisms may contribute to VEGF secretion. Smooth muscle cell numbers in the wall of a prosthetic vascular graft are likely to be far lower than those in confluent culture, making the contribution of stimuli which induce VEGF expression, such as cyclic stretch, more pronounced over long-term baseline secretion. In addition, the binding of VEGF by heparin in the basement membrane *in vivo* may stabilise VEGF released by SMC (Park et al., 1993). Thus, even if the release of VEGF by SMC under cyclic stretch proves to be a transient event, sequestering and stabilisation by heparin binding would serve to prolong the activity of VEGF released by SMC under cyclic stretch in the environment of a compliant graft.

The cumulative VEGF secretory response due to bFGF in this study (1.5 fold compared to values from unstimulated controls as determined by ELISA experiments on conditioned media) is also modest. Although the increase in VEGF in the media of bFGF-stimulated SMC at 24 hours is significantly ($p < 0.05$) greater than VEGF in unstretched controls, it is a far cry from the 3 fold increase in expression after 4 hours at the mRNA level and indistinguishable ($p > 0.05$) from the low increase in levels of VEGF expression in stretched cells at 24 hours noted above. As with the response to cyclic stretch, the low levels of increase in VEGF levels in the media at 24 hours compared to unstimulated SMC may be due to high levels of VEGF secreted gradually by the unstimulated cells for reasons mentioned above. These high values of VEGF may swamp any increase in VEGF expression due to bFGF. Alternatively, the secretion of VEGF due to bFGF may decline as the time of exposure approaches 24 hours as seen in PDGF-B and TGF-β stimulated SMC (Brogi et al., 1994). As noted above with respect to the VEGF secretion response to cyclic stretch, more ELISA analysis is required in future studies to better characterise the VEGF secretion due to bFGF over time.

The fold increase in VEGF-mediated endothelial cell migration by conditioned media from bFGF-treated SMC over unstimulated controls found at 4 hours in this study is also surprisingly low considering the fold increase in VEGF mRNA expression at this time compared to unstimulated controls. Despite the similar (approximately 3 fold) increase in VEGF mRNA expression levels at 4 hours by SMC stimulated with bFGF and cyclic stretch, there appears to be a difference in VEGF secretion after 4 hours as evidenced by differences in VEGF-mediated endothelial cell migration. Although the medium from stretched SMC induces a 1.6 fold increase in VEGF-mediated migration compared to unstimulated controls (see above), endothelial cell migration in response to the conditioned medium of bFGF-stimulated SMC was almost the same as to the media from unstimulated controls. This infers that bFGF does not lead to an increase in VEGF secretion over untreated SMC after 4 hours. Once again, the low relative increase in VEGF secretion over untreated SMC may be due to the high background levels of VEGF released by untreated SMC during induction of quiescence. Alternatively, although previously shown to act more rapidly in rabbit SMC in other studies (Stavri et al., 1995a), VEGF secretion due to bFGF in bovine SMC in this study may occur after the 4 hour peak VEGF mRNA expression. Whatever the explanation behind the lack of increase in VEGF-mediated migration at 4 hours in this study, the migration results indicate a difference in VEGF secretion after 4 hours between bFGF-stimulated and stretch-stimulated SMC although the increase in VEGF mRNA expression at this time is similar.

A possible reason for the difference in protein secretion at 4 hours by stretched and bFGF-treated SMC found in this study may be distinct post transcriptional regulation of VEGF mRNA processing. Low levels of VEGF mRNA expression were noted in unstretched smooth muscle cell controls (Figures 9, 10 and 11) consistent with the demonstrations of basal levels of VEGF mRNA expression by unstimulated SMC in other studies (Couffinhal et al., 1997; Ferrara et al., 1991). Considering the constitutive expression in the present work it is possible that steady state VEGF mRNA may be rapidly increased due to cyclic stretch by a post-transcriptional mechanism to increase the half-life of the mRNA rather than as a result of increased transcriptional activation of the VEGF gene. Such a mechanism has been shown by other groups to be behind the increase in VEGF mRNA steady state levels due to hypoxia (Levy et al., 1996). However, mRNA stabilisation is a complex and poorly understood mechanism and the time-consuming expression of additional factors, which protect the mRNA from degradation, may take longer than the time course of expression found in the present study. Alternatively, cyclic stretch may alter the control of translation of the VEGF mRNA or release of VEGF from the cells, resulting in accelerated protein secretion. Either mechanism would account for the rapid production of levels of VEGF protein over

