Mechanisms of Arterial ~~Lesion~~ NEOINTIMA Formation:

Study of the Effects of Angiogenic Factors in ~~a Rabbit Model~~ VIVO

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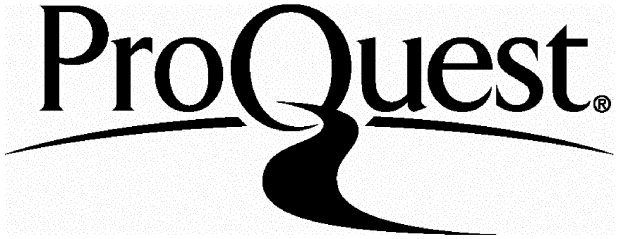

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ABSTRACT

Arterial thickening due to neointimal vascular smooth muscle cell (VSMC) and macrophage accumulation is an important early stage in atherosclerosis. Angiogenesis and angiogenic cytokines have been implicated in neointima formation, but their roles are not clearly understood. The aim of this thesis was to examine the effects of angiogenic factors on the growth and cellular composition of arterial neointimal lesions in an animal model.

Lesion formation was induced by the placement of a biologically inert collar around the carotid artery of rabbits. This induced the formation of localised lesions containing predominantly VSMCs with and without macrophages, in animals maintained on normal and hypercholesterolaemic diets, respectively. This model facilitates localized gene delivery and allows the biological influence of the endothelium to contribute to the observed effects, since it remains anatomically intact. Initially, the cellular composition of lesions was characterized on normal and cholesterol-enriched diets. Transfer of genes encoding angiogenic factors was performed using either low efficiency liposome-mediated or high efficiency adenoviral (Ad-) gene transfer, and mRNA and protein expression of transgene products was evaluated.

Low efficiency liposome-mediated gene transfer of the potent angiogenic factor, vascular endothelial growth factor-A (VEGF-A), reduced intimal thickening, macrophage infiltration and endothelial vascular cell adhesion molecule (VCAM-1) expression in hypercholesterolaemic rabbits without significantly increasing angiogenesis. In contrast, high efficiency Ad.VEGF-A transduction of collared arteries caused little significant change in either intimal thickening or macrophage content of lesions. Transfer of Ad.Placental Growth Factor-2 (PlGF2), a factor related to VEGF-A, increased neointimal macrophage infiltration and thickening, endothelial VCAM-1 expression and angiogenesis in the collared arteries of hypercholesterolaemic rabbits.

The effect of proline-arginine rich peptide (PR39), a natural mammalian peptide that induces angiogenesis via inhibition of proteasome-mediated degradation of hypoxia inducible factor, was also studied. Ad.PR39 increased angiogenesis and increased intimal thickening. Inhibition of both VEGF and fibroblast growth factor pathways abolished the

effects of PR39. Though a striking correlation was observed between the degree of intimal thickening and adventitial neovascularisation, the results indicate that PR39-induced neointima formation in this model has both angiogenesis-independent and -dependent phases. A direct chemotactic effect on VSMCs by both VEGF and PR39 was also demonstrated.

In summary, periadventitial gene delivery of angiogenic factors exerted profound effects on neointimal lesion formation in the collar model, as follows: (i), A concentration-dependent effect of VEGF-A in the regulation of neointima formation, (ii), PlGF induces atherogenic changes in the arterial wall and (iii), Studies with PR39 suggest that intimal thickening has two phases, an angiogenesis-independent phase and a later angiogenesis-dependent phase. These results reveal a complex interaction between angiogenic factors and neointima formation and are likely to have implications for the therapeutic use of angiogenic cytokines for ischaemic cardiovascular disease.

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ABBREVIATIONS

AMLV	Amphotropic Murine Leukemia Virus
Ang-1	Angiopoietin-1
Apo	Apolipoprotein
BSA	Bovine Serum Albumin
CAR	Coxsackie Adenovirus Receptor
CDK	Cyclin Dependent Kinase
CETP	Cholesteryl ester transfer protein
CHD	Coronary Heart Disease
CMV	Cytomegalovirus
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	ethylene diamine tetra acetate
EEL	External elastic lamina
ERK	Extracellular-signal-regulated kinase
EtOH	Ethanol
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR1-DN	Fibroblast Growth Factor Receptor- Dominant Negative
Flk-1	Fetal liver kinase-1

Flt-1	Fms-like tyrosine kinase-1
GFP	green fluorescent protein
GT	Gene therapy
GTA	Glutaraldehyde
HDL	High Density Lipoprotein
H&E	Haematoxylin & Eosin
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HL	Hepatic Lipase
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Cell Adhesion Molecule-1
IEL	Internal Elastic Lamina
Ig	Immunoglobulin
ITR	Inverted Terminal Repeats
IL-1	Interleukin-1
IGF	Insulin-like growth factor
I/M	Intima to Media Ratio
KDR	Kinase-insert Domain-containing Receptor
LDL	Low Density Lipoprotein
MAPK	Mitogen-activated Protein Kinase
MMP	Matrix Metalloproteinase
MΦ	Macrophage

MCP	Monocyte Chemotactic Protein
M-CSF	Macrophage-Colony Stimulating Factor
MOI	Multiplicity of Infection
MRI	Magnetic Resonance Imaging
NF-κB	Nuclear Factor κB
NRP	Neuropilin
NZW	New Zealand White
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ODN	Oligodeoxynucleotide
RTK	Receptor Tyrosine Kinase
PBS	Phosphate Buffered Saline
PECAM	Platelet Endothelial Cell Adhesion Molecules
PGI_2	Prostacyclin
PAI-1	Plasminogen Activator Inhibitor-1
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PFH	Paraformaldehyde
Pfu	Plaque forming units
PI3K	Phosphatidyl inositol 3'-kinase
PlGF	Placental Growth Factor
PLCγ	Phospholipase Cγ

PMN	Polymorphonuclear
PTCA	Percutaneous Translumenal Coronary Angioplasty
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
SRA	Scavenger Receptor A
TBS	Tris-Buffer Saline
TIMP	Tissue Inhibitor of Metalloproteinase
TGFβ	Transforming Growth Factor β
TNFα	Tumour Necrosis Factor α
TSP	Thrombospondin
Ub	Ubiquitin
uPA	Urokinase Plasminogen Activator
UTR	Untranslated Region
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VEGFR-1 /-2 /-3	Vascular Endothelial Growth Factor Receptor-1 /-2 /-3
VHL	Von Hippel Lindau
VLDL	Very Low Density Lipoprotein
VSMC	Vascular smooth muscle cell
vWF	von Willebrand Factor
WHHL	Watanbe Heritable Hyperlipidaemic

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It is good to have an end to journey towards; but it is the journey that matters, in the end.
Ursula Le Guin

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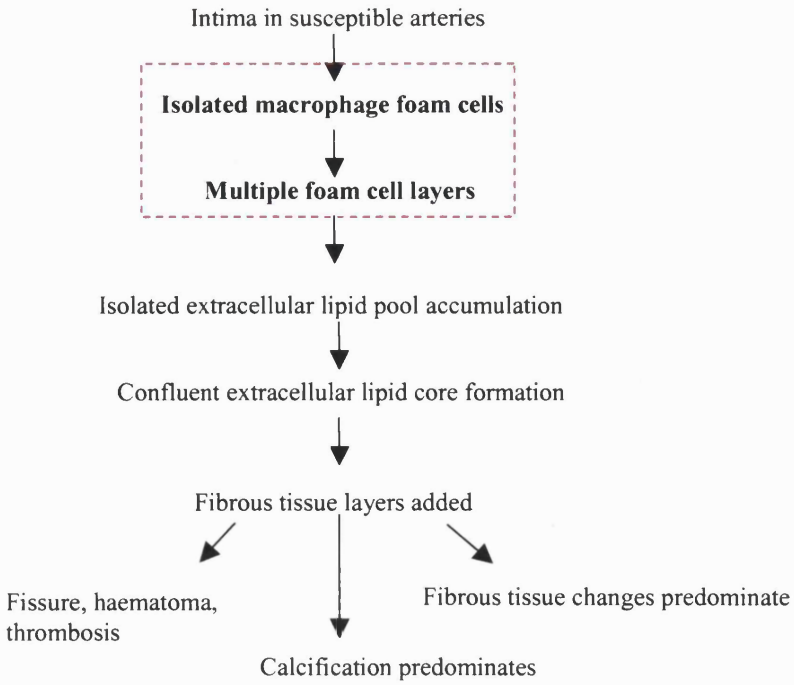
CHAPTER 1

1. INTRODUCTION

1.1. Atherosclerosis

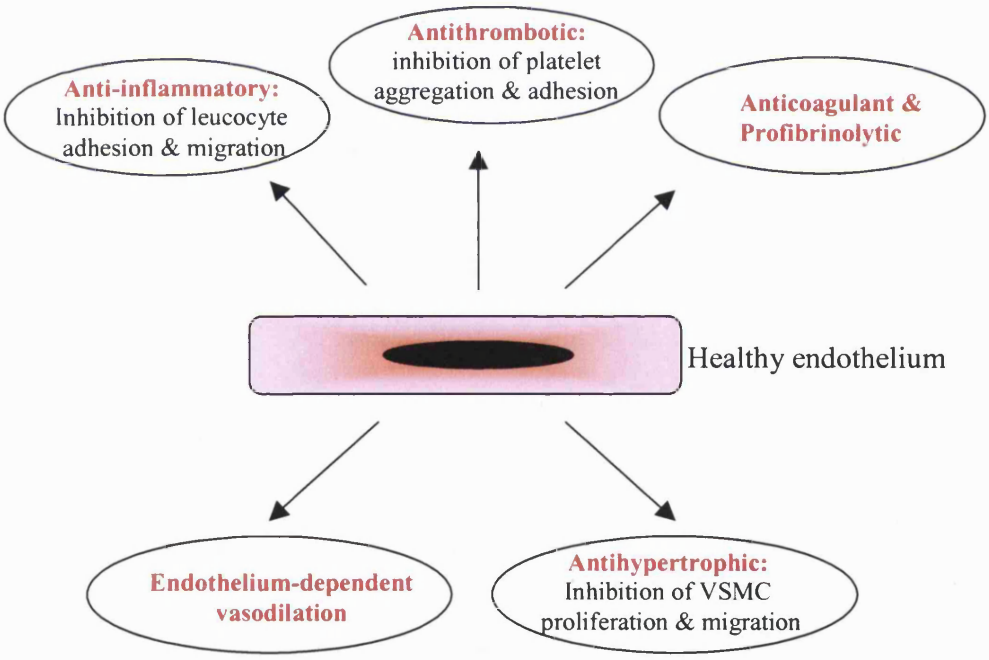
Atherosclerosis, a chronic inflammatory disease of arteries, is the principal cause of inadequate vascular supply to tissues (ischaemia) and most commonly affects the heart. It is accountable for the majority of deaths attributable to heart disease worldwide, and is the major cause of mortality and morbidity in the industrialised world. The prevalence of heart disease globally is increasing, and by 2010, cardiovascular disease is predicted to be the leading cause of death in developing countries (www.who.int/cardiovascular_diseases/). The process starts in early childhood and is characterized by the accumulation of lipids and lipoproteins within the vessel wall, together with macrophage infiltration and vascular smooth muscle cell (VSMC) proliferation (**Fig 1.1**).

Figure 1.1. Pathway of atherosclerosis progression: Histological Characteristics



The intact, healthy vascular endothelium is a monolayer that represents the principal barrier between blood constituents and the artery wall, and is responsible for regulating vascular tone and structure. Endothelial cells (ECs) form a highly selective permeability barrier, provide an anti-thrombotic surface inhibiting platelet and leucocyte adhesion to the vascular surface and, in response to injury, an inflammatory stimulus or disease, maintain a balance of profibrinolytic and prothrombotic activity (Libby 2002). The endothelium is also an active metabolic tissue constitutively generating vascular protectants such as nitric oxide (NO) and prostacyclin (PGI_2) (**Fig 1.2**).

Figure 1.2. Summary of the functions of healthy endothelium



Endothelial dysfunction constitutes one of the earliest phases of atherosclerotic disease (Ross et al. 1977) and traditional risk factors predisposing to ischaemic heart disease, such as elevated low density lipoprotein (LDL) cholesterol, hypertension, diabetes mellitus, smoking-induced free radicals and genetic factors are all thought to adversely affect the homeostatic function of the endothelium. An ongoing exposure to inflammatory stimuli

results in plaque development and thickening of the arterial wall, which compensates by gradual dilatation to ensure lumenal diameter is preserved (positive remodeling) (Glagov et al. 1987). Increased recruitment and multiplication of activated macrophages, with a concomitant release of hydrolytic enzymes, cytokines, chemokines and growth factors (Libby 2002) induce further damage and lead to focal necrosis. Cycles of VSMC proliferation, macrophage accumulation and deposition of fibrous tissue lead to advanced atherosclerotic lesions, comprising a fibrous cap overlying a core of lipid and necrotic tissue. Vulnerability to rupture and thrombotic complications may result in total arterial occlusion and serious clinical sequelae.

1.2. Macrophages, LDL cholesterol and Early Atherosclerosis

From an early age, LDL cholesterol level is a well established independent risk factor for arterial disease (Ross 1993). In individuals at moderate to high risk for coronary heart disease (CHD), intensive treatment with statins (HMG CoA reductase inhibitors in the cholesterol biosynthetic pathway) reduces plasma LDL cholesterol by 30-50% and reduced CHD mortality to a similar extent by 5yrs (1994; 2002).

The observation that hypercholesterolaemic mice become extremely resistant to atherosclerosis if they are bred with macrophage deficient mice represents one of several lines of evidence incriminating macrophages in the initiation of lesion formation and progression (Smith et al. 1995). Monocyte migration and LDL accumulation within the arterial intima define one of the earliest events in atherogenesis. Once resident within the arterial intima, monocytes acquire the morphological characteristics of macrophages (Fig 1.3). Increased expression of scavenger receptors for modified lipoproteins, which include scavenger receptor A (SRA) (Kodama et al. 1988) and CD36 (Endemann et al. 1993) mediate internalisation of the lipoproteins, such that cholesteryl esters accumulate in cytoplasmic droplets. Gene deletion and bone marrow transplantation experiments indicate that these receptors make quantitatively important contributions to the progression of atherosclerosis (Febbraio et al. 2001). These activated, lipid-laden macrophages, referred to as 'foam cells', characterize early atherosclerosis. Paradoxically, incubation of neither

monocytes nor macrophages with native LDL *in vitro*, increases their cell cholesterol content substantially (Goldstein et al. 1979). LDL must be modified before its pathogenic potential to induce foam cell formation is realized. Macrophages, EC and VSMCs can all promote LDL oxidation *in vitro*. Although the precise mechanisms are uncertain, lipoxygenases, myeloperoxidase, inducible nitric oxide synthase and NADPH oxidases have all been implicated because they can mediate LDL oxidation *in vitro* and are expressed, within macrophages, in human atherosclerotic lesions. Some foam cells are derived from smooth muscle cells because these can express scavenger receptors when appropriately activated (Pitas 1990). The secretion of pro-inflammatory cytokines, matrix metalloproteinases and reactive oxygen species from these activated macrophages propagate the local inflammatory milieu and may ultimately be responsible for the thrombotic sequelae of atherosclerosis. Macrophage colony stimulating factor (M-CSF) is thought to play a key role in this activation phase, but also serves as a survival and co-mitogenic stimulus (Rosenfeld et al. 1992). Modified LDL is chemotactic for other monocytes and is able to up-regulate M-CSF gene expression (Rajavashisth et al. 1990) and EC derived monocyte chemotactic protein-1 (MCP-1) production (Leonard and Yoshimura 1990). Inflammatory mediators, specifically tumour necrosis factor α (TNFα), IL-1 and M-CSF, can have a reciprocal effect by enhancing binding of LDL to ECs and VSMCs and promoting LDL receptor gene expression (Stopeck et al. 1993).

FIGURE 1.3

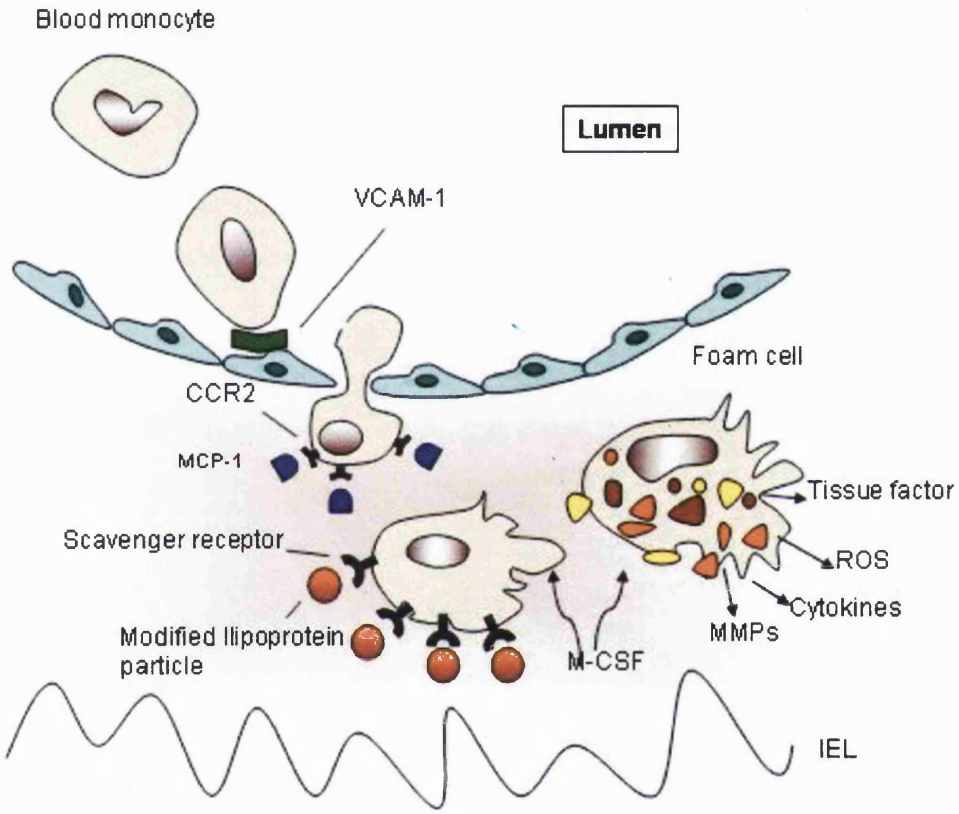


Fig 1.3. The Role of the Blood-Derived Monocyte in Atherogenesis.
Modified from Libby P, Nature, 2002.

The normal arterial endothelium resists prolonged contact with leucocytes. Endothelial dysfunction and its associated inflammatory activation lead to upregulated VCAM-1 expression. Adherent monocytes diapedese between intact ECs and penetrate the tunica intima. This directed migration requires a chemoattractant gradient and various chemokines participate in this process, particularly the interaction of MCP-1 with its receptor CCR2. Once resident within the intima, monocytes acquire the characteristics of the tissue macrophage. Expression of scavenger receptors allows internalization of oxidized and glycosylated lipoproteins, thus generating the foam cell. Continued secretion of pro-inflammatory cytokines and the generation of reactive oxygen species (ROS) propagate the local inflammatory response and plaque growth.

1.3. Endothelial Cell Adhesion Molecules

There is abundant evidence that cell adhesion molecules participate in atherogenesis. Selectins (P, E and L) and their ligands (mainly P-selectin ligand) are involved in the rolling and tethering of leucocytes onto the endothelium. Intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs) as well as some of the integrins mediate firm adhesion at the EC surface whereas platelet endothelial cell adhesion molecules (PECAMs) are involved in the extravasation of cells from the blood into the tissue compartment. Soon after the initiation of an atherogenic diet, endothelial dysfunction / 'activation' ensues and leucocytes attach to the endothelium (Poole and Florey 1958). ICAM-1 binds the integrin heterodimer CD11a/CD18 found on all leucocytes and the CD11b/CD18 heterodimer found on monocytes. VCAM-1 is transcriptionally induced on activated ECs and is thought to be one of the earliest events at lesion-prone sites, preceding macrophage accumulation (Sakai et al. 1997; Truskey et al. 1999); adhesion molecule expression and monocyte adhesion are also affected by local fluid dynamics (Walpola et al. 1995), as well as normal (Lin et al. 1996) and oxidized LDL concentrations. VCAM-1 interacts with integrin $\alpha_4\beta_1$, also known as very late antigen-4 (VLA-4) (Chan et al. 2001) and monocyte engagement with VCAM-1 induces a signaling cascade that triggers alterations in EC shape allowing leucocyte transmigration (Weber and Springer 1998; Matheny et al. 2000). Endothelial adhesion molecules mediate the attachment and transendothelial migration of mononuclear leucocytes at vulnerable sites of atheroma formation, a phenomenon thought to be critically dependent upon VCAM-1 gene dosage (Dansky et al. 2001). Expression of VCAM-1 (CD106) (O'Brien et al. 1993; Nakashima et al. 1998; Cybulsky et al. 2001), ICAM-1 (CD54) and L-selectin (CD62L) has been consistently observed in human atherosclerotic plaques. Constituents of modified lipoprotein particles, including oxidized phospholipids and short chain aldehydes, can induce transcriptional activation of the VCAM-1 gene, mediated in part by nuclear factor-κB (Collins and Cybulsky 2001). Pro-inflammatory cytokines, which are known to be present within atheromatous plaques, such as interleukin (IL)-1β or tumour necrosis factor (TNF)-α, also induce VCAM-1 expression via nuclear factor κB (NF-κB) and may explain its association with hypercholesterolaemia (Collins and Cybulsky 2001). Consistent with

this notion is the finding that statins attenuate VCAM-1 expression in cultured HUVECs through a reduction in NF-κB activity (Rasmussen et al. 2001).

1.4. Angiogenesis

The need of tissues for a blood supply is fulfilled by angiogenesis, typically defined as the sprouting of capillaries from pre-existing vessels and subsequent stabilisation of these sprouts by mural cells (Fig. 1.4) (Risau 1997). ECs are elongated, thin and fragile, yet they create channels that do not collapse and which efficiently distribute blood to target tissue. With the appropriate trigger, quiescent EC undergo coordinated and directed growth by sensing changes in blood flow and pressure, and through dynamic interaction with the internal cytoskeleton and surrounding extracellular matrix (ECM). When ECs migrate during vessel sprouting, mechanical tightness and permeability is transiently compromised as intercellular/junctional contacts are dissolved, but later re-established once new sprouts are assembled. CD31 (PECAM) and intercellular communication through connexins in gap junctions are also crucial for neo-vessel formation and maintenance. The ECM provides necessary contacts between ECs and the surrounding tissue to prevent collapse. In quiescent vessels, a basement membrane of collagen IV, laminin and other components encase vascular cells; an interstitial matrix comprising collagen I and elastin between cells further provides visco-elasticity and strength. Proteolytic degradation of the ECM during angiogenesis also enables an active participation in angiogenesis as new cryptic epitopes in ECM proteins, such as collagen IV, are unmasked. Structural transitions from monomeric to fibrillar collagen occur, inducing EC and VSMC migration (Hangai et al. 2002). Integrins are cell surface receptors of specific ECM molecules that bidirectionally transmit information between the outside and inside of cells, thus coordinating them with their immediate points of anchorage (Hynes 2002). In particular, the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins positively regulate the angiogenic switch. Key roles for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in EC migration and survival *in vivo* have been inferred from studies using pharmacological antagonists of these integrins, which were shown to suppress pathological angiogenesis (Brooks et al. 1994; Friedlander et al. 1995). Paradoxically, mice deficient in either β_3 (Hodivala-Dilke et al. 1999) or β_3 and β_5 (Reynolds et al. 2002), are viable and exhibit no defects in

vascularisation, but instead show enhanced tumour angiogenesis and increased VEGFR2 expression (Reynolds et al. 2002). These findings provocatively challenge the assumption that α_v integrins play a pro-angiogenic role and have led to the alternative hypothesis that these integrins may act to inhibit angiogenesis, at least in some situations (Hynes 2002). An essential role of other integrins in angiogenesis is more clearly established. Mice deficient in the fibronectin receptor, $\alpha_5\beta_1$, die during embryogenesis and exhibit major vascular abnormalities (Yang et al. 1993), while antibodies against the collagen receptors, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, inhibit VEGF-driven angiogenesis (Senger et al. 1997).

Remodeling of the ECM during angiogenesis requires breakdown by proteases such as plasminogen activators (urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), heparinases, chymases, tryptases and cathepsins (Luttun et al. 2000). Proteinases also facilitate EC sprouting by liberating matrix-bound angiogenic mitogens (FGF, VEGF and TGFβ) and chemokines (IL-1β). Proteolytic remodelling needs to be finely balanced, such that there is neither insufficient breakdown to prevent vascular cell migration nor excessive breakdown to hinder critical support or guidance cues for migrating cells. The pleiotropic activities of proteinases extends to resolution of the angiogenic process, as they liberate matrix bound inhibitors of angiogenesis (TSP-1, tumstatin, endostatin, platelet factor (PF)-4).

Establishment of a functional vascular network and the avoidance of vessel regression requires the maturation of nascent vessels into more durable, pericyte-coated vessels (Jain 2003). Platelet derived growth factor (PDGF)-BB and its receptor, PDGFR-β, are essential for stabilizing vessels by recruiting mesencyhmal progenitors. PDGF-B deficient mice, characterized by the insufficient recruitment of mural cells, results in endothelial hyperplasia, vessel enlargement, fragility, changed cellular distribution of certain junctional proteins and increased transendothelial permeability (Hellström et al. 2001). In addition, treatment with a combination of PDGF-BB and VEGF results in the formation of more mature vessels than monotherapy with either factor, a finding with important implications for therapeutic angiogenesis strategies (Cao et al. 2003). The angiopoietin/Tie-2 receptor signaling system also contributes to vessel growth and stabilisation. Even though trapping angiopoietins suppresses pathological vascularisation

(Takagi et al. 2003), their role is incompletely understood and appears context-dependent. Angiopoietin-1 (Ang-1) stimulates vessel growth presumably because it is an EC survival factor and mobilizes endothelial progenitor cells and haematopoietic stem cells (Hattori et al. 2001), but a capacity to inhibit angiogenesis has also been reported (Visconti et al. 2002). This anti-angiogenic effect of Ang-1 may relate to the need for EC to relax their intercellular contacts since Ang-1 decreases vessel permeability by affecting junctional molecules (Thurston et al. 2000) and by promoting EC-mural cell interaction as an adhesive protein and also by recruiting pericytes (Carlson et al. 2001).

FIGURE 1.4

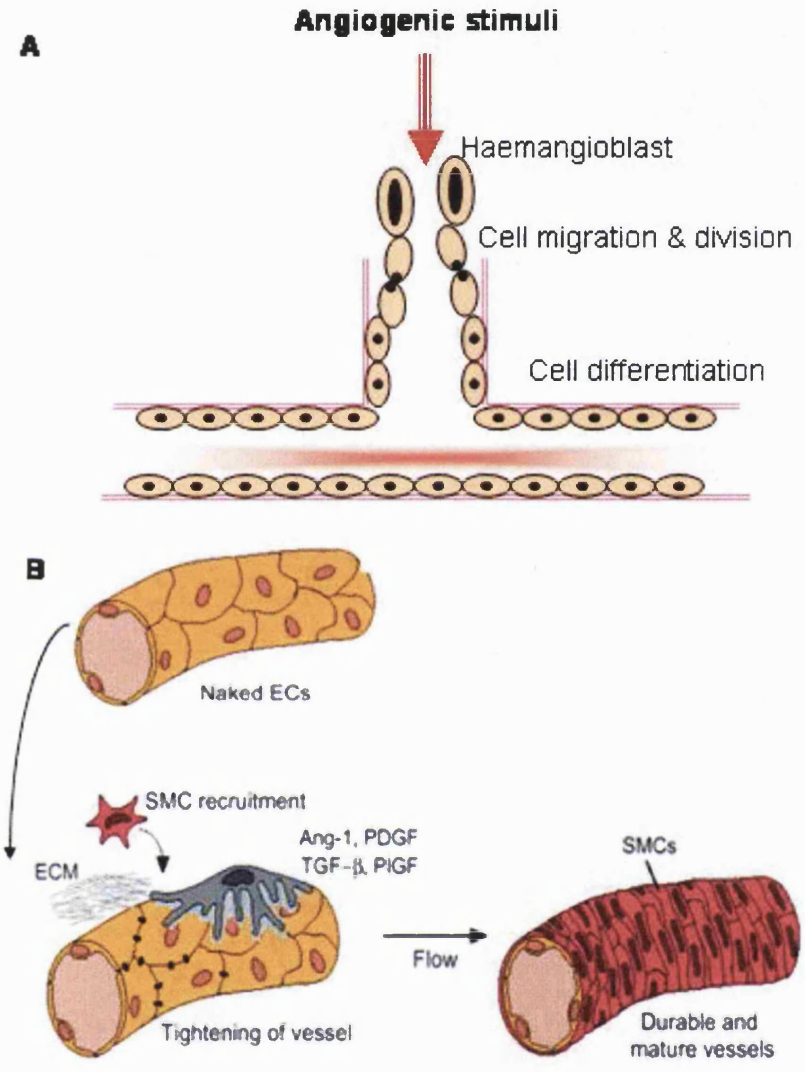


Figure 1.4. Angiogenesis and vessel maturation

A, Angiogenesis describes the growth and remodelling of a primitive vascular plexus into a more complex, functional network. The process involves the migration and differentiation of haemangioblasts (EC precursors)
B, **Adapted from Carmeliet P, Nat Med 2003a**. Maturation of nascent vessels (EC tubes) into more durable vessels requires 'vascular myogenesis' which also prevents vessel rupture or regression and stimulates the production of ECM. The muscular pericytes also bestow visco-elastic and vasomotor properties, necessary to accommodate the changing needs in tissue perfusion

1.4.1. Occlusive Vascular Pathology and Angiogenesis

The relationship between vessel wall pathology and neo-angiogenesis is not clearly understood, but the potential importance of vessel wall angiogenesis as a substrate for the development of proliferative vascular lesions has been recognized for many years (Geiringer 1951; Barger et al. 1984). Normal muscular arteries have no vessels within the inner media or intima, but large vessels possess an adventitial vasa vasorum responsible for the oxygen and nutrient supply to the media. The critical threshold distance between tissues and a capillary (or vessel lumen) above which hypoxia will occur has been calculated to be ~100 μm (Torres Filho et al. 1994). When this distance is exceeded, a hypoxic environment will form in the interior of the artery. The arterial wall oxygen supply is impaired after balloon injury but later compensated by the formation of adventitial vasa vasorum (Zemplenyi et al. 1989). The adventitial vascular plexus has been considered to play a role in vascular wall homeostasis to maintain normal arterial structure and function, whereas intimal/medial vasa vasorum may contribute to growth of the atherosclerotic plaque and plaque rupture and by serving as a conduit for inflammatory cell influx. However, little is known about the contribution of vasa vasorum or adventitial neovascularisation during arteriosclerotic remodeling or neointimal hyperplasia. Emerging novel molecular targeting strategies such as magnetic resonance imaging (MRI) of $\alpha_v\beta_3$ integrin-targeted paramagnetic nanoparticles may enhance detection of vessel wall micro-vasculature (Winter et al. 2003), and make exploration of this process more accessible. It has been proposed that the importance of angiogenesis is dependent upon the stage of lesion development, being a critical 'trigger' in the early phase, but receding within aging lesions (Ross et al. 2001).

Much of the work attributing importance for angiogenesis to the pathophysiology of occlusive vascular disease has been done in rodent models of atherosclerosis. Atheromata develop microvascular channels as a result of neo-angiogenesis; these new vessels are both fragile and prone to haemorrhage. Intra-plaque deposition of fibrin, fibrin-split products and haemosiderin, provide evidence of intra-plaque haemorrhage. Thrombosis *in situ* leads to thrombin generation which potently triggers the release of PDGF, further stimulating the migration and proliferation of VSMC. Activated platelets also elaborate transforming

growth factor-β (TGF-β), the most potent stimulus known for interstitial collagen synthesis by VSMC. Hence plaque neovascularisation and subsequent silent microvascular haemorrhage could conceivably contribute to growth spurts in the evolution of plaque growth. Microvessels may also have a nutritive function, which may contribute to plaque growth. Clinically, there is a higher incidence of intra-lesion angiogenesis in culprit lesions that culminate in unstable angina (Tenaglia et al. 1998).

Supporting evidence that angiogenesis accelerates the progression of early atheromatous lesions has been shown by intraperitoneal VEGF protein administration to hyperlipidaemic mice doubly deficient in apolipoprotein (apo) E and apoB100 (Celletti et al. 2001). Conversely, work from Folkman's group investigated the effects of two endothelial-specific inhibitors of angiogenesis, endostatin and TNP-470, on atherosclerosis in the hypercholesterolaemic apoE-deficient mouse model. These agents reduced plaque area by 85% and 70% respectively (Moulton et al. 1999). Later work from the same group demonstrated that blockade of plaque angiogenesis by angiostatin retards plaque growth, an effect which has been related to diminished macrophage infiltration in apoE-deficient mice (Moulton et al. 2003). Macrophages in the plaque and around the vasa vasorum were reduced. Activated macrophages stimulate angiogenesis that can further recruit inflammatory cells and more angiogenesis. Thus, inhibition of angiogenesis has been proposed to interrupt a positive feedback cycle and may have beneficial effects on plaque stability. A significant caveat to recent work supporting a causal role of angiogenesis in plaque progression, is the suitability of the mouse model for studying plaque neovascularisation. The incidence of lesions with intimal vessels reported by Moulton et al. was relatively low: no vessels occurred in early 'fatty streak' lesions and intimal vessels were detected in only 15 (13%) of 114 advanced aortic lesions. Even in the largest plaques (>250 μm thick), only 13 out of 46 lesions contained vessels.

Allograft-accelerated transplantation arteriosclerosis is the leading cause of late graft failure and death in patients with solid organ transplantation (Weis and von Scheidt 1997). A strong correlation has been documented between intragraft VEGF immunoreactivity and intimal thickening in rat cardiac allografts (Lemström et al. 2002). Secondly, in the same report, intracoronary perfusion of cardiac allografts with adenovirus encoding mouse $VEGF_{164}$ in cholesterol-fed rabbits enhanced the formation of arteriosclerotic lesions,

possibly secondary to intimal neovascularisation and increased intragraft influx of macrophages. Circulating bone-marrow derived progenitor cells have been shown to be a source of endothelial replacement and for neo-angiogenesis within the allograft arteriosclerotic lesions (Hu et al. 2002). In contrast to the aforementioned results, other investigators have reported neointima-reducing effects of angiogenic factors such as VEGF (Asahara et al. 1995; Laitinen et al. 1997b). Overall, the role of angiogenesis in atherogenesis and arterial neointimal VSMC hyperplasia is a highly controversial subject. Moreover, the role of angiogenesis in neointimal VSMC proliferation in non-hyperlipidaemic models and in larger models of hypercholesterolaemia was poorly defined at the outset of this thesis. The relationship between neo-angiogenesis within the vessel wall and occlusive vascular disease has become an issue of great practical as well as biological relevance in this era of growth factor gene therapy trials, where safety issues are a primary concern (Simons et al. 2000).

1.4.2. Atheroprotection and Growth Factors

Regions of the arterial tree protected from atherosclerosis usually experience laminar shear stress, due to blood flow. Several genes with potentially atheroprotective properties possess shear-stress response elements in their promoter regions. Such genes include superoxide dismutase (SOD) which may modulate inflammation by combating oxidative stress (Laukkanen et al. 2002). Signaling via the VEGFR2 pathway has been shown to enhance endothelial cell survival, and intermediates within the pathway have also been demonstrated to be upregulated by fluid shear stress in microvascular and large-vessel derived endothelial cells (Urbich et al. 2003).

Macrophages have essential functions in all phases from development of the fatty streak to processes that ultimately contribute to plaque rupture. Pharmacological approaches to modulating macrophage activity, specifically inhibiting leucocyte adhesion and/or chemotaxis, are a natural target to explore (Li and Glass 2002).

1.5. Intimal Hyperplasia

Intimal hyperplasia was first characterized almost a century ago (Carrel 1983). It is a structural change defined as the abnormal migration and proliferation of VSMC with associated deposition of ECM and is a key process of atherosclerosis. Indeed, focal accumulation of VSMCs within the intima may serve as a precursor to lesion development (Schwartz et al. 1995). The intima is located between the endothelium and internal elastic lamina (IEL) of an artery or between the endothelium and the medial smooth muscle cell layer of a vein graft. An artery adjusts to normal asymmetries in fluid mechanical forces along its course around its circumference through adjustments in thickness of the intima (and thus the lumen). Thus, arteries would naturally expect to have varying wall thickness to maintain an optimal blood flow equally at all points along the artery's course. Increases in intimal thickness begin to develop in foetal life and, although variable in degree, are found in everyone at birth (Stary et al. 1992). A thick intima is strongly associated with regions at and near bifurcations of arteries, where it is usually focal and eccentric. A thick intima is also found at some sites that are not obviously related to a branch vessel, and where it is more diffuse. These thick segments are called adaptive intimal thickening. Accumulation of lipid and macrophage foam cells occurs preferentially, but not exclusively, in these adaptive thickenings. Although a proportion of these lesions reach an equilibrium, stabilize and do not evolve, some will mature into clinically unstable plaques. It could be argued that regression and prevention of progression of these lesions are the optimum therapeutic ambition.

The precise origin and function of VSMCs in atherosclerosis is contentious and may depend upon the maturity of the lesion. Intimal thickening is apparent during normal development, detectable within neonatal coronary arteries (Ikari et al. 1999). This spontaneously developing intima is considered to be an adaptation to mechanical wall stress. The VSMCs reputedly display monoclonal lineage (Schwartz and Murry 1998), which suggests that the neointima arises from the proliferation of resident pre-existing clonal VSMCs. However, extensive replication within atherosclerotic lesions has not been a consistent finding (Katsuda et al. 1992), but this does not preclude the possibility of episodic spurts. Embryonic EC are reportedly able to transdifferentiate into mesenchymal

stem cells expressing smooth muscle cell actin (DeRuiter et al. 1997). Animal model data support the notion that in neointimal lesions associated with conditions as diverse as hyperlipidaemia-induced atherosclerosis, post-angioplasty restenosis, and graft vasculopathy, a substantial percentage of VSMCs may originate from subpopulations of bone-marrow and non-bone marrow derived circulating cells (Hillebrands et al. 2001; Sata et al. 2002). The contribution of these cells to human atherosclerosis has yet to be proven but circulating smooth muscle progenitors have been identified in human peripheral blood. The artery wall may also harbour a population of resident cells with multi-lineage developmental potential (Tintut et al. 2003).

Several vascular diseases involve VSMC proliferation as the primary pathophysiologic mechanism, including in-stent restenosis, transplant vasculopathy and vein bypass graft failure (Dzau et al. 2002). Percutaneous translumenal coronary angioplasty (PTCA) is a widely used therapeutic procedure for occlusive coronary artery disease. However, restenosis secondary to cellular hyperplasia within the neointima has emerged as an important clinical burden, affecting around 30 % of patients within 6 months of an apparently 'successful' procedure (McBride et al. 1988). Stent deployment reduced the failure rate to about 20% (Serruys et al. 1991), which is still unacceptably high. Emergence of drug- (sirolimus, paclitaxel) eluting stents has dramatically reduced the restenosis rate to 8.6% (Moses et al. 2003), but the biology of the process remains a vexing issue. Intimal thickening is a highly complex phenomenon and is characterized by cell-cell interactions, cell-matrix interactions, redox state, ligand-receptor interactions, tyrosine kinases and transcription factors. Gene array and differential mRNA display analyses have provided further insight in identifying novel genes that may be up- and down-regulated in intimal hyperplasia (Zohlnhofer et al. 2001).

Further understanding of intimal hyperplasia has been derived from animal models of vascular injury. Migration and proliferation of VSMCs with the secretion of extracellular matrix is a key phase in this process. Numerous growth factors have also been shown to play a crucial role, including basic fibroblast growth factor (bFGF or FGF2) (Lindner and Reidy 1991) or vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) (Nabel et al. 1993) and heparin-binding epidermal growth factor (HB-EGF). Subsequently, inhibition of VSMC proliferation by blocking the activity of growth factors

has been explored as a strategy to reduce restenosis. Loss of growth-inhibitory factors, secondary to impaired EC-derived NO release, inactivation of NO by reactive oxygen species or altered heparin sulphate proteoglycan synthesis, may also enhance the proliferative and migratory potential of VSMCs (Ignarro et al. 1987). Mitogenic growth factors share a final common signaling pathway: the cell cycle. Quiescent (G_0) cells enter a gap period (G_1), during which the factors necessary to DNA replication for the subsequent synthetic (S) phase are assembled. After DNA replication, the cells enter another gap phase (G2) in preparation for mitosis (M). Restriction points at the G_1-S and G_2-M interphases ensure orderly cell cycle progression (Elledge 1996). Several studies have utilized pharmacological agents or genes which tightly regulate transit through the cell cycle inhibit intimal thickening (Table 1.1; reviewed in Nabel 2002).

VSMCs can synthesize and secrete biologically active mediators that regulate contraction and relaxation, inflammation, apoptosis and matrix composition (Fig. 1.5). These processes, including differentiation and a commitment to hypertrophy and hyperplasia (Jacks and Weinberg 1998; Braun-Dullaeus et al. 1999), also appear to be linked by the apparatus governing the cell cycle. Cyclin dependent kinases (CDKs) regulate transcriptional gene activation by NFκB, providing a mechanism for coordinating adhesion molecule expression with cell cycle expression (Perkins et al. 1997). Macrophage migration inhibitory factor, which is essential in several inflammatory conditions, stabilizes the cell cycle-inhibitory protein $p27^{KIP1}$ and blocks proliferation.

Table 1.1

Targets of cell-cycle inhibition in vascular proliferative disease (modified from **Dzau et al. 2002**)

Pharmacological Approach	**Site of Action**	**Animal model**	
Rapamycin (sirolimus)	Protein translation	Porcine coronary stent	(Suzuki et al. 2001)
Paclitaxel	Microtubule assembly	Porcine coronary stent	(Heldman et al. 2001)
Flavopiridol	ATP-site inhibitor of CDKs	Rat carotid injury	(Ruef et al. 1999)
CVT-313	ATP-site inhibitor of CDKs	Rat carotid injury	(Brooks et al. 1997)
Irradiation			
Brachytherapy	DNA damage, p53	Numerous	(Fischell and Virmani 2001)
Gene Therapy			
Antisense oligodeoxynucleotide	CDK1 and CDK2		Reviewed in Lee et al. 1999.
	MYC	Porcine coronary injury	(Kipshidze et al. 2004)
	MYB	Porcine coronary injury	(Lambert et al. 2001)
	PCNA	Porcine coronary injury	(Robinson et al. 1997)
Ribozymes	PCNA	Porcine coronary stent	(Frimerman et al. 1999)
Transcription factor decoys	E2F	Rabbit vein graft, Rat carotid injury, porcine coronary injury	(Nakamura et al. 2002)
Gene Transfer	p21 ^{CIP1}	Rat carotid injury	(Ueno et al. 1997)
	p27 ^{KIP1}	Rat carotid injury	(Chen et al. 1997)
	NOS	Porcine coronary injury	(Varenne et al. 1998)
	p53	Rat carotid injury	(Scheinman et al. 1999)
	Retinoblastoma	porcine femoral, rat carotid injury	(Claudio et al. 1999)
	GAX	Rabbit iliac imjury	(Maillard et al. 1997)
	GATA-6	Rat carotid injury	(Mano et al. 1999)

FIGURE 1.5

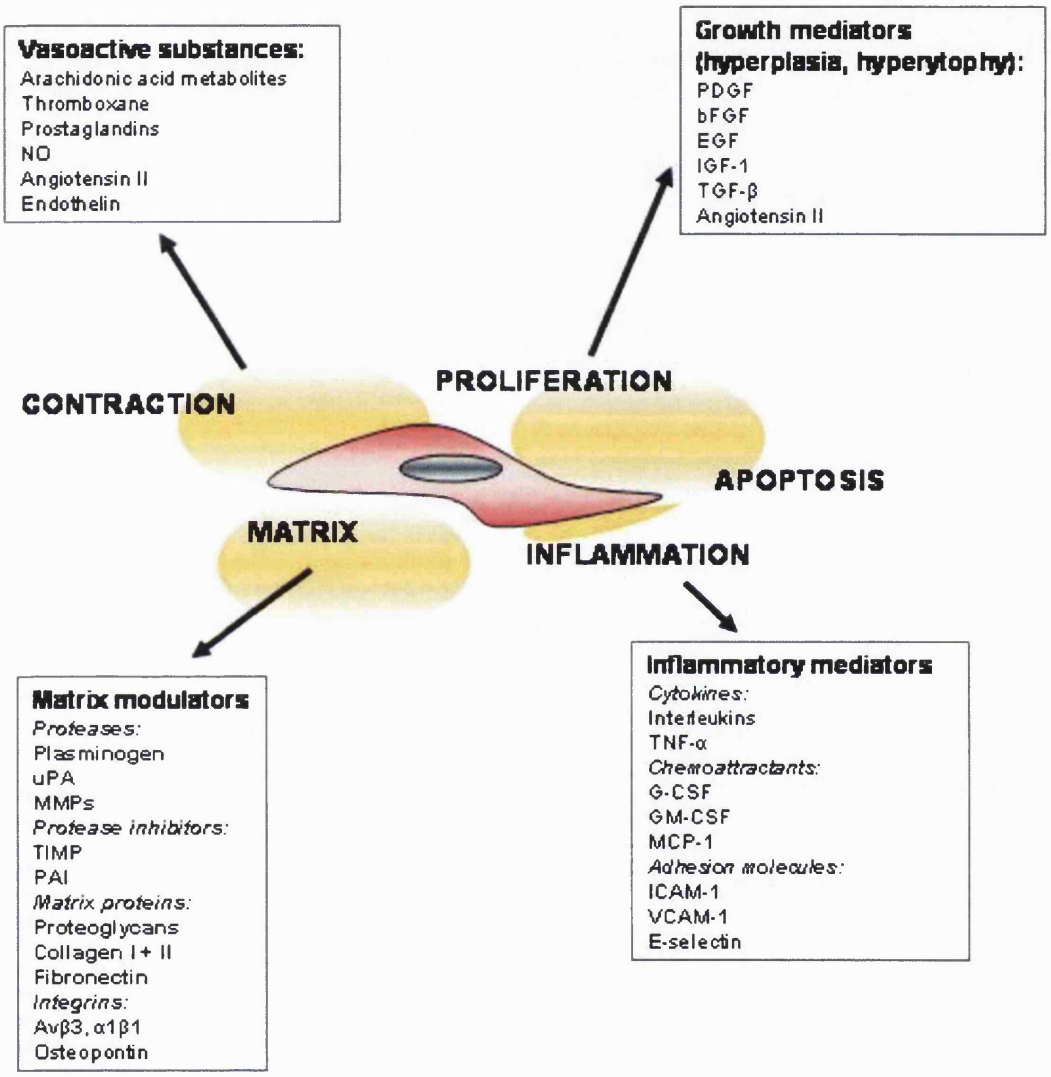


Figure 1.5. Multiple stimuli control VSMC proliferation including factors involved in inflammation, matrix alterations and contraction.

1.6. Animal Models

1.6.1. Intimal Hyperplasia

To dissect the cellular and molecular mechanisms underlying the origins of intimal hyperplasia, there is a need for appropriate animal models of a rapidly induced and controlled neointima formation. In the majority of models, an injurious stimulus is directly inflicted upon the normal, intact artery to facilitate intimal thickening. Intravascular techniques include endothelial cell denudation using a balloon embolectomy catheter, the distension of the artery by balloon angioplasty, implantation of a stent and dessication by blowing a stream of air through the lumen (De Meyer and Bult 1997). These strategies may induce arterial changes that mimic the cellular processes characterising the 'response to injury' restenosis following percutaneous, catheter-mediated interventions in patients (Schwartz and Henry 2002). They also incur a degree of damage to the vessel wall media and may induce the complications that are also encountered in the clinical setting such as thrombosis, dissection and, rarely, wall rupture. Perivascular techniques, which directly damage the native endothelium, are also employed such as electrical stimulation and application of a rigid (polyethylene) collar. However, the periadventitial placement of a biologically inert, flexible silicone collar (or cuff) around the common carotid artery in the rabbit, referred to hereafter as the 'collar model', is also increasingly used (Booth et al. 1989). This strategy involves minimal trauma to the vessel wall and generates a neointima that leaves the endothelium intact. Endothelial dysfunction is a key pathological process that underlies occlusive vascular pathology and so contributory effects of the endothelium can also be assessed in this model. Variations of the cuff approach to induce neointima formation have successfully been extended to the rat (Volker et al. 1997), mouse (Dimayuga et al. 1999), and porcine carotid arteries and mouse femoral artery (Hainaud et al. 2001; Hollestelle et al. 2004).

1.6.2. Atherosclerosis

Atherosclerosis research within animal models historically focused upon insights gained from rabbits, with a lesser number of studies in pigs and non-human primates. They have been pivotal in defining the cellular events in the initiation and development of lesions (Rosenfeld et al. 1987). The recent explosion of *in vivo* studies to study atherogenic mechanisms is attributable to the use of mouse models. The small size of the mouse and budgetary considerations facilitates the use of large group numbers and robust statistical analysis. A general advantage over other species is the extensive genetic information database available on the numerous inbred strains, facilitating versatile genetic manipulations (gene and gene mutant insertion in place of the wild type allele, gene knockouts) which have generated mechanistic insights not readily obtainable from larger animal models. A range of lesion pathology, from simple macrophage foam cell morphology to more complex plaques, comprising acellular lipid cores, fibrous caps and calcification is achievable in the mouse. One limitation, which applies to all animal models, is the lack of a mouse model displaying the features of lesion rupture or erosion that defines acute cardiovascular events in humans (Majesky 2002). The emergence of bone-marrow derived vascular stem cell biology and its association with occlusive vascular disease have enabled researchers to enlist mice for bone marrow transplantation studies (Linton et al. 1995). The characteristics of selected mouse models are summarized in Table 1.2.