4 hours of cyclic stretch capable of increasing endothelial cell migration found in this study. Future work could use nuclease protection assays and conditioned media ELISA studies at shorter time courses to determine the role, if any, of mRNA stabilisation in the regulation of VEGF expression in response to cyclic stretch.

The increased VEGF mRNA steady state levels in SMC due to bFGF treatment in this study could, by contrast, result from transcriptional induction and leads to a more gradual albeit more long term response as seen in studies by another group (Stavri et al., 1995a). Basic FGF has been shown by other groups to have profound effects on SMC behaviour (D'Amore and Smith, 1993; Slavin, 1995) and it is possible that the stimulatory effects of bFGF on SMC may reduce or delay the secretion of VEGF into the media in this study. The addition of anti-VEGF antibodies failed to completely inhibit the endothelial cells migratory response to media from bFGF-stimulated cells in this study, in a similar fashion and at similar levels to the attenuations observed in the endothelial cells migratory responses to anti-VEGF treated media samples from stretched and unstretched SMC (Figure12). This indicates that factors other than VEGF may play a role in endothelial cell migration due to conditioned media from bFGF-treated cells in this study although the bFGF - added to the smooth muscle cell media – has been shown to contribute to endothelial cell migration by other studies (Montesano et al., 1986) and may influence endothelial cell migration in this study.

Previous studies have shown that the levels of VEGF required to induce proliferation (Weatherford et al., 1996) and migration (Wang et al., 1999) in EC are similar to those required to increase endothelial cell survival by reducing apoptosis (Gerber et al., 1998; Nor et al., 1999). Thus, considering the values of VEGF secreted by SMC under cyclic stretch in this study, in terms of both concentration after 24 hours and biological activity after 4 hours, it appears that SMC under cyclic stretch secrete sufficient VEGF to promote endothelial cell survival. Following the development of a number of commercially available *in vitro* apoptosis assays, the direct anti-apoptotic effect on EC of conditioned media from stretched SMC could be tested in future studies.

Our pilot RT-PCR study indicates that VEGF may not be the only cytokine whose expression is found in SMC under cyclic stretch (Figure 14). Although the results are too preliminary to draw any conclusions from, future quantitative studies may elucidate the diverse downstream possibilities of VEGF, bFGF and TGF-β expression by SMC under cyclic stretch and the effect which cyclic stretch has on control of their expression. Furthermore, although many similarities exist between the physiologies and functions of SMC and pericytes (Nicosia and Villaschi, 1995), they have been shown to differ in their responses to cytokines such as PDGF-B and TGF-β (D'Amore and Smith, 1993). In the scenario of capillaries migrating through a fibrin network within the wall of an implanted synthetic vascular graft, the support cell for the EC would be the pericyte. Pericytes have been shown in other studies to be instrumental in VEGF-mediated endothelial cell survival in capillaries (Benjamin et al., 1998; Goede et al., 1998; Reynolds and Redmer, 1998). VEGF has also been shown to be produced by the specialised mesangial pericyte (Schlondorff, 1987) under cyclic stretch (Gruden et al., 1997). However, no work has investigated the effect of cyclic stretch on microvascular pericyte function. The isolation and culture of pericytes by a number of groups (Antonelli-Orlidge et al., 1989; D'Amore and Smith, 1993; Kim et al., 1998; Nicosia and Villaschi, 1995; Swinscoe and Carlson, 1992; Nehls et al., 1994) has enabled the *in vitro* study of this cell type although they are an ill-defined group of cells (Shepro and Morel, 1993), which is elusive to isolate and phenotypically unstable in culture. It would nevertheless be worthwhile to determine the effect of cyclic stretch on pericyte expression of VEGF and other cytokines such as the angiopoietins and TGF-β which have been shown to be important in capillary stabilisation (Darland and D'Amore, 1999). This may provide an *in vitro* indication of how stretch-induced pericyte production of these cytokines would affect the transmural migration of capillaries within a healing compliant porous prosthetic vascular graft.