Table 1.2. Mouse models of Atherosclerosis

Model	**Characteristics**	Reference
C57Bl/6	Small lesions formed only in aortic root (despite prolonged hyperlipidaemic diet)	(Paigen et al. 1987)
Transgenic Models		
Human apoB100 overexpression	Requires modified diet to form simple morphology lesions in aortic root	(Purcell-Huynh et al. 1995)
apoE Variants (apoE3-Leiden, human apoE2)	Respond to modified diets to form simple lesions in aortic root (MΦ foam cell rich)	(Leppanen et al. 1998; Sullivan et al. 1998)
Gene targeted		
$ApoE^{-/-}$ (strain-dependent, usually C57Bl/6) [1]	Mice are hyperlipidaemic (VLDL rich) with accelerated lesions upon modified diet. Lesions progress to intermediate complexity of MΦ foam cells, necrotic cores and fibrous caps ± intraplaque haemorrhage. Occur in several vascular beds.	(Plump et al. 1992; Zhang et al. 1992; van Ree et al. 1994)
LDL $receptor^{-/-}$	Modest hypercholesterolaemia, but very susceptible to modified diet. Lesions are predominantly early, with an abundance of MΦ foam cells.	(Ishibashi et al. 1994)
Compound genetic manipulations		
Apo $Bec^{-/-}$ x LDL $receptor^{-/-}$	Hypercholesterolaemic (LDL rich) with pronounced atherosclerotic of varying morphology in many vascular regions.	(Powell-Braxton et al. 1998)
ApoB100 transgenic x LDL $receptor^{-/-}$	Hypercholesterolaemic (LDL rich) with pronounced atherosclerotic of varying morphology in many vascular regions.	(Sanan et al. 1998)
ApoCIII transgenic x LDL $receptor^{-/-}$	Lesions require modified diet to induce. Currently no description of lesions available.	(Masucci-Magoulas et al. 1997)

Abbreviations used: MΦ, macrophage;

1.7. Rabbits as Models for Atherosclerosis Research

Several characteristics of the New Zealand White (NZW) rabbit make it an excellent model for assessment of the effects of transgenes on atherosclerosis susceptibility. Rabbit apolipoprotein (apo) B-containing lipoproteins are similar in composition and apoprotein content to human counterparts (Chapman 1980). Rabbit liver does not edit apoB mRNA and therefore produces very low density lipoproteins (VLDL) containing apoB100 as do

[1] Most plasma cholesterol is VLDL, whereas LDL predominates within humans.

humans (Greeve et al. 1993). Cholesteryl ester transfer protein (CETP), which plays a central role in the atherosclerotic process is abundant in both human and rabbit plasma (Nagashima et al. 1988). Finally, rabbits are very susceptible to diet-induced atherosclerosis (see later). The rabbit also displays fundamental differences from the most widely used transgenic model, the mouse. In the mouse, apoB mRNA is extensively edited by the liver, manufacturing apoB-48-containing lipoproteins (Greeve et al. 1993). CETP is absent and high density lipoproteins (HDL) are predominant in the plasma, conferring resistance to diet-induced atherogenesis. However, the rabbit lacks an analogue of human apoA-II and is relatively deficient in hepatic lipase (HL) (Warren et al. 1991), neither of which is the case for the mouse. The HL present in the rabbit is bound to the vascular endothelium, as it is in the human, whereas the majority of murine HL is circulating. Watanabe rabbits have a naturally occurring LDL receptor mutation, with greatly elevates plasma cholesterol concentrations predisposing them to premature atherosclerosis, which parallels the human disease (Buja et al. 1983). Thus, the rabbit provides a useful system within which to study the effects relevant to human atherosclerosis. The development of transgenic rabbit technology promises to yield valuable insight into the effects of individual genes and on lipoprotein metabolism and atherogenesis (Brousseau and Hoeg 1999).

1.7.1. The 'Collar Model'

The collar model was originally described by Booth et al. (1989), and has since established itself as a useful means to investigate neointima formation and other atherogenic events (leucocyte infiltration, VSMC proliferation, extracellular matrix deposition) without damaging the endothelium. Both carotid arteries are surgically exposed and one artery is enclosed by a flexible, hollow silicone collar. The free edges of the collar come into contact with each other and form a seal. Variations to induce a more definitive seal include gluing the edges together or tying sutures circumferentially around the collar. Sham operation (surgical manipulation in an identical fashion for the same time as the experimental carotid artery) of the contralateral artery in the same animal serves as a natural control. MRI of the collared artery demonstrates that the vessel has a slight curvature but is not occluded

(Carpenter et al. 1991). There is also no evidence that filling the space between the collar and periadventitia with saline causes any significant compression of the arterial wall. A subendothelial neointima comprising α-actin positive VSMCs orientated longitudinally relative to the direction of blood flow, is consistently induced within 5 days in rabbits fed a normal chow-diet. Lesions can be either concentric or eccentric. The VSMCs have a synthetic phenotype (Kockx et al. 1993) and in this respect, the collar-induced intima bears a resemblance to the normal human intima and intimal areas adjacent to atheromata (Mosse et al. 1985). An intact endothelium is preserved, as confirmed by anti-CD31 staining and scanning electron microscopy, with no macroscopic morphological change identifiable in the vascular endothelium in the length of vessel encased by the collar within 6 hours or at 1, 3 and 14 days (Kockx et al. 1993). Functionally, the intima-bearing (collared) arterial segments exhibit impaired vasodilation to the endothelium-dependent vasodilator, acetylcholine, which acts by enhancing NO release from the endothelium. This impairment is evident before intimal thickening occurs and persists after development of the lesion (De Meyer et al. 1991).

Although numerous explanations have been suggested to explain collar-induced intimal thickening, all are speculative and the mechanism is not really known (De Meyer et al. 1997). Proposed mechanisms include the loss of perivascular innervation, hypoxia secondary to occlusion of vasa vasorum, the induction of shear stress or turbulent flow and hindrance of transmural flow. Transmission electron microscopic changes affecting the endothelium have been described but exactly how this translates to dysfunction by sensitizing the subendothelium to serve as a substrate for migrating VSMC is not clear.

In the few hours after surgery, electron microscopy demonstrated small foci of EC denudation measuring 5 to 200μm in diameter that had regenerated to form a continuous layer by 24 hours (Donetti et al. 2002). In these areas, platelets and occasional leucocytes are present on the basal lamina (Kockx et al. 1993). It is likely that these early changes are the immediate result of distension injury from surgical manipulation but this is insufficient by itself to induce neointima formation. Intimal hyperplasia is preceded by a transient influx of leucocytes, predominantly polymorphonuclear (PMN) cells, in the collar model (Kockx et al. 1992; Donetti et al. 2002). Most PMNs disappear by day 3, as evidenced by scanty CD14 immunoreactivity. Their infiltration does not appear a prerequisite for early intimal

proliferation in this model, which is mostly due to SMC migration. More recently, transmission electron microscopy studies reported that the disappearance of these PMNs could be due their emperipolesis i.e. internalisation, by medial VSMCs, which show ultrastructural features between the true contractile and fully synthetic phenotype (Donetti et al. 2002). After this initial early phase of PMN infiltration, it has been proposed that medial SMC proliferation/migration followed by a final phase of intimal SMC proliferation is the cause of neointima formation in this model (Kockx et al. 1992). However, the pathophysiological role of PMNs may be model-dependent, since they appear to be critically involved in the balloon angioplasty model of iliac restenosis also within the rabbit (Welt et al. 2000).

Despite being morphologically intact in the initial 24-72 hours after collar placement, transmission electron microscopy showed that EC lost their normal flattened shape and became cuboidal. The endothelium becomes 'activated,' characterized by pronounced rough endoplasmic reticulum, free ribosomes and swelling and exhibit intense von Willebrand factor (vWF) immunoreactivity (Kockx et al. 1993), compared with the granular pattern of normal ECs. ECs typically express vWF, a platelet ligand essential for platelet adhesion and aggregation in response to vascular injury. In normal arteries, ECs secrete vWF both in the plasma and toward the subendothelial space, where a minimal deposition of this glycoadhesive protein is present in the basal lamina (Houdijk et al. 1986). It is likely that, after collaring, ECs directly synthesize and release the vWF, rather than platelets since the endothelium is always continuous with a notable absence of adhering platelets. The importance of increased deposition of vWF is undetermined. It is possible that it is merely a marker of endothelial activation, but its deposition after balloon angioplasty and within stenosed venous bypass grafts suggests an aetiological role.

Over the last decade, the collar has been used as a vehicle for localized perivascular drug or gene delivery targeting different cell types within the vessel wall, predominantly to investigate their therapeutic potential in abrogating neointima formation. Substances can be conveniently injected into the space between the collar and the arterial wall or be infused into the collar via a surgically implanted mini-osmotic pump. One major advantage of the collar approach over alternative models of neointima formation is the ability to exploit the endothelial dependence of prospective therapeutic candidates. Table 1.3 summarizes the

studies employing the collar model in the NZW rabbit carotid artery investigating the efficacy of prospective therapeutic agents on intimal thickening.

Nitric oxide (NO) plays a pivotal role in vessel wall biology, exerting potent restorative effects on the endothelium and inhibitory effects on VSMC proliferation, platelet aggregation and leucocyte adhesion. It is produced by a constitutive endothelial NO synthase (eNOS) in response to various stimuli, including fluid shear stress and exposure to the neurohumoral factors acetylcholine, bradykinin, serotonin and substance P. NO is an ephemeral but potent vasodilator that induces VSMC relaxation through the activation of soluble guanylate cyclase. Inhibition of endothelium-dependent relaxation due to reduced bioavailability of NO is the most prominent feature of endothelial dysfunction. In contrast to the injury/denuding models of intimal hyperplasia, the collar model allows endothelial dependence of NO production to be evaluated in both innate and exogenous mediators of vasculoprotection (Zachary et al. 2000). Both overexpression of either eNOS or iNOS have successfully inhibited intimal hyperplasia in numerous animal models (Janssens et al. 1998; Varenne et al. 1998). In addition, several NO donors have been investigated for their arterioprotective effects in the collar model (see Table 3). Collar placement *per se* also been reported to enhance inducible NOS (iNOS) expression within the neointima leading to a higher basal release of NO (Arthur et al. 1997). Whether this constitutes a protective response, albeit inadequate, mounted by the vessel wall is debatable. There is evidence in different models, including iNOS knockout mice, that neointimal VSMC iNOS expression, is associated with increased lesion formation (Chyu et al. 1999). Conversely, it has also been reported that iNOS expression in the collar model is absent from the neointimal and medial VSMC and inhibition of adventitial iNOS expression inhibits intimal thickening (De Meyer et al. 2000).

Table 1.3. Summary of studies investigating the effects of periadventitial drug and gene delivery in the rabbit collar model (1987-2002).

Pharmacological Agent	Study Duration (days), Diet	Effect on Neointima	I/M change	Reference
ACE Inhibitor				
Perindopril	**14, S**	↓	0.11±0.02 v 0.05±0.01	(Hickey et al. 1996)
NO donor				
Spermine-NONOate	**14, S**	↓	(74% reduction)	(Yin and Dusting 1997)
FK409 (10mg/kg/day)	**10**	↓	0.19±0.04 v 0.04±0.01	(Yasa et al. 1999)
SPM-5185	**14, S**	↓	0.11±0.02 v 0.05±0.01	(De Meyer et al. 1995)
PAF antagonist				
WEB 2170	**10, S**	↓	0.21±0.02 v 0.07±0.01	(Yin et al. 1998)
ET receptor antagonist				
Bosantan	**14, S**	↓	0.13±0.02 v 0.29±0.04	(Marano et al. 1998)
Ca^{2+} channel blocker				
SR 33805 (5mg/kg/day)	**30, S**	↓	0.36±0.06 v 0.14±0.07 *	(Hainaud et al. 2001)
Verapamil	**14, S**	↔	0.31±0.07 v 0.32±0.09	(Ustunes et al. 1996)
Lercanidipine (3mg/kg/wk)	**10 weeks, S then HC**	↓	2±0.42 v 0.32±0.1	(Soma et al. 1998)
Type C-NP (10µmol/L)	**7, S**	↓	0.16±0.01 v 0.06±0.01	(Gaspari et al. 2000)
Anti-oxidants				
Probucol, Carvedilol	**14, HC**	↓	1.10±0.14 v 0.51±0.1; v 0.52±0.1	(Donetti et al. 1998)
LDL, oxLDL (7µg/hr)	**14, S**	↑	0.13±0.03 v 1.77±0.08; v 0.92±0.05	(Matthys et al. 1997)
VEGF-A plasmid (25µg)	**14,S**	↓	0.44±0.2 v 0.25±0.08 NS	(Laitinen et al. 1997b)
AGE-BSA (15µg/hr)	**14, S**	↑	0.52±0.1 v 1.14±0.2	(Crauwels et al. 2000)
Statin				
Lovastatin; Simvatin; Fluvastatin; Pravastatin	**14, S**	↓	0.36±0.04 v 0.24±0.03; 0.2±0.03; 0.17±0.03; 0.32±0,03 (NS)	(Soma et al. 1993)
Fluvastatin 5mg/kg/day	**14, HC**	↓	0.25±0.07 v 0.19±0.11 (NS)	(Baetta et al. 2002)
Policosanol	**15, S**	↓	0.97±0.05 v 0.02±0.01	(Noa et al. 1998)
ApoA-I Milano dimer	**10, HC**	↓	0.63±0.11 v 0.26 ±0.19	(Soma et al. 1995)
Cytochalasin D (10^{-5}M)	**14, S**	↑	0.2±0.06 v 0.4±.14 (NS)	(Bruijns and Bult 2001)

Diet: S, Standard low-cholesterol; HC, High Cholesterol.
ACE, angiotensin converting enzyme; PAF, platelet activating factor; ET, Endothelin; LDL, low density lipoprotein; oxLDL, oxidized LDL; TypeC-NP, Type C-natriuretic peptide; AGE-BSA, advanced glycation end products-bovine serum albumin.
↓, Neointima-reducing effect; ↑, Neointima-increasing effect.
I/M values are expressed as mean ± s.e.

1.7.2. Intimal Hyperplasia Induced by Collar Placement: Variation of Cellular Composition

Feeding rabbits a cholesterol-enriched (0.3 to 2.5% cholesterol) diet generates lesions that bear a resemblance to the fatty streaks observed in human arteries (Verbeuren et al. 1986). These lesions predominate in the aortic arch and the proximal part of the thoracic aorta. In the abdominal aorta and pulmonary artery, fewer lesions are present. This distribution contrasts with that found in human atherosclerotic disease, and the extremely high levels of serum cholesterol achieved by cholesterol-supplemented diet in the rabbit (>20mmol/l) are rarely seen in patients. Nevertheless, perivascular collaring of the carotid artery in cholesterol fed rabbits generates neointimal lesions comprising VSMC and macrophages in the collared region. The normal endothelium does not generally support the binding of leucocytes. However, it has been demonstrated that soon after the initiation of an atherogenic diet, endothelial decompensation ensues and patches of arterial endothelial cells express adhesion molecules that bind cognate ligands on leucocytes, enhancing their migration into the intima (Libby 2002).

1.8. Gene Transfer to the Vasculature

Gene delivery to the vasculature has been performed mainly via intravascular and perivascular routes using the naked gene, liposomes and a variety of viral vectors. Targeted delivery to the native vessel wall has generally involved direct physical methods, including catheter-mediated local perfusion, direct intramuscular injection and coated or drug-eluting stents. Successful gene transfer and expression has been achieved after implantation of polymeric stents impregnated with adenovirus encoding LacZ (Ad.LacZ) in rabbit carotid arteries (Ye et al. 1996). For bypass grafts, adventitial approaches or *ex vivo* treatment have also had limited success (Ylä-Herttuala and Alitalo 2003). Advances in molecular phenotyping of organ-specific endothelial cells may further enhance site-specific targeting of genes to small arterioles and capillaries. The lack of tissue specificity of vectors may be overcome by using either hypoxia-inducible or tissue-specific promoters. Examples of EC-specific promoters include thrombomodulin (Weiler-Guettler et al. 1996), vWF (Aird et al.

1995) and Tie-2 (Minami et al. 2003). The latter is a receptor tyrosine kinase receptor for angiopoietins, essential for the regulation of vasculogenesis and remodeling (Schlaeger et al. 1997). Use of the SM22α promoter successfully directed uptake of adenoviral β-galactosidase specifically into the VSMCs of balloon-injured rat carotid arteries (Kim et al. 1997). Emerging regulated gene expression systems such as the doxycycline-regulated lentiviral vector may overcome the difficulties in regulating protein dosage levels after in vivo gene transfer (Koponen et al. 2003). This vector allows the transgene to be specifically turned on/off in response to doxycycline.

1.8.1. Vectors

1.8.1.1. Cationic lipid formulations

Experiments conducted in the early 1990s suggested that naked or cationic lipid-associated DNA could be used as a gene transfer tool. However, the promise of such formulations for transfer to the vessel wall has been undermined by low rates of transgene transfection and expression. Only a small fraction enters the cells and subsequently the nucleus, where it remains extrachromosomal and directs a transient expression normally lasting 1-2 weeks (Tripathy et al. 1996). Commercially available liposome formulations are limited by the need to use low concentrations, which is necessary to avoid toxicity effects, observed when higher concentrations of DNA-liposome complexes are exposed to cells (Stewart et al. 1992). Although improved liposomal formulations can produce further increases in transgene expression, it has remained least 10 times less than that achievable by adenoviral transduction in the vasculature. This low rate of expression may be related to the method of cationic liposome uptake by the cells. They are absorbed through non-specific interactions with the phospholipid bilayer of the cell membrane, in contrast to receptor-mediated, specific targeting obtained with the use of certain viral vectors (Wivel and Wilson 1998). Intravascular injection of naked plasmid DNA under high pressure to rat skeletal muscle markedly enhanced gene transfer efficiency to around 10%, and other methods such as electrical stimulation-induced membrane permeabilisation or electro-sonoporation (a combination of electric pulse and ultrasound) increase transfection efficiency substantially

(Mathiesen 1999; Yamashita et al. 2002). The clinical use of these approaches involves significant tissue damage, which itself enhances gene uptake. Traditionally, however, transfer of plasmid DNA has not been associated with major safety concerns, but 39% patients that received intracoronary plasmid-liposome complexes suffered a transient fever (Hedman et al. 2003).

1.8.1.2. Adenoviral (Ad-) vectors

Adenoviruses encode linear double-stranded DNA of approximately 36 kilobase pairs (kbp) surrounded by a capsid. Wild type human adenoviruses, existing in at least 51 different serotypes divided in 6 sub-groups, are a general cause of respiratory and gastrointestinal infections (Yeh and Perricaudet 1997). They offer several advantages which make them the most commonly used gene transfer vectors to investigate cardiovascular biology, in particular the angiogenic process, and as therapeutic adjuncts within clinical cardiovascular trials. They are readily engineered and easily introduced into accessible target tissue to infect quiescent and dividing cells (St George 2003). The adenoviral genome remains episomal within the infected cell, thus preventing the risk of insertional mutagenesis. This means, however, that each cell cycle leads to the loss of transgene expression (Dai et al. 1995). Cytokine expression begins within 24h and expression levels can be adjusted by altering the dose of injected virus.

Most adenoviral serotypes enter cells via a coxsackie-adenovirus receptor (CAR) and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which mediate the primary attachment and internalisation, respectively (Bergelson 1999). Of note is that the interaction of integrins exerts only a minimal effect on the overall transduction efficiency, at least *in vitro* (Mizuguchi et al. 2002). After internalisation, the viral DNA is released from the disassembled capsid in endosomes and it reaches the nucleus within 45 min. The transduction efficiency of unmodified adenoviral vector is dependent upon the presence of CAR and integrins on target cell membranes. CAR is a component of the tight junction (Cohen et al. 2001), and is abundantly present in myocardium, hepatocytes and epithelial cells but is down-regulated in skeletal muscle during maturation, which hampers transduction of mature skeletal

myocytes (Nalbantoglu et al. 1999). Intralumenal gene transfer of adenoviruses encoding a reporter gene into isolated, intact rabbit carotid arteries demonstrated exclusive transduction of EC (Gruchala et al. 2004). Penetration and transduction of medial SMC was described after mechanical injury to the IEL.

Problems associated with first generation adenoviral vectors pertain to the immunological and inflammatory reactions following transduction (St George 2003), triggered by the expression of viral genes. The adenoviral genome comprises gene sequences organized into early (E1-E4) and late regions (L1-L5) based on their expression before or after initiation of viral DNA synthesis flanked by inverted terminal repeats (ITR), which act as origins for replication, promoters, and packaging (psi or ψ) domains (Fig. 1.6). In the first generation vectors, the E1 and part of the E3 regions are deleted in order to make room for the transgene expression cassette (mammalian promoter and transgene), and to prevent viral replication. The second and third generation adenovirus vectors contain additional deletions in the E2 and/or E4 region which creates more space for the expression cassette and further decreases immunogenicity.

Transgene expression with viral heterologous promoters such as cytomegalovirus (CMV) and rous sarcoma virus (RSV) results in strong expression in many cells and tissues. Phase 1, uncontrolled trials have ascertained that local administration of low and intermediate doses of adenoviral gene transfer vectors appear to be well tolerated to individuals with a spectrum of co-morbid conditions (Crystal et al. 2002). Typically, transgene expression is maintained at steady-state levels for ~2 weeks within immunocompetent animals. Thereafter, expression declines rapidly, permitting studies of the effects of cytokine withdrawal. This early decline in gene expression may be due to inactivation or loss of vector DNA, by means other than cellular and humoral attack upon transduced cells. The repeated administration of adenovirus to skeletal muscle is effective in mice but not in larger animals because of the formation of neutralizing antibodies. Notably, the only death directly attributable to human gene therapy was caused by a very high adenoviral titer administered to an immunocompromised patient (Lehrman 1999) but recently published clinical phase I/II trials testify to the safety of Ad-gene transfer, despite an occasional transient fever (Grines et al. 2002; Rajagopalan et al. 2003).

FIGURE 1.6

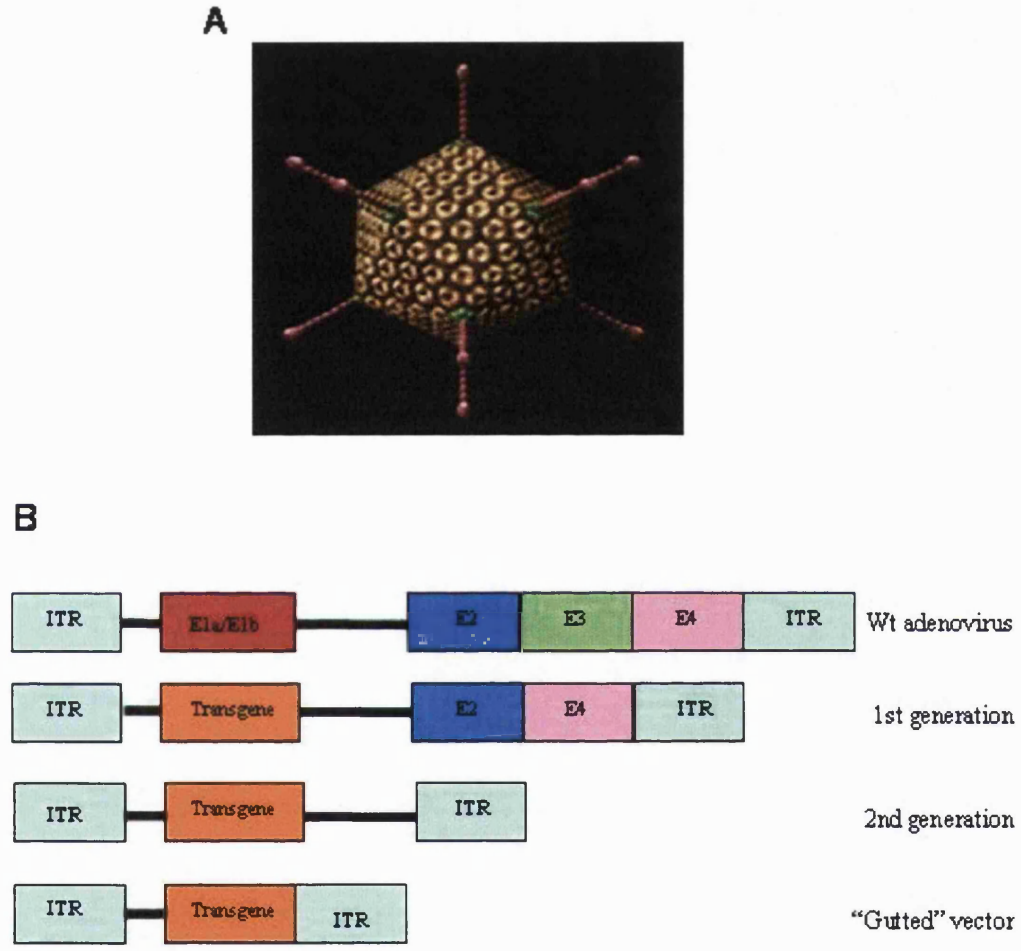


Figure 1.6. Summary of Adenovirus structure and their adaptation for use as gene delivery vectors.

A, **Adapted from Russell Kightly Media, Inc.** Adenoviruses are non-enveloped icosahedral particles approximately 70-100nm in diameter. The outer surface of the mature adenovirus comprises proteins which form and stabilize the capsid shell including the hexon, penton base and fibre proteins. Penton fibres that project from each apex mediate the attachment to the cell surface. **B**, Within 1st generation adenoviral vectors, the treatment (or marker gene) is usually Inserted in the deleted E1 region of the adenoviral genome. The E3 region is also partially or totally deleted. Within 2nd and 3rd generation vectors, E2 and/or E4 is also deleted allowing a larger transgene insert. The CMV promoter is most often used to drive the expression of the foreign gene. The capacity of the expressed gene is <7 kbp (in 1st generation vectors), depending upon the extent of the E3 deletion. ITR= inverted terminal repeat.

1.8.2. Adventitial Gene Transfer

The adventitia is a target for site-specific vascular wall therapy and has been evaluated in several animal models (Rios et al. 1995; Kullo et al. 1997). This allows more direct study of the role of the adventitia in neointima formation after injury since adventitial myofibroblasts have been shown to contribute towards vascular remodeling (Scott et al. 1996). It can easily be accessed for therapeutic means during bypass operations, prosthesis and anastamosis surgery and endarterectomies. Gene transfer vectors can be applied to the adventitial surface using a biodegradable collar or gel or by the more recently described needle injection catheter which allows direct gene transfer to the adventitia or media (Huehns et al. 1999). The latter is achieved by multiple small needles arranged linearly along the surface of the balloon that penetrate the vessel wall as the balloon is inflated.

Comparison of replication-deficient adenoviruses, VSV-G pseudotyped retroviruses and plasmid/liposome complexes in the collar model demonstrated significant differences in gene transfer efficiency (Laitinen et al. 1997a). More recently, the efficacy of *ex vivo* mediated transfer of autologous VSMC, transduced *in vitro* with retrovirus or feline immunodeficiency virus (FIV) was evaluated (Kankkonen et al. 2004). Specific advantages of the collar over alternative adventitial targeted approaches include its capacity to act as a reservoir to mediate localized transfection. The gene therapy field needs to address concerns regarding the dissemination of a transgene to non-targeted tissue where its expression may impart deleterious effects. LacZ positive cells were detected in the testis and liver (<0.01%) after periadventitial adenoviral LacZ transfer using the collar model, indicating some systemic leakage from the periadventitial space, but significantly less than was detectable after intravascular delivery (Hiltunen et al. 2000). Secondly, any intralumenal manipulation with interruption of blood flow and endothelial disruption is avoided. Thirdly, concomitant arterial VSMC proliferation enhances retroviral gene transfer efficiency.

1.8.3 Choice of Candidate Genes for Vascular Gene Transfer to Modulate Neointimal Hyperplasia

Gene therapy (GT) can be defined as the transfer of nucleic acids to the somatic cells of an individual with a resulting therapeutic effect (Ylä-Herttuala and Alitalo 2003). The effectiveness of GT is determined by the efficacy of gene delivery to the target tissue, the entry of genetic material into cells and the expression level of the transduced gene in the target cells. Modulation of localized arterial lesions requires site-specific gene targeting and to develop effective molecular therapies, it is important to understand its biology. Although inhibition of SMC proliferation and migration are possibly the main mechanisms, other factors listed in Table 1.4 are also potential therapeutic targets. Gene therapy options range from antisense oligodeoxynucleotides (ODNs) to transcription factor decoys to overexpression of specific genes through gene transfer to achieve a cytostatic effect, as listed earlier in Table 1.1. Antisense ODNs, designed to inhibit the expression of cell cycle regulatory genes (*c-myc*, *c-myb*, proliferating cell nuclear antigen (PCNA)) have been used successfully in models of vascular lesion formation. However, a recent trial in humans testing the intracoronary administration of antisense ODNs against *c-myc* failed to demonstrate efficacy against in-stent restenosis (Kutryk et al. 2002). Encouraging results from directly targeting E2F, which is responsible for the induction of multiple cell cycle-dependent genes, using transcription factor decoy ODNs, to inhibit neointimal hyperplasia (Nakamura et al. 2002) led to the first clinical trial using genetic engineering techniques to inhibit cell cycle activation in vein grafts. A prospective, randomized control led trial (PREVENT I) demonstrated both safety and efficacy of intra-operative transfection of human peripheral arterial bypass vein grafts with E2F decoy ODNs (Mann et al. 1999). The PREVENT II trial is a randomized, double-blind, placebo-controlled trial investigating the safety and feasibility of E2F decoy ODNs in preventing autologous vein graft failure after CABG surgery (Dzau 2003). Interim results have confirmed safety and feasibility. Analysis of the secondary endpoints using quantitative coronary angiography and 3-dimensional intravascular ultrasound demonstrated increased patency and positive vascular remodeling (inhibition of neointimal size and volume) in the treated group at 12 months. Adequately powered phase 3 studies in coronary and peripheral vessel disease, to determine definitively the extent and duration of clinical benefit, are in progress with scheduled

completion by 2005 (Dzau 2003). Restenosis and graft failure are localized and rapidly generated vasculoproliferative processes in contrast to atherosclerosis. The present approaches with proven efficacy for the former clinical states would probably not be effective for atherosclerosis. Conversely, inhibiting VSMC proliferation could potentially be deleterious, if these cells which comprise the stabilizing fibrous cap are specifically targeted.

Table 1.4. Restenosis has several pathogenic components which potentially can be modulated.

Targets to modulate neointima formation
VSMC proliferation and migration
Matrix elaboration
Angiogenesis
Inflammatory cell adhesion
Endothelial dysfunction
Apoptosis
Thrombosis
Intimal lipid accumulation
Haemopoietic progenitor cell mobilisation and differentiation

1.8.4 Growth Factors and Pathophysiology of Intimal Hyperplasia and Early Atheroma Formation

The most common invasive treatments of atherosclerosis - angioplasty, stenting and bypass surgery - are complicated by restenosis of the treated vessel segment (Serruys et al. 1988). Post-procedural restenosis, characterized by neointimal hyperplasia, has been compared with the general wound healing process and is histopathologically distinct from primary atherosclerosis and, as such is a fundamentally different pathophysiological process. The procedure results in severe arterial damage, including removal of the endothelium and lacerations throughout the neointimal plaque and media, which leads to a series of complex cellular and molecular events. De-endothelialisation after injury exposes a highly

inflammatory, pro-thrombotic subendothelial substrate to circulating blood constituents. Several growth factors and matrix modifying enzymes (elastases, proteases, MMPs, cathepsins), released from platelets and infiltrating monocytes, macrophages and lymphocytes, mediate the migration of smooth muscle cells towards the lumenal surface of the vascular wall and initiation of cell proliferation. The highest replicative activity of these proliferating VSMC occurs a few days after injury and may continue for many months, ultimately becoming clinically significant. In contrast, the neointimal hyperplasia in the presence of an intact, dysfunctional endothelium typifies the earliest lesion of atherosclerosis, occurring at susceptible sites (regions of adaptive thickening) within the arterial system, and comprising predominantly synthetic, proliferative VSMCs. Adaptive thickenings, which are present at constant locations in everyone from birth, do not obstruct the lumen and represent adaptations to local mechanical forces (Stary et al. 1992). They are sites of macrophage/foam cell accumulation that constitute the type I atherosclerotic lesion.

Numerous studies testify to the undisputed contribution of growth factors to the process of intimal hyperplasia in several animal models (Nabel et al. 1993). Inhibition of growth factors, including PDGF, TGF-β (Smith et al. 1999) and FGF2 (Francis et al. 2003), insulin-like growth factor (IGF) (Bayes-Genis et al. 2000), hepatocyte growth factor (HGF) (Hayashi et al. 2000), have proven to successfully alleviate negative remodeling and a consequent reduction in intimal mass and an increased lumenal volume.

1.9. Angiogenic Factors

1.9.1. Vascular Endothelial Growth Factor (VEGF)

1.9.1.1. Biological Activities of Different Isoforms

VEGF or VEGF-A is a secreted, highly conserved, disulphide-bonded dimeric 46kDa protein that is an endothelial cell specific mitogen (Ferrara et al. 2003). VEGF-A belongs to a family comprising several members including VEGFs-A, B, C, D, E, and placental growth factor (PlGF). The human VEGF-A gene is organized as 8 exons, separated by 7 introns. There is also a putative exon 9 encoding a VEGF-A_{165b} isoform. Alternative exon splicing was initially shown to result in the generation of four different isoforms ($VEGF_{121}$,

$VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$), having respectively 121, 165, 189 and 206 amino acids after signal sequence cleavage. (Mouse VEGFs are one amino acid shorter compared to the corresponding human isoform). All splice variants are glycosylated and expressed as dimeric proteins, but have distinct roles in both vascular development and angiogenesis (Carmeliet et al. 1999). $VEGF_{165}$ is the predominant isoform and lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. $VEGF_{121}$ is an acidic polypeptide that does not bind heparin, whereas $VEGF_{189}$ and $VEGF_{206}$ bind to heparin with high affinity. $VEGF_{121}$ is a freely diffusible protein but $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (ECM). $VEGF_{165}$ is both secreted and able to bind heparin. The ECM bound isoforms may be released in a diffusible form by plasmin cleavage at the C-terminus, which generates a bioactive fragment. $VEGF_{165}$ is the most commonly expressed and biologically potent VEGF-A isoform. Loss of the heparin-binding domain results in a significant weakening of the mitogenic property of VEGF, which suggests that $VEGF_{165}$ has optimal characteristics of bioavailability and biological potency.

The biological profile of VEGF has been the focus of intense study for over a decade and an increasing picture of its importance in both physiological and pathological environments is emerging. The *in vitro* activity of VEGF to promote growth of vascular endothelial cells, derived from arteries, veins and lymphatics is well documented (Ferrara et al. 2003). VEGF is a potent secreted angiogenic mitogen, which has been well characterized in diverse animal models (Leung et al. 1989). In addition, $VEGF_{165}$ recruits bone-marrow derived endothelial progenitors to sites of actively occurring angiogenesis (Hattori et al. 2001). As discussed later, neo-vascular growth and maturation are a highly complex and coordinated process, requiring the sequential activation of numerous receptor-ligand interactions, but VEGF signaling often represents a critical rate limiting step. It is a survival factor for ECs, both *in vitro* and *in vivo*, which is mediated by the phosphatidyl-inositol 3'-kinase (PI3K)-Akt pathway (Gerber et al. 1998). The anti-apoptotic proteins Bcl-2 and A1 in ECs are also up-regulated by VEGF (Gerber et al. 1998). VEGF was originally identified as a permeability factor, based on its ability to induce vascular leakage (Dvorak et al. 1995), which is now appreciated to contribute to both the inflammatory response and pathological angiogenesis. Consistent with a regulatory role in vascular

permeability, VEGF induces endothelial fenestration in some vascular beds (Roberts and Palade 1995).

VEGF plays a critical role in embryonic and postnatal development. Detailed characterisation of the vascular anomalies within mouse models, in which single components of the VEGF family or related receptors have been deleted, has provided valuable information concerning their biological functions. This is summarized in Table 1.5. Disruption of even a single VEGF-A allele in mice leads to severe vascular abnormality, providing one of the only known examples of lethality due to haploinsufficiency (Ferrara et al. 1996). Thus, it is apparent that VEGF-A is a potent and critical angiogenic regulator, the concentration of which must be regulated to avoid embryonic lethality.

Table 1.5. Summary of phenotypes in VEGF and related knockout mouse models[2].

Growth Factor	+/-	-/-	Phenotype	Reference
VEGF-A	Lethal at 9-11 dpc	Lethal	Abnormal vessels, absence of vasculature	(Carmeliet et al. 1996; Ferrara et al. 1996)
VEGF-B	Viable	Viable	Heart size reduced, impaired recovery from myocardial ischaemia	(Bellomo et al. 2000)
PlGF	Viable	Viable	Impaired pathological angiogenesis / arteriogenesis	(Carmeliet et al. 2001)
VEGFR1 (Flt-1)	Viable	Lethal at 8.5 dpc	Excessive EC proliferation, abnormal vascular channels	(Fong et al. 1995)
VEGFR2 (Flk-1/KDR)	Viable	Lethal at 8.5 dpc	Reduced haematopoietic precursors, absence of vasculature	(Shalaby et al. 1997)
Neuropilin-1	Viable	Lethal at 13 dpc	Abnormal peripheral nervous system and abnormal cardiovascular development	(Kawasaki et al. 1999)
Neuropilin-2		Viable	No discernable abnormal vascular phenotype; defective cranial nerve development.	(Chen et al. 2000)
Neuropilin-1/ Neuropilin-2		Lethal	Totally avascular yolk sacs	(Takashima et al. 2002)
HIF1α		Lethal at 8.5 dpc	Striking vascular regression and abnormal remodeling within cephalic region along with marked mesenchymal cell death	(Kotch et al. 1999)

[2] There is a wide phenotypic variation in mutant mice with varying genetic backgrounds, which clearly illustrates the powerful impact of modifier genes within the system

VEGF expression levels progressively decrease postpartum and are minimal in most adult tissues, when endothelial cells become quiescent, except at sites of actively occurring angiogenesis such as in the ovaries, uterus and skin (during follicular growth). Alternative non-angiogenic roles, such as EC protection, may also be important (LeCouter et al. 2003).

VEGF signaling is mediated by three receptor tyrosine kinases (RTK), called VEGFR-1, VEGFR-2 and VEGFR-3 (Flt-1, Flk-1/KDR and Flk-4 respectively) (Zachary 2003). VEGFR-1 and -2 receptors are characterized by seven extracellular immunoglobulin (Ig)-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain. VEGFR-3 is a member of the same family but selectively binds VEGF-C and VEGF-D. Fig 1.7 illustrates the binding characteristics of the different VEGF family members to their receptors. VEGFR-2 is believed to be the major mediator of mitogenic, prosurvival and angiogenic properties of VEGF.

FIGURE 1.7

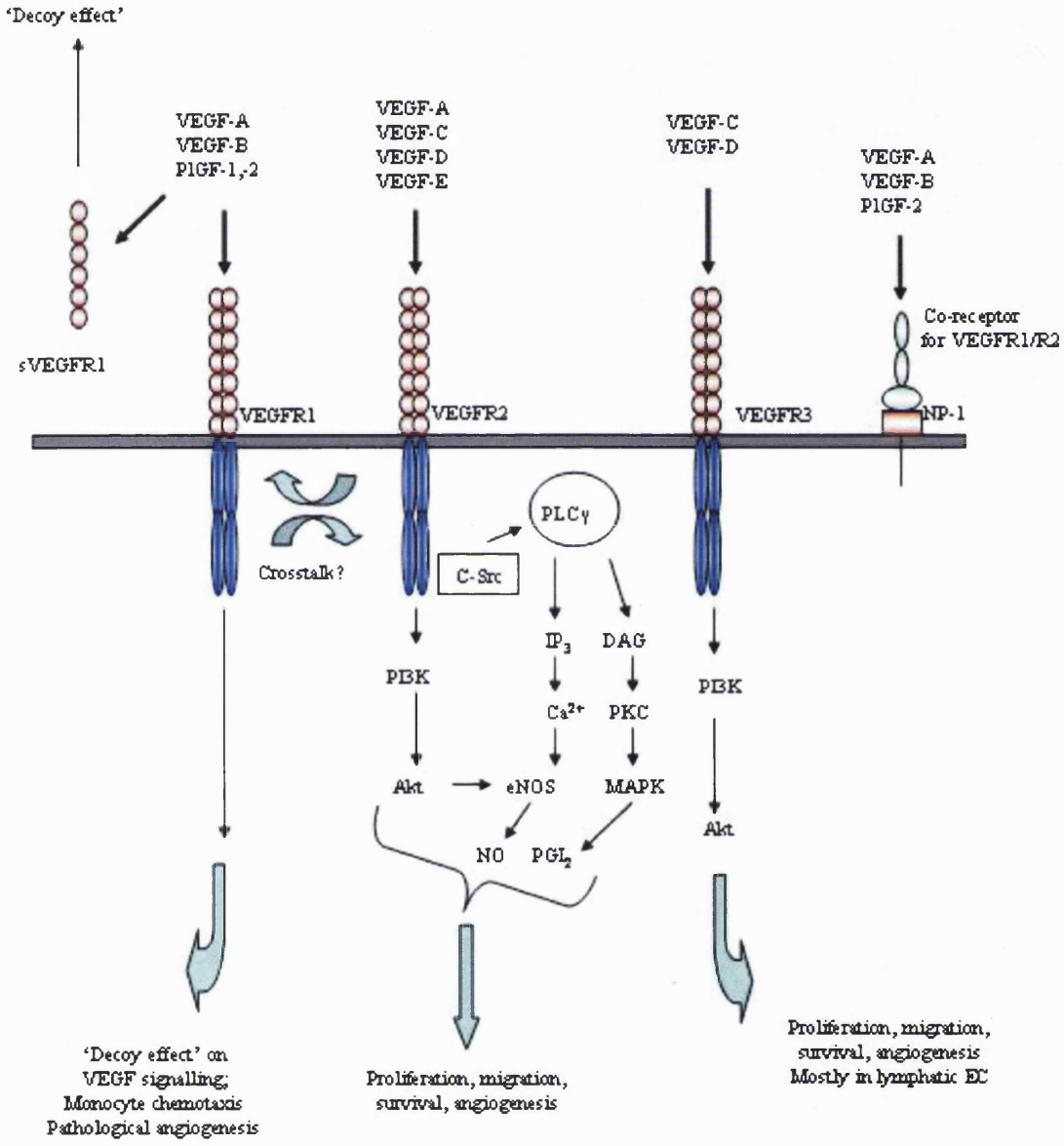


Figure 1.7. Summary of binding characteristics of different VEGF family members to their receptors.

1.9.1.2. Hypoxic Regulation of VEGF gene expression

The formation of blood vessels is triggered by a diminished supply of oxygen and nutrients, occurring when intercapillary distances exceed 100μm (i.e. exceeding 3-7 cell layers) (Torres Filho et al. 1994). Oxygen tension exerts a significant effect, both *in vitro* and in numerous pathophysiological circumstances, towards the regulation of VEGF gene expression (Lee et al. 2000). The hypoxia-inducible factor (HIF) family (HIF1α, HIF1β, HIF2α, HIF3α) mediate the hypoxic response within a range of physiological oxygen concentrations (0.5-20%) (Semenza 1998). A 28bp sequence, corresponding to the HIF-1 binding site, has been identified in the 5'-promoter of the human VEGF gene (Shweiki et al. 1992). Hypoxia-inducible genes involved in the different steps of angiogenesis are listed in Table 1.6. There are multiple ways in which hypoxia regulates VEGF as outlined in Fig 1.8.

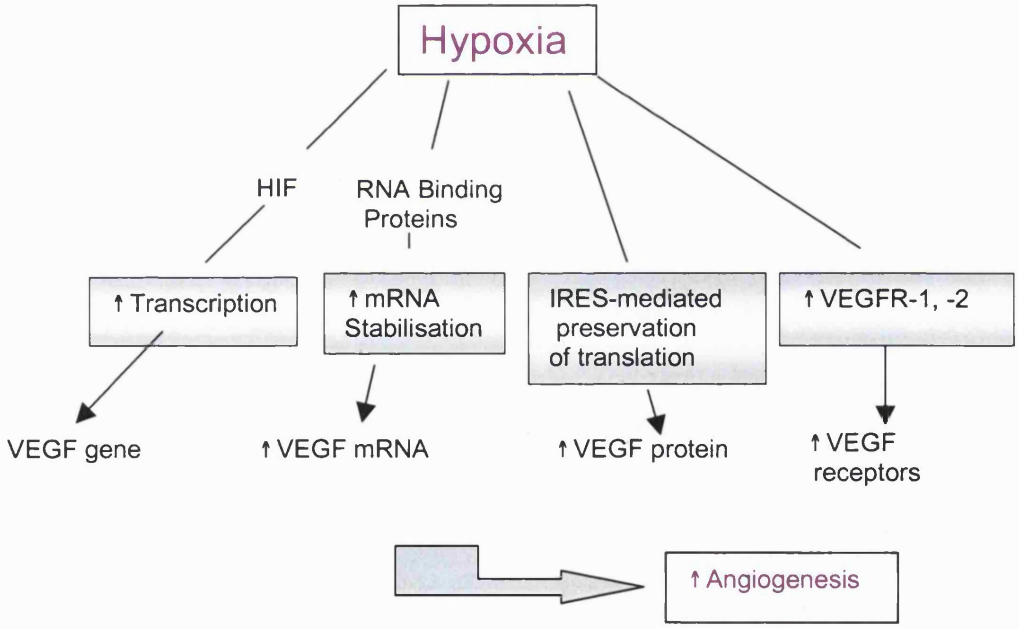


Figure 1.8. Summary of mechanisms whereby hypoxia influences the angiogenic process; IRES – internal ribosomal entry site

In addition to erythropoietin and VEGF, the target genes for transcriptional activation by HIF-1 include VEGFR1, insulin-like growth factor-2 (IGF-2), inducible nitric oxide synthase (iNOS) and PAI-1 (Forsythe et al. 1996; Semenza 2002). HIF-2α regulates at least

VEGF, VEGFR2 and eNOS expression (Kappel et al. 1999; Coulet et al. 2003). At normal oxygen tensions, hydroxylation of HIF-1α at two prolyl and asparaginyl residues, respectively leads to rapid proteasomal destruction of HIF-1α via interaction with the product of the von Hippel Lindau (VHL) suppressor gene (Pugh and Ratcliffe 2003). During hypoxia, these hydroxylases are inactive allowing HIF-1α to escape inactivation through proteasomal destruction. The VHL protein is part of the E3 ubiquitin ligase complex that targets HIF-1 subunits, after covalent attachment of a polyubiquitin chain and thus negatively regulates VEGF and other hypoxia-inducible genes (Vincent et al. 2002). The VHL gene is inactivated in patients with VHL disease, which is characterized by retinal and cerebellar capillary haemangioblastomas. Increased VEGF mRNA stability has also been shown to be an important post-transcriptional component in the response to hypoxia, mediated by the 3'-untranslated region (UTR) (Levy et al. 1997).

Cellular levels of HIF-1α are determined primarily by the rate of its ubiquitin-proteosome-dependent degradation (Maxwell et al. 1999). This allows for hypoxia-dependent and –independent means to regulate the expression of HIF-1α –inducible genes (Haddad 2002), including VEGF, potentially through the Shc-Ras signaling pathway (Abe and Berk 2002).

Table 1.6. Summary of action of hypoxia on some molecules involved in the different stages of angiogenesis; adapted from Pugh and Ratcliffe (2003).

ANGIOGENESIS STEP	Stimulatory Factor	Inhibitory Factor
Vasodilatation	Nitric oxide synthases	
Increased Vascular permeability	VEGF	Angiopoietin-1 †
	VEGFR1	
	VEGFR2 †	
Plasma protein extravasation (fibronectin, vitronectin, fibrinogen, coagulation factors)	VEGF	Angiopoietin-1 †
Endothelial sprouting	Angiopoietin-2 *	
	Tie-2 *	
ECM Degradation	Balance between MMP (MMP-2 †) and TIMP (TIMP-1)	PAI-1
	Collagen prolyl-4-hydroxylase	
Growth Factor Liberation	uPA Receptor	Thrombospondin-1 *
EC Proliferation/Migration	Interplay between VEGFs, Angiopoietins * and FGFs *	
	MCP-1 *	
	PDGF *	
Pericyte &VSMC recruitment	PDGF *	
Endothelial assembly & lumen acquisition	VEGF121/165	VEGF189
	Angiopoietin-1 †	Thrombospondin-1 *
	Intergrins	
Vessel stabilisation	PAI-1	
Maintenance, differentiation & remodeling	Angiopoietin-1 †; Tie-2 *	Angiopoietin-2 *; Tie-1 *

* Hypoxia-responsive genes in which HIF or related pathways are indirectly implicated by responses to cobalt, iron chelators or VHL inactivation.
† Responses not so far known to be connected to the HIF pathway

1.9.2. Placental Growth Factor (PlGF)

PlGF was originally isolated from a human placental cDNA library (Maglione et al. 1991). Alternative splicing of the PlGF primary transcript generates three forms of the mature human PlGF protein (Maglione et al. 1993), termed PlGF1, PlGF2 and PlGF3 (also $PlGF_{131}$, $PlGF_{152}$ and $PlGF_{203}$ respectively). PlGF2 differs from the other 2 isoforms by the insertion of a highly basic carboxy-terminal 21 amino-acid sequence, which enables binding to the co-receptors neuropilin-1 and -2 (NRP-1 and NRP-2) and heparin. PlGF2 is also the only isoform present in mice (DiPalma et al. 1996).

The 3-dimensional crystal structures of VEGF-A and PlGF1 have been solved (Iyer et al. 2001). They exhibit close structural similarities to each other, despite only 42% amino acid sequence homology and important functional differences (Muller et al. 1997; Iyer et al. 2001). The most prominent structural feature is a cysteine knot motif, comprising an 8 residue ring, formed by one interchain and three intra-chain disulphide bonds, which is characteristically found in other related growth factors e.g.TGF-β2, PDGF-B and nerve growth factor (McDonald and Hendrickson 1993). Conformational differences between PlGF and VEGF-A occur at both the amino- and carboxy-terminal residues but also within the loop regions that form part of the receptor binding face in both molecules. These differences provide a possible structural explanation for the inability of PlGF to bind VEGFR2. Gene expression profiling studies have revealed that PlGF initiates its own cascade of angiogenic genes, while mass spectrometry showed that PlGF and VEGF each induce the phosphorylation of distinct tyrosine residues in VEGFR1. The affinity of VEGFR-1 for VEGF-A (K_d = 1-20pM) is higher than that for PlGF (K_d ~ 170pM) (Davis-Smyth et al. 1996; Sawano et al. 1996) and the recent report that both PlGF and VEGF-A bind to the same binding interface of VEGFR1 in a very similar fashion suggests that dissection of the differential downstream signaling events will be complex (Christinger et al. 2004).

Many cell types produce PlGF, including ECs, VSMCs and bone marrow cells, especially when activated or stressed. PlGF selectively binds VEGFR1 (see Fig 1.7) and despite its ability to induce VEGFR1 auto-phosphorylation (Sawano et al. 1996), *in vitro* studies have not yet conclusively established whether PlGF transmits angiogenic signals.

While some studies have shown PlGF to stimulate EC growth and migration (Ziche et al. 1997a), others have documented minimal effects on proliferation and migration of ECs (Park et al. 1994). This unresolved issue is most probably due to the fact that cultured wild-type ECs are a sub-optimal model to study the effects of PlGF, since an abundant endogenous production would saturate VEGFR1 and mask sensitivity to exogenous PlGF (Carmeliet et al. 2001).

$PlGF^{-/-}$ mice are viable and devoid of any apparent vascular defects. However, in pathological conditions such myocardial or hind limb ischaemia, these mice demonstrate a reduced capacity to respond to the ischemic insult through adaptive angiogenesis and arteriogenesis (Carmeliet et al. 2001). The infarcted hearts of $PlGF^{-/-}$ mice exhibit reduced neovascularisation within the border zone, 7-14 days post ligation of the left coronary artery along with a reduced macrophage infiltration. Notably, VEGF-A and VEGFR2 expression remained at comparable levels in both $PlGF^{-/-}$ and wild type control mice. Administration of recombinant human PlGF protein through a mini-osmotic pump is able to rescue the impaired neovascularisation response along with macrophage recruitment. After ligation of the femoral artery in $PlGF^{-/-}$ mice, there was no compensatory enlargement of the pre-existing collaterals, macrophage recruitment or fibronectin deposition, all of which occurred in controls to serve as a scaffold for subsequent arteriogenesis. Thus, in the presence of physiological concentrations of VEGF-A, the loss of PlGF inhibits the involvement of macrophages, implying a specific role for their mobilisation. The monocyte-macrophage lineage is defined by a population of cells in progressive stages of differentiation and it is conceivable that a subset expressing VEGFR1 are susceptible to the actions of PlGF. By displacing VEGF from VEGFR1, PlGF is thought to make more VEGF available to bind and activate VEGFR2, thereby enhancing VEGF-induced angiogenesis (Park et al. 1994). More recent evidence suggests that this PlGF-mediated amplification is through several co-operative mechanisms: (i) Interaction with VEGFR1 creates an intermolecular cross-talk between VEGFR1 and VEGFR2, which determines the activation status of VEGFR2 and the subsequent angiogenic response and (ii) PlGF, as a subunit of PlGF/VEGF heterodimers, stimulates angiogenesis by inducing formation of VEGFR1/VEGFR2 heterodimers which trans-phosphorylate each other in an intra-molecular reaction. PlGF is also capable of up-regulating VEGF expression, thereby further

amplifying the synergism. This may partly explain why many tumours that are abundant secretors of PlGF have a rich vascular plexus (Luttun et al. 2002a). The chemoattractant property of PlGF for VEGFR1 expressing monocytes combined with the increased production of PlGF by activated macrophages, establishes positive feedback, which may contribute to diseases associated with ischaemia and inflammation.