3. Effect of Cyclic Stretch on KDR Phosphorylation in Endothelial Cells

Time Course of KDR Phosphorylation

The migratory and anti-apoptotic effects of VEGF on EC are mediated by the endothelial cell-specific receptor KDR (Gerber et al., 1998; Waltenberger et al., 1994). Recently, biomechanical forces have been shown by other groups (Hu et al., 1998; Osawa et al., 1997; Yano et al., 1996a) to result in phosphorylation of receptors in vascular cells in the absence of their respective ligands. This has led to speculation that these receptors may possess a dual role as receptors for their ligand cytokines and as mechanoreceptors for biomechanical forces (Hu et al., 1998). It has been previously proposed that receptor phosphorylation in response to biomechanical forces may initiate signalling pathways and thus responses usually triggered by the cytokine ligand (Hu et al., 1998). As KDR is highly and constitutively expressed by EC *in vivo* (Couffinhal et al., 1997; Quinn et al., 1993) it was considered worthwhile investigating any effect of cyclic stretch on its expression and activity. This was carried out by immunoprecipitation and western blotting of stretched EC for KDR and KDR phosphorylation using unstretched EC as a negative control and VEGF-stimulated EC as a positive control for KDR phosphorylation. It was hoped that this would give an indication of the ability of EC under cyclic stretch to respond to the VEGF released by SMC under cyclic stretch. Alteration of KDR expression by EC or KDR phosphorylation due to cyclic stretch may occur *in vivo* which might alter the effects of VEGF and levels of VEGF required to influence the EC.

For this study, KDR was chosen over Flt-1 due to the involvement of KDR in the anti-apoptotic effect of VEGF on EC (Gerber et al., 1998). In addition, KDR has been shown to work as a mechanoreceptor for shear stress and it is possible that this function extends to the detection of cyclic stretch (Chen et al., 1999). Although the results do not indicate a statistically significant change in KDR phosphorylation up to 120 minutes due to cyclic stretch at the 95% confidence interval ($p > 0.05$), this appears to be due to the contribution of a few outlying results and the overall trend in KDR phosphorylation is too pronounced to assume that stretch has no biological effect on phosphorylation of KDR over time (Figure 13). Therefore, some of the possible implications of the results are considered below. The results demonstrate an initial minor decrease in KDR phosphorylation in stretched EC to 60 minutes under cyclic stretch followed by an overall increase, although there was some variation between individual experiments, to an average of 2.5 fold of unstretched controls at 120 minutes. One important finding of this study is that KDR expression by the EC, which has previously been shown in other studies to be changed due to other stimuli (Brogi et al., 1996; Mandriota et al., 1996), was not altered due to cyclic stretch over time (Figure 13B). This is consistent with *in vivo* observation of constitutive expression of the receptor in the elastic artery (Quinn et al., 1993) and indicates that any KDR-mediated response to VEGF by the EC may occur within an environment under cyclic stretch.

Levels of KDR Phosphorylation

Given that the high (50 ng/ml) VEGF concentrations used in this study as a positive control for KDR phosphorylation caused an average 12.3 fold increase in KDR phosphorylation after 20 minutes, the 2.5 fold upregulation of KDR phosphorylation after 120 minutes represents a dramatic response. Other studies using human umbilical vein EC (HUVEC) have previously confirmed this degree of KDR phosphorylation to result from to 1 – 10 ng/ml of VEGF (Waltenberger et al., 1994). This range of VEGF concentration has furthermore been shown to be sufficient to lead to dose dependent VEGF-mediated reductions in apoptosis in HUVEC via the phosphorylation of KDR (Gerber et al., 1998; Nor et al., 1999). As there is only one tyrosine phosphorylation site on the KDR receptor (Heldin, 1995; Kroll and Waltenberger, 1997) it is thus possible that any phosphorylation response will initiate similar signal transduction responses to VEGF-mediated phosphorylation. Therefore, it is conceivable that the values of phosphorylation observed in this study after 120 minutes may be sufficient to affect the response to VEGF in EC under cyclic stretch. However, the differences in time courses and magnitudes of the response to VEGF (12.3 fold increase at 20 minutes) and cyclic stretch (2.5 fold increase at 120 minutes) observed in this study seem to infer that the two phosphorylation responses would only affect each other if VEGF was added at a later period than used in the present study. In any case, considering the diversity of endothelial cell responses to cyclic stretch (Sumpio, 1989; Sumpio et al., 1988a) and the number of signalling pathways triggered by KDR phosphorylation (Kroll and Waltenberger, 1997), further work is required to determine if the possible KDR phosphorylation under cyclic stretch noted here leads to altered response of EC to VEGF.

Possible Mechanisms behind KDR Phosphorylation

Although the response of KDR phosphorylation seen by EC under cyclic stretch in this study is of a similar magnitude to the responses seen in other receptors subject to biomechanical forces by other groups (Hu et al., 1998; Osawa et al., 1997; Yano et al., 1996a), the time course of phosphorylation is late compared to the rapid profile of phosphorylation found in most responses to biomechanical forces by other receptors. PECAM phosphorylation in EC under shear stress (Osawa et al., 1997), the phosphorylation of the PDGF receptor-α by cyclic stretch in SMC (Hu et al., 1998) and focal adhesion kinase phosphorylation in EC due to cyclic stretch (Yano et al., 1996a) all occur in under 30 minutes. Although it seems unlikely in the light of the finding of these studies, it cannot be ruled out that the initial lag in KDR phosphorylation up to 90 minutes in the present study may be due to a period of adjustment to the force regimen by the EC after which phosphorylation responses are seen. However, considering the relatively high (1.8 fold) initial values of KDR phosphorylation found after 10 minutes of stretch in preliminary time course experiments, the ensuing decrease in phosphorylation until 90 minutes followed by a subsequent increase in phosphorylation appears to be more consistent with a biphasic response to stretch in which an initial phosphorylation response to stretch up to 30 minutes is followed by a second and more pronounced response after 90 minutes. The initial response may be better characterised by future studies analysing KDR phosphorylation before 30 minutes but the delayed response raises questions as to whether the KDR phosphorylation after 90 minutes shown in this study is directly due to cyclic stretch or is mediated by the stretch-induced release of VEGF consistent with an autocrine loop.