1.9.3. Fibroblast Growth Factor (FGF)

Currently, the fibroblast growth factor (FGF) family comprises 23 closely related proteins sharing 30-70% amino acid sequence homology, and the ability to interact with specific receptors (Ornitz and Itoh 2001). The prototypical members of the family human FGF1 (acidic FGF) and FGF2 (basic FGF), were cloned in 1986 (Abraham et al. 1986; Jaye et al. 1986). FGFs elicit diverse biologic effects on numerous cell types including fibroblasts, ECs, VSMCs and keratinocytes, among others. These effects include stimulation of growth, proliferation, migration, differentiation and anti-apoptosis.

FGF1, FGF2 and FGF9 differ from all other FGFs in that they lack a signal peptide that would otherwise channel their secretion into the extracellular matrix by the classic endoplasmic reticulum/Golgi/vesicle pathway. These FGFs bind to the basement membrane and extracellular matrix and are liberated upon tissue injury. FGF2 signaling up-regulates endogenous VEGF and VEGFR2 antagonists inhibit FGF2 induced angiogenesis (Stavri et al. 1995; Tille et al. 2001). FGF1 and FGF2 have been studied the most in clinical trials to induce therapeutic angiogenesis (Simons and Ware 2003), but animal models and chick chorioallantoic membranes assays have demonstrated that FGF3, FGF4 and FGF5 also have a positive regulatory effect on angiogenesis (Giordano et al. 1996). The angiogenic capacity of the remaining FGFs has not been defined. One of the difficulties in establishing the importance of particular biologic functions, especially complex ones such as angiogenesis, is the high degree of compensatory activity among FGF family members.

Members of the FGF family have crucial but complex roles during embryonic development such as mesoderm induction, organogenesis, bone growth as well as limb outgrowth and patterning. Deletions of either FGF3, FGF4 or FGF8 are embryonic lethal

(Xu et al. 1999), whereas disruption of FGF1 or FGF5 results in only mild phenotypic changes. Despite its diverse biologic profile, FGF2 gene disruption in mice leads to a relatively mild phenotype characterized by decreased VSMC contractility, lower blood pressure and thrombocytosis (Zhou et al. 1998). They survive and are fertile suggesting a functional redundancy amongst the protypical FGFs, but compensation by FGF1 alone does not explain the $FGF2^{-/-}$ phenotype (Miller et al. 2000). Furthermore, only subtle impairments in wound healing and haematopoiesis and a normal ischaemia-induced angiogenic response were reported in $FGF2^{-/-}$ mice (Sullivan et al. 2002). FGF2 is a 16.5 kiloDalton (kDa) 146-amino-acid peptide that binds with high affinity ($K_d \sim 10^{-9}$M) to cellular and extracellular matrix heparan sulphates and with even higher affinity ($K_d \sim 10^{-11}$M) to its specific tyrosine kinase receptors (Nugent and Edelman 1992). Heparan sulphate binding of FGF2 prolongs the effective tissue half-life of the peptide and enhances the binding to its high affinity receptors. FGF2 is present in significant amounts in most normal tissues, including the myocardium (Casscells et al. 1990). It is upregulated by haemodynamic stress and to some extent, by hypoxia (Bernotat-Danielowski et al. 1993; Kuwabara et al. 1995). The mechanism of hypoxia-induced increase in FGF2 expression is not clear; it is not mediated by hypoxia inducible factor-1α (Fang et al. 2001). Recent studies *in vitro* demonstrated that hypoxia enhances EC sensitivity to FGF2 by upregulation of heparan sulphate FGF2 binding sites (Li et al. 2002).

1.9.4. VEGFR1 (fms-like tyrosine kinase, Flt1)

Although VEGFR1 was the first VEGF receptor to be identified (de Vries et al. 1992), its precise role in the mediating the complex biology of VEGF is not fully understood. VEGFR1 is essential for embryonic angiogenesis but mice expressing only the extracellular domain are phenotypically normal (Hiratsuka et al. 1998). The main defect in VEGFR1-deficient mice appears to be overproduction of endothelial progenitors leading to disorganisation of vessels. These findings are consistent with a role for VEGFR1 in the negative regulation of VEGF signalling via VEGFR2. VEGFR1 undergoes weak tyrosine autophosphorylation in response to VEGF (Waltenberger et al. 1994). A repressor motif has

been identified in the juxtamembrane region of VEGFR1 which impairs PI3K activation in response to VEGF (Gille et al. 2000), and VEGFR1 may directly regulate VEGFR2 function (Zeng et al. 2001).

Other studies have indicated induction of a signal transduction cascade and in certain circumstances, the generation of a mitogenic signal via VEGFR1 (Maru et al. 1998). VEGFR1 is thought to mediate the chemotactic response of VEGF to monocytes, which requires the tyrosine kinase domain (Barleon et al. 1996). It has also been reported that VEGFR1 may transmit a survival signal in ECs and macrophages by induction of the anti-apoptotic gene survivin (Adini et al. 2002). More recently, its role in haematopoiesis and endothelial progenitor cell recruitment has been emphasized (Hattori et al. 2002). Another novel function for VEGFR1 was also recently described in liver sinusoidal EC (LSECs). Activation of the receptor resulted in the paracrine release of HGF, IL-6 and other hepatotrophic molecules by the LSECs, such that mature hepatocytes were stimulated to proliferate when co-cultured with LSECs (LeCouter et al. 2003). This trophic capacity was shown to protect the liver from toxic insults, despite the inability of VEGFR1 agonists to stimulate LSEC proliferation. This suggests that VEGFR1 signaling in the vascular endothelium can induce the release of tissue-specific growth factors, independent of a monocyte-mediated angiogenic role.

1.9.5. VEGFR2 (fetal liver kinase, Flk-1 / kinase domain region, KDR)

VEGFR2 is believed to be the major mediator of mitogenic, survival, angiogenic and vascular permeability properties of VEGF on mature ECs (Ferrara et al. 2003). VEGFR2 binds VEGF with a K_d of approximately 75-125pM (Terman et al. 1992), upon which the receptor dimerizes followed by tyrosine phosphorylation. VEGFR2 is expressed on haemangioblasts (the common precursor of endothelial and haematopoietic cells) and may be implicated in their mobilisation from the bone marrow and migration to sites of actively occurring angiogenesis (Peichev et al. 2000). VEGF up-regulates VEGFR2 in an autocrine manner via the PI3K-mediated activation of the serine-threonine kinase Akt (PI3/Akt) pathway (Jiang et al. 2000), so hypoxia-induced VEGF expression ultimately also leads to

VEGFR2 upregulation and amplification of the angiogenic effects (Brogi et al. 1996). VEGFR2 expression can also be induced in a ligand-independent manner, by fluid shear stress leading to NO production (Jin et al. 2003).

The intracellular signaling cascades initiated upon ligand binding to VEGFR2 have been extensively studied and a highly complex picture is emerging, as summarized in Fig 1.9. Activation of mitogen-activated protein kinase (MAPK) and the downstream Raf-Mek-Erk pathway, increases DNA synthesis, EC proliferation and migration (Kroll and Waltenberger 1997). Stimulation of the PI3K/Akt pathway also promotes EC migration but also contributes to prolonged survival, by up-regulating anti-apoptotic proteins, Bcl-2 and A1 (Gerber et al. 1998). Akt-dependent and independent mechanisms (through a rise in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$) lead to eNOS activation and subsequently increased nitric oxide (NO) production by EC (Kroll and Waltenberger 1998; Dimmeler et al. 1999). In addition, prostacyclin is released in response to VEGFR2 activation via PKC-mediated MEK and ERK activation (Wheeler-Jones et al. 1997).

FIGURE 1.9. ***Legend:***

Activation of VEGFR2 occurs through ligand-induced dimerisation and receptor autophosphorylation at multiple tyrosine residues. Several intracellular proteins such as VEGFR-associated protein (VRAP), Sck and phospholipase C (PLC)-γ bind to specific tyrosine residues via their Src homology-2 (SH2) domains, leading to the phosphorylation and activation of these proteins. A major mitogenic signalling mechanism for VEGF is the PLC-γ pathway resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate, generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, and subsequent mobilisation of Ca^{2+} from intracellular stores, and PKC activation. PKC mediates activation of ERKs 1/2 via Raf-1 and MEK, and this pathway is a major mediator of both mitogenesis and $cPLA_2$ activation leading to generation of COX-derived prostanoids (PGs), including PGI_2 and PGE_2. Ca^{2+} signalling is also important for eNOS activation and NO generation. VEGF-dependent endothelial cell survival is mediated in part via PI 3-kinase (PI3K)-mediated activation of the anti-apoptotic kinase Akt, though the mechanism of PI 3-kinase activation is unclear. Akt phosphorylates and inhibits the pro-apoptotic protein Bad, leading to inhibition of caspase activity. Akt also causes Ca^{2+}-independent eNOS activation through phosphorylation, and this pathway may also be essential for migration. Increased tyrosine phosphorylation of FAK, mediated in part through Src, is a point of convergence for VEGF and integrin-mediated survival and migration signalling.

Abbreviations: $cPLA_2$, cytosolic phospholipase A2; Erk, extracellular regulated kinase; HSP27, heat-shock protein 27; MAPKAP 2/3, MAPK-activating protein kinase-2 and 3; PGI_2, prostacyclin; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; Sck, Shc-like protein; SPK, sphingosine kinase.

FIGURE 1.9

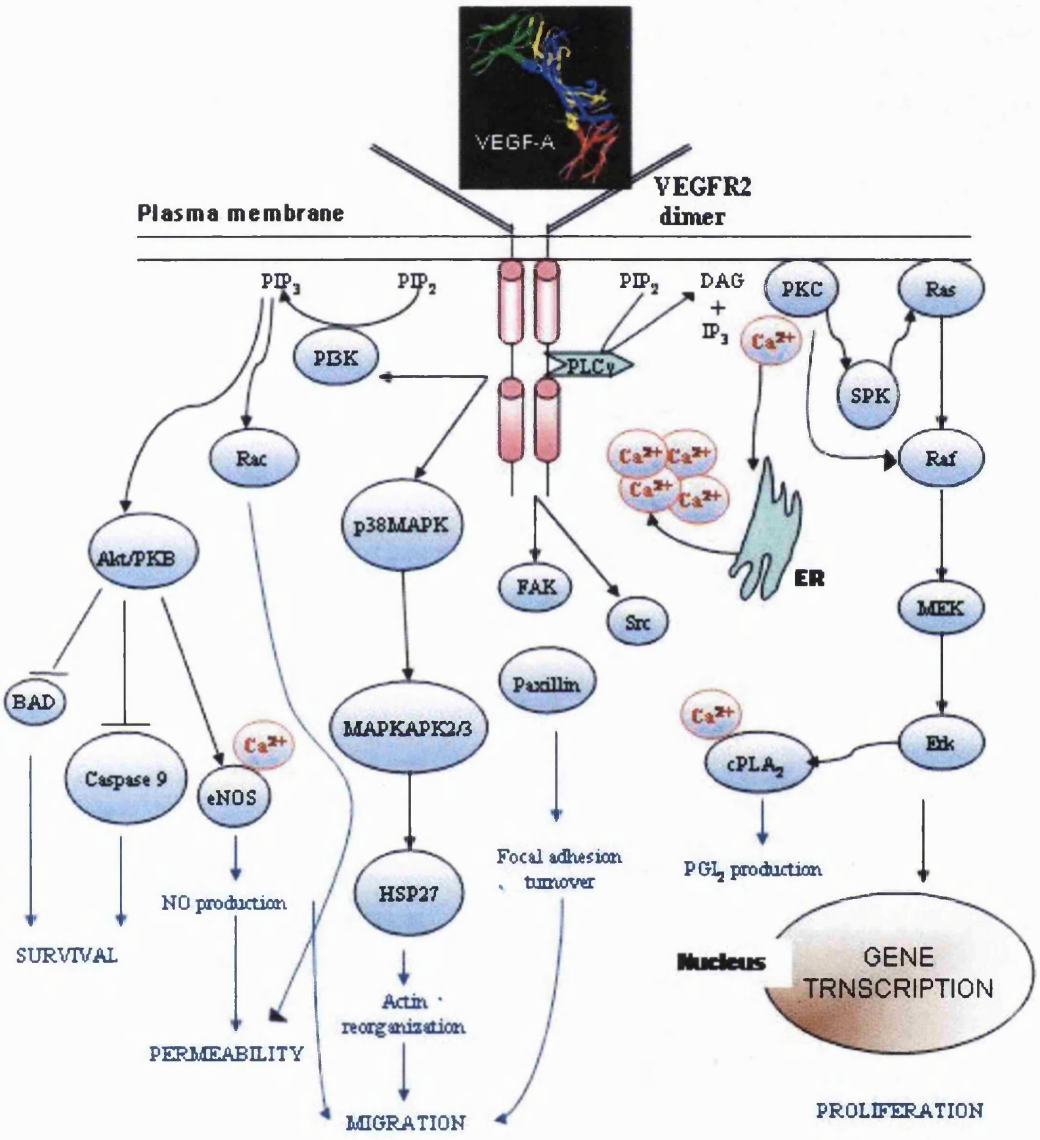


Figure 1.9. Schematic illustration of VEGFR2 intracellular signalling. Adapted from Cross MJ et al., Trends Biochem Sci 2003.

1.9.6. Neuropilins – Coreceptors for the VEGF Family

Neuropilin-1 (NRP-1) and -2 were originally identified as receptors for the semaphorins, factors with a key role in neuronal axon guidance (Neufeld et al. 2002). The extracellular part of NRP-1 and NRP-2 possesses 2 short complement binding domains, 2 coagulation factor V/VIII homology domains, a MAM (meprin, A5) domain, which are important for homo- and hetero-dimerisation of NRP (Renzi et al. 1999), a transmembrane segment and a short cytoplasmic domain lacking enzymatic activity. NRP-1 is a receptor for $VEGF_{165}$, PlGF2, VEGF-B and VEGF-E, but not other VEGF-A or PlGF isoforms or other VEGF family members. $VEGF_{165}$-induced migration of cells expressing VEGFR2 was enhanced in the presence of NRP-1 (Soker et al. 1998). Similarly, NRP-2 behaves as a splice-form specific VEGF receptor, being capable of binding both $VEGF_{165}$ and $VEGF_{145}$, but not $VEGF_{121}$ (Gluzman-Poltorak et al. 2000). This may partly explain the reduced biological potency of $VEGF_{121}$ compared to $VEGF_{165}$ (Whitaker et al. 2001). Heparin binding capability appears to be significant prerequisite for a growth factor to form a complex with NRP receptor. Both PlGF2 and VEGF-B are selective ligands for VEGFR1, which was recently found to form complexes with NRP-1 and NRP-2 (Fuh et al. 2000), suggesting that this receptor may play a key role in NRP associated signaling. Like VEGFR1, NRPs exist as soluble 'decoy' forms, generated by alternative splicing, and which may negatively modulate angiogenesis and vasculogenesis (Neufeld et al. 2002).

1.9.7. Fibroblast Growth Factor Receptors

The cloning of FGF receptors (FGFRs) has identified four distinct genes encoding four transmembrane tyrosine kinase receptors. Recently, existence of FGFR5 was also reported, but it lacks a tyrosine kinase domain, suggesting a role as a decoy receptor (Sleeman et al. 2001). The four principal FGFRs share 55% to 72% homology (Johnson and Williams 1993). Endogenous heparan sulphate proteoglycans, such as syndecan-4 are required to enhance receptor binding of FGFs (Klint and Claesson-Welsh 1999). The structural motifs that constitute the transmembrane FGFR are three extracellular immunoglobulin (Ig)-like

domains (designated IgI, IgII and IgIII), an acidic region between IgI and IgII, a transmembrane domain and an intracellular tyrosine kinase domain (for a review, see (Powers et al. 2000). Alternative splicing of the IgIII domain generates another source of variation for FGFRs 1, 2 and 3, which are designated IgIIIa and IgIIIb and IgIIIc, with different affinity profiles towards FGFs. The FGFR4 gene is unique in that there are no splice variants (Klint and Claesson-Welsh 1999). During development, FGFR1 is expressed in the mesenchyme, FGFR2 in several epithelial tissues, FGFR3 predominantly within the central nervous system and FGFR4 in several tissues of endodermal and neuroectodermal origin (Klint and Claesson-Welsh 1999). Deletion of FGFR1 or FGFR2 in mice results in early embryonic lethality due to a lack of mesoderm-inducing signals (Xu et al. 1999).

The precise expression pattern and role of FGFRs in physiological and pathological conditions in adults is barely known. FGFR1 and FGFR2 appear to be the main mediators of biological responses to FGFs; FGFR1 appears to trigger EC proliferation, migration and tube formation whereas FGFR-2 selectively mediates migration (Javerzat et al. 2002).

Signal transduction of FGFRs is less extensively studied than the VEGFR system. As with other receptor tyrosine kinases, binding of ligands to FGFRs leads to receptor dimerisation and tyrosine kinase autophosphorylation. Thereafter, mitogenic effects are elicited via MAPK involving Raf but independently of phospholipase Cγ (PLCγ) and Ca^{2+} (McLaughlin and De Vries 2001). The involvement of the PI3K pathway and the need for nitric oxide generation to mediate the vascular effects of FGFs is under debate (Ziche et al. 1997b).

1.9.8. Proline Arginine Rich Peptide, PR39

PR39 is a naturally occurring antibacterial peptide, originally isolated from pig intestine and identified in neutrophil azurophilic granules and macrophages (Shi et al. 1996). A tentative human counterpart with antibacterial properties, designated FALL-39, has been identified by probing a human bone marrow cDNA library (Agerberth et al. 1996). RNA blot analyses disclosed that the gene for FALL-39 is expressed mainly in human bone marrow and testis. PR39 is secreted as a prepro-peptide incorporating a canonical leader

sequence and the amino (NH_2)-terminal is rapidly cleaved to yield the mature 39 amino-acid proline- and arginine-rich peptide:

Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Pro-Phe-Phe-Pro-Pro-Arg-Leu-Pro-Pro-Arg-Ile-Pro-Pro-Gly-Phe-Pro-Pro-Arg-Phe-Pro-Pro-Arg-Phe-Pro

Recently, PR39 was found to induce syndecan expression in mesenchymal cells and to influence cell motility and metastatic potential in wound repair (Gallo et al. 1994). PR39 carries a highly positive charge and despite the general consensus that molecules with highly positive charges do not efficiently penetrate cell membranes, recent studies have showed that arginine is an important amino acid that enables proteins to enter the nucleus. Arginine-containing peptides are able to penetrate cell membranes in an energy-independent pathway (Tung and Weissleder 2003). A recent report also showed that synthesized PR39 enters ECs and localizes to the cytoplasm (Wu et al. 2004). There, it reversibly binds and allosterically inhibits the non-catalytic $\alpha 7$ subunit of the 20S proteasome (Gaczynska et al. 2003). This binding influences the interaction between the 20S proteasome and the 19S complexes, ultimately affecting the assembly and catalytic function of the 26S proteasome. Intranuclear ubiquitination and the subsequent degradation of hypoxia inducible factor, HIF-1α, and IκBα are blocked. In contrast to the competitive inhibitors, allosteric inhibitors allow for a more precise, substrate-specific regulation of proteasome activity. The functional effects of PR39 treatment are mediated by stimulation of HIF-1α dependent gene expression (Li et al. 2000) and by inhibition of NF-κB dependent gene expression (Gao et al. 2000). The shortest functional sequence derived from PR39 retaining an allosteric inhibitory effect consists of 11 NH_2-terminal residues, including three essential NH_2-terminal arginines (Gaczynska et al. 2003). PR39 and related peptides have been shown to stimulate effective angiogenesis (Li et al. 2000b) and by virtue of blocking NFκB activation, it dramatically abrogates the production of inflammatory mediators and leucocyte adhesion molecule expression conferring protection against ischaemia-reperfusion injury (Gao et al. 2000).

1.10. Proteasomes and Intimal Hyperplasia

The **proteasome**, or multicatalytic proteinase, is responsible for the majority of non-lysosomal proteolysis in eukaryotes (Voges et al. 1999). The enzyme is a large assembly of multiple subunits with a modular structure. Its 28 subunits of the 20S catalytic core are arranged into four stacked rings, each containing seven subunits. The outer α rings comprise only non-catalytic α subunits whereas each of the two inner β rings harbour three active centres concealed inside a barrel-like structure, with NH_2-terminal threonines as catalytic residues. These 3 active centres possess distinct specificities (trypsin-like, chymotrypsin-like and post-glutamyl peptidyl hydrolytic) that cleave polypeptides on the COOH-terminal sides of hydrophobic, basic or acidic amino-acid residues. The 20S proteasome alone is capable of degrading small peptides and unfolded proteins. The 20S complex with attached two 19S complexes forms the 26S (2500 kDa) proteasome responsible for recognition and degradation of proteins tagged with polyubiquitin chains.

Ubiquitin (Ub), comprising 76 amino acids, is one of the most conserved peptides in eukaryotic systems, with only a 3 amino acid difference between yeast and human homologues. It is conjugated to other proteins by an energy-dependent enzymatic pathway (Fig 1.10), allowing the tagged protein to be recognized by the proteasome. Ub-dependent proteolysis of key regulatory proteins impacts upon various cellular processes such as cell cycle progression, transcription, antigen presentation, receptor endocytosis, fate determination and signal transduction (Voges et al. 1999). Hence, targeting the proteasome has emerged as an attractive target for drug development in diverse pathologies, from cancer and autoimmune disease treatment to arterial restenosis (Yeh 2002). Local application of a pharmacological proteasome inhibitor, MG132, resulted in significant inhibition of intimal hyperplasia in a rat carotid artery balloon injury model (Meiners et al. 2002). MG132 inhibited cell proliferation, blocked activation of NFκB, and induced apoptosis in rat vascular smooth muscle cells. However, the vast array of proteins that might be affected by non-specifically targeting the proteasome would inevitably incur unacceptable systemic toxicity. Specific inhibitors of ubiquitination are required that would more precisely disrupt a single pathophysiological process, with potential for local application.

FIGURE 1.10

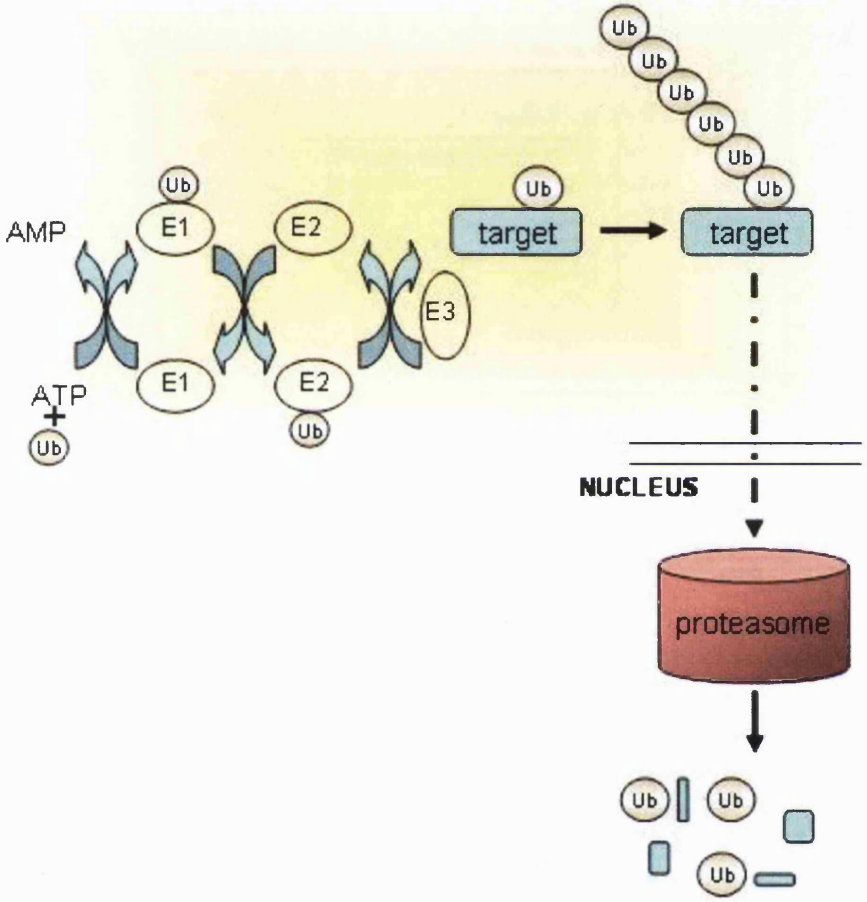


Figure 1.10. Schematic summary of Ubiquitin (Ub) -dependent proteolysis.

Ub-activating enzyme, E1, activates Ub which forms a high energy Ub-thiol ester bond in the presence of ATP. Ub is transferred to the ubiquitin conjugating enzyme, E2. E2 attaches Ub onto the substrate through an isopeptide linkage between the conserved C-terminal glycine residue of Ub and the ϵ-amino group of the lysine residue of the substrate, with the aid of ubiquitin ligase, E3. The polyubiquitinated substrate translocates to the nucleus and is recognised by the 26S proteasome, resulting in degradation and recycling of ubiquitin.

1.11. AIMS OF THE THESIS

The goal of this thesis was to explore the effects of localized angiogenic growth factor gene transfer to the adventitia of a rabbit collar model of neointimal hyperplasia, sustained on normal or cholesterol-supplemented diets. More specifically, this thesis pursued the following aims:

1. Verification of the feasibility of inducing neointimal hyperplasia and localized gene transfer, by placement of a silicone collar around the carotid artery of rabbits.

2. Exploration of the effects of low-efficiency and high-efficiency VEGF-A gene transfer on intimal thickening and neointimal macrophage composition, using liposomal and adenoviral vectors, respectively.

3. Examination of the effects of adenoviral gene transfer of PlGF2, a selective VEGFR1 ligand, upon intimal thickening and neointimal macrophage composition.

4. Investigation of the effects of PR39, a novel potent angiogenic factor that inhibits the proteasomal degradation of HIF-1α, upon intimal hyperplasia.

CHAPTER 2

METHODS AND MATERIALS

2.1 Plasmid Preparation

The mouse $VEGF_{164}$ and LacZ plasmids used for this study incorporated a CMV promoter and were produced at the Gene Vector Laboratory, AI Virtanen Institute, Kuopio, Finland (kind gifts of Professor S Ylä-Herttuala) (**Appendix 8.1**). The pCMV-$VEGF_{164}$ plasmid contained mouse VEGF cDNA nucleotides 1-583 (Breier et al. 1992) and the pCMV-LacZ plasmid contained *Escherichia coli* LacZ cDNA nucleotides 1-3,100 (Kalnins et al. 1983). They were isolated using Qiagen Mega Columns (Qiagen, CA) and purified using three phenol/chloroform extractions and one ethanol precipitation. Plasmid concentrations were adjusted to 1 μg/μl and analyzed to be free of any microbiological/endotoxin contamination using the Limulus assay (Jorgensen et al. 1973).

Upon transfer to UCL, additional stocks of the LacZ and $VEGF_{164}$ plasmid cDNAs were generated. An aliquot of the plasmid cDNA was diluted with deionized water in a chilled microfuge tube. 10pg of each plasmid was used to transform 40 μl electro-competent DH5α *E. Coli* (Gibco-BRL) on ice in a sterile electroporation cuvette (BioRad Gene Pulser: 1.8kV, capacitance 25 μFarad) following the supplier's protocol. 500 μl of S.O.C medium (Invitrogen; **Appendix 8.2**) was added to the transformed cells and the mixture placed in a shaker-incubator for 30 min. 200 μl of this culture was spread on to LB Agar Plates, containing 50 μg/ml ampicillin and incubation was allowed to proceed overnight at 37°C. The remainder of the transformed cells were stored at 4°C and plated later, if required. Individual colonies were picked and used to inoculate 10 ml starter cultures of LB Broth containing 50 μg/ml ampicillin starter cultures, which were placed at 37°C for 16 hrs in a shaker-incubator. 100 μl aliquots from the starter cultures were used to inoculate 500ml LB Broth containing 50μg/ml ampicillin in an Erlenmeyer flask placed in a shaker-incubator (225rpm) overnight. The cells were transferred to two 250 ml bottles and harvested by centrifugation using a GSA rotor (Sorvall centrifuge) at 5000 rpm at 4°C for 10 min. A plasmid maxi-kit (Qiagen) was then used to purify endotoxin-free plasmid

DNA according to the manufacturers' instructions. After isopropanol extraction, the DNA was re-suspended in nuclease-free water and quantified by measuring the absorbance at 260 nm in a spectrophotometer (Cecil, Aureus). The concentration of each plasmid was adjusted to 1 μg/μl. Before use in animal experiments, the identity of the plasmids were verified by restriction digest mapping using BamH1 & Hind III or EcoRI restriction enzymes (Promega), for LacZ and $VEGF_{164}$ plasmids, respectively (see **Appendix 8.3** for example digest gels). Restriction digestion reaction reactions were performed at 37°C for 1 hr in a waterbath, using 0.25 μg cDNA, 1 unit restriction enzyme (1u/μl) and 10x enzyme buffer, following the manufacturer's protocol.

2.2 Generation of Adenoviruses

Adenoviral constructs contained a first generation adenoviral backbone in which the E1 and E3 early regions of the double stranded DNA viral genome were deleted to prevent transactivation of viral genes required for viral replication and to make room for foreign DNA inserts. Adenoviruses containing the cDNAs for either green fluorescent protein (GFP) (Ad.GFP), PR39 (Ad.PR39) and a dominant negative FGFR1 construct, containing a GFP insert (Ad.FGFR1DN-GFP), were generated for these studies. These transgene cassettes contain the human cytomegalovirus (CMV) immediate-early promoter and β-globin polyadenylation (polyA) signals. Adenoviral vector preparations were titred by standard plaque assay on human 293 cells and purified by two cycles of caesium chloride (CsCl) gradient centrifugation. The final products were titred by the optical absorbance method (Maizel et al. 1968) and the results expressed as plaque forming units per ml. Recombinant viral stocks were kindly provided by the Vector Core Laboratory of the Harvard Gene Therapy Initiative or Ark Therapeutics, Kuopio, Finland. Adenoviral-gene preparations were stored at -80°C in a solution containing 50mM Tris-HCl (pH 7.4), 5mM EDTA, 1.4M CsCl, 50mM Magnesium Chloride ($MgCl_2$) and 25% Glycerol. The adenovirus preparations were desalted by centrifugation at 1100*g* for 4 min through G50 Sephadex columns (Boehringer Mannheim) immediately prior to their use in animal studies.

Adenoviral constructs encoding human VEGF-A_{165} (Ad.VEGF-A_{165}) (**Appendix 8.4**) and LacZ (Ad.LacZ) were provided by Professor S Ylä-Herttuala, (University of Kuopio, Finland) and adenovirus containing mouse Placental Growth Factor-2 (mPlGF2) cDNA prepared as previously described (DiPalma et al. 1996), was donated by Professor L Moons, (Catholic University of Leuven, Belgium). These adenoviruses were diluted with a sterile buffered solution (**Appendix 8.5**), prior to their use for animal studies.

2.3. Soluble Flt1 for *In Vivo* Use

Soluble flt1 protein comprising extracellular IgG domains 1 to 3 (0.6mg/ml), was obtained from Genentech Inc., San Fransisco, USA (Gerber et al. 2002), under a material transfer agreement.

2.4. Preparation of Normal and 1.5% Cholesterol Diet

Rabbits were fed on a normal chow diet (Teklad Global Rabbit Diet 2049, Harlan). A 1.5% cholesterol diet was prepared by dissolving powdered cholesterol (Sigma) in 10mls 1% olive oil, which avoided significant alteration of the final fat content. The cholesterol/oil paste was then thoroughly mixed with the correct pre-weighed amount of normal chow diet ensuring all pellets were coated.

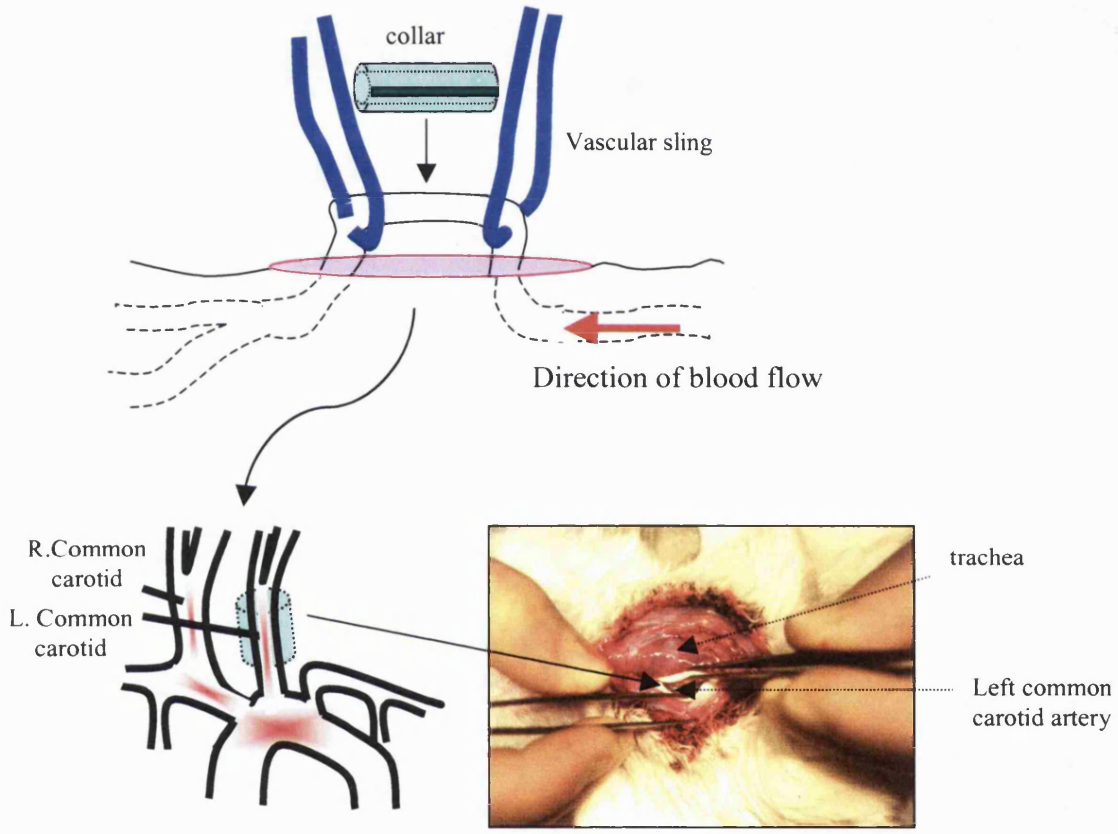
2.5. Rabbit Collar Model and Gene Transfer

All protocols involving animals were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986, and also conformed to the Animal Care and Use guidelines and Ethics Committees of University College London, UK and Dartmouth College, Lebanon, USA. Male New Zealand White (NZW) rabbits (**163** animals weighing 1.8-2.5kg each; Charles River, UK and Canada) were delivered to animal units one week prior to the first planned experimental procedure, to allow acclimatisation to their new environment. They were housed and maintained in standard conditions: individual cages, under temperature control (16 to 20°C) and under a 12 hr light/dark cycle.

Diet, prepared as above, and water were provided *ad libitum*. Animals in the hypercholesterolaemic subgroup commenced their 1.5% cholesterol diet 7 days prior to their first operation; other animals (normocholesterolaemic) were maintained on a normal chow diet. Prior to each procedure, rabbits were weighed to allow calculation of anaesthetic doses; they were anesthetized intramuscularly with ketamine (35 mg/kg body weight) and xylazine (3 mg/kg body weight). Diazepam (2mg/kg) was administered intravenously via the marginal ear vein. Alternatively, they were sedated using Hypnorm given intramuscularly (0.5 ml/kg body weight, Janssen Animal Health, Bucks.) and Diazepam (0.2mg/kg body weight; Phoenix Pharma Ltd., Gloucester) administered intravenously via the marginal ear vein. Baytril 2.5% (Bayer Ltd, Animal Health Business Group, Chapel Lane, Co. Dublin, Ireland) was given to the animal (1ml/5kg) before the operation commenced. The depth of anaesthesia was judged by assessing the papillary light reflex and the withdrawal response to gentle pinching of the rabbit's paw. Arterial oxygen saturation and pulse rate were constantly monitored by attaching a pulse oximeter to a shaved section of the tail. When the rabbit was fully anesthetised, its anterior neck (from cricoid process to sternal notch) was shaved free of fur and washed with betadine-soaked swabs to reduce the risk of wound contamination and facilitate operative access. The rabbit was then placed supine upon an operating table, covered with surgical drapes and kept warm by use of a heated pad below the animal. Rabbits were maintained under anaesthesia with oxygen and 0.5-2 % halothane.

All surgery was performed using aseptic technique. A midline neck incision was made and both common carotid arteries accessed by careful incision to the trachea, followed by teasing away the sternocleidomastoid muscle immediately lateral to the trachea. A 2-2.5 cm stretch of the left common carotid artery was blunt dissected away from neighbouring tissues (vagus nerve) and any small branches cauterized using a small hand-held battery operated cauterizer (John Weiss and Sons Ltd, Milton Keynes). The artery was elevated, using a friction-free vascular sling and a non-occlusive, biologically inert, flexible silicone collar (20 mm long; inlet/outlet diameter 1.8 mm; Ark Therapeutics, Kuopio, Finland) was placed around it, swiftly and gently to ensure minimal disruption to the blood flow or damage to the endolumenal surface (illustrated below in Fig 2.1).

Figure 2.1. Schematic and Photographic Illustration of Collar Placement around the left Common Carotid Artery in the Rabbit.



The contralateral artery was sham operated, i.e. isolated from surrounding connective tissue and exposed to similar stretch as the collared artery, as previously described (Booth et al. 1989). The operation site was then closed, using W501 4-0 black braided non–absorbable suture for the muscle layers and W9940 3-0 white braided absorbable suture to oppose the skin incision with a continuous subcuticular stitch (Johnson and Johnson, Belgium). The animals were given Vetergesic analgesic (Alsatoe Ltd, Melton Mowbray, UK) at 0.3–0.6mls / 10kg subcutaneously each day for 3 days. Five days later, the collar was surgically

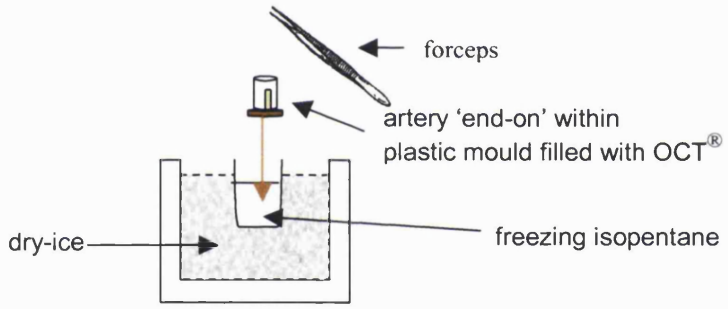
re-exposed via the original incision and 100 μl Lipofectamine-plasmid in Ringers' Lactate solution (Sigma), adenoviral gene solution (5 x 10^9 pfu) or protein solution was instilled into the periadventitial space encased by the collar. The compounds and their respective doses tested in this model are outlined in Table 2.1. Within two subgroups, soluble Flt1 (adjusted to 0.1mg/kg; Genentech Inc.) either alone or in combination with Ad.PR39 was transferred to the adventitia. Incubation was allowed for 10 min ensuring minimal leakage to surrounding tissue, before closing the site. Lipofectamine-plasmid solutions were prepared up to 1 hr prior to instillation, according to the manufacturer's instructions (Gibco-BRL). The animals were anaesthetized as part of the final procedure to harvest tissue, 4 or 9 days after gene or protein transfer. The operation site was accessed again via the original incision and after excision of the collared artery, a lethal dose of Euthatal Sodium Pentobarbitone (40mgl/kg; RhoneMerieux Ltd., Harlow, Essex) was given via the marginal ear vein and flushed with normal saline, [Code of Practice For The Humane Killing of Animals Under Schedule 1 to the Animals (SCIENTIFIC PROCEDURES) ACT 1986.] An equal length of the contralateral sham artery was then harvested. During the course of this project, 3 animals died peri-operatively, despite prolonged attempts at resuscitation, including adrenaline infusion and external cardiac compression. One fatality was due to vomiting and aspiration, but the precise mechanism of the other two deaths was not elucidated, though appeared not to be secondary to blood loss.

2.6. Tissue Harvesting and Processing

Upon harvesting, treated and control arteries were rinsed free of excess blood using ice-cold saline, cut transversely using a scalpel, and divided into three equal segments. In a subgroup (n=3) of Ad.VEGF-A treated animals, additional biopsies of organs (heart, lung, kidney, testis) were collected and snap-frozen in liquid nitrogen (N_2) and stored at -80°C for subsequent total RNA isolation. The harvested arteries were divided into three equal segments and processed as follows:

1. *Proximal (caudal) segment*: Immersion-fixed in 1% paraformaldehyde/phosphate buffered saline (PFH/PBS) (pH 7.4) for 30 min, rinsed in PBS, then embedded in OCT® compound (Sakura Finetek USA, Inc. Torrance, CA). Specimens were mounted end-on upon a cork mat (RA Lamb, UK), within a 0.5 cm diameter cylindrical mould (cut from a plastic 2ml syringe). The mould containing the arterial segment was filled with OCT® at room temperature, ensuring that the OCT® filled the lumen of the vessel, and the preparation plunged into freezing isopentane (BDH) as illustrated in Fig. 2.2. After solidifying, the plastic mould was detached and the OCT®-embedded specimen stored at –80°C for subsequent sectioning.

Figure 2.2. Embedding the caudal segment of the artery in OCT® Compound.

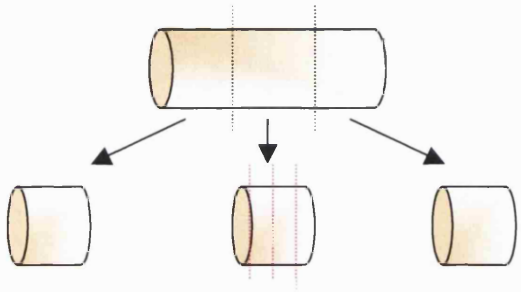


2. *Middle segment*: Immersion-fixed in 1% PFH/15 % sucrose (pH 7.4) for 6 hours, rinsed in 15% sucrose (pH 7.4) and transferred to 70% ethanol (**Appendix 8.6**). The specimens were progressively dehydrated for paraffin embedding using automated 20 hr cycle processors in the Departments of Histopathology at UCL, London and Dartmouth Hitchcock Medical Center, Lebanon, USA. The processed specimens were embedded in paraffin blocks 'end-on' and allowed to cool on a cold-block before sectioning.

3. *Distal (rostral) segment*: Snap frozen in liquid N_2, weighed and stored at –80°C in cryovials (Corning) for subsequent total RNA extraction.

The post-processing techniques performed on each segment of the majority of collared and sham-operated carotid artery is summarized in Fig. 2.3 below.

Figure 2.3. Summary of processing procedures upon harvesting the treated and contralateral sham carotid arteries.



OCT® Compound:
Immunohistochemistry
Confocal fluorescence
OR
β-galactosidase staining

Paraffin:
H&E – morphometric analysis.
Sections cut at 100μm incremental intervals along the length of the segment – a, b, c, etc (at least 6 sections cut).
Immunohistochemistry

Snap Frozen:
Total RNA extraction

Table 2.1. Summary of the study plan for collar model experiments (total **n= 163**):

Experimental agent	Concentration	n	Diet	Duration of collar/gene/ peptide delivery (days)
Collar alone	-	3	Normal	1
Collar alone	-	3	Normal	3
Collar alone	-	3	Normal	5
Collar alone	-	3	Normal	7
Collar alone	-	3	Normal	14
Collar alone	-	3	Normal	16
Collar alone	-	3	1.5% C	5
Collar, PKH-26	0.1 μM	2	Normal	3
Collar, PKH-26	0.1 μM	2	Normal	5
Collar, Normal saline	-	2	Normal	9
Collar, Lp-LacZ	25μg	10	Normal	9
Collar, Lp-VEGF-A_{164}	25μg	10	Normal	9
Collar, Lp-LacZ	25μg	10	1.5% C	9
Collar, Lp-VEGF-A_{164}	25μg	10	1.5% C	9
Collar, Ad.GFP	5 x10^9 pfu	11	Normal	9
Collar, Ad.PR39	5 x10^9 pfu	12	Normal	9
Collar, Ad.PR39	5 x10^9 pfu	6	Normal	4
Collar, Ad.FGFR1DN-GFP	5 x10^9 pfu	7	Normal	9
Collar, Soluble Flt1 peptide	0.1mg/kg	6	Normal	9
Collar, Ad.PR39 + Ad.FGFR1DN-GFP	5 x10^9 pfu each	6	Normal	9
Collar, Ad.PR39 +sFlt1	5 x10^9 pfu + 0.1mg/kg	6	Normal	9
Collar, Ad.LacZ	5 x10^9 pfu	6	Normal	9
Collar, Ad.VEGF-A_{165}	5 x10^9 pfu	6	Normal	9
Collar, Ad.PlGF2	5 x10^9 pfu	6	Normal	9
Collar, Ad.LacZ	5 x10^9 pfu	8	1.5% C	9
Collar, Ad.VEGF-A_{165}	5 x10^9 pfu	8	1.5% C	9
Collar, Ad.PlGF2	5 x10^9 pfu	8	1.5% C	9

Abbreviations: Lp- Lipofectamine; 1.5% C, 1.5% cholesterol-supplemented diet

2.7. Plasma Cholesterol Assay

A 1 ml blood sample collected into a syringe containing 60 µl 5% EDTA was taken from the marginal ear vein in both normal and 1.5% cholesterol groups (n=10 each) prior to collar placement, and also 1 day before initiation of the cholesterol-supplemented diet and a final sample was taken immediately prior to euthanasia. The sample was immediately centrifuged at maximum speed (15,000*g*) at 4°C for 2 min (Beckman Tabletop). The plasma supernatant was promptly extracted, divided into aliquots and rapidly frozen on dry-ice before transferring to storage at -80°C. Plasma samples were thawed and analyzed for total, HDL and LDL cholesterol fractions by an automated enzymatic assay (Department of Chemical Pathology, University College London Hospitals, UK).

2.8. Total RNA Isolation

Bench surfaces were adequately prepared for RNA work, by liberal cleansing with RNase Away (Invitrogen) and gloves worn at all times during handling of experimental material. Total arterial RNA was isolated for reverse transcription (RT) -PCR and real time RT-PCR experiments. Different isolation procedures were used during the course of this thesis, for work done on lipofectamine-gene transfer (UCL, UK) and adenoviral-gene transfer studies (UCL, UK and Dartmouth College, USA).

2.8.1. Lipofectamine-plasmid transfer experiment:

Total arterial RNA was extracted from 100-150 mg (pooled 4 carotids) frozen tissue using Trizol reagent (Gibco-BRL). The frozen tissue was ground with a pestle and mortar on a bed of dry ice; thawing was prevented by frequently pouring liquid N_2 into the mortar. 1 ml Trizol reagent (phenol and guanidine isothiocyanate) was added to the powdered tissue, followed by 200 µl chloroform (i.e. 1/5 volume of Trizol) and and mixed by repeatedly inverting the tube for 15 sec. The sample was incubated at room temperature for 5 min and then centrifuged at 12,000*g* for 15 min, which allowed separation of the solution into an

aqueous phase and an organic phase. RNA remained exclusively in the aqueous phase. After careful removal of the aqueous phase into a fresh eppendorf tube, the RNA was recovered by precipitation with 500 µl isopropanol. The preparation was centrifuged at 12,000*g* for 10 min in the cold room (4˚C) and the supernatant removed to leave the RNA pellet. The pellet was washed with 500 µl 70% EtOH, by centrifugation at 7,500*g* for 5 min at 4°C and then allowed to air dry for 10 min at room temperature. The pellet was dissolved in 50-100 µl Diethyl Pyrocarbonate- (DEPC; Sigma) treated water (**Appendix 8.7**). Genomic DNA was removed by treatment with RNase-free DNase I (Promega) at 37˚C for 30 min (**Appendix 8.8**). The DNase was then inactivated by incubation at 65°C for 10 min. A260/A280 absorbance ratios were determined by spectrophotometry (Cecil, Aurius) to ascertain RNA quality and quantity. 500ng RNA was run on a 1.2% agarose gel at 80-100V (constant voltage) for 30-60 min or until the bromophenol blue dye front was at the bottom of the gel (BioRad Apparatus), to confirm lack of degradation by visualisation of discrete 28S (~5 kb) and 18S (~2kb) bands (**Appendix 8.9**). The procedure for agarose gel analysis is decribed later in Chapter 2, section 2.11. For short-term storage, RNase-free water (with 0.1 mM EDTA) or TE Buffer (10 mM Tris, 1mM EDTA, pH 7.5) was used as diluents[3].

2.8.2. Adenoviral-PlGF2, -VEGF-A and -LacZ gene transfer experiments:

Total RNA was isolated from 30-50 mg (pooled from 2 carotids) frozen tissue using an RNAeasy spin-column method, adapted by the manufacturer to optimize RNA yields from fibrous tissue by incorporating an additional Proteinase K digestion step (RNAeasy Fibrous Tissue Mini Kit, Qiagen). The tissue was first disrupted and homogenized using a rotor-stator (Polytron 3100). An on-column RNase-free DNase-1 treatment was also included in the protocol to eliminate any contaminating genomic DNA, before eluting the total RNA in RNase-free water. RNA yields and A260/A280 absorbance ratios were determined by spectrophotometry (CE2041, Cecil Technologies), and non-degradation was confirmed by running a 500ng aliquot on a 1.2% agarose gel.

In some experiments, total RNA was isolated from 100-150 mg (pooled from 4 carotids) frozen tissue using Micro- to Midi- Total RNA Purification Systems (Invitrogen)

[3] RNA is generally stable at -80°C for up to a year without degradation.

according to the manufacturer's instructions and then treated with RNase-free DNase (Ambion) for 1 hour at 37°C, to remove contaminating genomic DNA. The quality and quantity of the isolated RNA was determined using RNA 6000 Nano LabChip technology with a Bioanalyzer (Agilent). The detection principle of biomolecules in the Bioanalyzer is reliant upon laser-induced fluorescence translated into an electropherogram (rather then UV absorbance), so that an A260/A280 absorbance ratio is not generated. During chip preparation, a dye concentrate is mixed with a gel. With the help of the priming station, the channels of the chip are filled with the gel-dye mix. During the chip run, the dye intercalates directly with the analytes (DNA or RNA).

2.9. Confirmation of Transgene expression by RT-PCR

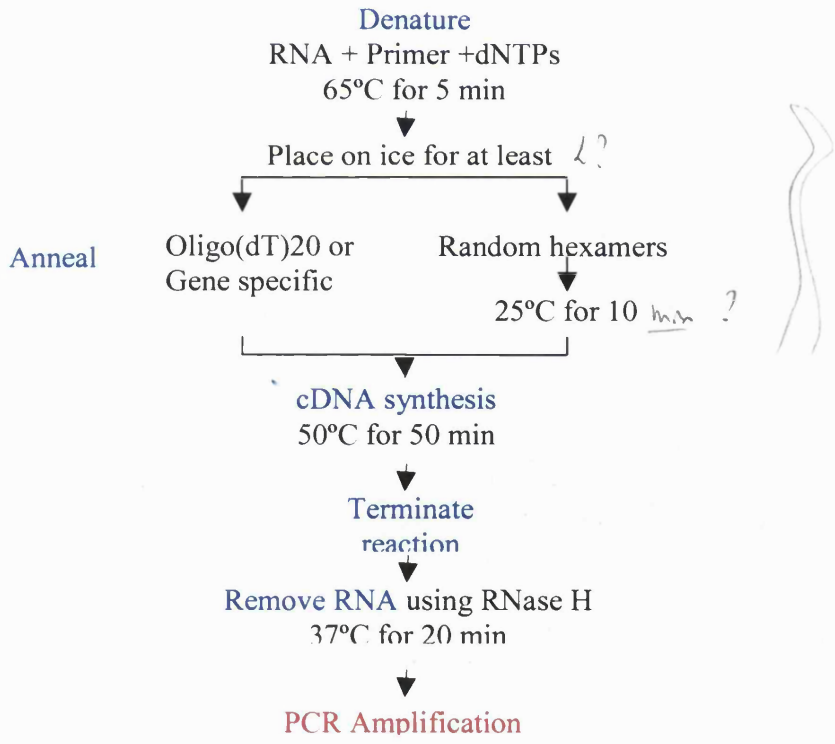


Figure 2.4. Procedural principles of PCR (specific volumes and concentrations slightly vary) were according to the kit used. Products of PCR reactions, where the reverse transcriptase was omitted, served as negative controls.

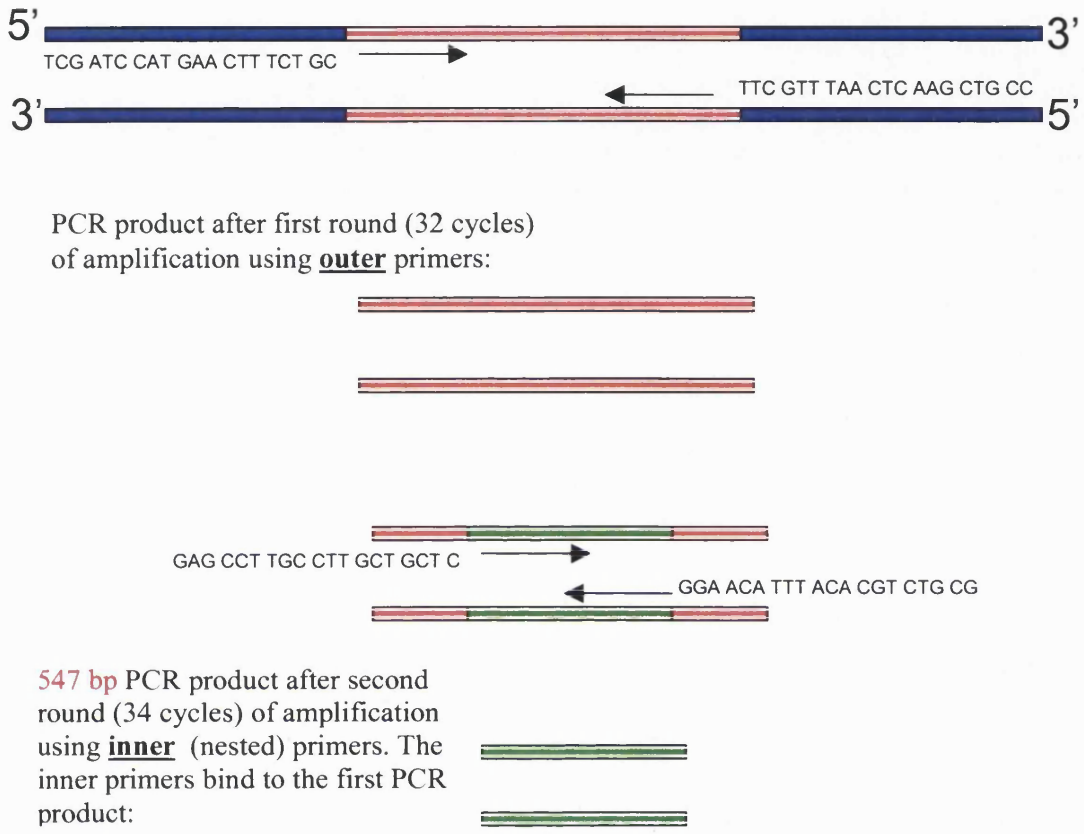
2.9.1. Lipofectamine-plasmid transfer experiment.

1.5μg total RNA was reverse transcribed using Amphotropic Murine Leukemia Virus Reverse Transcriptase (AMLV-RT) and random hexamers (0.5μg/μl) according to the manufacturer's instructions (Perkin Elmer). PCR was performed in a thermal cycler (PTC-100, MJ Research Inc.) using DNA Taq polymerase (Boehringer Mannheim) and primers for the pCMV-$VEGF_{164}$ plasmid (**see Table 2.2**). The following PCR cycle parameters were used: 4 min at 96°C (hot start, enzyme omitted), 35 sec at 96°C, 40 sec at 53°C, 90 sec at 72°C for 39 cycles and the last extension step was continued for 11 min. Products of each reaction were run on a 1.2% agarose gel, alongside 100kb DNA markers (Promega). A 0.5 μg aliquot of the plasmid cDNA was used as a positive control.

2.9.2. Adenoviral gene transfer experiments.

500ng of total RNA was reverse transcribed using Superscript III RT (Invitrogen) which is a version of Moloney murine leukemia virus RT (M-MLV RT) that has been engineered to reduce RNase H activity and provide increased thermal stability) and random hexamers (50 ng/μl), according to the manufacturer's instructions. For PlGF2, PCR was performed using Platinum® Taq DNA Polymerase (Invitrogen) and transgene specific primers (5' primers selected from the CMV promoter and 3' primers from the coding region of the inserts as shown in Table 2.2) with the following PCR cycle parameters: 1 min at 94°C, followed by 34 cycles comprising 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C and the last extension step was continued for 7 min at 72°C. A 280 base pair (bp) PlGF2 amplicon was generated. A 2 stage nested PCR as illustrated in Fig. 2.5, was used for the amplification of VEGF-A, requiring 32 and 34 cycles of amplification for the outer and inner primer pairs (see Table 2.2), respectively, with the annealing step performed at 56°C for 45 sec. A 547 VEGF-A bp amplicon was produced. Products of each reaction were run on a 2% agarose gel, alongside 50kb DNA markers (Invitrogen). 200ng PlGF2 and VEGFA plasmid cDNA aliquots derived from HUVECs, transfected with Ad.PLGF2 and Ad.$VEGF\text{-}A_{165}$ respectively, served as a positive controls.

Figure 2.5 Principles of Nested PCR Procedure for Detection of Ad.VEGF-A_{165}



For detection of PR39, 500 ng total RNA was reverse transcribed to cDNA, with 5 units AMV RT, and amplified in a one step 15µl reaction (AccessQuick, Promega) containing 100nM each of forward and reverse primer (Table 2.2). The RT step was carried out at 48°C for 45 min. After an initial denaturation step at 95°C for 2 min, 38 cycles of PCR were performed with denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The last extension was at 72°C for 7 min. Ad.PR39 PCR products of the predicted 601 bp size were confirmed by electropheresis on a 1% agarose gel. A positive control was prepared by transfecting 5 x 10^6 rat SMC early passage (Cell Applications, Inc, USA), and grown on a non-coated 6 well plate (Falcon) with Ad.PR39 (4 x 10^{10} pfu/ml

with a multiplicity of infection = 100), for 48hrs before extracting the total RNA (Invitrogen) for further analysis.

2.10. Real-time RT PCR

The mRNA expression of endogenous rabbit VEGF, FGFR1 and β-actin in Ad.PR39, Ad.GFP treated and sham control carotid arteries was quantified by real-time RT PCR. Total RNA (2μg) was reverse transcribed in a 20 µl reaction system using Superscript® first-strand synthesis kit (Invitrogen) under conditions described by the supplier. PCR was then performed using 12.5ng of reverse transcribed total RNA with 50nM of both forward and reverse primers (Invitrogen) in a final volume of 30μl, using SYBR Green PCR Core Reagents (Applied Biosystems) according to the manufacturer's instructions using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). SYBR Green contains AmpliTaq Gold® DNA polymerase. PCR cycles comprised an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. PCR products were confirmed as single bands using gel electrophoresis (4% E-Gel, Invitrogen) against a 10bp ladder (Invitrogen).

Standard curve generation: A standard curve was constructed by sub-cloning the freshly prepared amplicon into a pCRII-TOPO cloning vector (TA Topo Cloning Kit, Invitrogen). 100 µl competent E. Coli (One Shot® TOP10, Invitrogen) were then transformed by gently mixing 1-10 ng DNA, followed by a 30 min incubation on ice. The cells were heat-shocked for 45 sec in a 42°C water bath then placed on ice for 2 min. 0.9 ml of room temperature S.O.C medium (Invitrogen; see Appendix) was added and the mixture shaken at 225rpm at 37°C for 1 hr. 100 µl of the mixture was then spread onto LB Agar plates, containing 50 µg / ml ampicillin and 50 µg / ml X-gal (Sigma) and the plates incubated overnight at 37°C. Blue-white selection was used to pick colonies for subsequent inoculation of a starter culture (containing ampicillin, 50µg/ml). The plasmid DNA was harvested by a spin-column preparation according to the manufacturer's instructions (Qiagen Maxiprep). The plasmid containing the insert was verified by sequencing (Molecular Biology Core Facility, www.dartmouth.edu/~mbcf/). The reference plasmid was digested using BamH1 restriction endonuclease and its concentration measured using chip technology (Agilent Bioanalyzer).

Serial dilutions of the stock plasmid DNA were prepared, using 100ng/μl herring sperm DNA (Promega) as a carrier. Amplification by PCR using SYBR Green (Applied Biosystems) following the manufacturer's instructions was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). The number of copies in each experimental sample was then calculated[4] with respect to the calibration curve, which was run in duplicate with each analysis. Every PCR experiment also included two non-template controls. The expression of VEGF, FGFR1 and β-actin mRNA within each sample was calculated according to the standard calibration curve. The final values are expressed by normalizing each cDNA load to the relative quantity of housekeeping β-actin gene within each experimental sample (n=3).

Primers used in RT- and real time PCR were designed using Primer Express® software v2.0/v3.0, such that one primer crossed an exon junction where relevant. The primers would thus amplify mRNA (or cDNA made from it), but not genomic DNA. The primers were custom synthesized by Invitrogen Life Technologies. Upon arrival, the desalted DNA primers were reconstituted in RNase-free water to a stock concentration of 100nmol/l and stored at -20°C. Stock concentrations were further diluted to 10nmol/l prior to their use in RT- and real-time PCR reactions. For PR39 and $VEGF_{164}$, 5'primers were selected from the CMV promoter and the 3' primers from the coding regions of the gene inserts.

[4] The number of copies/μl was determined using the formula: Number of copies/μl = g/μl x [1/(330g/mol x n)] x 6.023 x 10^{23}, where 330 g/mol is the average molecular weight of a nucleotide, n is the number of nucleotide pairs (3973 bp for the plasmid plus the size of the insert) and 6.023 x 10^{23} is Avogadro's constant (number of molecules/mol).

Table 2.2. A summary of the primer pairs used in both RT- and real time PCT experiments.

	Primer Sequence	Gene Bank Accession No.
* pCMV-VEGF$_{164}$ f 5'	TCG ATC CAT GAA CTT TCT GC	**AB020216**
* pCMV-VEGF$_{164}$r 5'	TTC GTT TAA CTC AAG CTG CC	
¶ PR39 F 5'	CTC TAC CGC CTC CTG GAG CT	**NM_214450**
¶ PR39 R 5'	GGC CCT TCA TAA TAT CCC CCA	
VEGF-A f 84 5'	CTG CAA TGA TGA AAG CCT GGA	**AF022179**
VEGF-A r 145 5'	TGA TCT GCA TGG TGA CGT TGA	
‡ VEGF-A161 5'	TCA ACG TCA CCA TGC AGA TCA	
‡ VEGF-A 226 5'	AGC TCA TCT CCC CTA TGT GCT G	
β-actin F185 5'	CTC ATG AAG ATC CCT CAC GGA GC	**AF000313**
β-actin R271 5'	GCA CAG CTT CTC CTT GAT GTC C	
† FGFR1 425 F 5'	GAC AAG GAC AAA CCC AAC CGT	**N/A**
† FGFR1 483 R 5'	GCA TCC GAC TTC AAC ATC TTC AC	
PlGF2 F 5'	GAT CTT GAA GAT TCC CCC CAA TCG G	**X80171**
PlGF2 R 5'	ATA GAG GGT AGG TAC CAG CAG GGA GGG	
VEGF-A F 5' (outer)	TCG ATC CAT GAA CTT TCT GC	**AB020216**
VEGF-A R 5' (outer)	TTC GTT TAA CTC AAG CTG CC	
VEGF-A F 5' (inner)	GAG CCT TGC CTT GCT GCT C	
VEGF-A R 5' (inner)	GGA ACA TTT ACA CGT CTG CG	

* Primers were used for detection of Lipofectamine-VEGF$_{164}$ transfer to yield a 547 bp amplicon. The 5' primer was derived from the CMV promoter region within the expression plasmid and the 3' (reverse) primer derived from VEGF. ‡ Primers used for real-time PCR of endogenous rabbit VEGF-A mRNA.

† Primers were derived and kindly provided by William A. Paznekas and Ethylin Wang Jabs (NIH P60 DE13078). The rabbit BAC containing the FGFR1 gene was isolated and sequenced by the Berkeley PGA (grant HL66728 of NIH-NHLBI Programs for Genomic Applications)

¶ PR39 primers: forward primer for PR39 sequence 163-183, and reverse primer in human β-globin poly A following PR39 insert, in construct.

2.11. Agarose Gel Analysis of RNA and PCR Products

Depending upon the anticpitaed sizes of the molecular products being analysed, a 1-2% agarose gel was prepared. 1-2 g of agarose (Sigma) was dissolved in 105 mls 1X Tris-Acetate-EDTA (TAE) Buffer (**Appendix 8.9**), within a 500 ml conical flask. The mixture was microwaved for 1 min, or until the agarose was fully dissolved. The solution was allowed to cool at room temperature and when it cooled below 60°C, 4-6 µl ethidium bromide[5] (10mg/ml; Invitrogen) was added. The gel was poured into a tray, containing a comb of the required size. The gel hardened within 10-15 min and was used immediately or transferred to the cold room (4°C) for storage to be used within 24 hrs.

2.12. Tissue Analysis

2.12.1. Cryosectioning

OCT®-embedded sections stored at -70°C were brought to -20°C in the cutting chamber of a Cryotome (Model AS 620 SME, Shandon Scientific Ltd, UK). OCT® compound was used to 'glue' the cork mat on to the cryostat chuck, so that the long axis of the vessel was perpendicular to the upper surface of the chuck. The excess OCT® was shaved, until it was evident that complete cross-sections of the artery were being obtained. Initially, cross-sections are incomplete since the cryostat blade meets the comparatively rough-cut end of the artery. The anti-roll plate was adjusted so that it was exactly parallel to the knife edge and the micrometer screw turned such that 6-8 µm cryosections were cut. Two to three serial sections were transferred to each polylysine coated slide and then allowed to dry at room temperature. The slides were either used immediately for staining or stored at -20°C. If fluorescence was to be investigated, the slides were wrapped in aluminium foil and stored at -20°C.

[5] Ethidium bromide is a commonly used fluorescent dye to detect nucleic acids in agarose gels. It is stored by being protected from light. Ethidium bromide can be added directly into the gel, after allowing it to cool down and before pouring it into the mould to set. It fluoresces under UV light (254 nm) and allows the visualisation of the bands on the gel. Pictures are then taken for subsequent analysis. Ethidium Bromide is a potent carcinogen and is always handled wearing gloves.

2.12.2. Haematoxylin and Eosin (H&E) Staining

The paraffin blocks were cooled face-down on ice and then positioned on a Finesse 325 microtome (Shandon Scientific Ltd, UK). Excess surface paraffin and tissue was first trimmed, then 5μm sections were cut in rapid succession, forming ribbons. They were carefully picked up with the point of a 25 gauge needle and transferred to a tissue flotation bath (Shandon Scientific Ltd, UK) maintained at 50°C. Two to three sections were transferred onto one polylysine coated microscope slide and then air dried at room temperature, before storage. Slides were placed in a stainless steel slide rack and into a heat chamber at 42°C overnight or 55°C for 1 hr. Sections were deparaffinized and rehydrated by transferring the slides through a series of xylenes (2 x 5 min) and alcohols [100% ethanol (EtOH) (2 x 5 min), 90% EtOH (1 x 2 min), 70% EtOH (2 x 2 min)] and finally into deionized H_20. Sections of carotid artery required for morphometric analysis were stained with haematoxylin and eosin as follows. The slides were immersed in Harris' Haematoxylin (BDH) for 5 min, rinsed in deionized H_2O and transferred to a tray with tap water running into it for 5-10 min, which allowed the blue stain to develop. The slides were dipped 5-10 times in rapid succession into a 1% acid-alcohol (70 % EtOH, 1 ml concentrated HCl). After rinsing in deionized H_2O, the slides were immersed in eosin (BDH) for 1-2 min, followed by a rinse in tap water. The slides were dehydrated [(70%, 90% and 2 x 100% EtOH (1 min each)], then immersed in 2 changes of xylene (or histoclear) (1 min each). Cover slips were mounted on the slides using DPX, as decribed later in section 2.12.5.

RESULTS:

- Nuclei stain blue-black
- Cytoplasm appears as varying shades of pink
- Muscle fibres stain deep pinky red.
- Fibrin appears deep pink

2.12.3. β-Galactosidase staining

Carotid arteries transduced with Ad.LacZ or Lipofectamine-LacZ, and contralateral sham operated carotid arteries were randomly selected for β-galactosidase staining. The LacZ gene encodes for the enzyme ß-galactosidase and is a bacterial gene often used as a reporter construct for determination of transfection efficiency as well as histochemical localisation following transfection of eukaryotic cells. The LacZ gene product ß-galactosidase catalyzes the hydrolysis of the substrate X-gal (5-bromo-4-chloro-4-indolyl β D-galactopyranoside; Sigma) to produce a blue color that is easily visualized. After fixation of the artery as described earlier in 1% PFH/15 % sucrose (pH 7.4), for 6 hrs, the specimen was rinsed with a sodium phosphate buffer (pH 7.2) (**Appendix 8.10**) and transferred to β-gal staining solution (**Appendix 8.11**). Detection of transgenic β-galactosidase activity was performed by overnight incubation of whole arterial segments or 6 μm thick paraffin sections on a staining tray in the dark at 37°C. Macroscopic photographs were taken of stained artery (Hitachi KP-113 Colour Camera), before they were processed and embedded in paraffin as described earlier. The arteries were sectioned and counterstained with eosin as described above, before examination under a light microscope.

2.12.4. Immunohistochemistry

Immunohistochemical staining was performed on either paraffin embedded sections or cryosections, depending upon the specification of the primary antibody. Primary antibodies and their optimized conditions necessary for staining are summarized in **Table 2.3.** All primary and secondary antibodies (donated or commercial) were stored at 4°C, unless specified otherwise and freshly prepared prior to staining by dilution in Tris-buffered saline (TBS) (Sigma; **Appendix 8.12**), pH 7.2, containing 1% bovine serum albumin (BSA). To optimize the efficacy of certain primary antibodies or to avoid false negative staining results, PFH-fixed deparaffinized sections were required to undergo an antigenic retrieval procedure (referred to in Table 2.3). Heat induced epitope retrieval is a clinically accepted method whereby tissue sections are boiled in a citrate buffer to retrieve antigens (Shi et al.

1991). Slides were placed evenly in a rack and immersed in 200 ml 10mM Sodium Citrate buffer[6], pH 6.4, and placed in a pressure cooker for 20 min. The slides, within the citrate buffer, were then cooled by running tap water for 15 min, rinsed with distilled/deionized water and transferred to tap water. The slides were washed 2 times with TBS/0.1% Tween (**Appendix 8.12**), 5 min each and endogenous peroxidases (found in haemoproteins such as haemoglobin, myoglobin) were blocked with a 10 min incubation in 0.3% H_2O_2/methanol. Excess wash buffer from the sections was removed with an absorbent wipe and the section encircled by a wax PAP pen (hydrophobic barrier pen), care being taken to prevent dehydration of the sections. Sections were then incubated at room temperature with primary antibody for the indicated times, in a humid chamber, followed by 3 washes, 3 min each, with TBS/0.1% Tween. Application of an appropriate secondary antibody was performed using either a Vectastain Elite ABC Kit (anti-murine IgG, PK-6102, Vector Laboratories) or a Dako Envision + Kit (K4006, DAKO).

Vectastain Avidin/Biotin (ABC) Technique:

Prior to application of the primary antibody, sections were also incubated with protein block® (Dako) for 20 min. The secondary antibody was prepared by adding 1 drop of concentrated biotinylated horse anti-mouse IgG to 10 mls PBS, pH 7.4 and this was applied to the sections for 30 min at room tempearature. During this incubation, the ABC reagent was prepared by adding 2 drops *Reagent A* to 10 mls PBS, pH 7.4, mixed immediately, and then 2 drops *Reagent B* was added to the same mixing bottle. After the secondary antibody incubation, the sections were washed with PBS and the excess drained off. The slides were placed in a humid chamber at room temperature and the pre-prepared ABC reagent was applied for a 60 min incubation. The slides were then washed in twice with PBS, for 5 min each, before proceeding to the 3,3'-diaminobenzidine solution (DAB) substrate chromogen visualisation procedure.

[6] 10mM Sodium Citrate Buffer (2.94 g $C_6H_5Na_3O_7 \cdot 2H_2O$ in 1 litre deoinized water). Dissolve with stirring bar and pH to 6.0 with 10N sodium hydroxide (original pH will be in the 2-2.5 range). Buffer can be stored at room temperature or at 4°C for several months.

Dako Envision + Technique:

This is a single step system based on a horseradish peroxidase (HRP)-labeled polymer which is conjugated to a goat anti-mouse secondary antibody, in TBS solution. The sections required a 30 min incubation at room temperature, before proceeding to DAB substrate chromogen visualisation procedure.

For visualisation, sections were incubated with DAB substrate, pH 7.5, containing H_2O_2 (DAKO; Biogenex) for 5-10 min. DAB chromogen results in a brown coloured precipitate at the antigen site. The sections were counterstained with pre-filtered Harris' haematoxylin, as described in section 2.12.2, or with methyl green (DAKO). The slides were rinsed in tap water and immersed in Methyl Green staining solution (DAKO) at room temperature for 10 min. The slides were then subjected to three changes of distilled water (dipped 10x in the first, then 30 sec in the subsequent two). The slides were immersed in three changes of 100 % N-Butanol (Sigma) (dipped 10x in the first, then 30 sec in the subsequent two) before proceeding to xylene (or histoclear) dehydration.

RESULTS:

- Nuclei stain green-blue

Sections were mounted in DPX, as described later in 2.12.5, before examination under a light microscope. Additional steps required for specific primary antibodies are described in later sections.

Frozen sections were retrieved from -20°C storage and fixed for 10 min in acetone also at -20°C and then air-dried. Immunostaining procedures were done exactly as described above for paraffin embedded sections. After application of the DAB chromogen and counterstaining, the slides were dehydrated, cleared with histoclear and mounted with an aqueous glycerol-based medium (Glycergel, DAKO), which was heated to at least 50°C immediately prior to use.

Negative controls for all immunostainings were performed by incubations under the same conditions (incubation time, concentration, diluents) using irrelevant antibodies

belonging to the same subclass and animal species as the primary and incubations in which the primary antibody was omitted.

Table 2.3. A summary of the primary antibodies used and their optimized conditions.

Antibody	Type	Dilution	Tissue processing	Antigen retrieval / duration	Clone	Source
CD31	monoclonal anti-human (IgG1, kappa)	**1:500**	Paraffin	CB, PC; 1 hr	JC/70A	DAKO
RAM-11 Macrophage-specific	monoclonal anti-rabbit (IgG1)	**1:50**	Paraffin	CB, PC; 1 hr	RAM11	DAKO (M0633)
α-actin (HHF-35) VSMC specific	monoclonal anti-human (IgG2a)	**1:50**	Paraffin	CB, PC; 1 hr	HHF-35	DAKO
ICAM-1†	monoclonal anti-rabbit (IgG1)	**1:100**	Frozen; acetone	None, 1 hr	Rb 3/9	Gift from M. Cybulsky
VCAM-1†	monoclonal anti-rabbit (IgG1)	**1:100**	Frozen; acetone	None, 1hr	Rb 1/9	Gift from M. Cybulsky
VEGF-A	monoclonal anti-human (IgG2a)	**1:200**	Paraffin	None, 1hr	sc-7269	Santa Cruz (sc-7269)
VEGFR-1	monoclonal anti-mouse (IgG1)	**1:50**	Paraffin	None, overnight	sc-316	Santa Cruz
VEGFR-2*	polyclonal (rabbit) anti-human	**1:500**	Paraffin	None, overnight	sc-6251	Santa Cruz
PlGF2†	polyclonal rat anti-mouse	**10μg/ml (in TNB)**	Frozen; acetone; TSA	None, overnight	62526.111	R&D Systems

* Antibody worked on paraffin sections but sensitivity was enhanced if used on frozen sections with tyramide signal amplification.
† Stored in aliquots at -20°C to avoid repeated freeze-thaw cycles.
Abbreviations: TNB: 0.1M Tris-HCl, pH7.5. 0.15M NaCl, 0.5% Blocking reagent (provided in kit); **TSA**, Tyramide signal Amplification; **CB**, 10mM Citrate Buffer pH 6.0; **PC**, Pressure cooker

PlGF2 Immunostaining: Secondary antibody incubation was with an HRP linked rabbit anti-rat secondary (DAKO, 1:300) for 1hr. An additional tyramide signal amplification (TSA) step was performed according to the manufacturer's instructions (Perkin Elmer). The TSA Biotin system uses HRP to catalyse the formation of biotin-labelled tyramide, resulting in the deposition of numerous biotin labels immediately adjacent to the

immobilized HRP enzyme. Chromogenic visualisation was accomplished via a 30 min streptavidin-HRP conjugate incubation (provided in the kit) followed by the application of DAB.

2.12.4.1 Mounting sections in DPX

After dehydration of the sections in graded ethanols and xylene, the slides were placed on a sheet of fibre-free paper. The appropriate size coverslip (Fisher) was selected and a drop of DPX (RA Lamb, UK) applied to it. DPX is a mixture of distyrene, a plasticizer, and xylene, and is a colourless synthetic resin mounting medium which preserves the stain and dries quickly. Slides were lifted from the xylene tray and the excess was drained off. Each slide was gently lowered onto the drop of DPX so that the section was sandwiched between the slide and the coverslip. The slide was turned over, allowing a film of DPX to form between the section and coverslip. The surplus DPX was wiped from around the coverslip and the slides were air-dried for 1 hr.

2.12.5. Confocal Imaging

For immunofluorescence detection in arteries transduced with Ad.GFP or Ad.FGFR1DN-GFP, 6μm thick sections cut from OCT®-embedded tissue were fixed in acetone for 10 min at -20°C. Sections were mounted in an aqueous anti-fade medium (Biomeda GelMount, Foster City, CA) and visualized using a BioRad (Hercules, CA) MRC-1024 Krypton/Argon laser confocal system with blue-through-far red fluorescence excitation (488 nm, 568 nm, and 647 nm) and three PMT detectors. A 522DF35 emission filter (fluorescein isothiocyanate, FITC) was used for GFP visualisation.

To investigate whether adventitial cells contribute to neointima formation in the collar model, PKH-26 (Sigma), a fluorescent, lipophilic dye was delivered to the periadventitial space. PKH-26-labelled carotid cut frozen sections were mounted in an anti-fade medium containing 4'-6-diamidino-2-phenylindole to counterstain DNA (Vectashield with DAPI, Vector Labs). Red fluorescence was visualized with a Nikon Eclipse E1000

Micrsope with Biorad Radiance 2000 Argon-laser confocal system (excitation (551 nm) and emission (567 nm) characteristics compatible with rhodamine). DAPI excites at about 360nm and emits at about 460nm when bound to DNA producing a blue fluorescence.

2.12.6. Scanning Electron Microscopy (SEM)

Collared (n=2) and Ad.GFP (n=2) treated arteries with corresponding sham operated arteries were harvested at 5 days and washed with ice-cold PBS (pH 7.4) and immersion fixed for 4 hr at room temperature in freshly prepared 2% glutaraldehyde ($C_5H_8O_2$,GTA; Sigma) in 0.1M Sodium Cacodylate[7] (Sigma), pH 7.4. Fixative volume was a minimum of 15 x volume of the artery/buffered saline. The artery was then sliced longitudinally to expose the lumen and placed in fresh fixative at 4°C. Samples were coated with gold-palladium (2 min at 10mAmp sputtering current in a Hummer IV) prior to analysis at 15 kilovolts (Zeiss DSM 962, Rippel Electron Microscope Facility, Dartmouth College).

2.13. Image Acquisition and Morphometric Analysis

Images of sections at x25, x100, x200 or x400 were acquired with a high resolution color camera (Zeiss microscope, Jenoptik Camera at UCL, UK; Olympus BH-2 microscope, Polaroid DMC-1 camera at Dartmouth College, USA) and analysed using automated image analysis software (Image J, National Institutes of Health). Intimas were defined as regions between lumenal endothelium and the innermost internal elastic lamina (IEL). The media was defined as the area between the internal and external elastic laminae (EEL). Sections obtained were cut serially at progressive 100 μm intervals along the length of the arterial segment, stained with H & E, then blindly analysed for each artery (n= 4-6 sections). The results were averaged and expressed as means ± s.e. Neovascularisation was quantified by counting the numbers of CD31-positive vessels. CD31 analysis was deemed a single positive count if a single cell or a mature vessel with a lumen was present in the adventitia.

[7] 0.1M Sodium Cacodylate (24.1g $C_2H_6AsNaO_2 \cdot 3H_2O$ in 1 litre of deionized water)

Total RAM-11 positive macrophages were counted in the neointima and adventitia and results were expressed per mm^2 neointimal and adventitial areas, respectively. VCAM-1 immunostaining was quantified on high resolution (1300 x1030 pixel) images using OpenLab 3.14 software (Improvision Ltd) and expressed as the number of pixels representing endothelial VCAM-1 immunostaining as a percentage of the total endothelial pixel count.

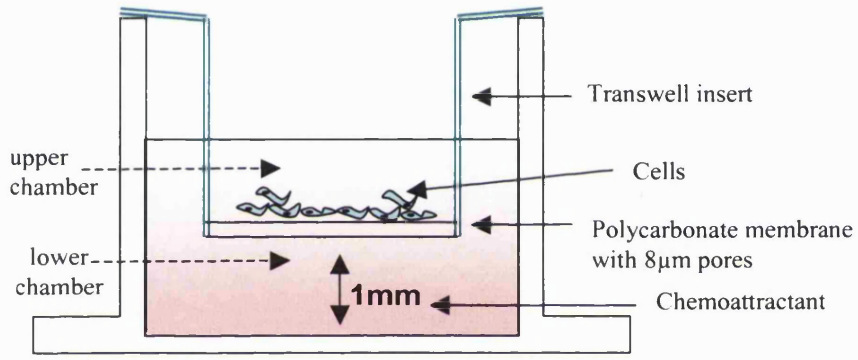
2.14. Transwell Migration Assay

2.14.1. Porcine Smooth Muscle Cell Migration

Porcine vascular smooth muscle cells were isolated using the explant-outgrowth method (Ross and Glomset 1973), and were donated by Dr. K Moodie, Dartmouth College, USA. Cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The porcine VSMCs (passage 3-7) were grown to 70-90% confluence on non-coated culture dishes in full serum (10%) containing DMEM. After an overnight starvation in 0.5% fetal calf serum (FCS) DMEM, the cells were washed twice with Ca^{2+}/Mg^{2+}-free DPBS, (Mediatech, Inc) detached using a non-enzymatic cell dissociation buffer (Sigma) to obtain single cell suspensions and re-suspended in DMEM containing 0.5% FCS at a concentration of 5 x 10^5 cells/ml. 100 µl aliquots of the cells (5 x 10^4 cells) were added to the upper chamber of a non-coated transwell PVF-free membrane with 8-µm pores in 24 well plates (Corning Costar Inc) and allowed to attach for 2 hrs. Cell attachment and spreading was enhanced by pre-incubating the inserts in medium prior to seeding. The upper chambers were then transferred to the lower transwell compartment containing 0.6 ml 0.5% FCS DMEM and either PR39 (Genemed Inc), PDGF-BB (Peprotech) or VEGF (Peprotech Inc.) in varying concentrations. Incubation was continued for 1 hr and 4 hrs hours at 37°C, after which the adherent cells were fixed with 4% formalin, washed three times with PBS and then stained with Toluidine Blue. The cells on the upper surface of the membrane were scraped off using a cotton bud and the membrane with fixed cells attached to the underside was detached from the transwell insert by carefully cutting around the

edges with a scalpel. The membrane was mounted onto a microscope slide and the number of cells that migrated to the lower surface of each membrane was counted in five separate high-power fields (HPFs) at a magnification of 200X (Olympus BH-2). Experiments were performed in quadruplicate and numbers of migrated VSMC were averaged and expressed as means ± s.e.

Fig. 2.6. Experimental Set Up of the Transwell Migration Chamber



2.14.2. Preparation of $CD14^+$ Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were prepared from peripheral venous blood of healthy volunteers, anticoagulated with EDTA. After Ficoll Hypaque (Sigma) density gradient centrifugation (1800 rpm for 30 min at room temperature), three separate bands appeared: red cells/PMNs at the bottom, PBMCs at the interphase of the Ficoll Hypaque and diluted blood, and plasma at the top. The $CD14^+$ PBMCs (buffy coat cells) were transferred into three new tubes using a sterile plastic transfer pipette and washed three times with normal saline. Positive selection for CD14 was performed by adding MACS® colloidal paramagnetic microbeads (Miltenyi) conjugated with monoclonal anti-human CD14 antibodies to cooled, freshly prepared PBMC preparations in MACS® buffer (PBS with 5mM EDTA and 0.5% bovine serum albumin, BSA) according to the manufacturer's instructions. Cells and microbeads were incubated for 15 min at 4°C. The separation

column was positioned in the MACS® high gradient magnetic field and washed with MACS® buffer at room temperature. The cells were washed with MACS® buffer, resuspended and loaded onto the top of the separation column. The eluent containing $CD14^-$ cells was withdrawn and after removal of the column from the magnet, trapped $CD14^+$ PBMCs were eluted with ice-cold MACS® buffer, centrifuged and re-suspended in medium containing 0.5% BSA (10 x 10^6 cells/ml). Freshly prepared $CD14^+$ PBMCs were subsequently used for monocyte migration assays.

2.14.3. Transendothelial Monocyte Migration

Monocyte chemotaxis was quantified across a HUVEC monolayer grown on fibronectin pre-coated transwell migration membranes, (24 well, 5 μm pore size; Beckton Dickinson). Pre-warmed Endothelium Cell Medium (EGM®, Clonetics), containing 2% FBS, was added to the transwell inserts 30 min prior to seeding with HUVECs, to hydrate the coated membranes. HUVECs (pooled, cryopreserved; Clonetics), passage 2-3, were harvested from culture plates with a non-enzymatic cell dissociation buffer (Sigma), washed three times with PBS and re-suspended in 2 % serum containing EGM®. Transwell membranes were then seeded with 3 x 10^5 HUVECs in 200 μl medium. The medium was replaced after an overnight incubation and every 48 hrs thereafter. Confluence on the transwell membrane was confirmed after 3-4 days of culture by taking one transwell insert and staining the upper surface with Toluidine Blue, which confirmed their typical cobblestone pattern. Transwell migration experiments were always carried out within 48 hrs after the HUVECs had achieved confluence.

The confluent HUVEC monolayers were then gently washed three times with sterile PBS and incubated with 100μl 0.5% serum DMEM, containing 5 x 10^5 freshly isolated human peripheral blood derived monocytes (prepared and CD14-enriched as described above). The transwell insert was then placed into the lower chamber containing 600 μl 0.5% serum DMEM and chemotactic reagents as follows: monocyte chemotactic peptide (MCP, 10ng/ml; Peprotech); VEGF (10-50ng/ml; Peprotech Inc.); L-NAME (10mM; Sigma); Pseudourea (100µM; Calbiochem); and Indomethacin (10µM; Sigma). Care was taken to avoid trapping air bubbles. Migration was assessed at 2 hrs, 8 hrs and 24 hrs in a

humidified incubator (37°C, 5% CO_2), at which point a 100 μl aliquot was removed from the lower chamber and the migrated monocytes counted with a Coulter counter (Beckman). At the earlier two timepoints, the 100 μl aliquot was replaced with an equivalent volume of 0.5% serum DMEM. Preliminary migration studies with Trypan Blue vital stain (0.4% w/v; Cambrex) were performed, and cells counted using a haemocytometer. Cell viability for the time-course examined was confirmed. All migration experiments were performed in triplicate.

2.15. Statistical Analysis

Values for different treatment groups were analysed using **SPSS** (Statistical Package for the Social Sciences). Differences in plasma total/LDL cholesterol levels and morphometric differences between different treatment groups were evaluated by ANOVA and Bon Ferroni Correction. $P<0.05$ was considered significant.

CHAPTER 3

CHARACTERISATION OF LESIONS INDUCED BY COLLAR PLACEMENT AROUND THE RABBIT CAROTID ARTERY AND USE OF THE COLLAR FOR LOCAL GENE TRANSFER

3.1 INTRODUCTION

Intimal thickening due to accumulation of VSMC and macrophages is a central feature of early atherosclerotic lesions, and a frequent cause of failure of arterial bypass grafting and post-angioplasty and stenting procedures. There is a need for animal models of rapidly induced and reproducible neointimal thickening. In this chapter, the induction of neointimal thickening by periadventitial placement of an inert silicone collar around the rabbit carotid artery is described. In the collar model, neointimal thickening is increased by collar placement over a period of up to 14 days without major damage to the endothelium or medial VSMC, and the endothelium remains essentially intact and continuous throughout the study period. There is extensive experience within this laboratory in the use of this model and the initial aim was to reproduce earlier findings and to validate the use of the collar model as a vehicle for localized gene transfer. The histological composition of lesions that developed in animals sustained on a normal diet and those on a 1.5% cholesterol-supplemented diet was characterised.

3. 2 RESULTS

3.2. 1. Collar Placement Alone Generates a Smooth Muscle Cell Rich Neointima in a Time Dependent Fashion

Placement of a biologically inert, silicone collar around the left common carotid artery generates a neointima, which increases with time (Fig 3.1A-E). The intima to media ratio (I/M) increased rapidly between 1 and 3 days after collar placement and subsequently increased more slowly, reaching a maximum after 14 days (Fig 3.1F). Representative haematoxylin & eosin (H&E) staining of a contralateral sham-operated control artery shows that there is no anatomically visible neointima at all time-points (Fig 3.2A). Immunostaining for α-actin demonstrates that the neointima comprises predominantly SMC (Fig 3.2B). No endolumenal manipulation is necessary during the operative procedure with minimal handling of the vessel using friction-free vascular slings when the collar is placed around the artery. Immunostaining of EC by CD31 (Fig 3.3A) and scanning electron microscopy (Fig 3.4A) demonstrated that the lumenal endothelium of the carotid artery remains essentially intact and does not suffer major damage after collar placement. It was observed in EM photomicrographs that protrusions, which resembled semi-spherical elevations, were apparent in collared but not sham-operated arteries. It was verified that the contralateral sham artery also retains an intact endothelium (Fig 3.3A, 3.4A). The ability of CD31 to stain vessels was confirmed in positive control human tonsillar tissue (Fig 3.3C). Histological and immunocytochemical analysis of encapsulated adventitia 5 days post-collaring demonstrated that **little adventitial neovascularisation** occurs alongside the development of a neointima (Fig 3.3D).

3.2.2. Adventitial Cells Do Not Contribute to Neointimal Cell Mass in the Rabbit Collar Model

Several recent studies suggested that adventitial cells could contribute to neointima formation in certain models. It was therefore of interest to examine whether adventitial cells were involved in neointima formation in the collar model. To test this possibility, the cell-permeable lipophilic fluorescent cell reporter, PKH26 (Consigny and Miller 1994) was

transferred to the adventitia during collar placement. Uptake and cellular distribution of PKH26 was then examined on frozen arterial sections, using a confocal microscope 2, 5 and 14 days after collar placement. To identify cell nuclei in the arterial wall, tissues were counterstained with DAPI, a DNA-binding dye. Fig 3.5A demonstrates an absence of PKH26 in the contralateral sham operated artery, confirming that delivery via the collar is localized. PKH26-labelled cells were detected exclusively in the adventitia at all time-points. No PKH26-positive cells were detected in either the media or neointima at 48hrs, 5 or 14 days (Fig. 3.5B-D). Interestingly, it can be seen that PKH26-positive cells migrated to the adventitial border of the external elastic lamina, but did not cross it, at 14 days.

3.2.3. Collar Model Can Be Successfully Used For Gene Transfer

The ability of the collar to serve as a vehicle to achieve localized periadventitial gene transfer was tested using the reporter gene LacZ. Incubation of the carotid artery during collar placement with either lipofectamine-plasmid LacZ (25 μg) or Adenoviral-LacZ (5 x 10^9 pfu) resulted in successful gene transfer, as determined by X-Gal staining of sectioned collared artery (Fig. 3.6A, B). Lipofectamine-mediated LacZ gene transfer elicited a low efficiency of gene transduction as indicated by sporadic X-gal positive cells and consistent with an efficiency of 0.5% or less, as reported in previous studies of liposome-mediated LacZ gene transfer in the same model (Laitinen et al. 1997a). In contrast, Ad.LacZ gene delivery produced a much higher level of arterial gene expression as indicated by the presence of abundant X-gal stained cells, consistent with a transduction efficiency of 5 %, as reported previously (Laitinen et al. 1997a). In the majority of experiments, positive X-Gal staining for β-galactosidase gene expression was localized to the peri-adventitia (3.6A, B). After 5 days of transfection using 5 x 10^9 pfu (the viral load used in all experiments), discrete cellular blue stain was also mostly confined to the adventitia but infrequently was also detected in the tunica media and neointima (Fig 3.6C, D, E).

3.2.4. Effects of Cholesterol-Supplemented Diet

All animals fed a 1.5% cholesterol diet developed hypercholesterolaemia at 21 days, with up to a 10-fold increase in total serum cholesterol (Fig. 3.7A), most of which comprised LDL. Indeed there was a 20-fold increase in LDL cholesterol compared with the normal diet control rabbits. However, there was only a 3-fold increase in serum HDL cholesterol. In contrast, serum levels of triglycerides did not significantly change throughout the entire experimental time-course (1.44 to 1.56 mmol/L).

3.2.5. Cellular composition of collar-induced neointimas

Collar-induced neointimal lesions in rabbits on a normal diet were composed largely of VSMC as shown by immunostaining with a VSMC-specific α-actin antibody, with no detectable macrophage involvement (Fig 3.7B). In a set of rabbits fed a 1.5% cholesterol diet, collar placement induced formation of neointimas composed of VSMC and abundant Ram 11-stained macrophages. The contralateral sham-operated carotid arteries in the cholesterol-fed rabbits displayed no evidence of either intimal thickening or increased Ram11 immunostaining. Immunostaining of endothelial cells with CD31 antibody indicated that neither collaring nor hypercholesterolaemia caused significant discontinuities in the endothelium, which remained essentially intact, in agreement with previously published findings. Furthermore, microscopic analysis of histological sections from collared arteries revealed no disruption of either the internal or external elastic lamina and no detectable damage to the tunica media.

FIGURE 3.1

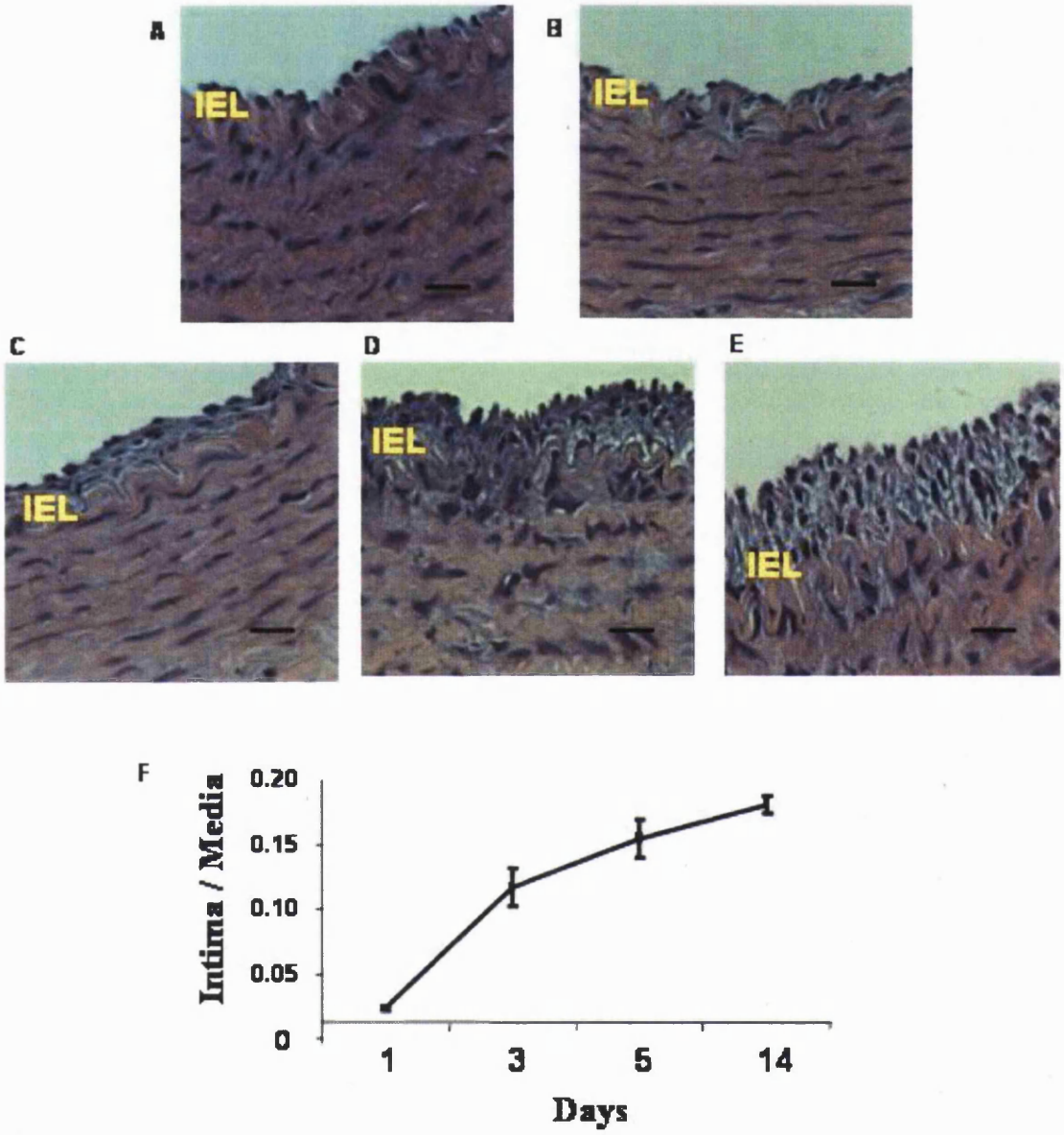


Figure 3.1. Characterisation of Intimal Thickening in the Rabbit Collar Model.

A-E, Representative H&E stains of carotid artery at A, 1 day, B, 3 days, C, 5 days, D, 7 days and E, 14 days after collaring; scale bar = 20 μm. **F**, Time-course of neointima formation over 14 days from collaring. Each time point represents the mean intima/media ratio in 6-10 sections from arteries (n=3). Sections were taken at 100 μm incremental lengths along the harvested artery.

FIGURE 3.2

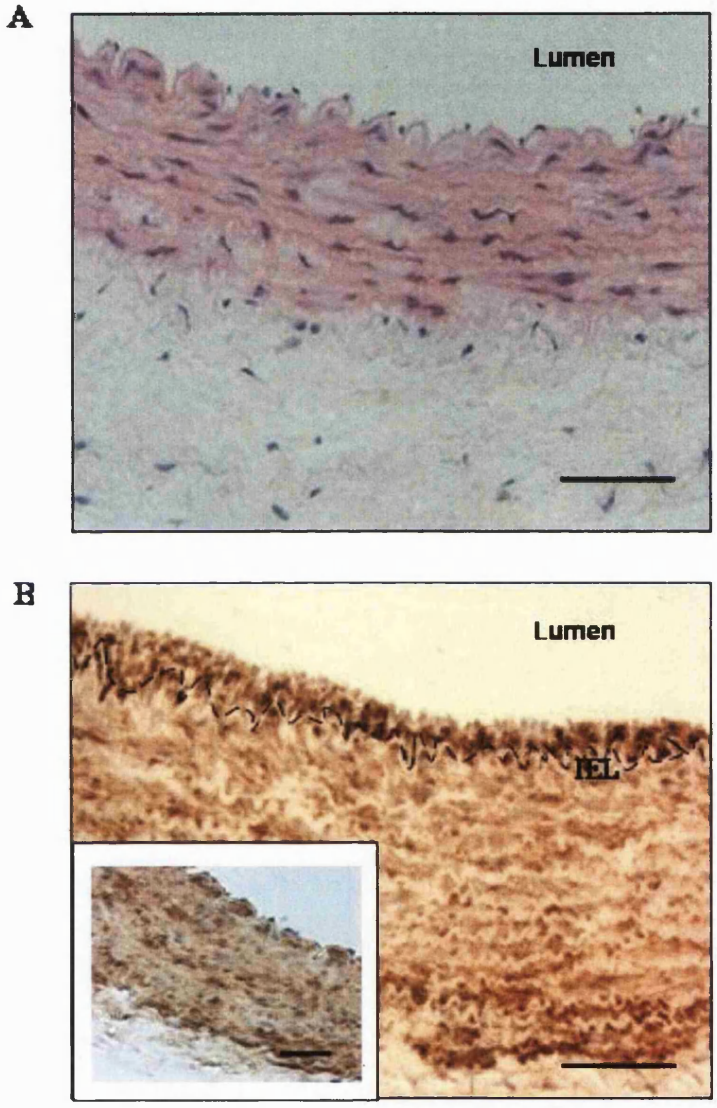


Figure 3.2 Contralateral Sham Artery and α-Actin Immunostaining

A, H&E staining of contralateral sham control after 5 days (x400 magnification). **B**, α-actin stain for VSMC of collared artery after 5 days (x400 magnification). Dotted line demarcates the Internal Elastic lamina (IEL). Insert: Sham control also at 5 days. Magnification bars represent 20 μm.