Although other studies have shown VEGF to be expressed in other cell types under cyclic stretch (Gruden et al., 1997; Seko et al., 1999), expression in cultured EC has previously been found to be undetectable (Williams et al., 1995) and information on expression of VEGF secretion by EC under cyclic stretch is lacking. However, future ELISA studies on conditioned media from stretched EC and anti-VEGF antibody-inhibitory studies on EC under stretch may show whether cyclic stretch leads to production of VEGF in EC after 90 minutes by a novel mechanism. Alternatively, placenta growth factor (PlGF) may be released by the EC due to cyclic stretch and influence the interaction of VEGF with KDR. This factor has been shown to potentiate the activity of low levels of VEGF in an autocrine manner though to be brought about by displacing Flt-1 bound VEGF (Park et al., 1994) allowing it to interact with KDR. If VEGF is not involved in the KDR phosphorylation observed in this study, it is possible that the activation of KDR represents a direct later response to cyclic stretch by EC. Such a response has been demonstrated in other studies by the PDGF receptor-α in SMC, which was found to be phosphorylated by stretch without PDGF-B release up to 4 hours in SMC. In EC, longer time courses of surface protein phosphorylation due to cyclic stretch have been shown in other studies – such as by paxillin, which is phosphorylated after 4 hours (Yano et al., 1996a) - and it is possible that the response by KDR observed in my work follows a similar late pattern. If this is the case, the mild (and statistically insignificant ($p > 0.05$) compared to unstretched controls) decrease in phosphorylation before 90 minutes in the this study may represent nothing more than transient minor fluctuations in KDR phosphorylation seen over time in culture. It is hoped that future work can determine whether the statistically insignificant ($p > 0.05$) difference between KDR phosphorylation levels over time in stretched EC and unstretched controls truly indicates that KDR phosphorylation does not occur due to cyclic stretch or whether the perceived increase in phosphorylation of KDR after 90 minutes noted in this study is biologically relevant. If the phosphorylation of KDR over time due to cyclic stretch is a genuine and functionally relevant response, it will be left to determine whether it is inhibitory, additive or synergistic to the anti-apoptotic effects of physiological levels of VEGF on the EC. A further role of KDR activation may be the release of NO by EC (Kroll and Waltenberger, 1999). Thus locally released VEGF from stretched SMC may also induce production by EC of the smooth muscle cell-relaxing molecule NO (van der Zee et al., 1997) to complement the levels already induced in EC by cyclic stretch (Awolesi et al., 1995), providing a compensatory pathway to alleviate pathologically high levels of stretch. Furthermore, it is hoped that future inhibitor studies will outline the regulation of signal transduction events following KDR phosphorylation in EC under cyclic stretch and thus determine whether the response shown in this study affects the expression of cellular signalling molecules, such as Bcl-2 and the caspases (Nor et al., 1999), associated with increased endothelial cell survival in response to VEGF.

Concluding Remarks

VEGF was once hypothesised to be involved in smooth muscle cell proliferation in intimal hyperplasia by increasing the vascularity of the vessel wall (Ferrara et al., 1991). However, constitutive expression of VEGF by SMC (Couffinhal et al., 1997) and the VEGF receptor KDR by EC (Quinn et al., 1993) have since been shown in arteries *in vivo* and appear to be indispensable to correct vessel function. An important function of this ligand-receptor expression is the dose-dependent anti-apoptotic role of VEGF in endothelial maintenance (Nor et al., 1999). VEGF has been shown to be instrumental in increased vascularity of prosthetic grafts (Masuda et al., 1997) and maintaining the endothelial layer (Benjamin et al., 1999) responsible for superior graft performance (Zilla et al., 1999). However, EC and accompanying SMC / pericytes are largely excluded from contemporary grafts due to a number of factors and consequently healing is poor (Davids et al., 1999). It is hoped that, amongst other developments, improved graft porosity will allow a higher level of cellular infiltration into the grafts. However, it is most likely that the levels of VEGF secretion seen in the native artery (Couffinhal et al., 1997) will not be achieved because of decreased cell density, which is due to the presence of the polymer scaffold and fibrous scarring. In addition, as shown in this study, there is a contribution of mechanical stimulation to the production of VEGF by the SMC and this functional stimulation is lacking in stiff contemporary grafts. It therefore appears that the production of VEGF by SMC in a porous vascular prosthesis can never be as high as the levels in the artery. In order to improve the functional production of VEGF by SMC within the walls of a synthetic vascular graft the design of the prosthesis should minimise the volume of scaffold in the wall. In addition, it should be determined whether pulsatile stretching can raise the levels of VEGF secretion from the basal levels observed in static culture and at least asymptotically lead to levels of VEGF required for physiological maintenance. This will give an indication of whether the beneficial effects of compliance on the release of cytokines are sufficient to lead to improved healing. If it can be shown that stretching significantly influences levels of maintaining VEGF, spontaneously healing grafts would need to be physiologically compliant.