FIGURE 3.3

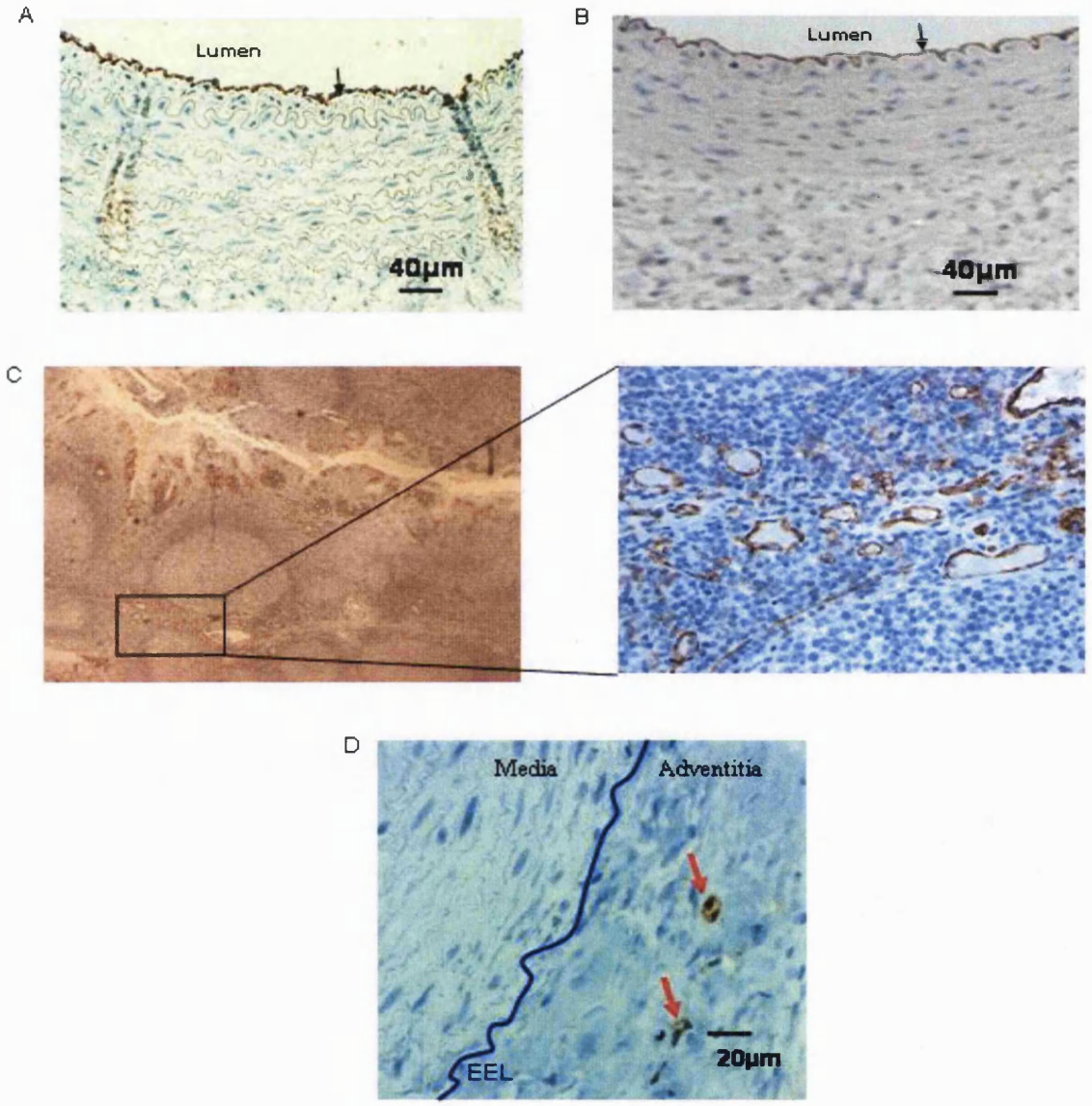


Figure 3.3. Characterization of EC staining after Collaring, by CD31 immunohistochemistry.

The luminal endothelium remains intact as determined by CD31 staining, **A**, 5 days post-collaring (methyl green counterstain) and in **B**, contralateral sham control carotid (haematoxylin counterstain). **C**, represents low power (left; x50) and higher power (right; x400) magnification images of human tonsillar tissue stained with CD31 which served as a positive control. **D**, There is little adventitial 'basal' vascularization (marked by arrows) within the adventitia 5 days post-collaring as determined by CD31 staining. The external elastic lamina dividing the media and adventitia is marked (EEL).

FIGURE 3.4

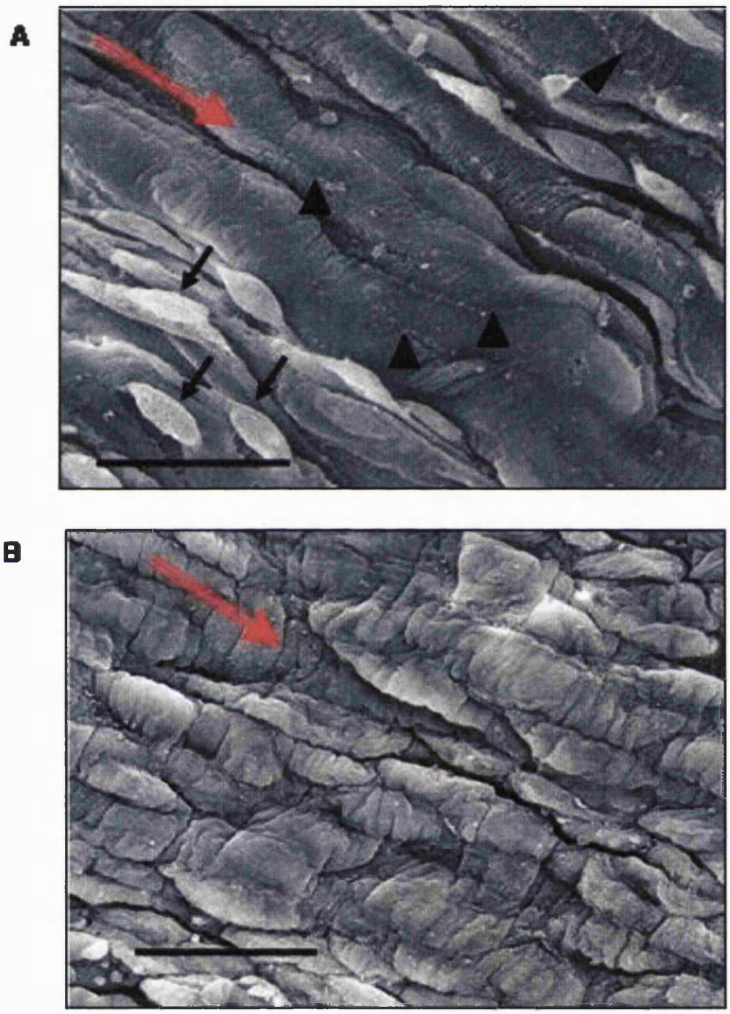


Figure 3.4. Scanning electron micrographs of sham control and collared carotid arteries.

Scanning electron photomicrographs of the luminal surface at 5 days confirms an intact endothelium in **A**, collared carotid and **B**, sham operated artery. Arrowheads mark the intercellular border of ECs and the smooth appearance of the surface was frequently disturbed by ridges of underlying tissue and semi-spherical elevations (arrows). In the sham vessels, the EC surface appears ruffled and ridges divide the cells, but there is an absence of semi-spherical elevations. Red arrow indicates direction of blood flow. Scale bar, 20μm.

FIGURE 3.5

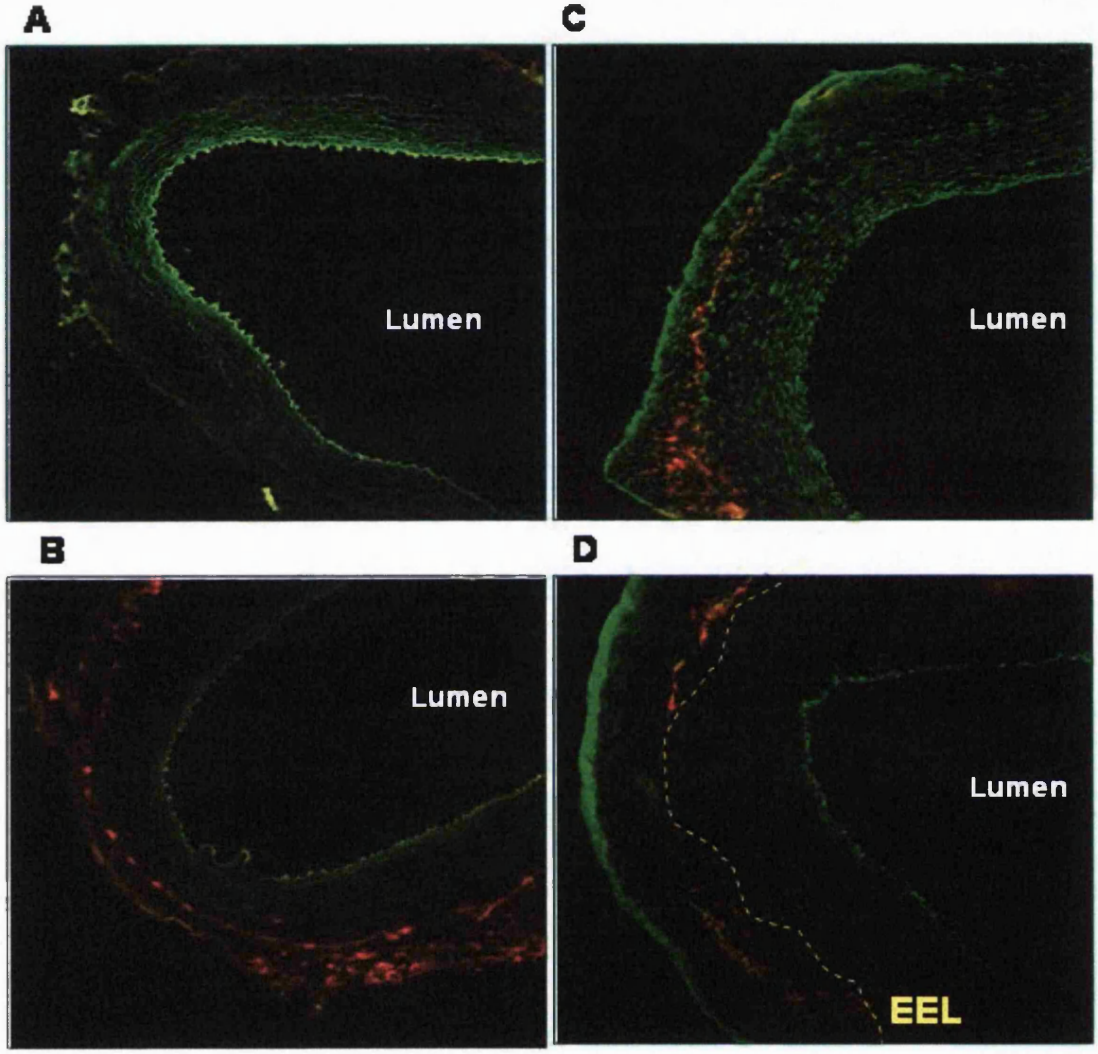


Figure 3.5. PKH26 delivery to the adventitia of collared arteries in the NZW Rabbit.

Confocal microscopy image (TRITC filter) of 10 μm thick cryosections of **A**, sham operated and, **B-D,** collared carotid arteries after **B**, 48 hrs, **C**, 5 days and **D**, 14 days after treatment with 200μl 0.1μM PKH26 (Sigma). Uptake and distribution of PKH-26 is confined to the adventitia with no staining evident in the sub-endothelial region at any timepoint. The dotted yellow line at 14 days demarcates the external elastic lamina (EEL). All images are at X100 magnification.

FIGURE 3.6

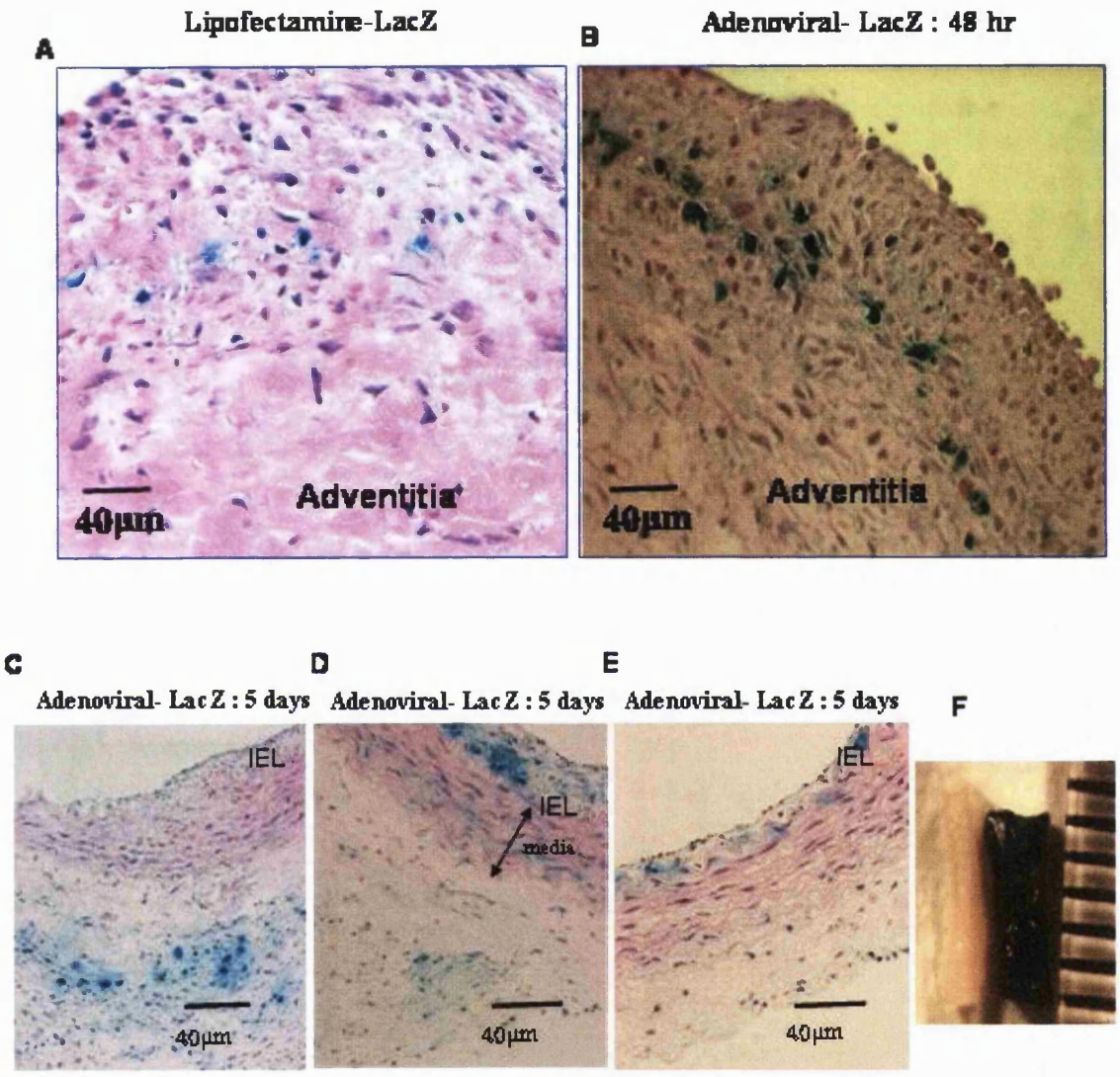


Figure 3.6. β-Galactosidase staining of collared carotid arteries transduced with the reporter Gene, LacZ.

A, Lipofectamine-mediated LacZ gene (25 µg) transfer results in occasional positive cells within the adventitia after 48 hrs. No positive staining is observed within the media or neointima. Ad.LacZ gene ($5x10^9$ pfu) transfer results in significantly higher positive staining within the adventitia after **B**, 48 hrs and **C**, 5 days. Positive β-galactosidase staining was also infrequently observed in **D**, the media and **E**, neointima after Ad.LacZ periadventitial gene transfer. IEL: internal elastic lamina. **F**, Macroscopic segment of artery successfully transduced with Ad.LacZ (right), compared to non-collared contralateral control in the same animal (left).

FIGURE 3.7

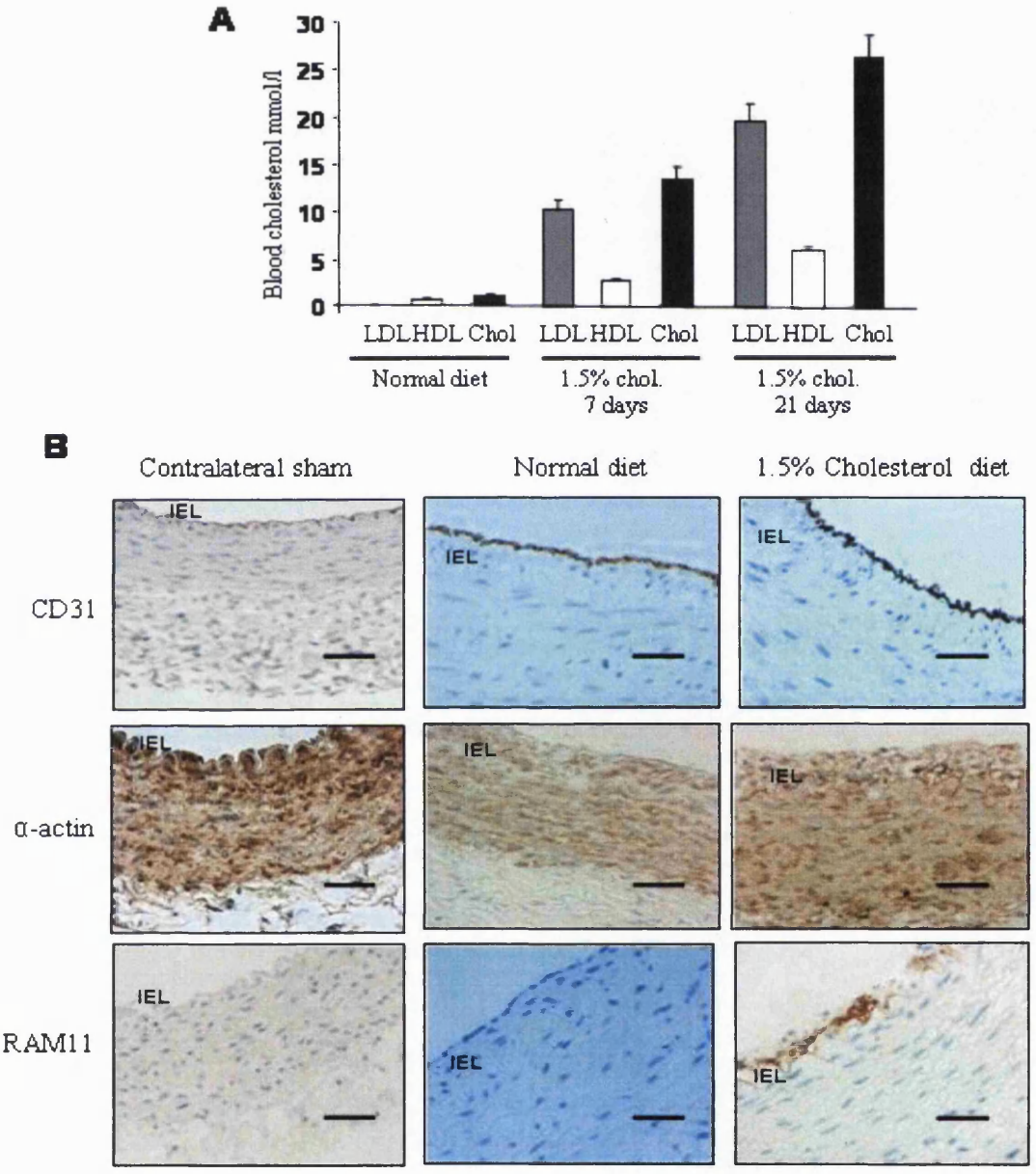


Figure 3.7. Effect of High Cholesterol Diet on Serum and Lesion Composition in Collared Arteries.

A, Levels of LDL-cholesterol, HDL-cholesterol and total cholesterol (Chol) were determined in serum from rabbits on a normal diet and a 1.5% cholesterol diet for 7 and 21 days. **B**, Rabbits were fed either a normal or cholesterol-rich diet for 7 days prior to collaring of the carotid artery, and 5 days after collaring, arteries were immunostained with antibodies to α-actin (VSMC), Ram 11 (macrophage) and CD31 (endothelial). Scale bar represents 20 μm; x400 magnification. IEL: Internal Elastic Lamina

3.3 DISCUSSION

VSMC activation from a quiescent state, followed by proliferation and migration to comprise a neointima occurs during normal development and aging, and as a response to 'injury'. Neointima formation in the VSMC-rich vessel wall has been compared to the fibrotic scarring response to injury in the fibroblastic dermis of the skin (Schwartz et al. 1995). The best studied model of neointimal formation is the response of the rat carotid artery to balloon angioplasty (Clowes et al. 1983). As with any model, extrapolation of results to explain cellular events in human atherosclerosis or restenosis must be made with caution. Unlike arteries in larger mammals, the rat carotid artery rarely possesses intimal cells. Secondly, although platelet adhesion occurs in injured rat arteries, thrombus formation and leucocyte infiltration are minimal. In contrast, these important events are prominent after vascular injury in rabbits, pigs and non-human primates. In this rabbit model, placement of a silicone collar involves minimal manipulation to the vessel coupled with no endolumenal injury which results in a VSMC-rich neointima underlying a structurally intact endothelium. CD31 (platelet endothelial cell adhesion molecule, PECAM-1) is a widely accepted surrogate marker for both mature endothelium and neo-vessel formation (DeLisser et al. 1997). CD31 positivity was present on the lumenal endothelia of both sham operated and collared carotid artery, from 24 hours to 14 days, i.e. the experimental time-course used throughout the experiments described in this thesis. CD31 mediates endothelial cell-endothelial cell and leucocyte-endothelial cell adhesion (Ilan and Madri 2003). It also mediates signaling functions within the adhesion cascade, culminating in the activation of integrins. It also has an important role in the diapedesis step of leucocyte emigration in inflammation (Muller et al. 1993). An intact endothelium was confirmed by scanning electron microscopy (SEM) of the intimal surface in both sham and collared arteries 5 days after collaring. It is not certain what the protrusions over the endothelium of the collared artery represent, but previous reports document similar findings and suggest they are adherent leucocytes (Nathan et al. 1999) or patches of exposed subendothelium (Asahara et al. 1995).

The origin of neointimal SMCs in models of restenosis is contentious and has not previously been studied in the collar model. PKH26, a lipophilic and inert fluorescent dye,

has been used to label cells to investigate the pharmacokinetics of drug delivery to the vessel wall (Consigny and Miller 1994). Using this method, labelled adventitial cells were not found to contribute to the neointimal accumulation of cells in the rat balloon-angioplasty model of restenosis (De Leon et al. 2001). Application of PKH26 to the adventitia at the time of collaring the artery resulted in labelling of cells in the outer adventitia after 48 hrs. A progressive movement of dye towards the inner layers was observed over the time-course studied and, at 14 days, PKH26 was predominantly located at the adventitial border of the EEL, but did not extend beyond it into the media. At 5 days and 14 days, imaging of the neointimal mass at higher magnification did not reveal a single cell to be stained with PKH26 dye suggesting that, in this model, adventitial cells do not contribute to neointima formation.

Consonant with previously published reports, it is shown that the collar can be used to locally deliver genes to the vessel wall (Laitinen et al. 1997a). Consistent with published reports, the transfection efficiency observed with lipofectamine-LacZ was very low, in contrast to the much higher efficiency seen with adenoviral transduction (Laitinen et al. 1997a). It can be seen that the adventitia is predominantly targeted and occasionally, the media and neointima.

Neointimal lesions induced by collar placement around the rabbit carotid artery are VSMC-rich with no detectable macrophage involvement. Though similar VSMC-rich lesions are a prominent feature of restenosis, vein graft stenosis and other vasculo-occlusive disorders characterized by excessive intimal thickening, intimal macrophage accumulation is characteristic of atherogenesis and also occurs within vein graft failure (Westerband et al. 1997). A major goal of this thesis was to examine the impact of angiogenic factors upon macrophage accumulation in collar-induced lesions. Since high blood VLDL- and LDL-cholesterol are major risk factors for atherosclerosis in humans and induce the formation of macrophage-rich lesions resembling the human disease process in a number of animal models, the effects of a high cholesterol diet on the macrophage content of collar-induced lesions were investigated. It is recognised that rabbits fed a 'high' cholesterol diet for 8-16 days have increased low density lipoprotein (LDL) levels in lesion prone regions of the aortic intima before macrophage foam cells appear (Schwenke and Carew 1989). To dissect out these early cellular processes, the collar model with the animal maintained on a

cholesterol-supplemented diet allows for this type of investigation. Placement of rabbits on a 1.5% cholesterol supplemented diet elicited a time-dependent increase in serum LDL and HDL cholesterol and triglyceride. The mean level of LDL cholesterol obtained (~20 mmol/l) at the end of the experimental time-course is comparable to that seen in familial hyperlipidaemias (Marks et al. 2003) and is appreciably greater than levels which are routinely treated in humans to improve their cardiovascular risk profile. Although not measured in this experiment, the principal serum lipid-fraction increased by dietary cholesterol supplementation in rabbits is very low density β-lipoprotein (VLDL) (Shore et al. 1974; Roth et al. 1983). In this study, macrophages accumulated in the arterial intima under conditions of hypercholesterolaemia and in contrast to contralateral sham controls and lesions in animals on a normal diet. These findings confirm previous studies in collared carotid arteries (Baetta et al. 2002). Other studies have shown the monocytes enter from the circulation (Gerrity 1981) and may be due to the chemotactic property of subendothelial LDL (Quinn et al. 1987). Studies of macrophage foam cells from rabbit lesions indicate that the majority of the cholesterol is esterified (Rosenfeld et al. 1991). Macrophage cell infiltration was also evident in the adventitia, but not in the media.

CHAPTER 4

EFFECTS OF PERIADVENTITIAL VASCULAR ENDOTHELIAL GROWTH FACTOR GENE TRANSFER IN HYPERCHOLESTEROLAEMIC RABBITS

4.1 INTRODUCTION

Vascular endothelial growth factor (VEGF or VEGF-A), is essential for endothelial cell differentiation (vasculogenesis) and angiogenesis during embryonic development, and plays a major role in neovascularisation in a variety of disease states. VEGF elicits an array of biological responses in endothelial cells via its major signalling receptor, KDR, including activation of multiple intracellular signalling cascades, cell proliferation, migration and survival, increased vascular permeability, and production of the potent vasodilators nitric oxide and prostacyclin.

VEGF has been championed as an attractive therapeutic approach to cardiovascular disease. Over the last decade, numerous clinical trials have tested the efficacy of VEGF to promote revascularisation of ischaemic tissue (Simons and Ware 2003). It has also been proposed that enhancement of protective endothelial functions by targeted arterial VEGF gene delivery may produce local protective effects in arteries (Zachary et al. 2000) and in support of this concept, earlier work from this laboratory demonstrated that liposome-mediated $VEGF_{164}$ gene transfer was found to inhibit collar-induced formation of a VSMC-rich neointima (Laitinen et al. 1997b). The results presented in Chapter 3 confirmed the potential of using the collar model for localized gene transfer, with an observed efficiency consistent with previous reports. In this study, it was tested whether local $VEGF_{164}$ gene delivery could reduce intimal thickening and macrophage involvement in the collared carotid arteries of hypercholesterolaemic rabbits.

4.2. RESULTS

4.2.1. Effect of $VEGF_{164}$ gene delivery on intimal thickening in normocholesterolaemic rabbits

Previous findings from this laboratory showed that periadventitial liposome-mediated $VEGF_{164}$ gene transfer into collared arteries significantly reduced collar-induced neointima formation (Laitinen et al. 1997b). Initially, it was important to reproduce these findings, by performing periadventitial VEGF-A_{164} gene transfer into carotid arteries 5 days after positioning of the collar in rabbits maintained on a normal low cholesterol diet. Intimal thickness and cellular composition in arterial sections was then measured a further 9 days after gene transfer. Consistent with previously published work, liposome-mediated $VEGF_{164}$ gene transfer caused a significant decrease in collar-induced intimal thickening as compared to a control liposome-mediated LacZ gene (Fig. 4.1).

4.2.2. Periadventitial $VEGF_{164}$ gene delivery in collared, hypercholesterolaemic rabbits

The effect of $VEGF_{164}$ gene delivery on collar-induced intimal thickening and macrophage accumulation in cholesterol-fed rabbits was determined next. Expression of the $VEGF_{164}$ transgene was verified in transduced carotid arteries by RT-PCR. An expected amplicon of 547 bps corresponding to the $VEGF_{164}$ transgene and vector was detected only in collared arteries transduced with the $VEGF_{164}$ vector (Fig. 4.2A). Immunostaining of sections of VEGF-transduced arteries with anti-VEGF antibody showed the expression of VEGF in the adventitia, media and intima including endothelium (Fig. 4.2B). Previous studies demonstrated that liposome-mediated gene transfer in the collared rabbit carotid artery results in a low efficiency of transfection (~0.05 %) (Laitinen et al. 1997a). Staining for β-galactosidase in arteries transduced with the control LacZ gene revealed a small number of strongly-stained cells in the adventitia consistent with a similarly low efficiency of gene transfer and in agreement with previous findings (see Fig 3.5, Chapter 3).

As shown in Fig.4.3A, $VEGF_{164}$ gene transfer for 9 days caused a marked, highly significant decrease in collar-induced intimal thickening in cholesterol-fed rabbits ($p < 0.001$ for VEGF versus LacZ). Immunostaining with Ram11 showed that VEGF also

reduced macrophage accumulation in the neointimas of collared arteries (Fig. 4.3B). Compared with LacZ-transduced arteries, periadventitial VEGF gene delivery significantly decreased the neointimal density of Ram11-positive cells (Fig. 4.3C). VEGF also decreased the neointimal density of Ram11-positive cells compared with saline, non-transfected collared arteries (224±66 versus 384±54 for VEGF and saline, respectively; $p < 0.05$).

4.2.3. Neovascularisation in VEGF-transduced collared arteries

Since angiogenesis is a major biological effect of VEGF *in vivo*, it was examined whether VEGF gene transfer increased neovascularisation in collared carotid arteries. Immunostaining of CD31 revealed the presence of new vessels specifically in the adventitia of both VEGF-transduced and Lac Z-transduced arteries (Fig 4.4A). Quantification of the adventitial density of CD31-positive vessels indicated that neovascularisation was increased in VEGF-transduced arteries compared with LacZ transfectants, but this effect was not significant (Fig. 4.4B). CD31-positive cells were not detected in the media and, apart from lumenal staining of the endothelium, were also absent from the neointima.

4.2.4. Cell Adhesion Molecule Expression in VEGF-transduced collared arteries

Upregulation of the endothelial cell adhesion molecule, VCAM-1, plays a central role in mediating increased monocyte adhesion to the endothelium and is therefore essential for transendothelial monocyte migration leading to neointimal accumulation of macrophages (Cybulsky et al. 2001; Dansky et al. 2001). VEGF-mediated modulation of endothelial VCAM-1 expression is therefore a possible mechanism that could explain the marked decrease in macrophage accumulation observed in the VEGF-transduced collared arteries of cholesterol-fed rabbits.

Endothelial expression of VCAM-1 was strikingly increased in LacZ-transduced collared arteries from cholesterol-fed rabbits compared with the sham-operated

contralateral control arteries in the same animals (Fig 4.5). VCAM-1 staining in the LacZ-transfected arteries of cholesterol-fed rabbits was also evident within the media and more sparsely in the adventitia. ICAM-1 was constitutively expressed on the endothelia of sham-operated arteries in cholesterol-fed rabbits and collaring caused no significant increase in endothelial ICAM-1 expression. In the VEGF-transduced collared arteries of cholesterol-fed rabbits, endothelial VCAM-1 staining was markedly reduced particularly where neointimal thickness exhibited the most striking decrease, and was also noticeably decreased in the media (Fig 4.5A). Quantification of VCAM-1 immunostaining showed that the total percentage of the endothelium positive for VCAM-1 immunostaining was significantly reduced in VEGF-transduced arteries compared to LacZ (Fig 4.5B). A similar marked significant reduction in VCAM-1 staining in VEGF-transduced arteries was found when total neointimal VCAM-1 staining was quantified. VEGF gene transfer did not significantly affect endothelial ICAM-1 immunostaining in hypercholesterolaemic rabbits.

4.2.5. Effect of VEGF on Monocyte Migration Assay over HUVEC monolayer

VEGF has previously been reported to stimulate monocyte chemotaxis acting via VEGFR1 / Flt1 (Barleon et al. 1996). The findings presented in Figs 4.3 suggested that VEGF gene transfer might inhibit monocyte migration *in vivo*. This prompted me to examine the effects of VEGF on the migration of $CD14^+$ monocytes through an EC monolayer. The effects of recombinant VEGF-A peptide on monocyte migration were investigated in a transwell assay. Monocytes were obtained from the peripheral blood of healthy volunteers and allowed to migrate through a confluent monolayer of HUVECs grown on a 5 μm pore-size transwell membrane (Fig 4.6A). VEGF exerted a significant chemotactic effect on $CD14^+$ enriched human monocytes, in accordance with previously published findings (Clauss et al. 1990; Heil et al. 2000). Nitric oxide (NO) is thought to mediate the arterioprotective mechanism of VEGF. It was found that the neointima reducing effects of periadventitial VEGF gene transfer was abrogated by placing the animals on a diet containing L-NAME (Laitinen et al. 1997b). The role of NO and PGI_2 on the stimulation of monocyte chemotaxis by VEGF was therefore examined. Monocyte migration was not significantly attenuated by the presence of either L-NAME or thiopseudourea or indomethacin alone,

compared to 0.5% foetal calf serum (FCS) suggesting that basal migration occurs independently of nitric oxide and prostacyclin. However, the monocyte pro-migratory effect of VEGF was abolished in the presence of either L-NAME or thiopseudourea, after 2 hrs incubation, but only thiopseudourea had a significant effect. A similar inhibitory effect was also seen with the prostacyclin inhibitor, indomethacin, but was not significant (Fig. 4.6B).

FIGURE 4.1

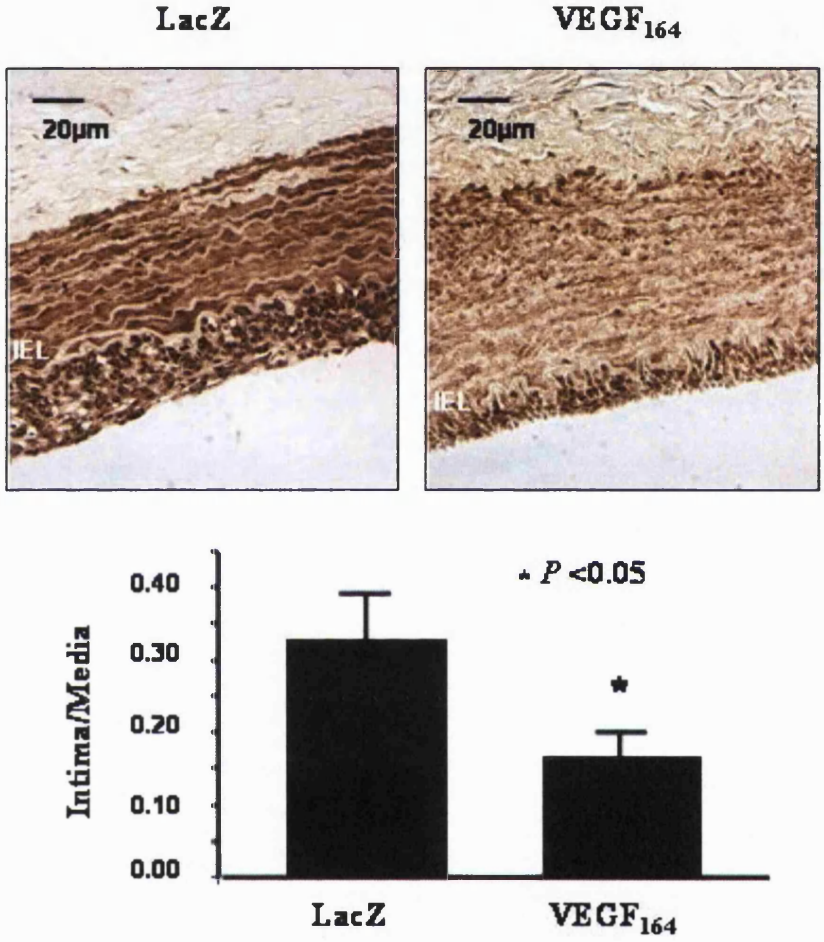


Figure 4.1. $VEGF_{164}$ gene transfer Inhibits Collar-induced Intimal Thickening in Normocholesterolaemic Rabbits

VSMC-specific α-actin immunostaining (top) and intima to media ratios (bottom) in collared carotid arteries transfected with LacZ (n = 10) or $VEGF_{164}$ (n = 10) in rabbits on a normal diet. The position of the internal elastic lamina (IEL) is indicated. Intimal thickening was significantly reduced in VEGF-transduced arteries; * $P< 0.05$ for VEGF versus LacZ.

FIGURE 4.2

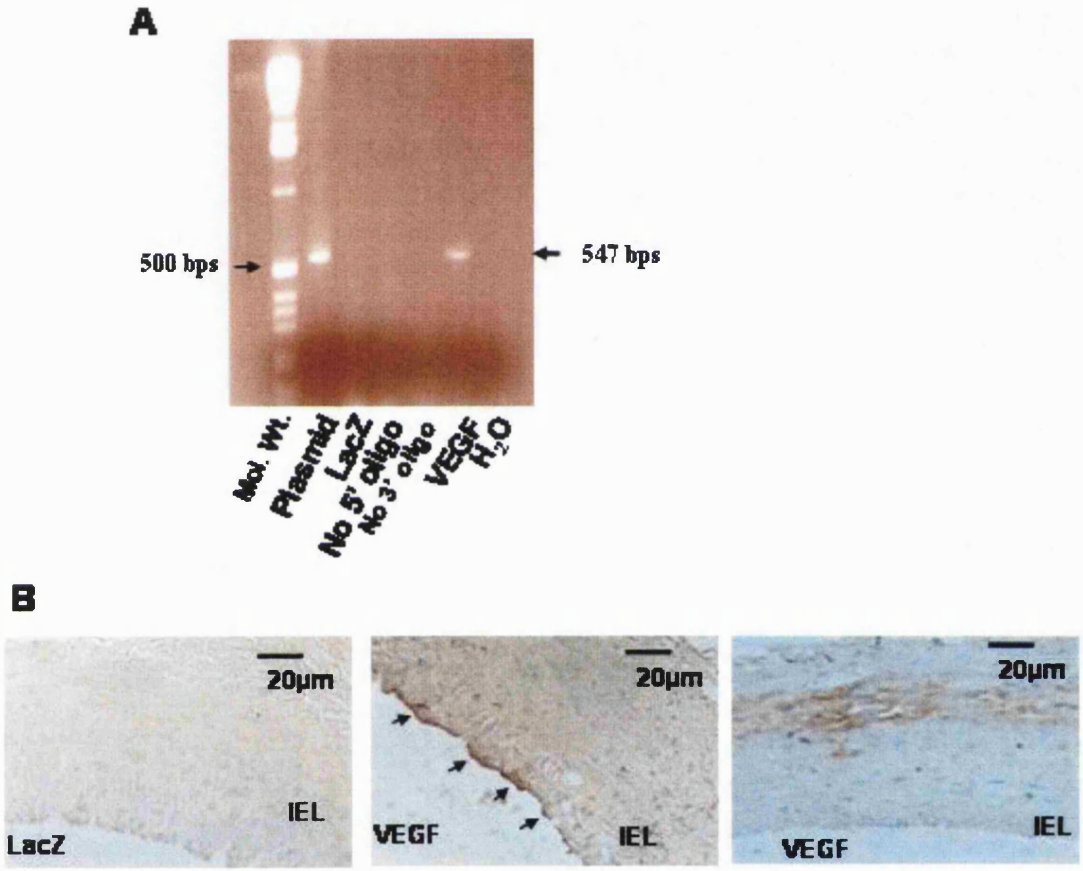


Figure. 4.2. Expression of $VEGF_{164}$ transgene in collared arteries.

A, RNA was extracted from collared transfected carotid arteries in cholesterol-fed rabbits, and $VEGF_{164}$ transgene expression was determined by RT-PCR. A predicted 547 bp PCR product was detected only in arteries transfected with the $VEGF_{164}$ plasmid and was absent from LacZ-transfected arteries, and in the absence of DNA, or either forward or reverse primers. RT-PCR of the $_{VEGF164}$ plasmid was used as a positive control (plasmid). **B,** Collared carotid arteries in rabbits on a high-cholesterol diet were transfected with LacZ or $VEGF_{164}$ and VEGF expression was detected by immunostaining with anti-VEGF antibody. VEGF immunostaining was detected in the endothelium and neointima (middle), the media and adventitia (right) in VEGF-transduced arteries, but was absent in LacZ-transfected arteries (left). The position of the IEL is indicated

FIGURE 4.3

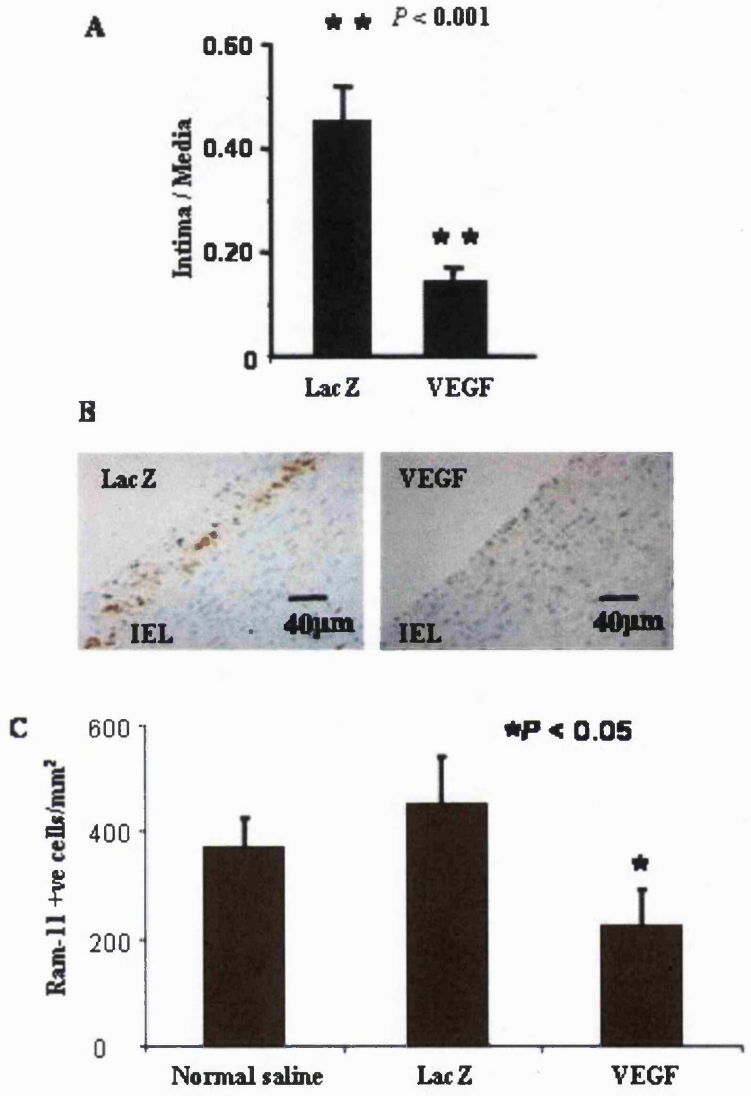


Figure. 4.3. Effect of $VEGF_{164}$ gene transfer on Collar-induced Intimal Thickening and Macrophage Accumulation in Hypercholesterolaemic Rabbits.

A. Collar placement and gene transfer were performed in cholesterol-fed rabbits and intima to media ratios were determined in collared carotid arteries transfected with LacZ (n = 12) or $VEGF_{164}$ (n = 12) as described in Materials and Methods. Intimal thickening was significantly reduced in VEGF-transduced arteries: ** $p < 0.001$ for VEGF versus LacZ. **B**, Collared carotid arteries in rabbits on a high-cholesterol diet were transfected with LacZ or $VEGF_{164}$ and macrophage content was determined by immunostaining with Ram 11. The position of the IEL is indicated. C. Macrophage density is expressed as neointimal Ram 11-positive cells ± s.e./mm^2. VEGF significantly reduced neointimal macrophage content. * $p < 0.05$ for VEGF versus either LacZ or saline controls.

FIGURE 4.4

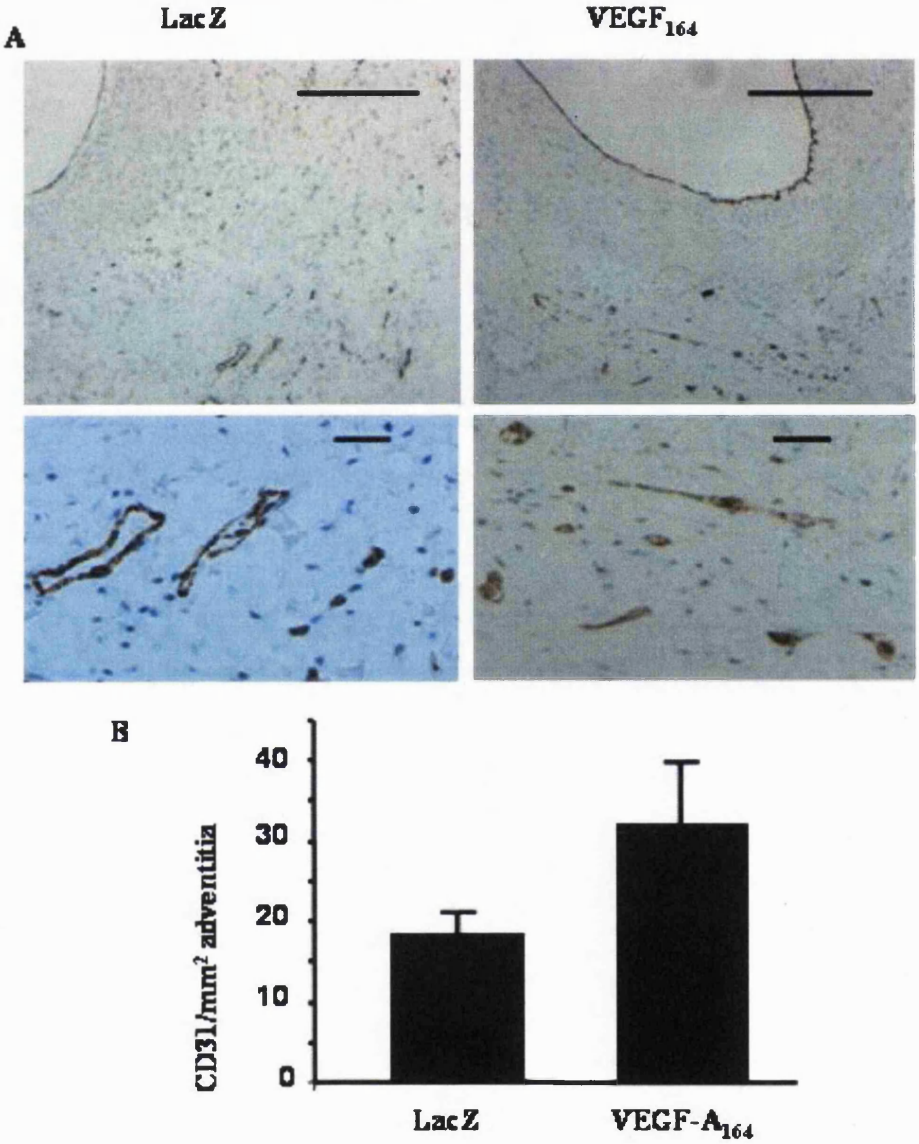


Figure. 4.4. Neovascularisation in Transfected Collared Arteries

A, Sections of collared carotid arteries transfected with LacZ or $VEGF_{164}$ were immunostained with CD31 antibody. CD31 staining is shown at x100 (upper photomicrographs) and x400 magnification (lower). CD31-positive microvessels are evident in the adventitia of arteries transfected with either LacZ or VEGF. Magnification bar represents 100 μm in top and 20 μm in bottom micrographs. **B,** The numbers of CD31-positive microvessels and cells in the adventitia were determined and results are presented as CD31-positive vessels/mm^2; $P>0.14$ for VEGF versus LacZ. VEGF did not significantly increase adventitial CD31 immunostaining.

FIGURE 4.5

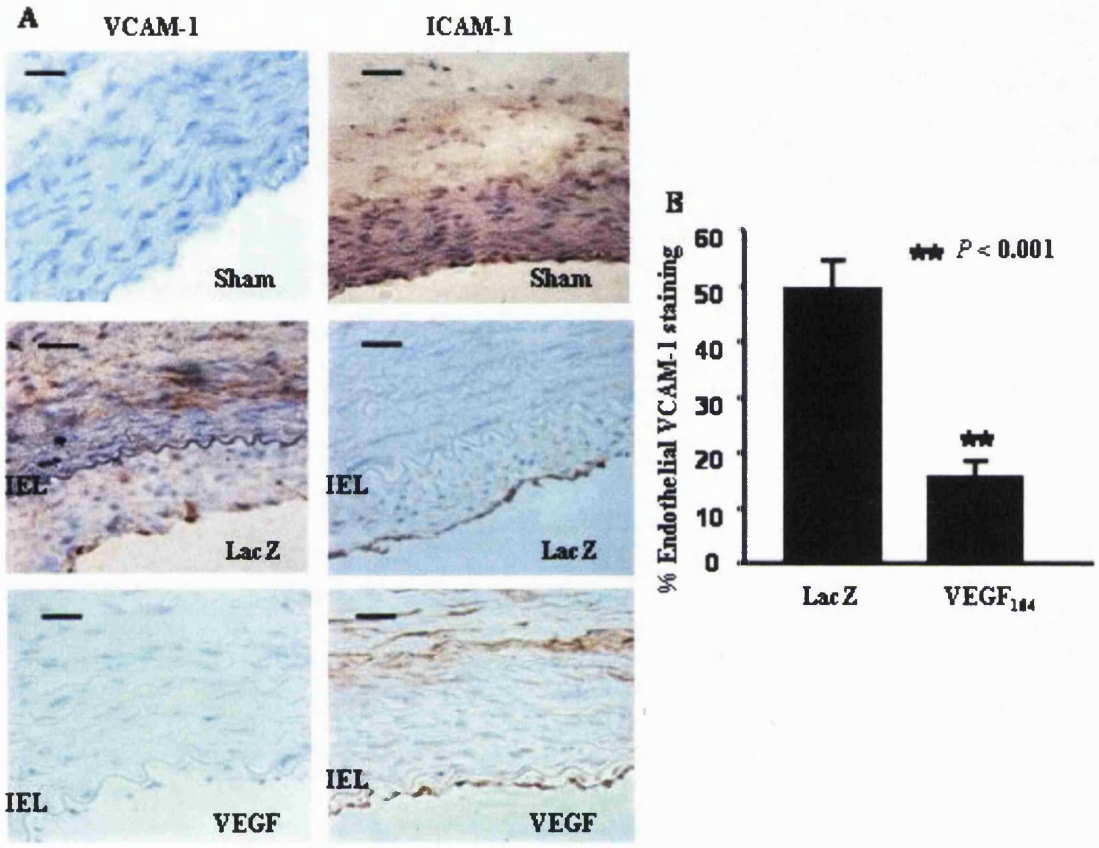


Figure. 4.5. VCAM-1 and ICAM-1 Expression in Collared Transfected Arteries

A, Immunohistochemical staining of VCAM-1 and ICAM-1 was performed in sections from sham-operated control arteries or contralateral collared arteries transfected with either LacZ or $VEGF_{164}$. In hypercholesterolaemic rabbits, endothelial VCAM-1 Immunostaining is reduced in $VEGF_{164}$-transfected arteries, whereas endothelial ICAM-1 immunostaining is not noticeably altered. The position of the IEL is indicated. Scale bar = 20 μm.
B, Quantification of endothelial VCAM-1 immunostaining shows that endothelial VCAM-1 immunostaining is significantly reduced in $VEGF_{164}$-transfected arteries (** $P < 0.001$).

FIGURE 4.6

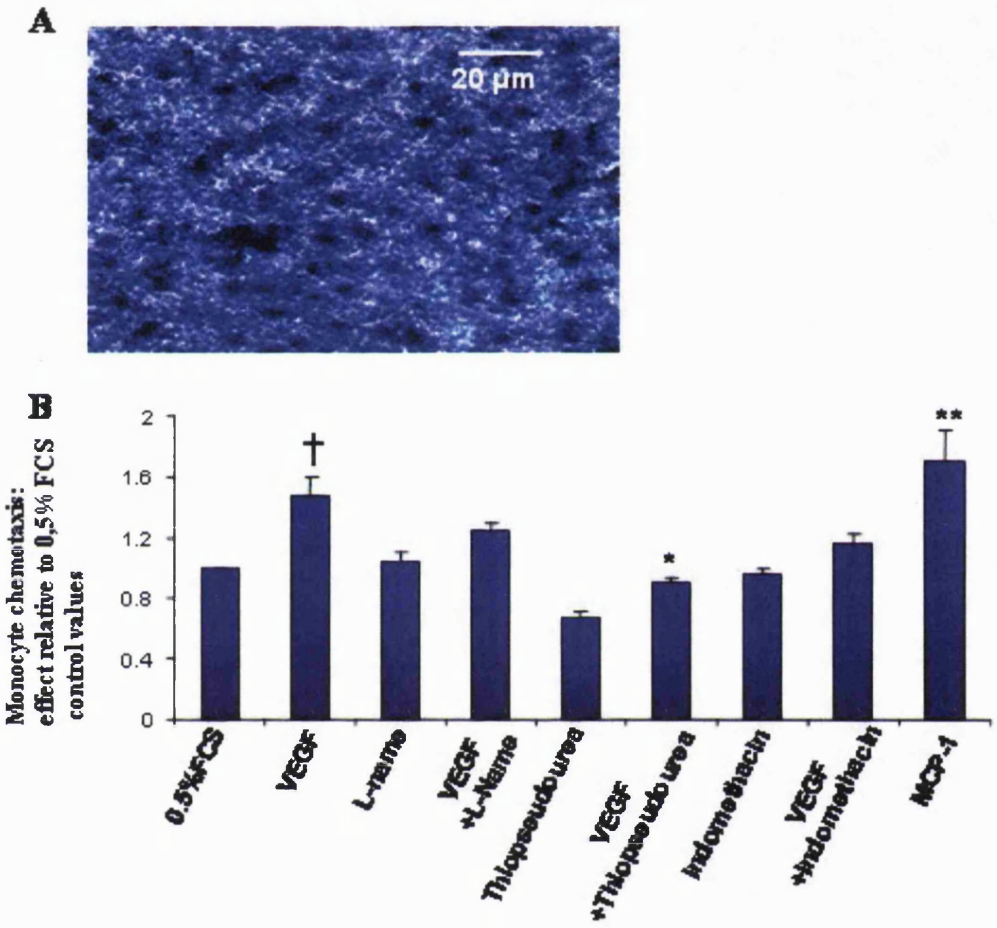


Figure 4.6. CD14$^+$ Monocyte Transwell Migration Assay.

A, Toluidine Blue staining after 72 hrs illustrating confluent monolayer of passage 2-4 HUVECs on a representative 5 µm pore-size fibronectin-coated transwell membrane (x25). Confluence is confirmed by the lack of acellular regions, denoted by unstained white patches on the membrane. **B**, Migration response of peripheral blood derived CD14$^+$ enriched human monocytes through fibronectin-coated transwell membrane carpeted with a HUVEC monolayer after 2 hrs at 37ºC. Results are expressed as a ratio relative to the migration response to 0.5% FCS (designated 1), and represent the mean ± s.e of 3 independent experiments for each treatment group. VEGF 20ng/ml, L-NAME 10mM, Thiopseudourea 400μM, Indomethacin, 10μM, MCP-1 10ng/ml. (†, $P<0.02$ and **, $P<0.001$ compared to 0.5% FCS; *, $P<0.05$ compared to VEGF alone)

4.3. DISCUSSION

The major finding of the results presented in Chapter 4 is that periadventitial liposome-mediated gene transfer of $VEGF_{164}$ inhibited collar-induced arterial intimal thickening and macrophage accumulation in cholesterol-fed rabbits. The fact that macrophage infiltration into carotid arteries was not detected in sham-operated vessels in hypercholesterolaemic rabbits indicates that the neointimal accumulation of macrophages in collared arteries is critically dependent on intimal thickening as well as high blood cholesterol. Therefore, it can be inferred that VEGF gene transfer inhibits macrophage accumulation in cholesterol-fed rabbits partly through an indirect neointima-decreasing effect. However, the finding that neointimal macrophage density was also significantly reduced in VEGF-transfected arteries suggests that VEGF may either directly or indirectly inhibit macrophage infiltration into the subendothelial region through other mechanisms. Since VEGF gene delivery did not significantly increase vessel wall neovascularisation in collared arteries, these results suggest that the inhibitory effects of liposome-mediated VEGF gene delivery on intimal thickening and macrophage influx are largely independent of its ability to stimulate angiogenesis. The weak angiogenic response induced in VEGF-transduced arteries is likely to be due to a low intravascular concentration of VEGF resulting from the low efficiency of liposome-mediated VEGF gene delivery, and the impairment of angiogenesis by hypercholesterolaemia (Kai et al. 2002).

Decreased VCAM-1 expression may partly be responsible for the inhibition of neointimal macrophage influx in VEGF-transduced arteries. In contrast to ICAM-1, which is expressed on the arterial endothelium in mice and rabbits with normal cholesterol levels, the cytokine-inducible cell adhesion molecule, VCAM-1, is upregulated in the arterial endothelium at atherosclerosis-prone sites in hypercholesterolaemic rabbits and mice (Iiyama et al. 1999). A critical role for VCAM-1 in early atherosclerosis, was revealed by studies showing that atherosclerotic lesion formation was impaired in LDL receptor-deficient mice expressing greatly reduced levels of VCAM-1, but was not affected by ICAM-1 deficiency (Cybulsky et al. 2001). Consonant with previous studies, we found that VCAM-1 expression was induced in the endothelia of collared arteries in hypercholesterolaemic rabbits. VEGF gene transfer caused a marked decrease in

endothelial VCAM-1 expression compared to LacZ-transfected arteries that was particularly marked where the neointima-reducing effect of VEGF was greatest. Other studies performed in this laboratory showed that VEGF did not significantly alter VCAM-1 mRNA or protein levels in cultured endothelial cells, suggesting that VEGF is unlikely to exert a direct inhibitory effect on VCAM-1 expression *in vivo* (I. Zachary, personal communication; A. Stannard and I. Zachary, manuscript in preparation). It is noteworthy that VEGF has been reported to up-regulate VCAM-1 expression in HUVECs (Kim et al. 2001). The mechanism of the reduced VCAM-1 expression we have observed in VEGF-transduced arteries is unclear, but it may be inferred from the inability of VEGF to inhibit VCAM-1 expression in cultured cells that this is likely to be complex and indirect, involving interactions between VEGF, the effects of high blood cholesterol, endothelial cells and monocyte/macrophages. Low density lipoprotein (LDL) increases VCAM-1 expression in endothelial cells (Allen et al. 1998), while lipid lowering drugs reduce endothelial VCAM-1 expression in atheroma in hypercholesterolaemic rabbits (Aikawa et al. 2002). Interestingly, other findings from this laboratory indicate that VEGF attenuates LDL uptake by HUVECs (S. Shafi and I. Zachary, unpublished findings), lending plausibility to the notion that VEGF might indirectly regulate VCAM-1 expression through a long-term effect on LDL interactions with the endothelium. This possibility warrants further investigation. Regardless of the mechanism(s) involved, given that VCAM-1 plays a key role in early atherosclerotic lesion formation and monocyte adhesion to the endothelium, our results indicate that impairment of endothelial VCAM-1 expression is at least partly responsible for the reduced neointimal macrophage content of VEGF-transduced arteries.

VEGF is recognized to exert a pro-chemotactic effect on monocytes via VEGFR1 (Flt1) (Clauss et al. 1996) and the results in Fig 4.6 confirmed these findings. While VEGF may be able to stimulate monocyte migration through an EC monolayer *in vitro*, the effect of VEGF on the migration of monocytes is likely to be complex and dependent upon several factors. For instance VEGF-induced upregulation of endothelial MCP-1 gene expression has also been reported (Yamada et al. 2003). A single VEGF concentration of 20 ng/ml was used in the transwell assay, which was chosen because this level has been reported to exert important and diverse biological effects in the literature. However, it is

difficult to ascertain what the prevailing locally available VEGF concentration in the arterial wall is and thus, whether the *in vitro* concentration is truly reflective of the *in vivo* situation. Secondly, the accepted paradigm of monocyte to macrophage foam cell transformation in atherogenesis suggests that results from assays of monocyte migration *in vitro* cannot easily be extrapolated to explain my *in vivo* observations in hypercholesterolaemic rabbits. In addition, while VEGFR1 mediates direct chemotactic effects of VEGF on monocytes, the effects of VEGF on endothelial VCAM-1 expression and macrophage accumulation *in vivo* may be mediated via VEGFR2 / KDR. Regardless of the reasons for the differences between observed effects of VEGF on monocyte migration *in vitro* and on neointimal macrophage accumulation *in vivo*, the results in Chapter 4 argue strongly that low level VEGF expression impairs arterial monocyte infiltration in hypercholesterolaemic rabbits, at least in part via a reduction in endothelial VCAM-1 expression.

The L-arginine/NO biosynthetic pathway has previously been shown to regulate human monocyte chemotaxis *in vitro* (Belenky et al. 1993) and was also examined in this chapter. Thiopseudourea is a highly potent competitive inhibitor of inducible, endothelial and neuronal NOS isoenzymes (Garvey et al. 1994), and this agent significantly abrogated the chemotactic effect of VEGF-A after 2 hours, whereas L-NAME, a selective eNOS inhibitor did not. This suggests that more than one NOS isoenzyme could be involved in mediating the chemotactic effects of VEGF. Alternatively, thiopseudourea is a much more potent inhibitor of eNOS than L-NAME and this alone may account for the observed additional reduction of monocyte migration, in the presence of VEGF. A longer time-course would be necessary to determine if these effects persist. The chemotactic property of VEGF is attenuated in patients with increased cardiovascular risk such as diabetes mellitus (Waltenberger et al. 2000). The protective effects of VEGF reported in this *in vivo* study were under conditions of hypercholesterolaemia. Although mildly oxidized LDL has also been shown to be chemotactic for monocytes (Ishikawa et al. 1997; Mine et al. 2002), it would be interesting to determine if there was an interaction between the effects of modified / oxidized LDL, VEGF and NO in this transwell assay. Oxidized LDL inhibited VEGF-induced EC migration by dephosphorylating Akt, with a resultant impairment of eNOS activation and NO bioavailability (Chavakis et al. 2001).

CHAPTER 5

EFFECTS OF PERIADVENTITIAL ADENOVIRAL PLACENTAL GROWTH FACTOR AND VASCULAR ENDOTHELIAL GROWTH FACTOR GENE TRANSFER IN NORMO- AND HYPER-CHOLESTEROLAEMIC RABBITS

5.1. INTRODUCTION

The results presented in Chapter 4 showed that liposome-mediated $VEGFA_{164}$ gene transfer to the collared carotid arteries had a neointima-reducing effect and decreased neointimal macrophage accumulation in hypercholesterolaemic rabbits. It is known that the concentration of VEGF is an important determinant of its biological function *in vivo* and *in vitro*. Furthermore, it was noted that liposome-mediated VEGF-A_{164} gene transfer was not sufficient to produce a significant neovascular response in the collared carotid artery. Since liposome-mediated delivery results in low efficiency gene expression and therefore a low concentration of the secreted VEGF protein product, it was important to compare liposome-mediated delivery with the effects of higher efficiency gene transduction likely to result in a higher local arterial concentration of VEGF. This was examined by transducing collared carotid arteries with an adenovirus construct encoding human $VEGFA_{165}$ (Ad.VEGF-A_{165}). In addition, the experiments presented in this chapter also compared the effects of Ad.VEGF-A_{165} with an adenovirus encoding PlGF2, a VEGF related factor that is a specific ligand for VEGFR1.

5.2. RESULTS

5.2.1. Periadventitial PlGF2 and $VEGFA_{165}$ adenoviral gene delivery in collared, hypercholesterolaemic rabbits

Similar to the results described in Chapter 3, collar placement around the carotid arteries of rabbits fed a 1.5% cholesterol diet induced the formation of neointimal lesions composed largely of VSMC with the involvement of Ram 11-stained macrophages (see Chapter 3, Fig 3.6B). The cholesterol-supplemented diet increased serum LDL-cholesterol and total cholesterol to, respectively, 10 and 14 mmol/litre after 7 days, and 20 and 26 mmol/litre after 21 days. The contralateral sham-operated carotid arteries in the cholesterol-fed rabbits displayed no evidence of either intimal thickening or increased Ram11 immunostaining (see Fig 3.6B). Immunostaining of endothelial cells with CD31 antibody indicated that neither collaring nor hypercholesterolaemia caused significant discontinuities in the endothelium, which remained essentially intact (see Fig 3.6B). Staining for β-galactosidase in arteries transduced with a control Ad.LacZ construct revealed abundant cells staining strongly for β-gal in the adventitia, consistent with a high efficiency of gene transfer (~5%) and in agreement with previous findings (Laitinen et al. 1997a). As mentioned in Chapter 3, LacZ expression was mostly confined to the adventitia, with occasional staining seen in the media and neointima.

Expression of the $VEGF\text{-}A_{165}$ and PlGF2 transgenes following periadventitial delivery of adenoviruses to carotid arteries was established by RT-PCR and immunohistochemistry. Amplicons with the predicted sizes, 270 and 547 base pairs, corresponding to PlGF2 and $VEGF\text{-}A_{165}$, respectively were detected only in RNA prepared from collared arteries transduced with Ad.PlGF2 and $Ad.VEGF\text{-}A_{165}$, respectively (Fig. 5.1A). Expression of β-actin was very similar in RNA prepared from all transduced arteries. No PlGF2 or $VEGF\text{-}A_{165}$ transgene expression was detected in Ad.LacZ-transduced arteries, or in contralateral non-collared control arteries or in other non-targeted tissues, including lung, liver, kidney and testis (Fig 5.1B). These results indicate that the perivascular collar was effective in targeting gene expression specifically to the collared region of the artery. Immunostaining of sections of transduced arteries with specific antibodies to murine PlGF or human VEGF showed strong expression of PlGF in the

adventitia of Ad.PlGF2-transduced vessels and additional expression in the neointima and endothelium (Fig. 5.2A), while VEGF protein expression was readily detected in the adventitia with sporadic intimal staining (Fig. 5.2B).

Delivery of either Ad.PlGF2 or Ad.VEGF-A_{165} treatment resulted in significantly enhanced neointima formation in the collared carotid arteries of rabbits fed a normal low-cholesterol diet (Fig. 5.3A). However, whereas delivery of Ad.PlGF2 to collared carotid arteries in cholesterol-fed rabbits significantly increased intimal thickening, Ad.VEGF-A_{165} had no significant effect on intimal thickness in hypercholesterolaemic rabbits (Table 5.1, Fig. 5.3B).

In hypercholesterolaemic animals, immunostaining with Ram11 showed that PlGF2 stimulated macrophage accumulation in the neointimas of collared arteries (Fig. 5.4A). Compared with either LacZ-transduced arteries or non-transfected collared arteries, periadventitial Ad.PlGF2 delivery significantly increased the neointimal density of Ram11-positive cells in hypercholesterolaemic rabbits (Fig. 5.4B). In contrast, Ad.VEGF-A_{165} had no significant effect on neointimal macrophage accumulation (Fig. 5.4A, B). It was observed that no neointimal Ram 11-positive macrophages were detected in the arteries of normocholesterolaemic rabbits transduced with either Ad.PlGF2 or Ad.VEGF-A_{165} (Table 5.1). In addition, a significant increase in adventitial macrophage staining occurred in response to transduction by either growth factor, compared to controls in both hypercholesterolaemic and normocholesterolaemic animals (Table 5.1).

PlGF2 is a specific ligand for VEGFR1 and does not bind to VEGFR2 / KDR (Selvaraj et al. 2003). We therefore examined the expression of VEGFR1 in Ad.PlGF2-transduced arteries. VEGFR1 expression was readily detected in neointimal cells and was expressed in patches of endothelium (Fig. 5.4C). Ram11 staining of serial sections showed close association between some areas of VEGFR1-positive staining and Ram 11-positive regions of the neointima, suggesting that lesion macrophages expressed VEGFR1.

5.2.2. Neovascularisation in Ad.PlGF2 and Ad.VEGF-A_{165}-transduced collared arteries

Angiogenesis is a major biological effect of VEGF-A and recent findings have discovered a novel role for PlGF in pathophysiological angiogenesis in adult animals (Luttun et al. 2002b). It was therefore examined whether VEGF-A or PlGF2 adenoviral gene transfer increased neovascularisation in collared carotid arteries. For the animal subset maintained on a cholesterol-supplemented diet, immunostaining of CD31 revealed the presence of new vessels specifically in the adventitia of both PlGF2-transduced and VEGF-A-transduced arteries, compared to controls (Fig 5.5A). Quantification of the adventitial density of CD31-positive vessels indicated that neovascularisation was increased in both Ad.PlGF2 and Ad.VEGF-A_{165}-transduced arteries (Fig 5.5B). CD31-positive cells or vessels were not detected in the media and, apart from lumenal staining of the endothelium, were also absent from the neointima.

5.2.3. VCAM-1 Expression

Upregulation of the endothelial cell adhesion molecule, VCAM-1, is thought to play a central role in mediating increased monocyte adhesion to the endothelium and trans-endothelial monocyte migration leading to neointimal accumulation of macrophages (Cybulsky et al. 2001; Dansky et al. 2001). It was previously described in Chapter 4 that endothelial VCAM-1 expression within the main vessel lumen was increased in collared arteries from cholesterol-fed rabbits compared with the sham-operated contralateral control arteries in the same animals. It was also shown in Chapter 4 that liposome-mediated $VEGF_{164}$ gene transfer decreased VCAM-1 expression in hypercholesterolaemic rabbits. In contrast to these results, Ad.PlGF2 delivery increased endothelial VCAM-1 staining compared with Ad.LacZ-transduced arteries, whereas Ad.VEGF-A_{165} had little detectable effect (Fig 5.6A). Quantification of VCAM-1 immunostaining showed that the total percentage of the endothelium positive for VCAM-1 immunostaining was significantly enhanced in PlGF2-transduced arteries compared to LacZ (Fig. 5.6B).

TABLE 5.1

A, Normal Diet		Ad. LacZ	Ad. PlGF2	Ad. VEGF-A
I/M ratio	Average	0.097	0.160 ***	0.197 ***
	SEM	0.006	0.018	0.018
Macrophages/ mm³ adventitia	Average	24.4	47.1 ***	44.4 ***
	SEM	3.3	4.1	4.1
CD31 / mm³ adventitia	Average	9.3	22.4 **	22.3 **
	SEM	2.7	4/4	2.9

B, High cholesterol Diet		Ad. LacZ	Ad. PlGF2	Ad. VEGF-A
I/M ratio	Average	0.15	0.28 ****	0.19
	SEM	0.02	0.01	0.02
Macrophages/ mm³ neointima	Average	246.1	470.2 *	263.4
	SEM	30	66	38
CD31/ mm³ adventitia	Average	6.7	28.7 ****	19.4 ****
	SEM	2.3	2.3	2.9
Macrophages/ mm³ adventitia	Average	13.5	22.5 **	13.3
	SEM	1.5	3.6	1.4
Endothelial VCAM-1 (%)	Average	24.00	47.83 *	28.5
	SEM	1.59	4.41	3.29

Table 5.1. Effects of Ad.PlGF2 and Ad.VEGF-A_{165} gene delivery on lesion formation in normal and hypercholesterolaemic rabbits.

Values represent means ± s.e for carotid arteries transfected with Ad.LacZ, Ad.PlGF2 or Ad.VEGF-A_{165} in rabbits fed either A, normal or B, 1.5% cholesterol diet. Neointimal macrophages and endothelial VCAM-1 staining were not detected in rabbits on a normal diet. Other experimental details are provided in Chapter 2 (Materials and Methods). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$, **** $P < 0.0001$, for Ad.PlGF2 and Ad.VEGF-A_{165} versus Ad.LacZ.

n=?

FIGURE 5.1

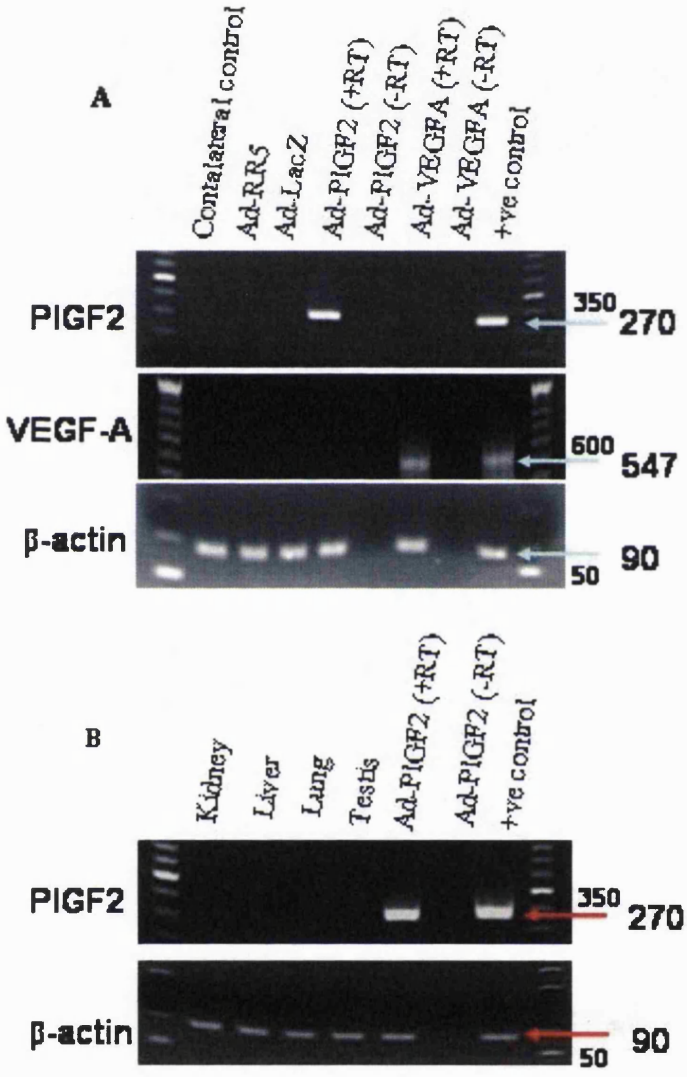


Figure 5.1 Detection of PlGF2 and VEGF-A_{165} transgene expression in collared arteries

A, PlGF2 and VEGF-A_{165} transgene expression was determined by RT-PCR in the presence (+) or absence (-) of reverse transcriptase using β-actin as a reference gene. Predicted PCR products of 270 bps and 547 bps corresponding to PlGF2 and VEGF-A_{165}, respectively, were detected only in arteries transfected with the appropriate adenovirus and were absent from Ad.LacZ-transduced arteries, or the adenoviral backbone (Ad.RR5), or in contralateral control arteries from the same rabbit. A product of 90 bps corresponding to β-actin was present at a similar level in all samples. RT-PCR using cDNA derived from HUVECs transfected with either PlGF2 or VEGF-A_{165} adenoviruses provided positive controls (+ve). **B**, PlGF2 transgene was not detected within RNA derived from non-targeted tissues.

FIGURE 5.2

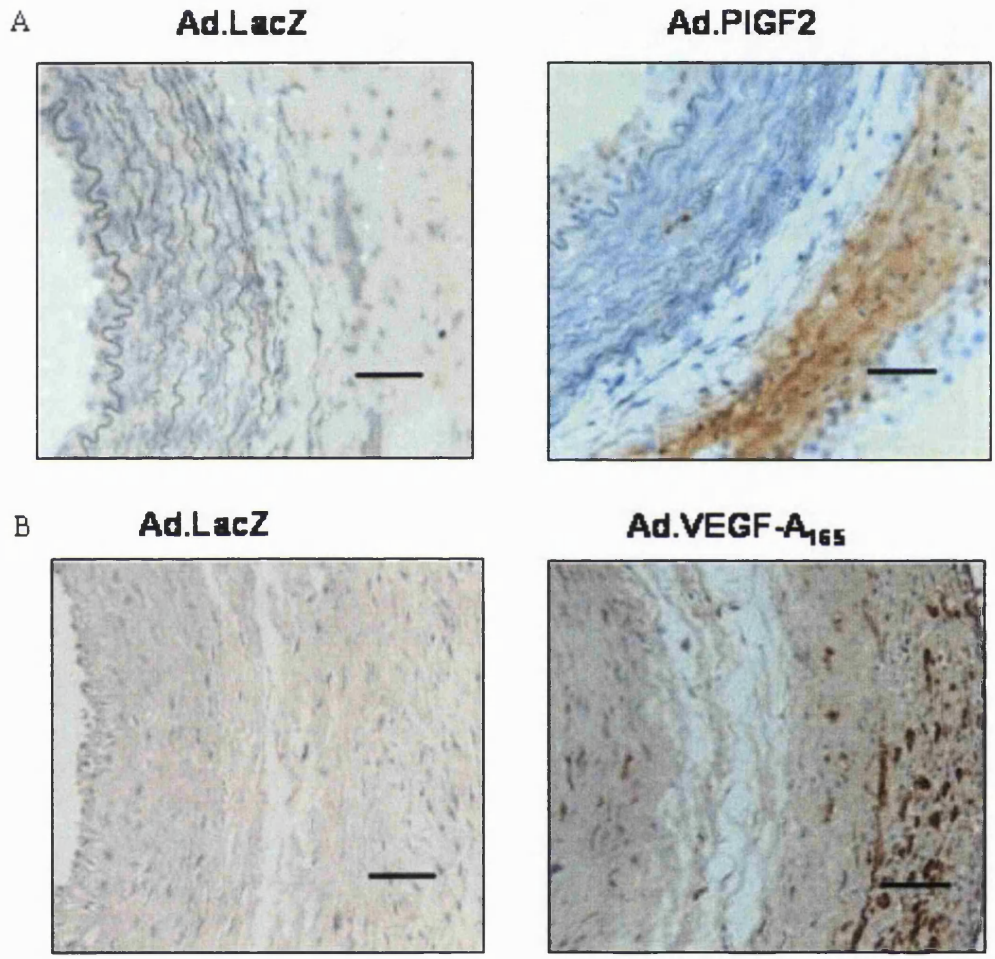


Figure 5.2 Detection of PlGF2 and VEGF-A_{165} protein by immunostaining of the collared artery.

A, Arterial PlGF2 and **B**, VEGF-A_{165} expression was detected by immunostaining with specific antibodies for mouse PlGF2 and human VEGF-A, respectively. Ad.LacZ-infected and uninfected sham-operated (control) arteries stained with PlGF of VEGF antibodies showed little detectable expression. PlGF2 immunostaining in Ad.PlGF2-transduced arteries was detected mainly in the adventitia, with some neointimal staining. VEGF staining was also detected predominantly in the adventitia and inner media.
Bar represents 40μm.

FIGURE 5.3

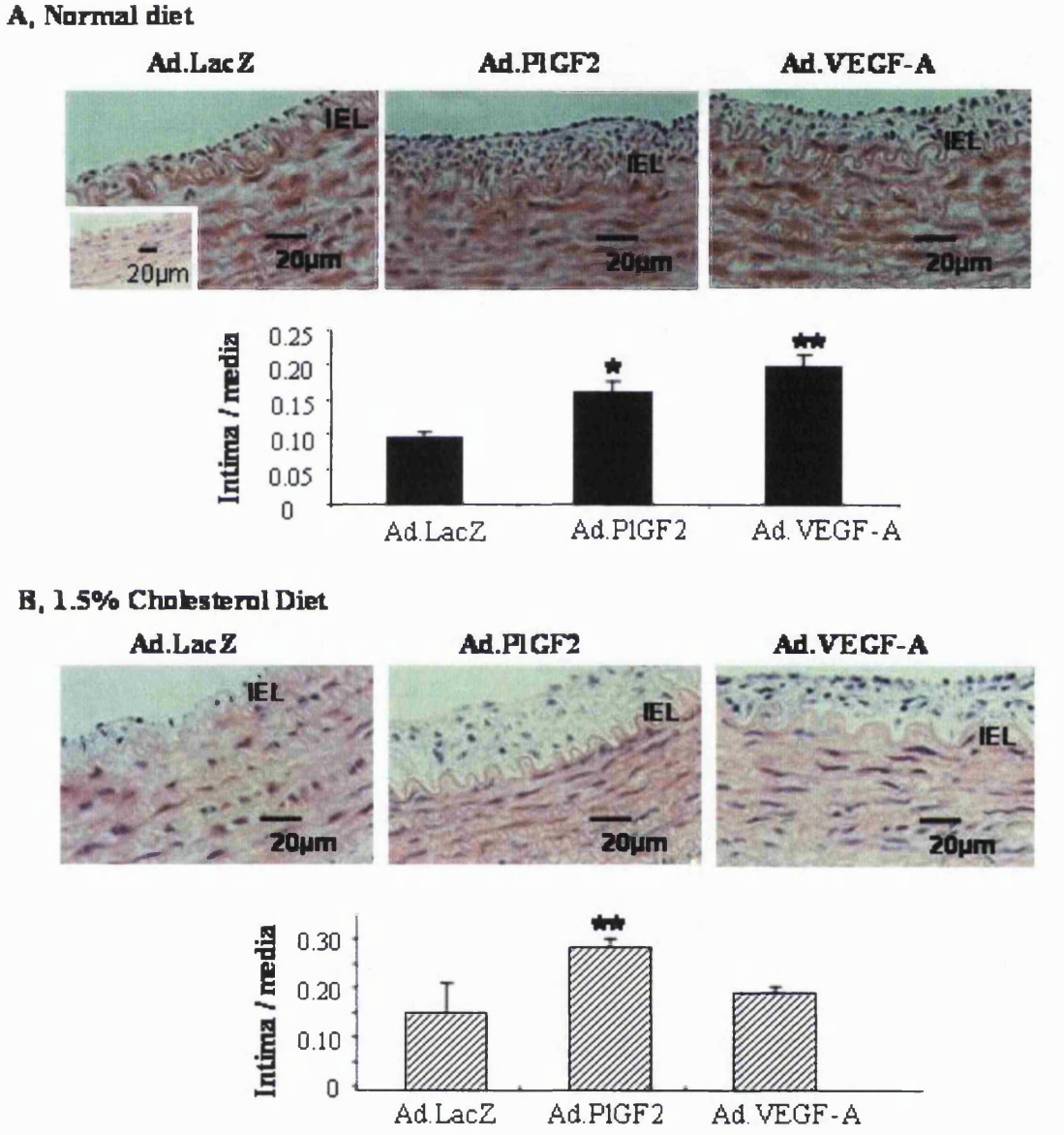


Figure 5.3. Ad.PlGF2 and Ad.VEGF-A_{165} increase intimal thickening.

A, Representative H&E sections indicating that Ad.PlGF2 and Ad.VEGF-A_{165} delivery to collared carotid arteries significantly increased intimal thickening in rabbits fed a normal diet: * $P<0.05$ for PlGF2 and ** $P<0.01$ for VEGF-A_{165} versus LacZ. Inset: Sham-operated contralateral carotid artery shows no intimal thickening.

B, Representative H&E sections indicating that Intimal thickening was significantly increased in Ad.PlGF2 and Ad.VEGF-A_{165} transduced arteries, in rabbits fed a high cholesterol diet : ** $P<0.01$ for PlGF2 versus LacZ. IEL= internal elastic lamina

FIGURE 5.4

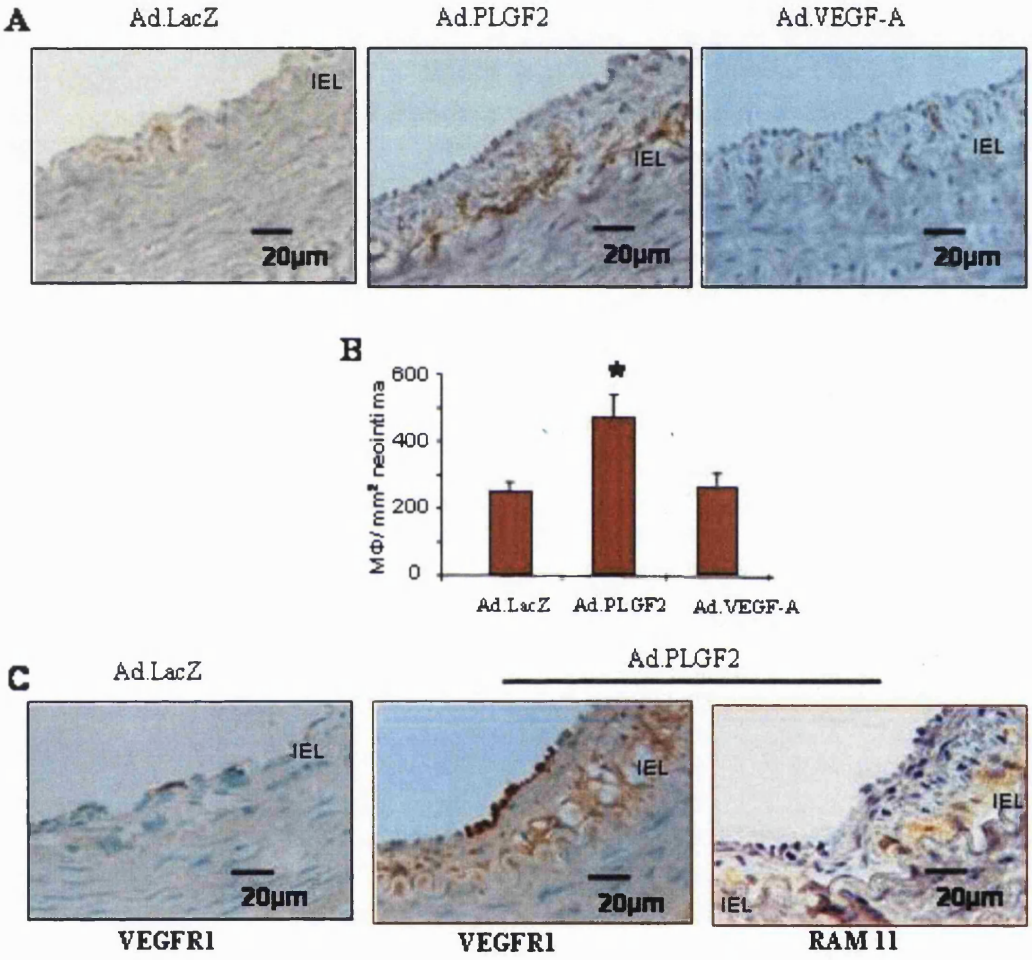


Figure 5.4. Effects of PlGF2 and VEGF-A_{165} on neointimal macrophage infiltration

A, Collared carotid arteries in rabbits on a high-cholesterol diet were transfected with Ad.LacZ, Ad.PlGF2 and VEGF-A_{165} and macrophage content was determined by immunostaining with Ram 11. **B**, Macrophage density is expressed as neointimal Ram 11-positive cells ± s.e./mm^2 total neointimal area. Ad.PlGF2 significantly increased neointimal macrophage content in hypercholesterolaemic rabbits (* $P<0.05$ for PlGF2 versus LacZ). **C,** Immunostaining of macrophages with Ram 11 antibody and VEGFR1 with a specific anti-VEGFR1 antibody was performed in serial sections from Ad.PlGF2-transfected collared arteries. Representative photomicrographs show abundant VEGFR1 immunostaining in the neointima parts of which overlap with regions of Ram 11-positive staining in a serial section.

FIGURE 5.5

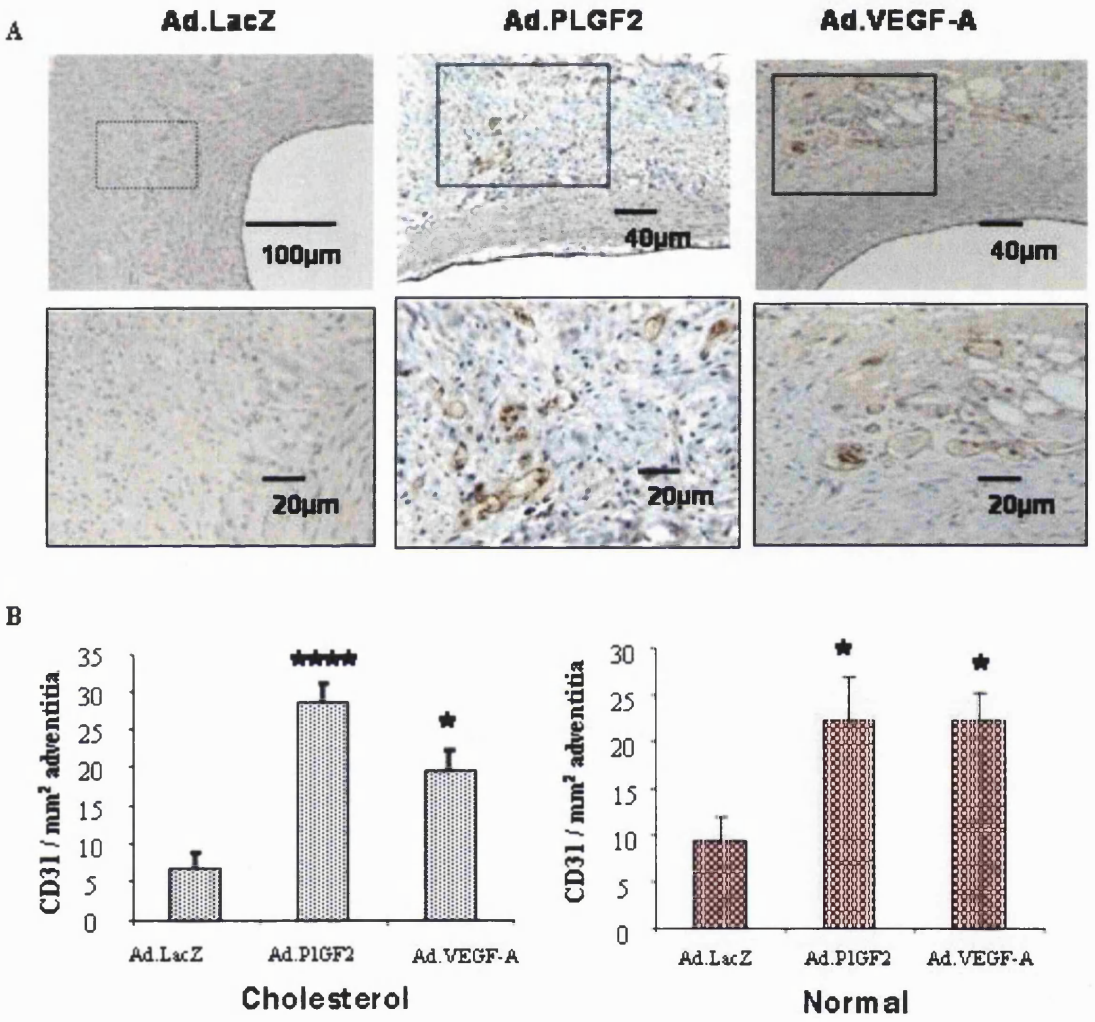


Figure 5.5. Angiogenic response to Ad.PlGF2 and Ad.VEGF-A_{165} in collared arteries.

A, Sections of collared carotid arteries in hypercholesterolaemic rabbits infected with Ad.LacZ, Ad.PlGF2 and Ad.VEGF-A_{165} were immunostained with CD31 antibody. An increase in CD31-positive microvessels occurred in the adventitia of arteries transduced with either Ad.PlGF2 or Ad.VEGF-A_{165}.

B, The numbers of adventitial CD31-positive microvessels and cells in in rabbits fed high cholesterol or normal diets were quantified and results are presented as CD31-positive vessels/mm^2; ****P<0.001 and *P<0.05 for Ad.PlGF2 and Ad.VEGF-A_{165} versus Ad.LacZ.

FIGURE 5.6

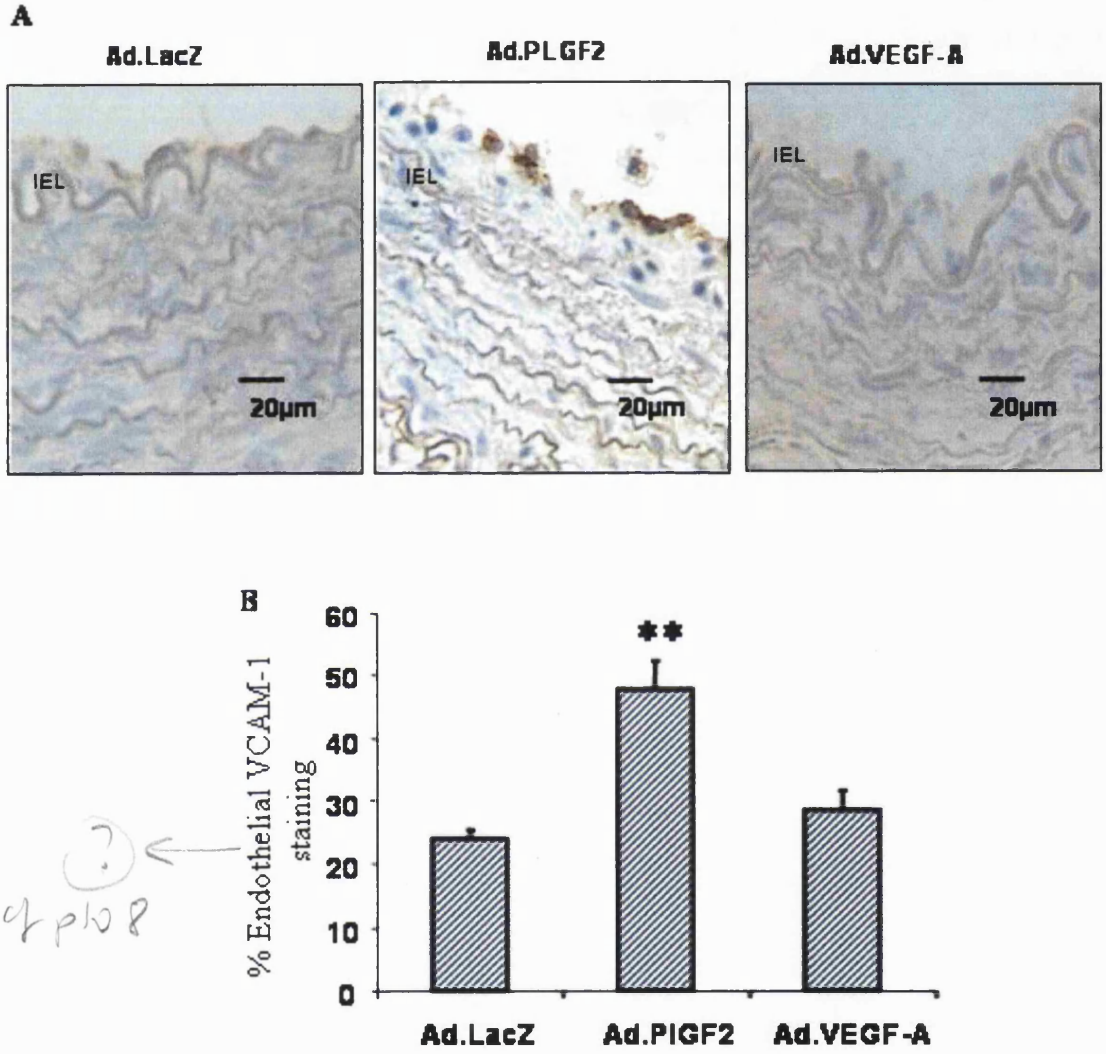


Figure 5.6. Ad.PlGF2 increases VCAM-1 Expression in collared Arteries.

A, Immunohistochemical staining of VCAM-1 was performed in sections from collared arteries infected with Ad.LacZ, Ad.PlGF2 or Ad.VEGF-A_{165}. In hypercholesterolaemic rabbits, endothelial VCAM-1 immunostaining is increased in Ad.PlGF2-transduced vessels. The position of the IEL is indicated. **B,** Quantification of endothelial VCAM-1 immunostaining shows that endothelial VCAM-1 immunostaining is significantly increased in Ad.PlGF2-infected arteries (**$P<$ 0.001 for Ad.PlGF2 versus Ad.LacZ). The values represent VCAM-1 staining on sections taken from 3 points in each treated artery (n = 8 arteries each for Ad.LacZ, Ad.PlGF2 and Ad.VEGF-A_{165}).

5.3. DISCUSSION

The major finding of the results presented in Chapter 5 is that targeted periadventitial delivery of adenovirus encoding PlGF2 increased a spectrum of atherogenic processes in the collared carotid arteries of cholesterol-fed rabbits, including arterial intimal thickening, endothelial VCAM-1 expression and macrophage accumulation. In contrast, Ad.VEGF-A_{165} significantly increased adventitial neovascularisation but did not significantly enhance neointima formation, and caused no significant increase in either neointimal macrophage accumulation or endothelial VCAM-1 expression in hypercholesterolaemic rabbits.

In rabbits fed a normal diet, Ad.PlGF2 enhanced intimal thickening without increasing neointimal macrophage accumulation, indicating that PlGF2 also has a neointima-increasing effect that is not due to intimal macrophage recruitment. It may be that PlGF2 influences neointimal cell accumulation by directly enhancing VSMC migration and/or proliferation. VEGFR1 expression on medial SMCs was shown to be upregulated with enhanced responsiveness to both VEGFA and PlGF (Parenti et al. 2002). The increased adventitial macrophage staining observed in Ad.PlGF2-transduced arteries in normocholesterolaemic animals does suggest, however, that PlGF2 can promote monocyte chemotaxis even in the absence of high blood cholesterol; this effect may reflect increased infiltration of macrophages via adventitial vasa vasorum. The significant increase in adventitial Ram 11-positive macrophages was also associated with increased endothelial VEGFR1 expression within the adventitial vasculature. Collaring alone does not elicit a prominent macrophage infiltration response, as previously discussed in Chapter 3.

VCAM-1 expression in lumenal endothelium is thought to precede subendothelial accumulation of macrophages (Hanyu et al. 2001) and has a prominent role in the pathogenesis of a neointima formation (Oguchi et al. 2000). The results of this study, as determined by VCAM-1 immunostaining, suggest that one possible mechanism to explain the pro-atherogenic profile of PlGF overexpression in this model is the upregulation of endothelial VCAM-1 expression within the main arterial lumen. Work to confirm whether the VCAM-1 mRNA is also increased in Ad.PlGF2 transduced vessels, can readily be investigated by quantitative real time PCR.

Periadventitial transfer of PlGF2 encoding adenovirus also induced a striking increase in adventitial neovascularisation in collared rabbit arteries. The fact that neovascularisation resulting from local PlGF2 expression was never observed in the neointima, where the majority of macrophages accumulated, but was confined to the adventitia strongly suggests that PlGF2 exerted a direct angiogenic effect which was not mediated by the increase in neointimal macrophage content. However, it cannot be precluded that the increased adventitial macrophage infiltration, also observed in Ad.PlGF2 transduced collared arteries, contributes to neo-vessel formation. It is noted in Chapter 6 that serial sectioning of Ad.PR39 treated vessels showed induced adventitial neovascularisation that was spatially concordant with the adventitial presence of Ram11 positive macrophages (see Fig. 6.7). Overall, the findings in this study further support recent reports that PlGF is a key angiogenic mediator in diverse models of adult pathophysiological angiogenesis. In accordance with the arteriogenic property of PlGF (Pipp et al. 2003), further assessment of the stability and longevity of the adventitial neo-vessels, particularly as compared to those induced by VEGF treatment, is warranted. Specifically, it would be interesting to know if there was increased pericyte coverage of the vessels by staining for SMCs.

Interestingly, periadventitial delivery of Ad.VEGF-A_{165} did not result in significantly increased intimal thickening or macrophage accumulation in the collared arteries of rabbits fed a high-cholesterol diet. Ad.VEGF-A_{165} did increase adventitial angiogenesis, but had a smaller effect than Ad.PlGF2. The role of VEGF in the pathogenesis of vessel wall disease has been hotly debated, some studies suggesting that either angiogenesis (Moulton et al. 1999) or VEGF-dependent recruitment of monocyte/macrophages (Celletti et al. 2001; Zhao et al. 2002) can promote atherosclerosis, while others have argued for an atheroprotective or non-atherogenic role for VEGF (Laitinen et al. 1997b; Isner et al. 2001). These issues are discussed at greater length in Chapter 7.

In contrast to the results in hypercholesterolaemic rabbits, Ad.VEGF-A_{165} delivery to rabbits on a normal diet did significantly enhance neointima formation as well as adventitial neovascularisation. The reasons for the differential effects of Ad.VEGF-A_{165} on intimal thickening in hypercholesterolaemic and normocholesterolaemic animals are unclear, but

the results suggest that high serum cholesterol impairs an effect of high efficiency Ad.VEGF-A_{165} expression that drives increased intimal thickening, a question that is discussed further in Chapter 7.

CHAPTER 6

THE ROLE OF ANGIOGENESIS IN COLLAR-INDUCED INTIMAL THICKENING: STUDIES WITH THE ANGIOGENIC PEPTIDE, PR39

6.1. INTRODUCTION

A number of factors are thought to contribute to neointima formation including proliferation of adventitial and medial smooth muscle cells, circulating smooth muscle progenitors and inflammatory processes in the media and adventitia. Several studies raised the possibility that vessel wall angiogenesis may be involved in the development of a neointima. In the $ApoE^{-/-}$ mouse, administration of VEGF promoted intra-plaque angiogenesis and the development of atherosclerotic plaques (Celletti et al. 2001), whereas anti-angiogenic drugs inhibited lesion formation (Moulton et al. 1999; Shigematsu et al. 2001; Moulton et al. 2003). Increased neovascularisation has been observed in the vessel wall at sites of intimal hyperplasia in models of arterial stenting (Shibata et al. 2001), angioplasty (Edelman et al. 1992; Kwon et al. 1998) and venous bypass graft failure (Westerband et al. 2000; Shigematsu et al. 2001). VEGF and FGF protein and gene transfer to the vasculature have been shown to influence vascular remodeling (Ylä-Herttuala and Martin 2000) with some, but not other studies, suggesting that VEGF enhances post-angioplasty restenosis (Asahara et al. 1995; Lazarous et al. 1996). It has been hypothesized that the importance of angiogenesis is dependent upon the stage of lesion development, being a critical 'trigger' in the early phase, but receding within ageing lesions (Ross et al. 2001). Overall, despite a considerable amount of experimental and clinical investigation, the specific role of angiogenesis in intimal thickening remains unclear. To explore the contribution of adventitial angiogenesis to neointima formation we used the rabbit collar model to deliver pro- and anti-angiogenic agents in a localized and controlled manner. Specifically, to establish the relationship between adventitial angiogenesis and the extent of neointima formation, we quantified the extent of both processes following intra-collar adventitial delivery of the angiogenesis stimulator, Ad.PR39.

6.2. RESULTS

6.2.1. PR39 Enhances Collar-Induced Neointima Formation and Adventitial Angiogenesis

In this study, adventitial gene transfer using the collar was confirmed using an adenoviral construct encoding Green Fluorescent Protein (Ad.GFP). Following Ad.GFP delivery, abundant GFP-expressing cells were detected primarily in the adventitia with little medial or intimal expression (Fig. 6.1A). Reverse-transcription PCR (RT-PCR), using primers specifically designed for the PR39 transgene (Chapter 2, Table 2.2), of total RNA extracted from 2 pooled vessels treated with Ad.PR39 confirmed successful and localized expression of PR39, within the collared segment of arteries, with no detectable expression in the neighbouring uncollared artery (Fig. 6.1B).

Administration of Ad.PR39 for 9 days after collar placement markedly increased intimal thickening compared to the Ad.GFP-transduced control (Fig. 6.2A, C). In contrast, PR39 transfer for only 4 days did not enhance collar-induced neointima formation (Fig. 6.2B). Ad.PR39 after 9 days increased the I/M ratio to 0.25±0.03 compared to an I/M ratio in control Ad.GFP-transduced arteries of 0.15 ± 0.02. The effect of Ad.PR39 was statistically significant ($P<0.001$) versus Ad.GFP. Ad.PR39 also increased neovascularisation compared to the Ad.GFP control (Fig. 6.2D, E). Ad.PR39 increased the number of CD31-positive adventitial vessels to 123±24 compared to 17±10 in Ad.GFP control arteries. This effect of Ad.PR39 was also highly significant ($P<0.001$ versus Ad.GFP). The region of neovascularisation stimulated by the growth factor, as determined by CD31 positive staining, was predominantly confined to the adventitia, with only occasional medial and intimal staining (Fig. 6.2D, E).