This study justifies this demand by showing that cyclic stretch increases the expression of VEGF by SMC under the possible mediation of other factors. This increased VEGF expression leads in turn to increased secretion of VEGF at sufficient levels to increase endothelial cell migration. The levels of VEGF required to affect endothelial cell migration have previously been demonstrated to be approximately equal to those sufficient to improve endothelial cell survival. This implies that the levels of VEGF secreted by the SMC are biologically relevant. The secretion of VEGF may then diminish slightly over time, although the levels of VEGF secretion by unstretched controls in my model made assessment of later secretion responses difficult. Furthermore, despite inducing similar VEGF mRNA expression responses, bFGF and cyclic stretch elicit different responses in terms of secretion of VEGF protein. This points to possible differences in the molecular mechanisms behind the increase in VEGF expression due to bFGF and cyclic stretch. Finally, cyclic stretch did not alter the expression of the KDR receptor although it is possible that cyclic stretch may influences the ability of the EC via this receptor to react to VEGF released locally by SMC. A greater understanding of the effect of cyclic stretch on VEGF-mediated communication between SMC and EC should provide information about the mechanical properties which optimise this interaction which can then be integrated into the design of future spontaneously healing vascular prostheses.

However, VEGF is not the only cytokine produced by SMC under cyclic stretch. Wilson et al (1993) show secretion of PDGF-BB and PDGF-AA by stretched SMC. Both of these isoforms are chemotactic for SMC (Ross et al., 1990), which may aid in recruitment of additional SMC to the walls of a compliant graft. However, the mitogenic action of PDGF-BB may lead to unrestrained proliferation of SMC. Thus, the beneficial effects of the increased production of VEGF on endothelial cell survival are balanced against the possibility of increased PDGF-mediated smooth muscle cell proliferation associated with intimal hyperplasia (Golden et al., 1991; Jawien et al., 1992). However, *in vivo*, other factors, such as the direct phosphorylation of PDGF receptor-α by cyclic stretch (Hu et al., 1998) which may alter the response of SMC to locally released PDGF via this receptor, might attenuate any proliferative effects of PDGF released by the SMC. Stabilisation of the SMC under conditions of cyclic stretch may also occur via the differentiating effects of cyclic stretch on smooth muscle cell phenotype (Reusch et al., 1996). The majority of SMC in the compliant artery, which have been shown to produce VEGF (Couffinhal et al., 1997), are found in this quiescent contractile state without overt proliferation (Mills and Sumpio, 1997). Thus cyclic stretch *in vivo* may promote the establishment of a stable population of SMC in the pores of a compliant graft.

Furthermore, the maintenance of the endothelium by increased production of VEGF under cyclic stretch may in turn lead to the release of factors by the EC which halt proliferation of SMC (Axel et al., 1997; Couffinhal et al., 1997). *In vivo* this stabilising function of EC will be additionally mediated by the biomechanical forces to which they are subjected and EC have been shown to decrease the production of PDGF-B (Malek et al., 1993) and increase the production of TGF-β (Cucina et al., 1998) in response to shear stress. TGF-β has been shown to be released by both EC and SMC in co-culture (Axel et al., 1997) and to induce quiescence in both EC (RayChaudhury and D'Amore, 1991) and SMC (D'Amore and Smith, 1993) which reduces the release of cytokines. This thus represents a shift from the release of stimulatory factors to a release of factors involved in inducing quiescent conditions (Axel et al., 1997). Finally, cyclic stretch in cardiac myocytes has been shown to lead to the release of VEGF via the mediation of TGF-β released by these cells (Seko et al., 1999). It is therefore possible that, if a similar response occurs in SMC, cyclic stretch may mute any possible PDGF-mediated smooth muscle cell proliferative response in a dose-dependant way by the release of TGF-β. Thus, although the presence of SMC in a compliant envronment brings with it the possibility of increased production of PDGF-B (Wilson et al., 1993), with subsequent smooth muscle proliferation characteristic of intimal hyperplasia, this is not observed in a healthy compliant artery where the endothelial-smooth muscle interaction appears to lead to stabilisation of both cell types (Axel et al., 1997). Production of VEGF by SMC under cyclic stretch leads to stabilisation of the endothelium, which in turn releases factors leading to the stabilisation of the SMC. Thus, the use of compliant graft materials in vascular grafts, which allow the SMC and EC to experience a measure of the cyclic stretch that they are subject to in the native artery, may also approach the natural function of the native artery.

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