6.2.2. PR39 gene transfer up-regulates VEGF and FGFR1 mRNA

Since PR39 is thought to mediate its pro-angiogenic effects via increased expression of VEGF and FGFR1, real time RT-PCR was used to examine induction of VEGF-A

and FGFR1 mRNAs in Ad.PR39-transduced arteries. Real-time PCR analysis of total RNA extracted from Ad.PR39-treated and control vessel segments demonstrated a 7-fold and 2-fold upregulation of VEGF-A and FGFR1 mRNAs, respectively, in Ad.PR39 treated arteries compared to levels measured in Ad.GFP-transduced arteries (Fig. 6.3).

6.2.3. Co-Administration of PR39 with Angiogenic Inhibitors Abrogates Intimal Thickening

To investigate whether the promotion of adventitial angiogenesis was causally involved in PR39-induced intimal thickening, arteries were co-administered with Ad.PR39 and inhibitors of VEGF and FGF angiogenic signalling. VEGF activity was inhibited using recombinant soluble Flt1 (sFlt1) protein and FGF signalling was blocked using a dominant negative FGFR1 construct, Ad.FGFR1DN-GFP. Recombinant sFlt1 acts as a 'sink' for VEGF-A_{165} and placental growth factor (PlGF) and is clearly established to be a potent and effective inhibitor of VEGF-induced angiogenesis (Holash et al. 2002; Luttun et al. 2002b; Marchand et al. 2002). A dominant negative FGFR1 construct, linked to eGFP and encoded within an adenoviral cassette, was used to inhibit FGF-dependent angiogenesis.

To assess the functional effect of the Ad.FGFR1DN construct, HUVECs were infected with Ad.FGFR1DN and the cells treated with FGF2. Efficient expression of Ad.FGFR1DN-GFP was confirmed by the GFP fluoresence (Fig 6.4A). Western blotting with antibody specific for activated ERKs 1 and 2 demonstrated that cells transduced with Ad.FGFR1DN, but not a control adenovirus, exhibited a complete abolition of FGF2-induced activation of ERKs 1 and 2 (Fig. 6.4B). For *in vivo* studies, Ad.FGFR1DN was injected under the collar and successful transduction of the arterial wall was confirmed by confocal microscopy (Fig. 6.4C).

Administration of either sFlt1 or Ad.FGFR1DN alone had little effect on intimal thickening, compared to Ad.GFP controls (Fig. 6.4 D-F). Co-administration of PR39 with either Ad.FGFR1DN or sFlt1 inhibited the neointima-increasing effects of PR39 in the adventitia (Fig. 6.4G-I). Intimal thickening was also reduced to levels that were comparable to controls (Figs. 6.2A, 6.4H, I). This strongly suggested that

the neointima-increasing effects of PR39 are mediated through upregulation of both VEGF-A_{165} and FGFR1.

6.2.4. Relationship between adventitial angiogenesis and neointima formation

The results in Fig. 6.4 indicated that PR39-induced neointima formation was mediated by angiogenesis. To investigate further the relationship between intimal thickening and adventitial neovascularisation, the correlation between the two processes was examined after Ad.PR39 delivery with and without co-administration of angiogenesis inhibitors. In addition, angiogenesis and intimal thickening were also quantified after Ad.PR9 delivery for 4 and 9 days. As shown in Fig. 6.5, sFlt1 and Ad.FGFR1DN inhibited the increase in both I/M ratio (Fig. 6.5A) and adventitial CD31 staining (Fig. 6.5B) induced by Ad.PR39. Furthermore, assessment of CD31 staining and I/M ratios in all the treatment groups demonstrated a strong overall correlation (r^2 = 0.88) between adventitial angiogenesis and intimal thickening (Fig. 6.5C). However, whereas Ad.PR39 delivery for 9 days induced intimal thickening and adventitial neovascularisation (Figs. 6.2 C, D and 6.5A, B), Ad.PR39 administration for 4 days caused no significant increase in I/M ratio (Fig 6.2B and 6.5A), but induced a striking increase in adventitial neovascularisation, similar to the effect of Ad.PR39 after 9 days (Fig. 6.5B). It was also of note that neither sFlt1 nor Ad.FGFR1DN, alone, significantly reduced the I/M ratio compared to the control (Fig. 6.5A).

6.2.5. PR39-induced Adventitial Neovascularisation is associated with Macrophage Infiltration

The monocyte/macrophage population of the vessel wall has long been thought to play a key role in restenosis (Moreno et al. 1996). Blood-derived monocytes and the chemokines they secrete also play a prominent role in the angiogenic response (Sunderkotter et al. 1994; Moulton et al. 2003). To assess the role of blood-derived monocytes in Ad.PR39-induced adventitial angiogenesis in the collar model, the occurrence of macrophages was studied in collared arteries following treatment with

angiogenic stimulators and inhibitors. Adventitial macrophages were rarely detected in control Ad.GFP-transduced arteries (Fig. 6.6A). While transduction of arteries with Ad.PR39 for 4 days caused no increase in the density of adventitial macrophages (Fig. 6.6B), Ad.PR39 delivery for 9 days induced a marked increase in adventitial macrophage infiltration (Fig. 6.6C, F). Moreover, adventitial macrophages were also reduced to levels that were not significantly different from Ad.GFP control levels (Fig. 6.6A), when Ad.PR39 was co-administered with either angiogenesis inhibitor (Fig. 6.6D, E, F). Macrophages were confined to the adventitia and outer media and, as with CD31-positive vessels, were never seen in the intima. Serial sections immunostained for Ram-11 and CD31 after Ad.PR39 transduction revealed a close association between localisation of macrophages and newly formed vessels (Fig. 6.7).

6.2.6. VEGF and PR39 Directly Stimulate Vascular Smooth Muscle Cell Migration

A potential mechanism that could also underlie neointima formation in response to PR39 angiogenic stimulation is the ability of VEGF and PR39 to induce VSMC migration. In support of this, previous findings suggest that VEGF can induce VSMC migration (Grosskreutz et al. 1999). In a transwell migration assay, both VEGF-A_{165} and PR39 exerted a significant pro-migratory effect on aortic VSMCs in a time- and concentration-dependent fashion (Fig. 6.8A, B). The effect of PR39 was substantially greater than the highest concentration of VEGF-A assayed, after both 1 and 4 hrs. The chemotactic effect of PR39 at the higher concentration was not significantly less than that of PDGF-BB, which is known to be a potent VSMC chemoattractant.

FIGURE 6.1

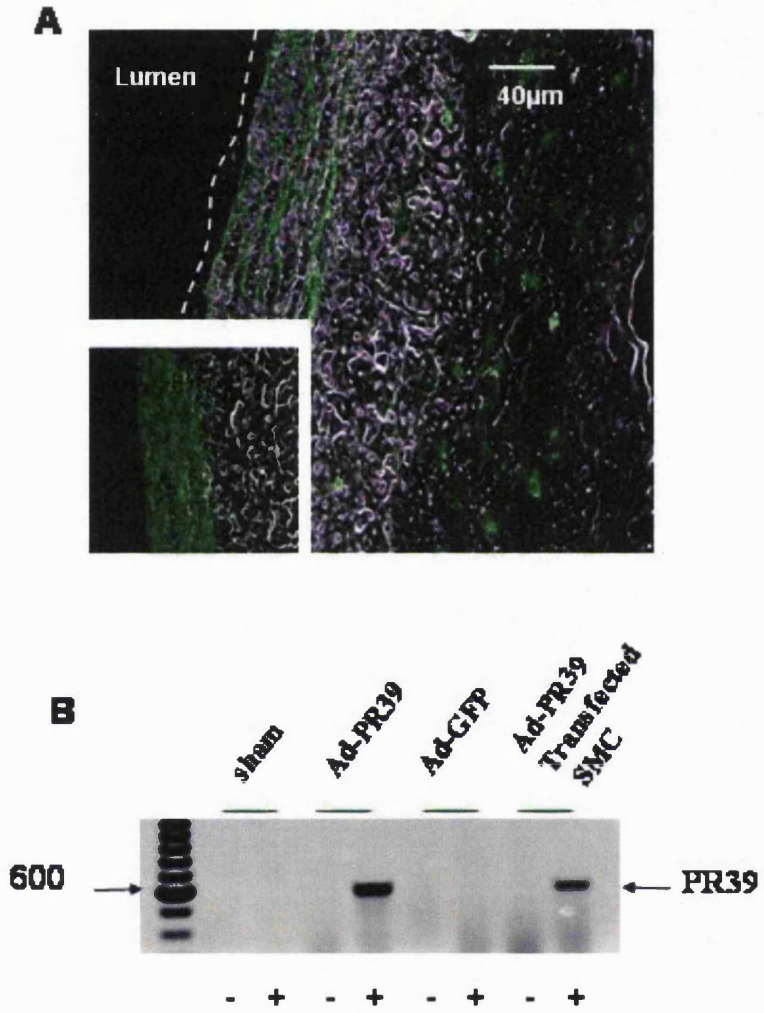


Figure 6.1. Expression of GFP Reporter Gene and PR39 Gene Transduction in the collared artery

A, Ad.GFP gene (5 x10^9 pfu) transduction demonstrates that adventitial cells of collared arteries are predominantly targeted. Endothelial and neointimal cells are not readily detected, though autofluorescence of the media may obscure medial cell transduction. *Insert*: Sham control illustrates autofluorescence within the elastin fibers of the media. **B**, Reverse-transcription (RT)-PCR analysis reveals the presence of PR39 (600bp band) in the total RNA obtained from pooled Ad.PR39 (n=2) treated arteries.
Left margin: molecular size marker; (-) without RT step, (+) with RT.

FIGURE 6.2

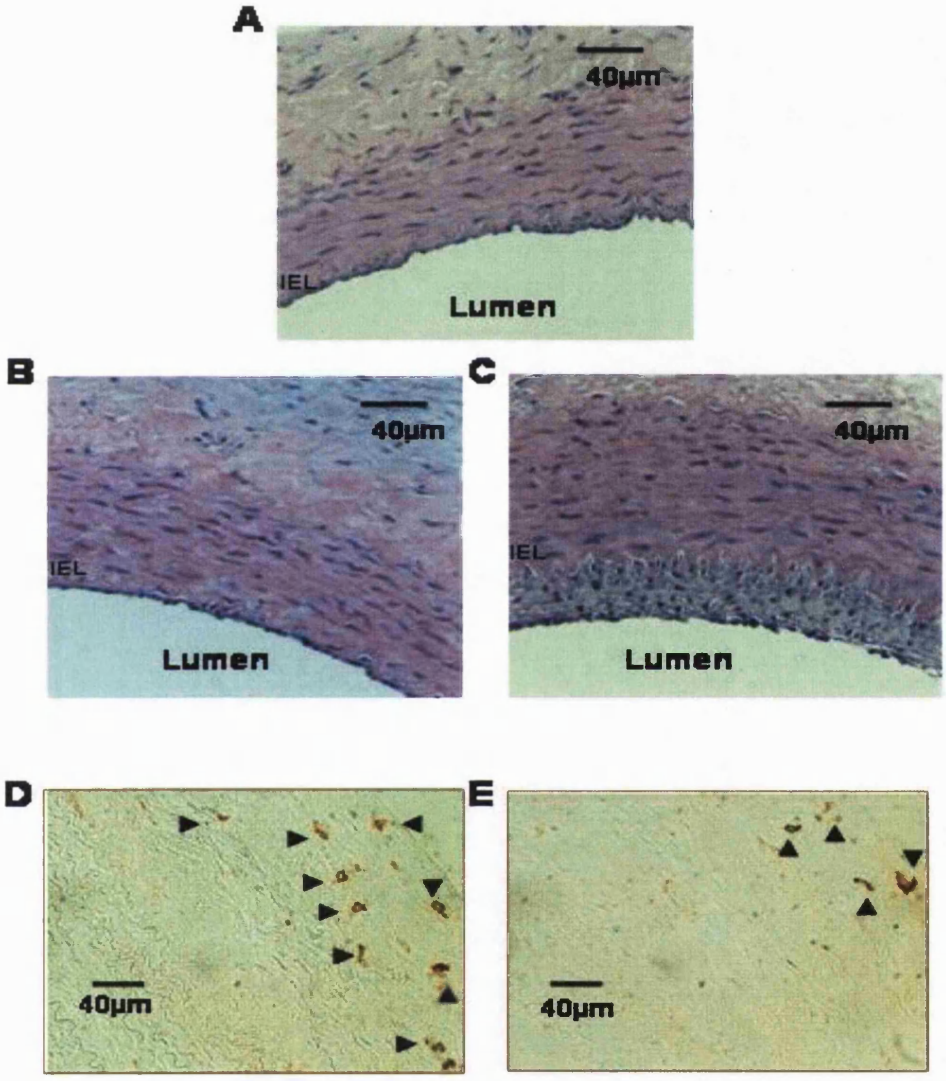


Figure 6.2. Effects of Ad-PR39 gene transfer on intimal thickening and adventitial angiogenesis

H&E sections demonstrating neointima formation after transduction with **A**, Ad.GFP (9days); **B**, Ad.PR39 (4 days); **C**, Ad.PR39 (9 days). IEL = internal elastic lamina. Ad.PR39 significantly increases adventitial angiogenesis (CD31 positive structures/mm^2 adventitia marked by arrowhead) at **E**, 9 days compared to **D**, Ad.GFP transduction (123 ± 24 vs. 17±10, $P< 0.001$).

FIGURE 6.3

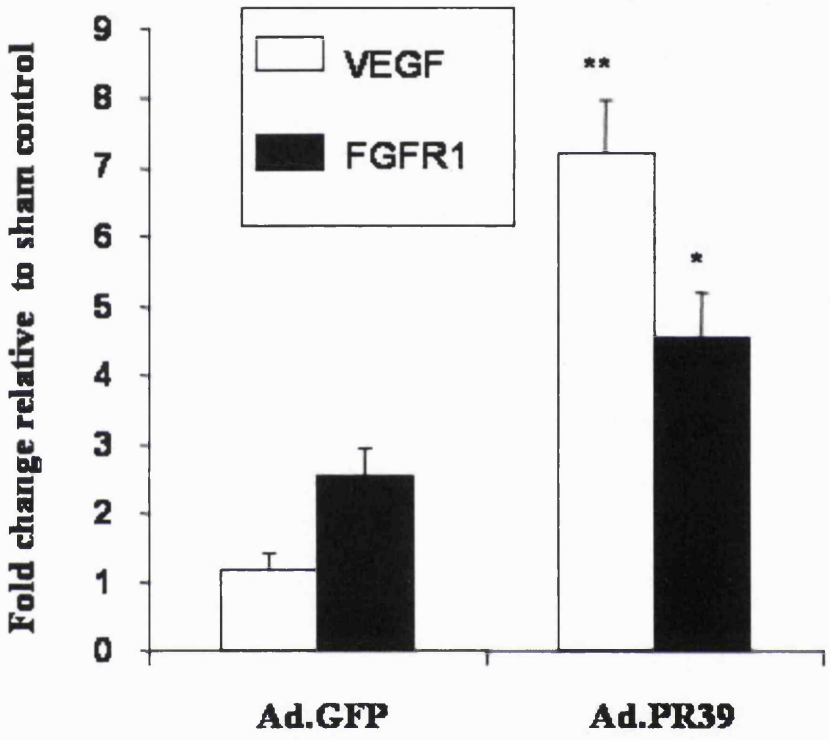


Figure 6.3. Effects of Ad.PR39 gene transfer on the expression of endogenous VEGF and FGFR1 mRNAs in the collared artery.

Real-time RT PCR analysis at 9 days shows that Ad.PR39 significantly increased levels of VEGF-A (7.21±0.78 vs 1.20±0.23, ** $P<0.001$) and FGFR1 mRNAs (4.54±0.66 vs. 2.55±0.4, * $P<0.03$), normalised to endogenous β-actin levels, relative to expression in Ad.GFP-transduced arteries. All values for mRNA expression in Ad.GFP- and Ad.PR39-transduced arteries were also normalised relative to corresponding mRNA levels in the sham-operated control.

FIGURE 6.4

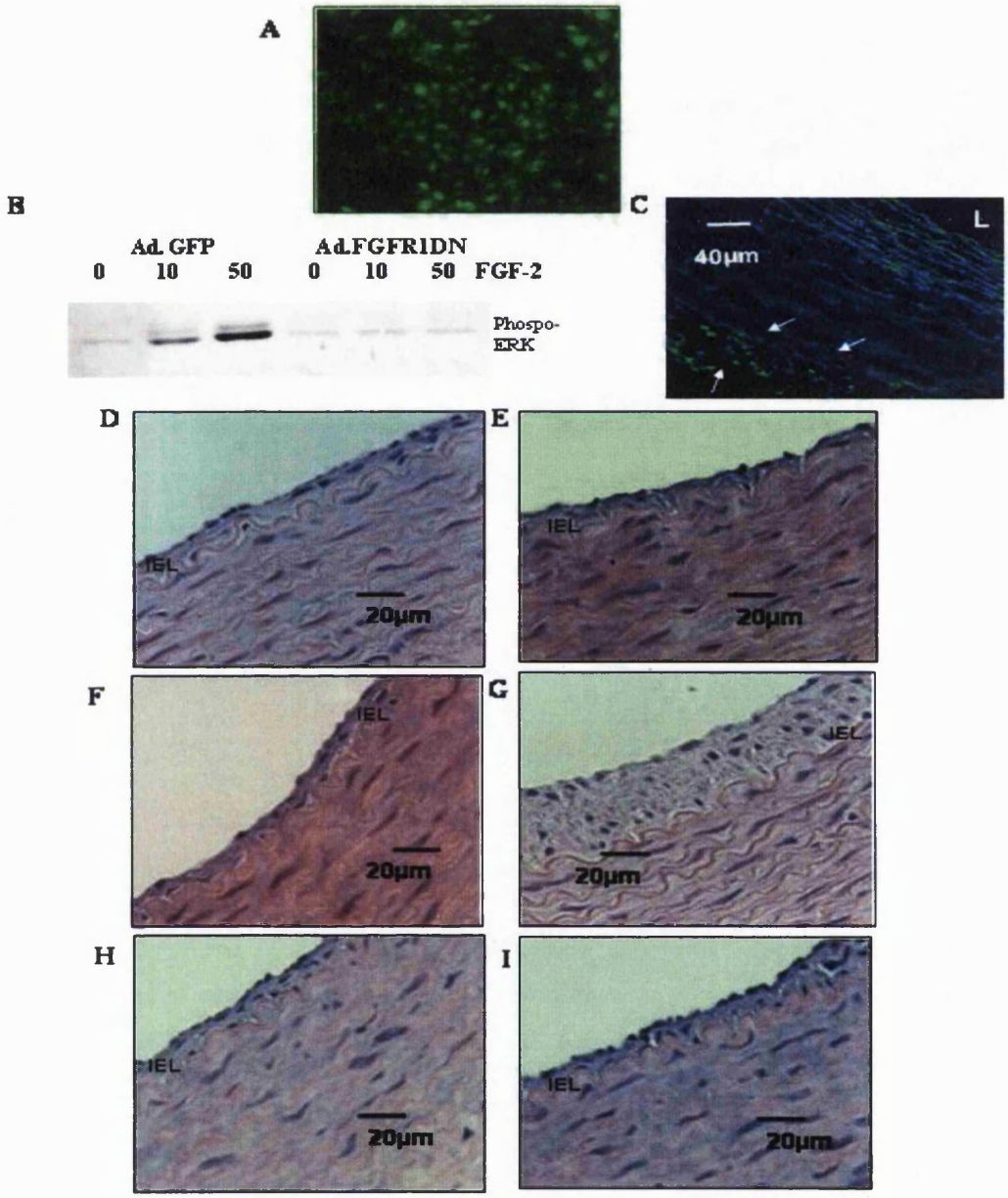


Figure 6.4. Effects of Ad.FGFR1DN-GFP and sFlt1 on Neointima Formation induced by Ad.PR39
A, HUVECs were efficiently transduced with Ad.FGFR1DN-GFP (MOI = 50; x50 magnification) **B**, HUVECs transduced with adenoviruses and serum starved (0.25% FBS, 0.2% BSA) for 16 hrs, then stimulated with FGF2 (10 or 50ng/ml) for 10 mins. Western blot for phospho-ERK 1/2 was performed and shows no response in the Ad.FGFR1DN–transduced cells after FGF2 treatment. **C**, Confocal immunofluorescent image of Ad.FGFR1DN-transduced collared carotid artery; arrows indicate adventitial location of GFP-expressing cells; L=lumen. At 9 days, H&E stains show that both **D**, sFlt1 and **E**, Ad.FGFR1DN transfer, alone, had no effect on neointima formation, compared to **F**, Ad.GFP, whereas **G**, Ad.PR39 increased intimal thickening after 9 days. Intimal thickening induced by Ad.PR39 was inhibited by co-transfection with **H**, sFlt1 or **I**, Ad.FGFR1DN, to levels comparable to control gene transfection (see Fig. 6.2A, F).

FIGURE 6.5

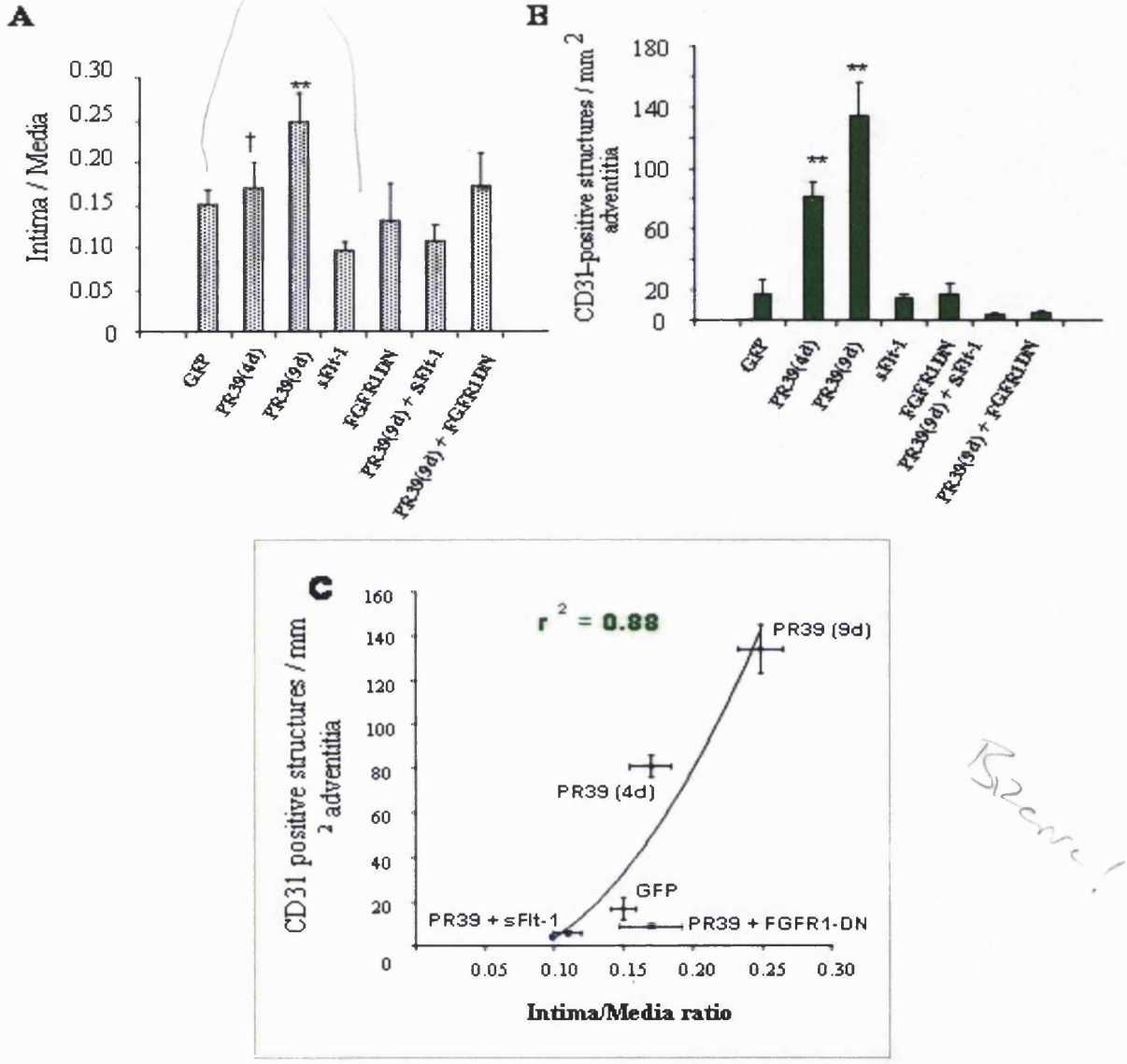


Figure 6.5. Quantification and Correlation of effects of Ad.PR39 and Angiogenesis Inhibitors on intimal thickening and adventitial angiogenesis.

A, Quantification of I/M ratios (**P*<0.01, ***P*<0.001, † *P*=NS) and **B,** Adventitial neovascularisation, shows that Ad.PR39 induced a marked intimal thickening and angiogenic response after 9 days that is inhibited by co-treatment with either sFlt1 or Ad.FGFR1DN (***P*<0.001). In contrast, transduction with Ad.PR39 for 4 days induces a striking adventitial neovascular response, but does not significantly enhance the I/M ratio **C**, Adventitial angiogenesis significantly correlates with the degree of intimal thickening in collared arteries after treatment with angiogenesis stimulators and/or inhibitors.

FIGURE 6.6

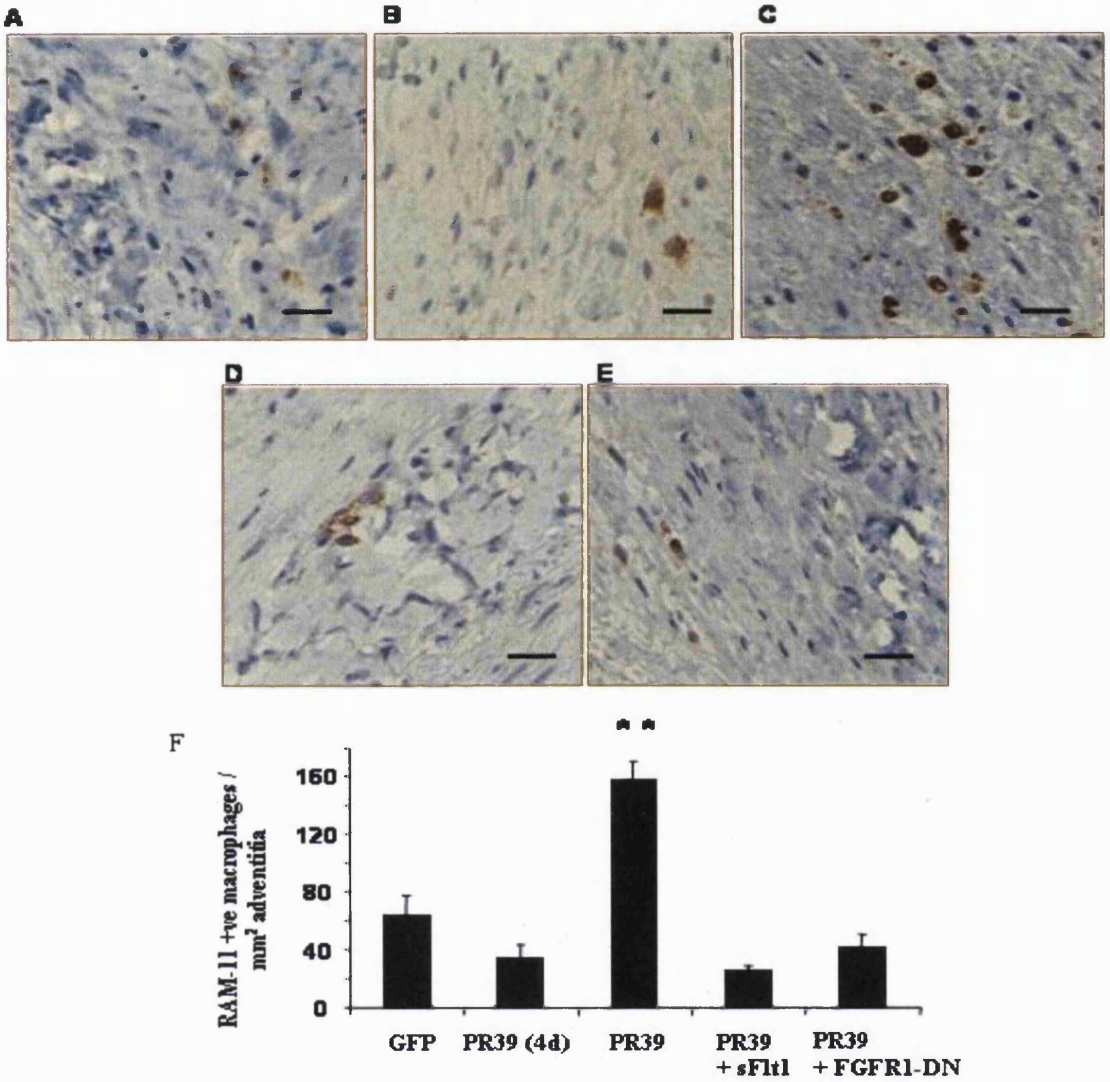


Figure 6.6. PR39-induced adventitial angiogenesis is associated with and mediates adventitial macrophage recruitment.

Adventitial macrophage (Ram-11) stain (hematoxylin counterstain) for **A**, Ad.GFP **B**, Ad.PR39 (**4d**) **C**, Ad.PR39 (**9d**) **D**, Ad.PR39 + Ad.FGFR1DN and **E**, Ad.PR39 + sFlt1 treated arteries. Ram-11 +ve macrophage recruitment is significantly increased by 9 day Ad.PR39 treatment compared to Ad.GFP control (157±13 vs. 64 ±14, $P<0.001$). This is not observed at the earlier time-point. Scale bar = 20μm.**F,** Quantitative morphometric analysis of adventitial Ram-11^{+} cell recruitment. Ad.PR39 significantly increased adventitial macrophage infiltration (** $P<0.001$) compared to Ad.GFP control, whereas recruitment is abolished to control levels by co-administration with either sFlt1 or Ad.FGFR1DN.

FIGURE 6.7

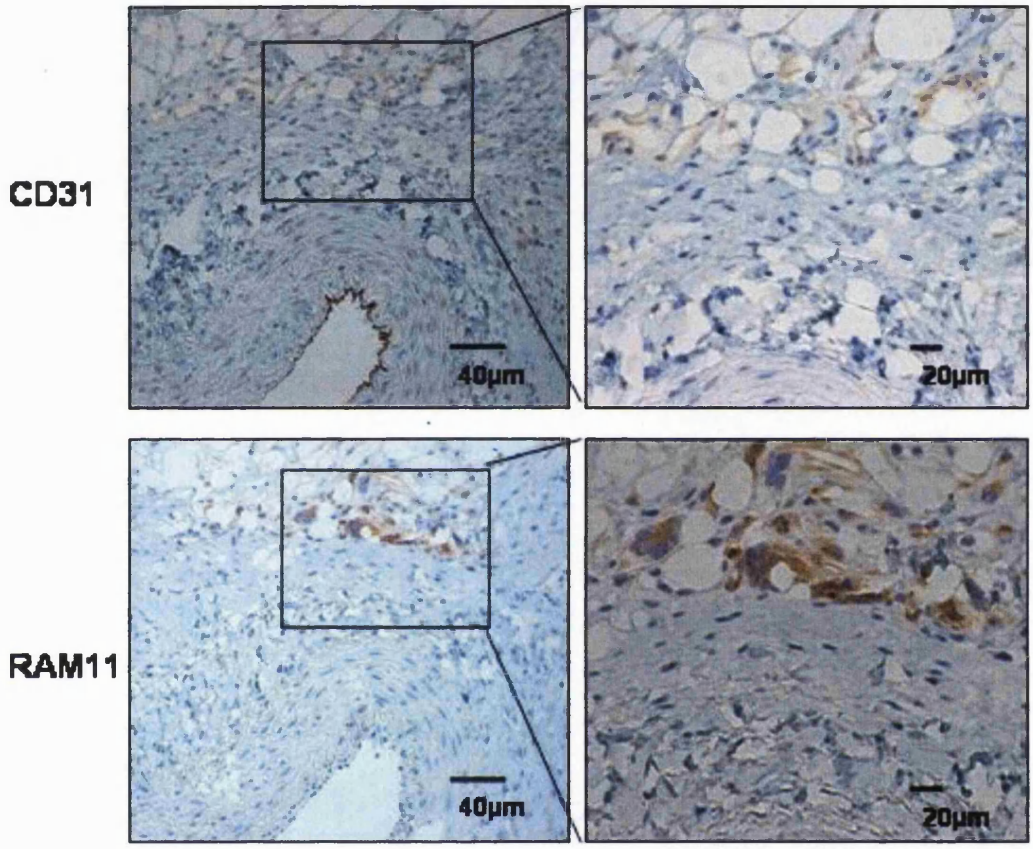


Figure 6.7. Association between adventitial microvessels and macrophages.

Representative sequential sections demonstrate a close association within the adventitia between regions of new vessel development (upper panels) and macrophage localization (lower panels). The panels on the right show high magnification images of the boxed areas in the left panels. Macrophages were rarely observed in the medial and neointimal layers.

FIGURE 6.8

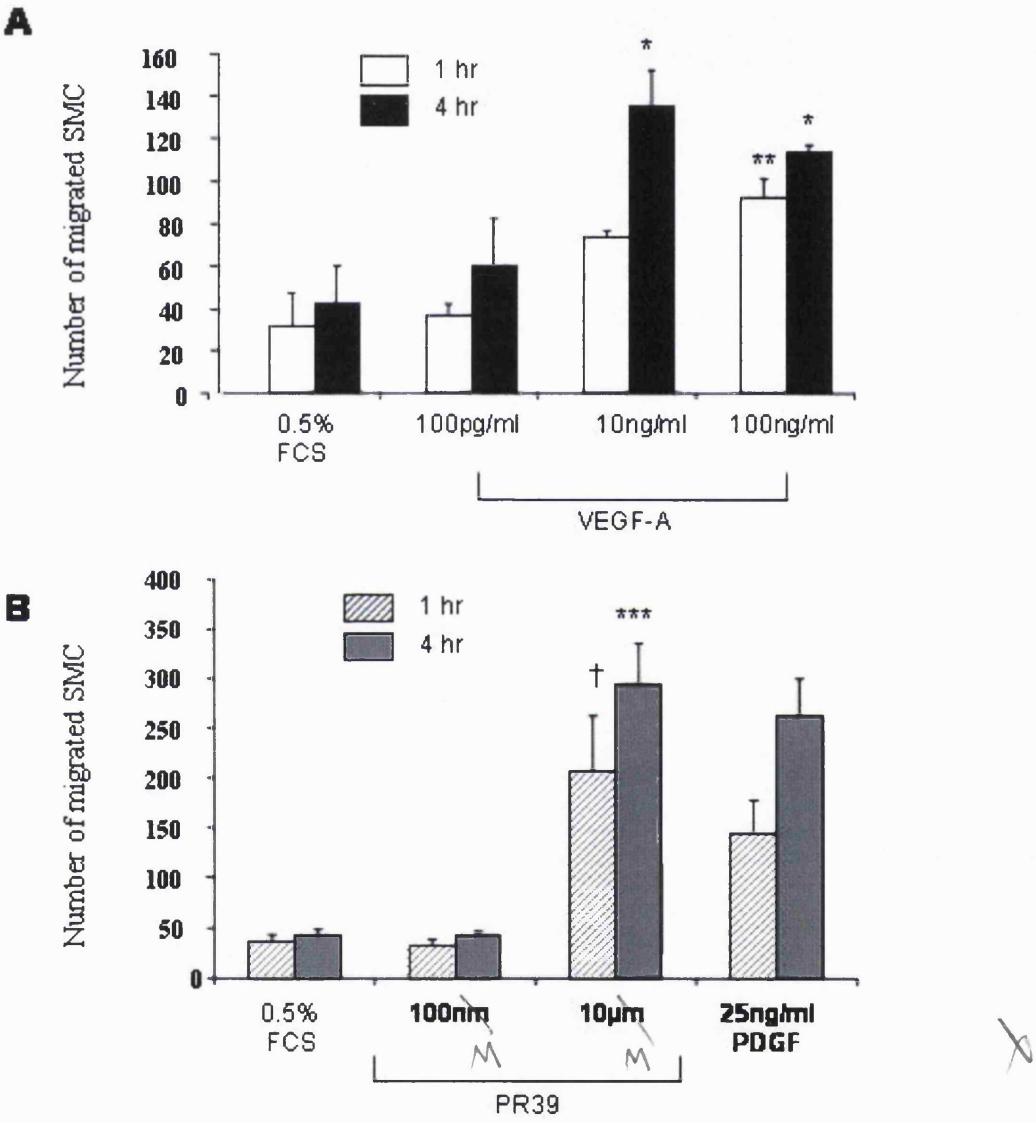


Figure 6.8. PR39 and VEGF-A_{165} stimulate SMC migration

A, VEGF-A significantly promotes the migration of aortic VSMC through an 8µm pore transwell membrane at the highest concentration after 1 hr (***P*<0.001). Migration is significantly enhanced after 4 hrs at both 10ng/ml and 100ng/ml (**P*<0.05). **B**, PR39 also exerts a significant pro-migratory effect at the higher concentration after both 1 and 4 hrs (****P*<0.0001). The effect of 10µM PR39 is greater than that of VEGF-A († *P*<0.01) at both the 1 and 4 hr time-points.

FIGURE 6.9

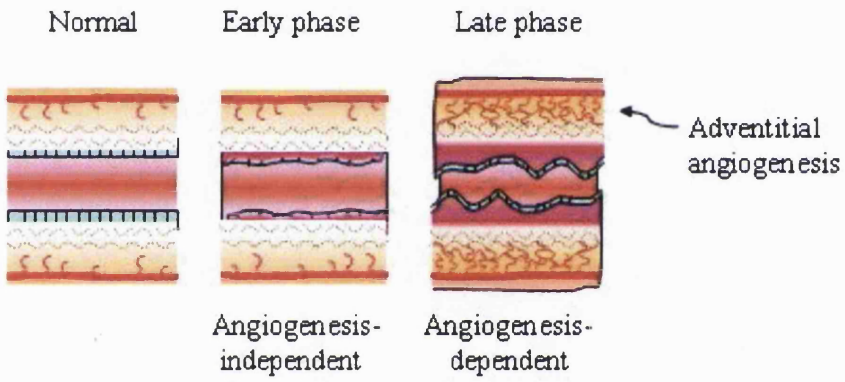


Figure 6.9. Diagram illustrating proposed 2 stage model of intimal thickening.

After an initial and angiogenesis-independent early phase, adventitial angiogenesis contributes to a later phase of intimal hyperplasia

6.3. DISCUSSION

The results presented in Chapter 6 suggest that neointima formation after collaring of an artery consists of angiogenesis-dependent and independent components (see Fig. 6.9). The placement of the collar around the artery by itself induces a mild adventitial angiogenic response (see Fig. 3.3D). The augmentation of this response leads to a greatly increased neointima formation while even full inhibition of adventitial neovascularisation does not eliminate neointima formation.

Several lines of evidence support these conclusions. Ad.PR39 induced a strong neovascular response specifically in the adventitia, and enhanced intimal thickening induced by periadventitial collar placement. Furthermore, a strong positive correlation was observed between the degree of adventitial angiogenesis and intimal thickening. Neointima-inducing effects of Ad.PR39 were completely abolished by co-administration of the angiogenesis inhibitors, sFlt1 and Ad.FGFR1DN. At the same time, administration of these inhibitors by themselves, in the absence of Ad.PR39, had no effect on basal collar-induced neointima formation.

While adventitial neovascularisation was responsible for promotion of intimal thickening by PR39, the early phase of neointimal VSMC accumulation was largely independent of angiogenic stimulation. Two lines of evidence support this conclusion. Firstly, transduction of collared arteries with Ad.PR39 for shorter times induced a striking adventitial neovascular response, but had little effect on neointimal thickness, suggesting that periadventitial angiogenesis was not required for the early phase of neointima formation. Secondly, as already mentioned, inhibition of angiogenesis using sFlt1 or Ad.FGFR1DN alone did not abolish basal intimal thickening induced in the absence of an angiogenic stimulus in the collar model. This supports the conclusion that angiogenesis is not a sufficient trigger for the initiation of neointima formation, but that pro-angiogenic agents can enhance or promote growth of an intimal lesion that was initiated by collar placement. Thus, there appears to be an initial angiogenesis-independent phase to collar-induced neointima formation.

Endogenous adventitial angiogenesis in the absence of an exogenous angiogenic stimulus was low and was not significantly reduced by either sFlt1 or

Ad.FGFR1DN. However, the fact that the much greater level of angiogenesis induced in PR39-transduced arteries was completely suppressed by these angiogenic inhibitors argues that basal angiogenesis does not play an important role in neointima formation in the absence of an additional angiogenic stimulus. Basal angiogenesis may reflect minor angiogenic pathways presumably independent of VEGF and FGF2 signaling.

The temporal and spatial concordance of macrophage recruitment with the development of new blood vessels may suggest an association between them. New blood vessels may provide the means for recruitment to the adventitia, or conversely, the arrival of macrophages may create a permissive environment for vascular growth.

The EC is the major target for biological effects of VEGF-A, and VEGF-A is conventionally regarded as an endothelial-specific factor. However, some recent findings suggest that VEGF-A can induce biological responses in VSMC (Grosskreutz et al. 1999; Ishida et al. 2001). The findings in Fig. 6.8 indicate that VEGF-A stimulates the migration of VSMCs, suggesting that a direct effect of VEGF on VSMC migration could contribute to the neointima-increasing effects of Ad.VEGF-A_{165} in the collar model. Porcine VSMCs were readily available for use in experiments during the time this work was conducted and the use of these cells, rather than rabbit VSMCs, is a valid concern of the results and its relevance for the findings in the rabbit collar model. Performing migration assays with rabbit VSMCs would be an interesting experiment to explore. The mechanisms mediating this direct chemotactic effect of VEGF-A are unclear, though VEGFR1 has been proposed to mediate VEGF-induced expression of metalloproteinases in human VSMCs (Wang and Kovanen 1999). The migratory effects of VEGF-A on VSMC warrant further study.

The appreciation of the contributory role of adventitial neovascularisation in the pathogenesis of vascular disease is of particular importance in the context of angiogenic cytokine gene therapy trials, where safety issues are a foremost concern (Simons and Post 2002).

CHAPTER 7

7.1. GENERAL DISCUSSION

The formation of neointimal lesions leading to intimal thickening and the excessive accumulation of VSMCs and lipid-laden macrophages are key features of atherosclerosis, vein-graft failure and restenosis after angioplasty. In spite of advances in the treatment of CHD, there remains an urgent need to develop new therapeutic approaches to combat the pathophysiological vascular changes associated with these and other related diseases. Work over the past decade has explored the potential for angiogenic cytokine therapy in cardiovascular disease – therapeutic angiogenesis or arteriogenesis – in the ischaemic heart or periphery. In addition, previous work from the laboratory in which this PhD research was conducted supported the concept that the angiogenic factor, VEGF-A, can augment protective arterial endothelial functions that exert a neointima-reducing effect. Together, this work has led to several clinical trials of angiogenic cytokines for ischaemic heart disease and other vasculoproliferative conditions (Simons and Ware 2003). However, recent findings have challenged the therapeutic value of angiogenic cytokines by demonstrating pro-atherogenic effects of VEGF-A in animal models of atherosclerosis, particularly genetically modified mice (Moulton et al. 1999; Celletti et al. 2001). Against this background, the primary purpose of this thesis was to investigate the effects of VEGF-A, and other angiogenic stimuli on neointima formation and atherogenic macrophage accumulation in the collar model of intimal thickening in hypercholesterolaemic and normocholesterolaemic rabbits. This work aimed to advance previous studies demonstrating an arterioprotective effect of VEGF-A in the collar model on rabbits fed a normal diet, and also examined the role of the efficiency of gene expression and, by implication, the local intra-arterial VEGF concentration, in determining biological outcome. In addition, these studies also examined the important and controversial question of the role of angiogenesis in intimal thickening,

The selection of the collar model for this thesis is justified for several reasons. This model, unlike other models of neointima formation, such as balloon injury does not depend on endolumenal damage and leaves the endothelium intact throughout the experiment. This

was an important consideration, since a key aim of this work was to examine the hypothesis that VEGF-A can reduce intimal thickening via an arterioprotective effect on the lumenal endothelium independent of endothelial regeneration. The collar model also made it possible to examine another key aim of this work, namely whether local VEGF-A delivery could reduce neointimal macrophage accumulation in hypercholesterolaemic animals. Furthermore, it was possible to compare, using the same model, the impact of VEGF gene delivery on neointima formation in rabbits on a normal and high cholesterol diet. In contrast, in other models, such as the ApoE deficient mouse, cytokines need to be delivered via other routes of administration (e.g. peritoneal or via the tail vein) and consequently, systemic or indirect effects of VEGF would be difficult to distinguish from direct arterial effects (Celletti et al. 2001). Recent reports suggest it is possible to place a periadventitial collar around the mouse carotid artery (von der Thusen et al. 2001) in conjunction with intravascular gene delivery, but this technique was not available when I embarked upon this research.

However, the collar model does raise several issues that merit further discussion. Perhaps the most important is that the mechanism underlying the initiation of neointima formation by collar placement is not understood. While the collar does not cause any major endothelial injury, some findings are consistent with an early effect of the collar on endothelial function. For example, an early wave of leucocyte infiltration into collared arteries from the lumen has been described (Donetti et al. 2002). These findings suggest that the most plausible initial trigger of collar-induced intimal thickening is some form of endothelial dysfunction. More work is required to establish the precise mechanism, however. An additional problem is that control or basal I/M ratios obtained in different experiments using the collar model, summarized in Table 1.3, were found to vary. Several factors may contribute to the observed results: Animal specific factors (source, age, strain, gender and diet), variation in collar manufacturer, and operator skill, could all help explain the variation in I/M values obtained in control gene transfer experiments, discussed in each chapter of results. It should be emphasized that animals were deliberately obtained from the same commercial source, Charles River Inc, for experiments done at Dartmouth College, USA and UCL, UK, to eliminate this source of variability and that collars were obtained from the same source and were of the same type and material throughout and that

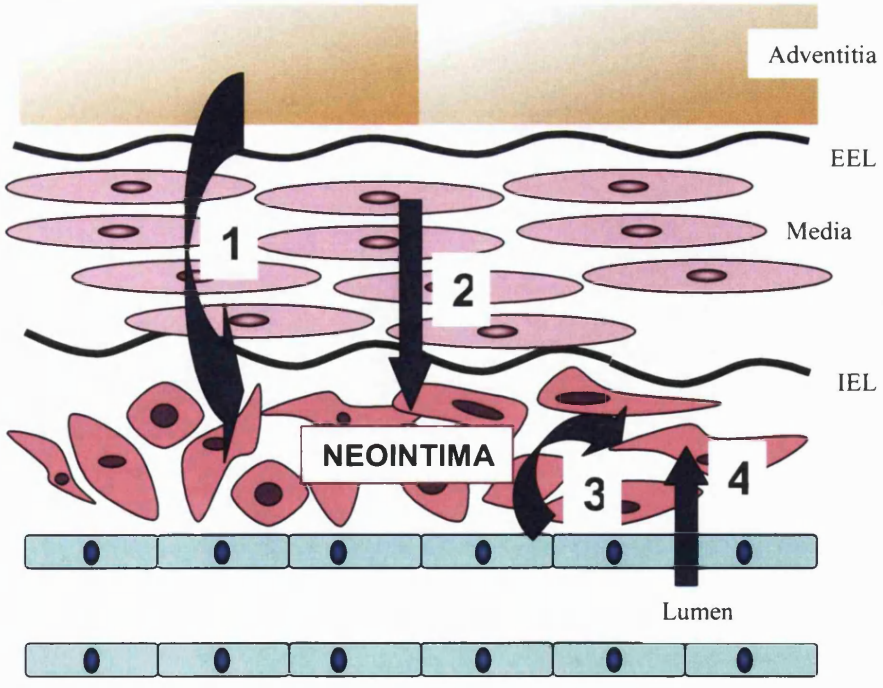
experimental techniques remained consistent in all experiments. Despite best efforts, however, it is not possible to preclude the possibility that subtle improvements and refinements in surgical and operator skill during the course of this thesis may be an underlying cause of variation in basal I/M values. However, despite these shortcomings, this model has been widely used by several laboratories (Chapter 1, Table 1.3).

The lipophilic, membrane staining dye, PKH26 was used to assess adventitial contribution to neointima formation in the collar model. In the results presented in Chapter 3, neither neointimal nor medial PKH26 staining was detected after collar placement demonstrating that cells resident within the adventitia are not responsible for any significant contribution to the subendothelial cellular mass within 14 days. These observations do not preclude adventitial cell involvement at much later time-points and this study did not address the role of circulating progenitor cells that may access the neointima via an adventitial vascular plexus that might avoid detection by the method used. Furthermore, the dye labels cell membranes indiscriminately and thus, specific cell types that may migrate lumenally cannot be directly identified without co-staining. Nevertheless, with these limitations, the results indicate that cells originating in the adventitia are unlikely to play a major role in neointima formation in the collar model. Moreover, these results are similar to the outer adventitial presence of dye and myofibroblast proliferation observed in the rat balloon angioplasty model (De Leon et al. 2001). The limited staining pattern is unlikely to be attributable to dilution or exclusion of the fluorescent dye or loss of cells, because intense adventitial staining was still evident. Adventitial myofibroblasts, either resident or circulating, are thought to play a role in the vascular remodelling process (Shi et al. 1996; Sartore et al. 2001), and after transduction of the adventitia with a reporter gene prior to balloon-angioplasty in the same rat model, fibroblasts were observed to migrate to the neointima (Siow et al. 2003). This study supported findings in which β-galactosidase expressing fibroblasts implanted to the adventitia of balloon-injured rat arteries also migrated to the neointima (Li et al. 2000a). However, contradictory results were found in a rabbit balloon angioplasty model, that argued against a role for the adventitia in neointima formation (Maeng et al. 2003). More recently, in a murine model of accelerated vein graft atherosclerosis, a population of progenitor cells, resident within the adventitia, were shown to contribute to the process (Hu et al. 2004). Thus, the contribution of adventitial cells to

vascular remodelling may depend upon several factors. These include whether the elastic laminae subtending the media are breached and the ability of an adventitial blood supply to serve as a conduit for circulating progenitor cells. The use of an external stent to mechanically restrict adventitial remodelling in a porcine model of vein graft failure has also been shown to reduce medial and neointimal thickening (Mehta et al. 1998), adding further evidence that adventitial cells and matrix are important for this process. Involvement of adventitial cells in intimal thickening may be dependent on the model and species investigated.

Neointimal cell expansion occurs rapidly and it is possible that collar-induced lesions arise either by migration of a medial cell or by a trapped intimal cell with high replicative potential. There is no direct evidence that medial SMC contribute to the neointimal mass in this model, but migrating SMC traversing the IEL were observed at higher magnification (Fig. 7.2). The source of neointimal SMC has been the subject of much research focus and may have multiple origins (Fig 7. 1).

Figure 7.1 Schematic illustrating potential sources of neointimal VSMC.



1= Migration of resident stem cells/myofibroblasts from the peri-adventitia and /or adventitial vasa vasorum; 2= Migration of VSMC / myofibroblasts from the media; 3= Trans-differentiation of EC or subendothelial resident stem cells; 4= Circulation-derived progenitors.

FIGURE 7.1

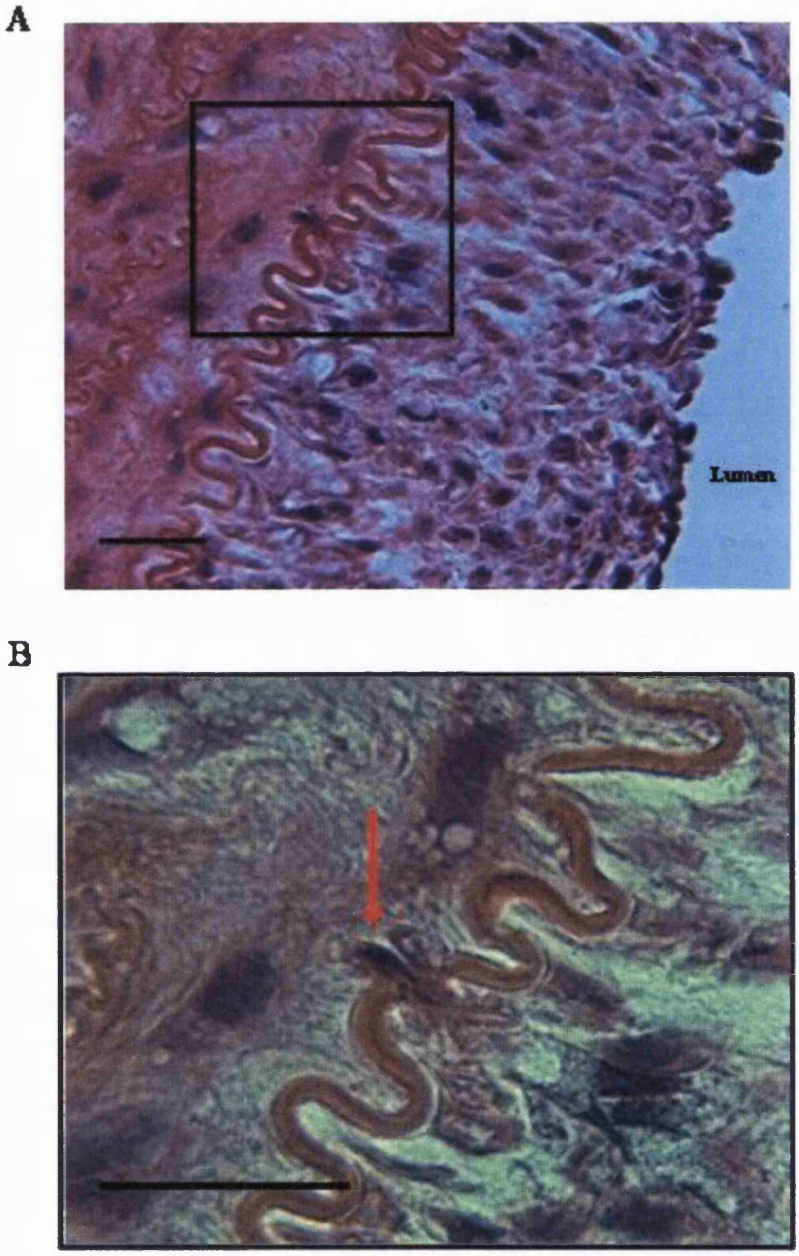


Figure 7.1. VSMC Crossing the Internal Elastic Lamina (IEL).

H & E stain captures a smooth muscle cell apparently crossing the IEL, suggesting that that the medial layer of VSMC is a potential source of cells that comprise the neointima in the collar model. Panel B represents a magnified image of the boxed area in panel A. A, x 400; scale bar = 20μm. B, x 1000 magnification; scale bar = 10μm.

Pathophysiological intimal thickening associated with restenosis after angioplasty, vein graft stenosis and other vasculo-proliferative complications, is often a focal process. Thus, there is a powerful argument for local arterial delivery of a therapeutic agent to the diseased region of the artery to achieve the desired neointima-reducing effect. The optimal method of delivery to achieve sufficient amounts of the desired agent to target the process is unresolved. Clinical trials, targeting restenosis, using different delivery modalities have proven inconclusive with respect to delivery methods (Ylä-Herttuala and Alitalo 2003). Perivascular delivery has been advocated for localised arterial disease and may have benefits over endolumenal strategies, particularly where open access to the vessel is required (restenosis post bypass grafting, transplant vasculopathy, haemodialysis access graft restenosis). Vasculitis, an inflammatory process affecting the arterial wall which results in adverse remodelling, may have its pathologic origins in the adventitia (Weyand and Goronzy 2003) and using adventitial delivery techniques allows a more site-specific study of the processes involved. Diverse delivery methods to target the adventitia have been reported including use of a needle injection catheter (Huehns et al. 1999), implantation of *ex vivo* expanded cells (Li et al. 2000; Kankkonen et al. 2004), and direct topical application (Rios et al. 1995). To date, the efficacy of therapeutic periadventitial gene delivery in human disease has not been demonstrated, but a phase II trial is in progress of periadventitial VEGF-D gene delivery using a biodegradable collar in patients undergoing haemodialyis access graft surgery (Fuster et al. 2001). This trial is designed to test the efficacy and safety of the collar and VEGF-D (designated TRINAM™) in preventing stenotic failure of haemodialysis access grafts.

The role of VEGF in the pathogenesis of vessel wall disease has been the subject of intense debate. Some studies have suggested that either angiogenesis (Moulton et al. 1999) or VEGF-dependent recruitment of monocyte/macrophages (Celletti et al. 2001; Zhao et al. 2002) can promote atherosclerosis. The model of collar-induced neointima formation in hypercholesterolaemic rabbits is distinct from those in which VEGF was reported to exhibit pro-atherosclerotic effects, and this may partly account for the divergence of our

results from previous findings in the apoE-deficient mouse model. In addition, local delivery of the VEGF gene directly into the collared carotid artery may produce biological effects distinct from those induced by administration of VEGF protein (Celletti et al. 2001), acting at sites distant from the biological target tissue. Another crucial determinant of the biological effect of VEGF *in vivo* is likely to be the local and/or sytemic concentration. It is possible that pro-atherosclerotic effects of VEGF may require at least transiently high systemic levels of VEGF and be mediated indirectly via non-endothelial actions of VEGF. VEGF stimulates monocyte chemotaxis via VEGFR1 (Clauss et al. 1996), and enhancement of plaque progression induced by intraperitoneal VEGF delivery was associated with mobilisation of bone marrow endothelial progenitor cells and monocyte/macrophages (Celletti et al. 2001). The lower efficiency gene transduction resulting from liposome-mediated gene transfer as used in Chapter 4, is likely to generate relatively modest VEGF levels that may be sufficient to cause neointima reduction via a by-stander effect, but insufficient to produce other biological effects such as angiogenesis. In support of this contention, results presented in Chapter 5 show that adenovirus-mediated VEGF-A_{165} delivery to collared arteries in cholesterol-fed rabbits produced a striking adventitial neovascular response, but had little effect on intimal thickening or macrophage accumulation.

It has been hypothesized that VEGF can elicit a local arterioprotective effect through its abilities to promote endothelial functions such as NO and prostacyclin production, which inhibit VSMC proliferation, and antagonize endothelial leucocyte adhesion (Zachary et al. 2000). The finding that local liposome-mediated VEGF gene transfer can reduce intimal thickening and macrophage accumulation in the hypercholesterolaemic rabbit identifies a novel mechanism through which VEGF can exert arterioprotective effects *in vivo*, and thus advances previous findings that periadventitial VEGF gene transfer inhibits collar-induced neointima formation (Laitinen et al. 1997b). Furthermore, a growing body of evidence supports the concept that VEGF can elicit diverse protective effects in blood vessels independent of angiogenesis, including the regulation of thrombogenic potential, (Kuenen et al. 2002) nitric oxide-dependent attenuation of leucocyte adhesion and transmigration in mesenteric venules (Scalia et al. 1999), protection against endothelial toxicity induced by oxidized LDL (Kuzuya et al. 2001), and

upregulation of decay accelerating factor, which counteracts complement-mediated cell injury (Mason et al. 2002). Given that increased VCAM-1 expression and macrophage infiltration are implicated in vein graft intimal thickening in hypercholesterolaemic rabbits (Zwolak et al. 1989; Hanyu et al. 2001), and that hypercholesterolaemia is a major risk factor for human vein graft atherosclerosis (Campeau et al. 1984), the results presented in Chapter 4 suggest that local, periadventitial VEGF gene transfer may be therapeutically useful for preventing vein graft failure.

The biological roles of both PlGF and its specific receptor VEGFR1 have remained enigmatic, because VEGFR1 does not have a clearly-defined signalling function and, during embryonic development is thought to act primarily as a regulator of VEGF-A functions mediated via VEGFR2. Recent findings have revealed a role for PlGF in post-embryonic angiogenesis in diverse pathophysiological settings and there is increasing evidence that mobilisation of bone marrow-derived haematopoietic progenitors is a key mediator of these biological effects (Carmeliet et al. 2001). Impaired pathophysiological angiogenesis in $PlGF^{-/-}$ mice can be rescued by transplantation of wild-type bone marrow and inhibitory anti-Flt1 antibodies block PlGF-induced neovascularisation in ischaemia, tumour growth and arthritis due to reduced mobilisation of bone marrow-derived myeloid progenitors (Luttun et al. 2002b). PlGF also stimulates haematopoiesis after bone marrow irradiation by recruiting VEGFR1-positive bone marrow-derived stem cells (Hattori et al. 2002). While previous findings have implicated VEGFR1 in atherogenesis (Luttun et al. 2002a), direct evidence for a role for PlGF in atherogenic recruitment of monocyte/macrophages has not been clearly provided. The findings presented in Chapter 5 indicate that local PlGF overexpression directly stimulates atherogenesis by increasing recruitment of monocytes / macrophages from the circulation. Since PlGF transgene expression was detected only in the collared region of the artery and was absent from other tissues, it is highly unlikely that the effects of PlGF in this model are attributable to increased systemic PlGF leading to increased mobilisation of bone marrow-derived stem cells. Furthermore, Ram 11-positive macrophages in collared arteries also expressed VEGFR1, suggesting that intra-arterial PlGF acted by directly inducing migration of VEGFR1-expressing monocytes into lesions. Macrophage foam cells in rabbit lesions have

been shown to have anaphase or metaphase nuclei and therefore have the capacity to synthesize DNA and therefore divide (McMillan and Stary 1968). Thus, it is possible that PlGF could increase intimal macrophage foam cell proliferation, though this was not directly addressed.

The study presented in Chapter 5 support the emergent view that in adult animals, PlGF is a functional cytokine particularly in pathophysiological settings, able to stimulate angiogenesis and atherogenic migration of monocyte/macrophages into the arterial wall. The finding that PlGF and its receptor VEGFR1 have a potential role in the pathogenesis of atherosclerotic disease also has clinical implications. Therapeutic strategies targeting either PlGF or its receptor VEGFR1 may be an attractive novel approach to the inhibition of inflammatory macrophage infiltration underlying atherosclerotic plaque formation (Autiero et al. 2003a). This notion is supported by previous findings that administration of anti-Flt1 antibody has an atheroprotective effect in atherosclerosis-prone ApoE-deficient mice, by reducing atherosclerotic plaque growth and plaque macrophage infiltration. Future studies examining the role of PlGF and VEGFR1 in other animal models of human atherosclerotic disease and in human lesions will be helpful in establishing the usefulness of such approaches. Work performed in parallel to my studies show that mice doubly deficient in PLGF and apoE ($PlGF^{-/-}$:$apoE^{-/-}$) results in decreased lesion growth and macrophage numbers compared to $apoE^{-/-}$ mice thus supporting a role for endogenous PlGF in atherosclerosis in the apoE-deficient mouse model (P.Carmeliet and L. Moons, **unpublished results**). PlGF2 also increased intimal thickening in normocholesterolaemic animals (Chapter 5), an effect which appeared to be independent of neointimal macrophage recruitment. Macrophages were not detectable by Ram11 staining in the neointima, but adventitial changes such as increased neovascularisation and macrophage infiltration were evident and may contribute to the observed effects. In addition PlGF might exert a direct effect on VEGFR1 expressing SMCs, inducing their proliferation and migration (Ishida et al. 2001).

Atherogenic effects of PlGF may be independent of angiogenesis, given that the ability of inhibitory anti-Flt1 antibody to reduce atherosclerosis in $ApoE^{-/-}$ mice was not due to the inhibition of plaque neovascularisation (Luttun et al. 2002b). The role of angiogenesis as a mediator of the neointima-promoting effects of PlGF2 in VSMC

hyperplasia induced by clinically-relevant interventions such as balloon injury or vein grafting is not known, however, and warrants further investigation.

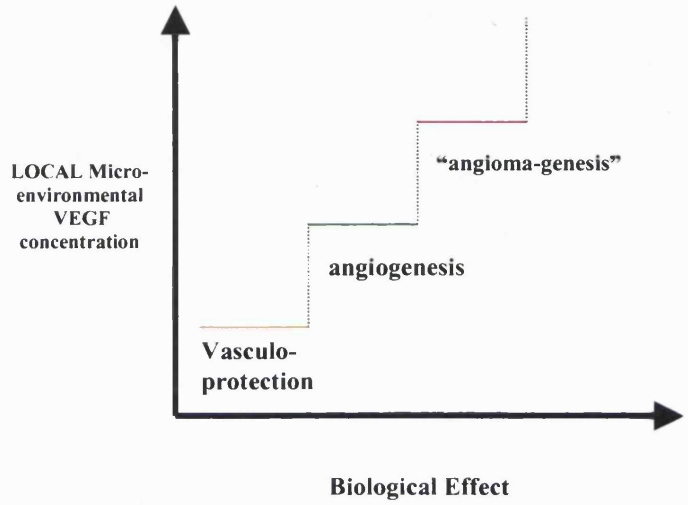
Upregulation of VEGFR1 mediated endothelial VCAM-1 expression was demonstrated to be one potential mechanism consistent with the pro-atherogenic profile of PlGF. However, the means by which VEGFR1 signalling influences VCAM-1 expression is unclear. VEGFR1 has, however, been reported to mediate signalling pathways in ECs though these findings are controversial and in general, VEGFR1 is thought to mediate only a weak signalling response. It is also plausible that in addition, or alternate to, direct signalling, VEGFR1 might indirectly increase VCAM-1 expression, via upregulation of inflammatory chemocytokines such as TNFα, IL-1β or MCP-1 (Selvaraj et al. 2003). PlGF2, unlike the human PlGF1 isoform, is also able to bind the neuropilin-1 (NP-1) receptor, which is thought to act as a co-receptor for VEGFR2 / KDR. Whether NP-1 is a co-receptor for VEGFR1 is unknown, but it cannot be precluded that angiogenic and monocyte effects of PlGF2 may be mediated in part by NP-1. These possibilities also require further investigation.

In contrast to Ad.PlGF2, Ad.VEGF-A_{165} did not significantly increase intimal thickening or macrophage infiltration in collared hypercholesterolaemic rabbits. The reasons for this are unclear. It can be speculated that the following non-exclusive mechanisms might contribute to these differences: 1) PlGF could act directly through VEGFR1 and induce unique intracellular signals that are distinct from those mediated through VEGFR2 by VEGF; 2) PlGF may act on distinct target cells expressing VEGFR1 (Park et al. 1994) ; 3) PlGF activation of VEGFR1 may induce a cross-talk with VEGFR2 on endothelial cells (Autiero et al. 2003b) with a biological effect distinct from that produced by activation of VEGFR2 alone. 4) The pro-atherogenic effect of transgenic PlGF2 may be mediated through modulation of the levels of endogenous PlGF or VEGF protein expression, but this was not assessed

The contrasting biological effect of Ad.VEGF-A_{165} and liposome-mediated VEGF-A_{164} transfer within the collared arteries of hypercholesterolaemic animals deserves further consideration. It was reported in Chapter 4 that local liposome-mediated $VEGF_{164}$ gene transfer reduced neointima formation and inhibited intimal macrophage infiltration in the

collared carotid arteries of hypercholesterolaemic rabbits. The interpretation of these findings given above, was that low level VEGF expression, arising from low efficiency liposome-mediated gene transfer, can exert an arterioprotective effect, whereas expression of higher levels of VEGF-A produced by high efficiency adenoviral gene transduction might cause an impairment of arterioprotective or possibly pro-atherogenic effects of VEGF-A. The findings presented in Chapter 5 are consistent with the notion that the effects of VEGF on intimal thickening and macrophage infiltration are concentration-dependent: a greater level of VEGF expression due to high-efficiency Ad.VEGF-A_{165} delivery did not inhibit either collar–induced neointima formation or increased neointimal macrophage accumulation, suggesting that the arterioprotective effects of VEGF-A observed after liposome-mediated gene transduction are impaired at higher local VEGF-A_{165} concentrations. The critical importance of local VEGF-A concentration for determining the biological outcome of VEGF-A treatment has recently been emphasized by the finding that low level VEGF production results in the growth of morphologically normal and stable capillaries, whilst VEGF production above a threshold level induces growth of aberrant leaky vessels and formation of haemangiomas (Ozawa et al. 2004). The likelihood that different efficiencies of gene transduction and resulting levels of local VEGF expression can produce markedly different consequences for pathophysiological changes in the arterial wall, may have an important bearing upon the therapeutic use of VEGF for ischaemic cardiovascular disease or other cardiovascular pathologies. Therapeutic strategies aimed to achieve the highest possible levels of VEGF expression may not always be optimal to achieve arterioprotective or anti-atherogenic responses. This theory is summarized below in Fig 7.3.

Figure 7.3 Hypothesis to Explain Concentration-Dependent Effects of VEGF



The arterioprotective effect of liposome-mediated $VEGF_{164}$ gene transfer in hypercholesterolaemic animals was independent of any significant angiogenic effect exerted in the adventitia. Hypercholesterolaemia is known to inhibit angiogenesis in atherosclerosis-prone ApoE-deficient mice, in the rat hindlimb ischaemia model and in hindlimb ischaemia within Watanabe Heritable Hyperlipidaemic (WHHL) rabbits (Van Belle et al. 1997; Duan et al. 2000; Jang et al. 2000). Since, and as the results in Chapter 6 indicate, a strong angiogenic response may in part mediate neointimal growth induced by Ad.VEGF-A_{165}, the impairment of VEGF-A_{165} stimulated angiogenesis by high prevailing concentrations of serum cholesterol may contribute to the inability of Ad.VEGF-A_{165} to enhance neointima formation in hypercholesterolaemic rabbits.

Although the results presented in Chapter 3 argued that adventitia-derived cells did not migrate to the neointima and therefore did not contribute significantly to the neointimal cellular mass directly, the results presented in Chapter 6 indicate that the adventitia may indirectly play a major role in vascular remodeling and intimal thickening (Wilcox et al. 1996). At least two mechanisms can explain the close association between adventitial

neovascularisation and intimal thickening in response to angiogenic stimuli. On the one hand, the local supply of nutrients and oxygen to the inner layers of the artery may become rate-limiting after the initial phase of neointimal growth and the formation of adventitial new vessels may create a microenvironment permissive of vascular remodeling (Stupack and Cheresh 2002). On the other, local production of growth and chemotactic factors, such as VEGF, FGFs and PDGF-BB may directly stimulate VSMC accumulation (Cao et al. 2003; Carmeliet 2003a). In accordance with the latter notion, both PR39 and VEGF-A exerted a direct chemotactic effect on VSMCs. Of note, while the ability of VEGF to induce smooth muscle cell migration has been previously reported (Grosskreutz et al. 1999; Ishida et al. 2001), the same has not been reported for PR39. This process may well be amplified by new vessels in the adventitia that provide an additional source of VSMC mitogens or circulating smooth muscle progenitor cells (Simper et al. 2002).

Most accounts of the formation of VSMC-rich neointimal lesions stress the role of either resident subendothelial or medial VSMC while the involvement of the adventitia has received relatively little attention. As discussed above, recent studies suggest that migration of myofibroblasts that are either resident within the adventitia or recruited as progenitors from the circulation, contribute significantly to neointimal VSMC accumulation (Sartore et al. 2001; Wilcox et al. 2001). The possibility that the adventitial vasa vasorum of human coronary arteries plays a role in the formation of atherosclerotic plaques was first advanced by Barger et al. in 1984, and it has subsequently been postulated that the density of newly formed vasa vasorum in response to injury, is proportional to vessel stenosis (Kwon et al. 1998).

Based on the results in Chapter 6, I propose a two step model of neointima formation: an early angiogenesis-independent phase initiated by collaring or perhaps other stimuli and a later angiogenesis-dependent phase during which the stimulation of adventitial angiogenesis further augments neointima formation (see Fig. 6.9).

Administration of angiogenic factors, such as VEGF, to the vessel wall has been shown to enhance re-endothelialisation and inhibit neointima formation in balloon-injured arteries (Asahara et al. 1995). However, the study described in Chapter 6 of this thesis does not necessarily conflict with these findings and does not negate the well recognized arterio-protective properties of the lumenal endothelium. The effects on neointima formation by

stimulation of adventitial neo-vessel formation were observed despite the absence of major structural damage to the endothelium. No major obvious change in the endothelial integrity occurred throughout the experimental time-course and therefore was not likely to contribute to the results.

The demonstration of an angiogenesis-dependent phase of neointima formation may explain progression of arteriopathy in a number of disease processes including atherosclerosis and may also explain a marked increase in post-angioplasty restenosis in patients treated with Granulocyte-Colony Stimulating Factor (G-CSF), a factor that stimulates the formation of bone-marrow derived stem cells, observed in a recent clinical trial (Kang et al. 2004). These findings may have particular relevance for vasculitis, a disease of the vessel wall associated with intimal hyperplasia and thought to be triggered by invasion of the outer media and adventitia by T cells, dendritic cells, monocytes and granulocytes (Weyand and Goronzy 2003). The pathogenic mechanisms underlying vasculitis, specifically the roles of adventitial cells and neovascularisation, have not been completely defined. Increased serum and local production of VEGF has been reported in Kawasaki's disease, a vasculitis preferentially affecting large vessels (Terai et al. 1999), but a direct pathogenic role of VEGF has not been demonstrated. Further investigation of the roles of angiogenesis and angiogenic cytokines in vasculitis could generate insight into the underlying pathogenic mechanism(s).

The contribution of adventitial macrophages to neointima formation and adventitial angiogenesis was also addressed in experiments presented in Chapter 6. The role of macrophages is further emphasized by the ability of monocyte chemoattractant protein (MCP-1) to induce angiogenesis and arteriogenesis (Arras et al. 1998). Macrophages provide a reservoir for matrix degrading enzymes (Galis et al. 1995) and chemoattractants (Assoian et al. 1987; Sukhova et al. 1998), which could prime the extracellular matrix for vascular growth. Gene therapy targeting monocyte chemoattractant protein-1 (Egashira et al. 2000) and inhibition of NFκB by overexpression of its natural inhibitor, IκBα (Breuss et al. 2002), have yielded promising results as a vasculo-protective strategy. Macrophage infiltration throughout all layers of the vessel wall has previously been correlated with an increased incidence of restenosis in a porcine stenting model (Moreno et al. 1996). Neovascularisation may serve to enhance local delivery of mononuclear cells to sites of

inflammation, thereby facilitating the release of mitogenic factors into the arterial wall. Such factors are also pro-angiogenic and thus, a positive feedback cycle could be propagated, whereby new vessels deliver inflammatory cells that further promote angiogenesis. In support of this concept, adventitial macrophage recruitment was also increased in Ad.PR39-transduced vessels. Their presence was correspondingly reduced in arterial walls treated with angiogenesis inhibitors. Recently, evidence has been put forward that vasa vasorum density and inflammatory cell infiltrates are tightly correlated with atherosclerotic plaque progression (Moulton et al. 2003). Monocytes/macrophages were not detected within the neointimas of normocholesterolaemic rabbits which suggests that at the time-points studied, the mature macrophage did not contribute to the neointimal cellular mass.

The results of Ad.PR39 transduction in Chapter 6 also need to be interpreted in the context of the findings obtained from adenoviral-VEGFA gene transfer presented in Chapter 5. VEGF upregulation was required for PR39-induced adventitial angiogenesis and neointima formation and Ad.VEGF-A_{165} delivery had paradoxical effects, depending on the prevailing serum cholesterol level. If VEGF is culpable, at least in part, for mediating the angiogenic and neointima-increasing effects of PR39, why is Ad.VEGF-A_{165} delivery by itself apparently not sufficient to significantly increase intimal thickening in hypercholesterolaemic rabbits? Firstly, the effects of Ad.PR39 were examined in normocholesterolaemic rabbits while Ad.VEGF-A_{165} was delivered in hypercholesterolaemic animals. Indeed, additional results reported in Chapter 5, show that treatment of collared arteries with Ad.VEGF-A_{165} in animals maintained on a normal diet did significantly increase intimal thickening. This suggests that high serum cholesterol abrogates the neointima-increasing effects of high level Ad.VEGF-A_{165} -mediated expression. As discussed above, hypercholesterolaemia has been widely reported to adversely affect the angiogenic process. A second consideration is that PR39 activates both VEGF and FGF signalling. Thus, while VEGF may be required for the angiogenic and neointima-enhancing responses induced by PR39, it may not by itself be sufficient to mimic the PR39 effects.

Angiogenesis and specifically the effects of VEGF-A, are altered under differing pathophysiological environments. Diabetes (Waltenberger 2001), hypercholesterolaemia

(Duan et al. 2000), smoking and specifically nicotine (Heeschen et al. 2001) and the aging process (Rivard et al. 1999) have all been shown to independently affect endothelial function and their propensity to develop new blood vessels. The studies performed for this thesis were in young and healthy NZW rabbits and as with most animal models, the results obtained should be interpreted with caution in terms of relevance to patient pathology. Patients often develop occlusive vascular disease in the setting of multiple risk factors which are difficult to address in animal studies of this type.

However, there have been reports in human pathological specimens associating extensive vessel wall neovascularisation and adverse vascular remodeling (Kamat et al. 1987). The hypothesis that angiogenesis contributes to the progression of lesion formation remains an attractive one, partly because it chimes with recent developments in understanding the role of hypoxia in regulating tumour angiogenesis. Theoretical arguments suggest that once vessel wall thickness exceeds a critical depth, due, for example, to intimal thickening induced by hypercholesterolaemia, the supply of oxygen and other nutrients to the media and neointima will be restricted by the increased distance either from the lumen or adventitial vasa vasorum. The critical threshold distance between tissues and a capillary (or vessel lumen) above which hypoxia will occur has been calculated to be 100 μm (Torres Filho et al. 1994). When this distance is exceeded, a hypoxic environment will form in the interior of the artery, which in turn will provide a stimulus for the accumulation of hypoxia-inducible transcription factors (HIFs), such as HIF-1α, which induce expression of VEGF and other angiogenic regulators. Secretion of VEGF stimulates angiogenesis, promoting plaque growth by increasing the oxygen supply to the media and neointima. Consistent with this hypothesis, expression of VEGF and HIF-1α has been demonstrated in atherosclerotic lesions (Khatri et al. 2004). It is important to emphasize that according to this model, angiogenesis does not initiate plaque formation, but acts as a permissive factor allowing later plaque growth once a critical arterial thickness has been reached. Determining the true contributory role of the angiogenic process in atherosclerotic disease has assumed greater urgency as angiogenic factors enter clinical trials for the treatment of cardiovascular diseases.

This thesis has focused specifically on the impact that overexpression of angiogenic growth factors has upon intimal lesion formation in an animal model. Many growth factors prevail at basal concentrations in the vascular system within humans; they display a diverse and complex biology on numerous cell types and appreciation of their role in vessel wall biology will remain an area of intense research activity for the foreseeable future. Clinical data is emerging to suggest that high circulating levels of angiogenic growth factors represent an adverse prognostic sign for the development of cardiovascular disease (Heeschen et al. 2003). Furthermore, as techniques and tools within the field of cellular and gene therapy to overcome vascular disease advance, it is clear that a more specific understanding of the molecular pathogenesis of atherogenesis is required to devise therapies targeting plaque development. The role of angiogenic growth factors will be central to this understanding. The experiments performed during the course of this thesis hoped to address this vital issue, but as with all scientific research, more questions have emerged and I will complete this thesis by discussing future directions of study that may further our understanding.

7.2. PERSPECTIVES AND FUTURE STUDY

The studies in Chapter 6 support a role for angiogenesis in neointimal growth, if not in the initiation of neointima formation. However, the relevance of adventitial neovascularisation for intimal thickening in other models or for neointimal macrophage accumulation is unclear. Supportive of a more general role of adventitial angiogenesis in neointimal growth, results obtained in parallel with my work with PR39 in the collar model show that PR39 has a similar effect on adventitial neovascularisation and neointima formation in the balloon-injured rat carotid artery (M. Simons and co-workers, **in press**). Further studies are required to extend the model of angiogenesis-dependent neointimal growth advanced here, to intimal hyperplasia in other species and different pathophyisological settings.

A striking feature of the results throughout this thesis was the absence of new vessels in the neointima. Intimal neovascularisation has been argued to play a key role in the development of atherosclerotic lesions, particularly in the ApoE-deficient mouse model.

The fact that neointimal angiogenesis could not be detected in the collar model indicates strongly that, at least early stages of arterial lesions in the rabbit are independent of intimal neovascularisation. Indeed, plaque neovessels are formed only in advanced lesions in ApoE-deficient mice when these exceed 250 μm in thickness, and are only found in 40% of advanced mouse lesions (Celletti et al. 2001). These findings, taken together, indicate that neointimal or plaque angiogenesis may only be a feature of late stage atherosclerosis and is probably not a causative factor in the development of these lesions, but rather a 'by-product' of the complex pathology in the late atherosclerotic milieu. The relevance of these findings for human atherosclerosis is unclear; studies of the role of intimal and adventitial neovascularisation in lesion formation in larger animals such as pigs may be helpful in addressing this question.

It would also be useful to prove that the adventitial new vessels were functional and sustained perfusion, using fluorescent *Ricinus communis* agglutinin I lectin. This lectin binds weakly to the lumenal surface of endothelium and strongly to the endothelial basement membrane (Thurston et al. 1996; Bhardwaj et al. 2003). Secondly, it would be interesting to determine the 3-dimensional architecture of the adventitial vascular plexus to clarify the precise orientation of the vessels in relation to eccentric thickening occluding the arterial lumen. Advanced imaging modalities such as microcomputerized tomography (micro-CT) (Gossl et al. 2003) could be utilized for this purpose.

Vascular and neural networks, although functionally distinct, have architectural similarities (Carmeliet 2003b). The vascular plexus within the vessel wall is often accompanied by a nerve supply and VEGF has been implicated in the 'cross-talk' between the two networks. In particular, VEGF has direct neurotropic effects through protecting and pre-conditioning neurons against hypoxic stress (Oosthuyse et al. 2001). Neural regeneration is also stimulated by VEGF, not only because of its angiogenic capacity but also through the stimulation of neuronal survival, proliferation and migration and has been demonstrated to improve ischaemic neuropathy (Schratzberger et al. 2000). Further evidence indicates that nerve-derived signals might affect angiogenesis. Sympathetic nerve fibres innervate blood vessels and might, by releasing noradrenaline and its co-transmitter neuropeptide Y (NPY), from nerve endings stimulate new vessel growth within the vessel wall (Zukowska et al. 2003). NPY mediates neurogenic ischemic angiogenesis at

physiological concentrations by activating Y2/Y5 receptors and eNOS, in part due to release of VEGF (Lee et al. 2003). The dependence of adventitial angiogenesis upon a neural component in the adventitia/vessel wall and the downstream effect on intimal thickening in the collar model, in the presence of angiogenic stimulators or inhibitors would be interesting to examine.

The angiogenic ligands investigated in this thesis, VEGF and PlGF, have been shown to have vasculogenic potential. They both potently mobilize progenitor cells from the bone marrow, which then incorporate into areas of neovascularisation within diverse animal models. Vasculogenesis originally referred to the formation of primary vasculature from precursor cells during embryonic development (Patan 2004), but has also been used to describe a similar process in the adult. However, more recently, it has been described that vascular progenitor cells may also be the source of VSMCs and that these progenitors are abundant within the adventitia. This resident adventitial progenitor cell population differentiated into VSMCs that contributed to atherosclerosis in an $ApoE^{-/-}$ mouse model (Hu et al. 2004). The collar model enables targeted adventitial delivery to investigate the effects on a localized region of affected artery. Thus, it is unlikely that bone-marrow derived progenitor cells are activated in the processes observed in the collar model, but this does not preclude activation of the resident vascular progenitor cells and their contribution to intimal thickening and /or adventitial neovascularisation. Both VEGFR1 and VEGFR2 signalling have been demonstrated to mediate vascular progenitor cell mobilisation (Yamashita et al. 2000; Burger et al. 2002; Eriksson and Alitalo 2002) and investigation of the significance of VEGF, PR39 and PlGF in this regard would be of interest.

It is difficult to measure accurately the local micro-environmental concentrations of growth factors, after either liposome or adenoviral gene transfer, in the vessel wall. A dose titration experiment using different quantities of Ad.VEGF-A_{165} would further distinguish the effects of this cytokine in the vessel wall, but also define the concentrations at which transitions in biological effect occur. This approach in a porcine heart model demonstrated that whilst lipofectamine mediated VEGF delivery exerted no detectable angiogenesis, the optimal dose elicited transmural myocardial angiogenesis and the highest doses provoked myocardial oedema (Rutanen et al. 2004). The molecular basis for the different concentration-dependent effects of VEGF is likely to reside in the complex array

of signalling events known to mediate the biological effects of VEGF via VEGFR2. The complex signalling pathways mediating the biological effects of VEGF allows for the integrated, yet functional versatility of this growth factor. Akt and extracellular-signal-regulated kinase (ERK), for example, are key junction points linking together signal transduction involved in survival and NO generation, and proliferation and prostanoid biosynthesis (Zachary 2003). Nitric oxide (NO) production ultimately appears a prerequisite (via eNOS activation) to mediate both angiogenesis and 'arterioprotection' (Laitinen et al. 1997b). However, low levels of VEGF may be sufficient to induce arterioprotective functions such as NO production but insufficient to induce the array of signalling events required to stimulate angiogenesis. A fuller understanding of the concentration-dependent effects of VEGF on signalling events and biological functions *in vitro* and *in vivo* is required to establish whether this is the case.

In summary, a major conclusion of this thesis is that the effects of VEGF-A on intimal thickening are dependent upon the efficiency of gene transduction and ultimately local concentration of angiogenic factor. In addition, my findings indicate that VEGF-A and PlGF have differential effects on intimal thickening and macrophage accumulation. Finally, studies with the novel angiogenic peptide PR39 revealed an unexpected role for adventitial angiogenesis in neointimal growth. While the effects of angiogenic factors are clearly complex, my results indicate that a strong angiogenic stimulus can promote but does not initiate neointimal development, whereas low levels of the angiogenic cytokine, VEGF-A, that are weakly angiogenic can exert an arterioprotective effect. What implications do these findings have for human angiogenic cytokine therapy? One implication is that delivery of angiogenic cytokines to diseased vessels at concentrations predicted to induce a neovascular response might have the potential to increase intimal thickening and therefore exacerbate disease. This consideration may not be an issue for advanced atherosclerotic disease in humans, where the contribution of angiogenesis remains an unresolved issue. For example, does intraplaque angiogenesis promote plaque growth and predispose to vulnerability or does angiogenesis enable stabilisation of the plaque? In an overview of angiogenesis trials, no excessive morbidity was attributed to an increase in unstable coronary syndromes (Simons et al. 2000) and, in the Kuopio Angiogenesis Trial, no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-

month follow-up (Hedman et al. 2003). In the VIVA (VEGF in Ischaemia for Vascular Angiogenesis) trial, a placebo-controlled double-blind study in 178 patients, intracoronary and intravenous infusion of recombinant human VEGF was safe, well-tolerated and resulted in significant improvement in angina after a 120-day follow-up (Henry et al. 2003).

Despite the excellent safety profile of angiogenic cytokines in cardiovascular disease, the results of this thesis suggest that the expression level of these molecules deserves closer consideration. Long term, but low level expression, could have greater therapeutic benefit and avoid unwanted and potentially pathogenic angiogenesis.

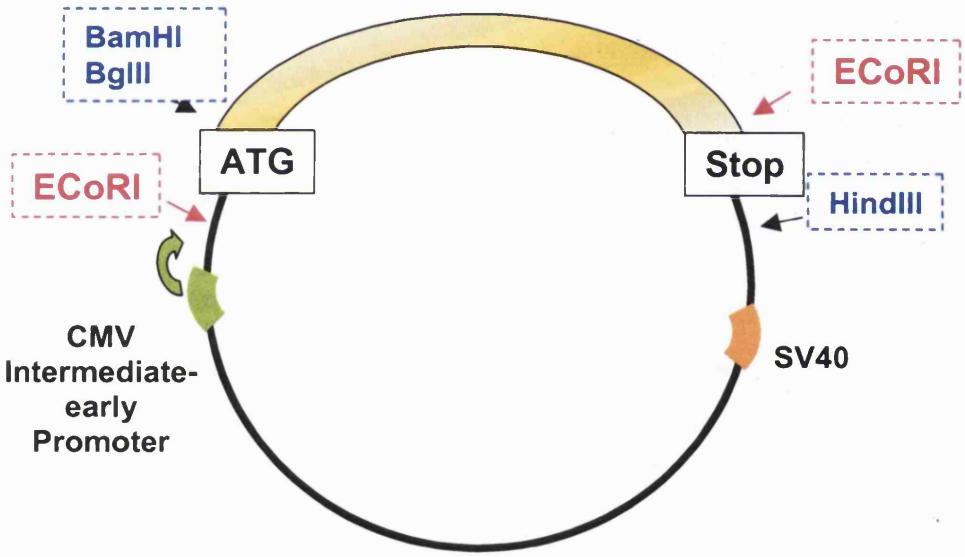
CHAPTER 8

APPENDIX

8.1. Plasmid Maps

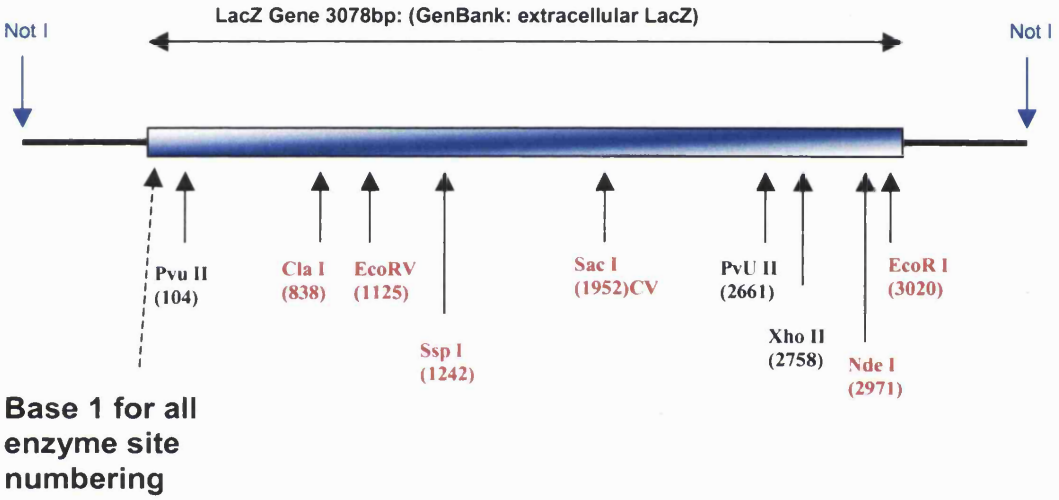
8.1.1. pCMV5-VEGF$_{164}$

Mouse VEGF$_{164}$ cDNA, 583 bp cloned into BglII-HindIII site of pCMV5.
Construct also has Ampicillin resistance gene.
Breier et al. Development 1992; 114, 521-32



8.1.2. pCMV- β Galactosidase

Construct also has Ampicillin resistance gene.
Red face indicates unique site in gene
McGregor et al. Nucleic Acids Res 1989; 17, 2365



8.2. S.O.C Medium (Invitrogen)

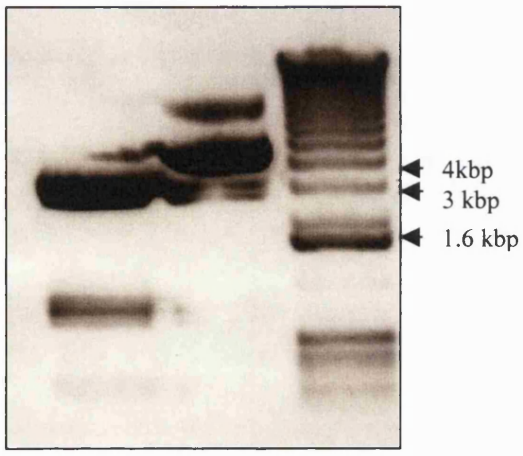
Composition:

2% tryptone
0.5% yeast extract
8.6 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
20 mM glucose

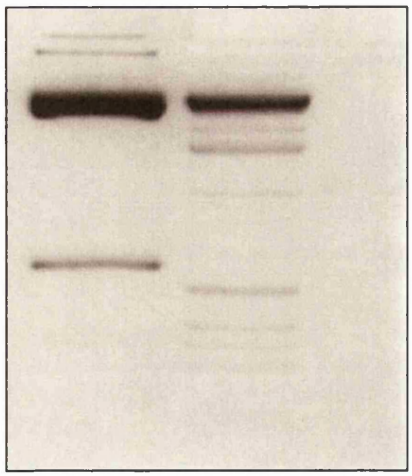
Adjust the pH to 7.0 with 5 M NaOH

8.3. Restriction Enzyme Digests of Plasmid DNA

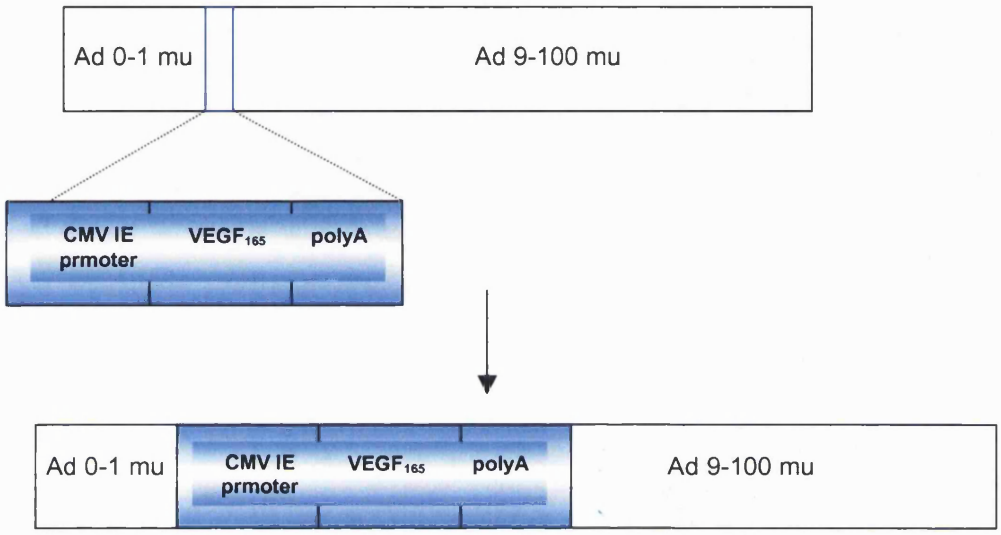
LacZ Plasmid



$VEGF_{164}$
Plasmid



8.4. Adenoviral construct encoding human VEGF-A_{165} (Ad.VEGF-A_{165})



mu = map unit

8.5. Virus Diluent Buffer

Stock	*Amount needed for 1 litre:*	*Final Concentation*
Tris HCl, 1M, pH 7.4	10 ml	10mM
NaCl, 5M	27.4 ml	137mM
KCl, 2.5M	2 ml	5mM
$MgCl_2$, 1M	1 ml	1mM

Filter solution in 0.22μm filter (Millipore, USA) and autoclave.
Stored at 4°C

8.6. Fixation Mediums After Tissue Harvest

1. ***1% Paraformaldehyde(PFA)-Sucrose, pH 7.4 (1 litre)***

Add 10 g paraformaldehyde (BDH) in 900 ml deoinized water
Add 4-5 pellets of NaOH (BDH)
Warm to 60-70˚C and mix until dissolves then cool to room temperature
Add 75 g sucrose, mix well
Add anti-oxidants: 0.5M EDTA, 4ml (Sigma); 10mM butylated hydroxytoluene, 5ml (Sigma)
Add deionized water until the final volume is 1 litre.
Adjust pH to 7.4 with HCl.

Store at 4˚C (and use within 1 month).

2. ***15% sucrose, pH 7.4 (1 litre)***

Add 150g sucrose to 1000ml deionized water
Adjust pH to 7.4 with HCl.

Store at 4 ˚C (and use within 1 month).

8.7 Diethylpyrocarbonate (DEPC) water (nuclease-free)

Stock	*Amount needed for 1 litre:*	*Final Concentation*
DEPC	1ml	0.1%

DEPC is stirred into solution and incubated for 2 hours at 37°C.
Solution autoclaved for at least 45 min, or until DEPC scent is gone.

8.8. DNase Treatment of RNA (Promega) – Prior to RT-PCR

The DNase digestion reaction was set up on ice as follows:

RNA (3µg) in water	1-15 µl
DNase x10 reaction buffer [8]	2 µl
RNase-free DNase (1 u/µl)	3 µl
RNase-free water to a final volume	20 µl

Incubate at 37°C for 30 mins in RNase-free tubes (Qiagen) within a Thermal Cycler.
1µl DNase Stop solution added to terminate reaction
Incubate at 65°C for 10 min to inactivate the DNase.

(Promega kit stored at -20 °C)

8.9. TAE Buffer

For 50 X TAE buffer:

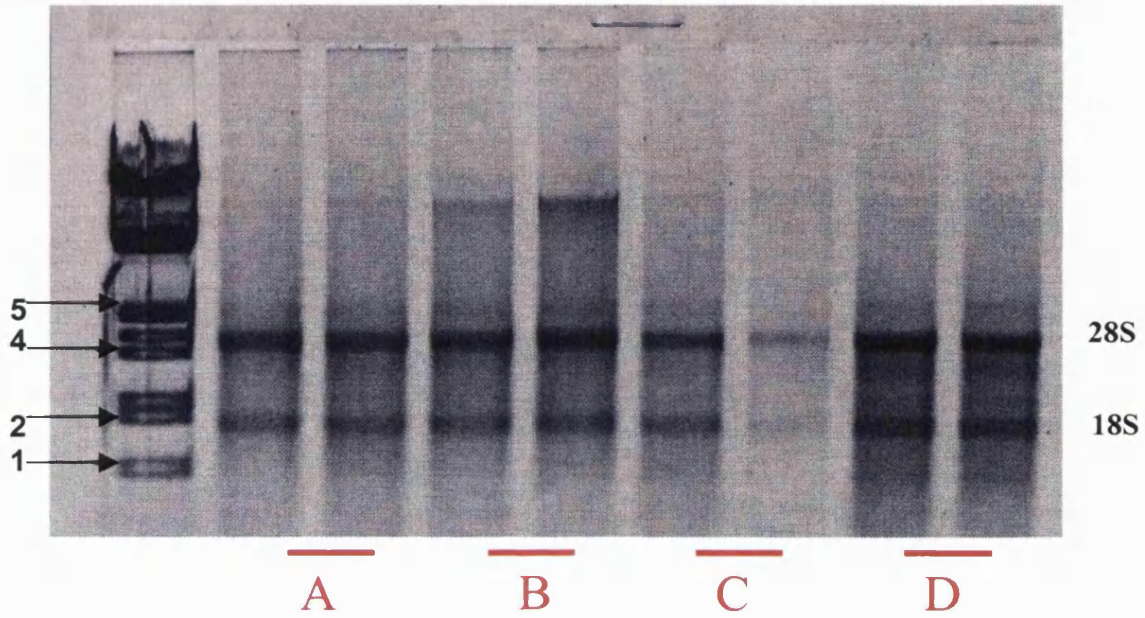
Stock	*Amount needed for 1 litre:*	*Final Concentation*
Tris Base	242g	2M
Glacial Acetic Acid[9] (17.4M)	57.1 ml	1M
Disodium EDTA (0.5M)	100 ml	0.05M

Add distilled/deionized H_20 to final volume of 1 litre and adjust pH to 8.0 with NaOH (1M)

[8] Reaction Buffer Composition:
400mM Tris-HCl, pH 8.0, 100mM $MgS0_4$, 10mM $CaCl_2$

[9] Glacial Acetic acid is 99.6% (w/v) acetic acid

Eg. RNA Analysis for Integrity Prior to Use in RT-PCR



Total RNA extracted from 4 pooled carotid arteries (100-150mg) for each treatment group using TRIZOL reagent. Each lane represents 500ng total RNA (in 10 µl RNAse-free water, 2 µl 6x Gel loading dye [10]).

Left lane represents marker – lamda HindIII/EcoR1 (0.5µg) (Promega).
A, B – RNA obtained from Lp-LacZ treated arteries.
C, D – RNA obtained from Lp-$VEGF_{164}$ treated arteries.
All samples are run in duplicate on a 1.2% agarose gel stained with ethidium bromide.

The integrity and size distribution of total RNA harvested from the carotid artery appear as sharp bands. 28S ribosomal RNA bands should be present at approximately twice the amount of the 18S RNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is completely intact.

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[10] ***Blue/Orange Loading Dye, 6X*** (Promega)
Contains 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0). It is provided in a premixed, ready-to-use form and certified free of nuclease activity.

[In a 0.5–1.4% agarose gel in 0.5X TBE, xylene cyanol FF migrates
at approximately 4kb, bromophenol blue at approximately 300bp
and orange G at approximately 50bp]

8.10. X-Gal Rinse [1 litre sodium phosphate buffer (pH 7.2)]

1M Na_2HPO_4, 68.4 ml and NaH_2PO_4 into 900 ml deionized water; Mix well

Store at 4 °C (and use within 1 month).

8.11. X-Gal Staining Solution

For a final working volume of *10 ml*:

6.75 ml 1x PBS, sterile filtered without $MgCl_2$ and $CaCl_2$
1ml Solution A
1ml Solution B
1ml Solution C
250 µl X-Gal[11]

Solution A (Stock 10X): 0.1% SDS in PBS

Stock	*Amount needed* for *100ml:*	*Final Concentration*
Igepal-CA360 (Sigma)	0.2 ml	0.2%
SDS	1ml of 10%SDS	0.1%
PBS	98.8 ml	

Solution B (Stock 10X):

Stock	*Amount needed* for *100ml:*	*Final Concentration*
0.5M $MgCl_2$ (Sigma)	4ml	20mM
PBS	96 ml	

Solution C (Stock 10X; protected from light to prevent oxidation):

Stock	*Amount needed* for *100ml:*	*Final Concentration*
Potassium Ferricyanide $K_3Fe(CN)_6$	3.28g	50mM
Potassium ferrocyanide $K_4Fe(CN)_6$	4.24g	50mM
PBS	100 ml	

[11] X-Gal (5-bromo-4-chloro-4-indolyl β D-galactopyranoside) (Sigma) was reconstituted according to manufacturer's instruction. Stock solution (100 mg/ml dimethylformamide, DMF) stored at -20 °C in aliquots and protected from light. Since DMF can dissolve plastic, DMF and the X-Gal stock solution were pipetted using polypropylene tips.

8.12. Tris-Buffered Saline (TBS) ± 0.1% Tween

10X TBS (0.5M Tris Base, 9% NaCl, pH 7.2):

61 g Trizma base (Sigma)
90 g NaCl (Sigma)

Mix to dissolve in 1 litre deoinized H_20 and adjust pH to 7.2 using concentrated HCl.
1ml Tween 20 added if required.
Solution stored at room temperature. Dilute 1:10 with deionized water before use and adjust pH if necessary.

CHAPTER 9

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