OSTEOPROTEGERIN:

A PATHOLOGICAL ROLE IN HUMAN ABDOMINAL AORTIC ANEURYSM

Thesis submitted by

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Corey Stephen Moran

October 2006

Declaration on Ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers H1464 and A964)

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Communications & Awards

1. CONFERENCES

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Moran CS, Golledge J. *Interaction between ang-II, OPG, and PPARγ in human AAA: A therapeutic pathway?* XIIIth meeting of the Australian Vascular Biology Society (AVBS); Gold Coast, Queensland; September 2006. (Poster presentation)

2. PAPERS

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3. AWARDS

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Abstract

Rupture of Abdominal Aortic Aneurysm (AAA) is the end-stage, catastrophic failure of the aneurysmal aortic wall and is associated with a mortality rate of up to 95 percent. Presently, surgery is the only treatment option available but carries with it a mortality rate of up to five percent and is usually reserved for repair of aneurysms showing high probability of rupture. What is required for the treatment of AAA, and essentially the basis of research in this area, is to understand the pathology of the disease well enough so that non-surgical intervention aimed at inhibiting small aneurysm progression can be developed.

The lack of non-invasive medical treatment for the disease, especially at the initial stages of development, stems from an incomplete understanding of its pathogenesis. Despite extensive laboratory and clinical research, the precise mechanisms leading to aneurysm formation remain unclear. The hallmark features of an aneurysmal aortic wall are degradation and fragmentation of the medial extracellular matrix (ECM), and significant reduction in smooth muscle cell (SMC) density, believed to be associated with the marked cellular inflammatory response also observed in the aneurysmal tissue.

A newly identified member of the tumour necrosis factor receptor superfamily known as osteoprotegerin (OPG) is constitutively expressed within the human artery wall and, under pathological conditions, is upregulated and associated with vascular disease. Elaboration on the involvement OPG of in AAA will determine its potential as a pharmacological target for the treatment of aneurysmal disease.

The focus of this study was to understand whether OPG might be important in the development of AAA. Two hypotheses were proposed:

1. Expression of OPG is upregulated in the aneurysmal aorta
2. Osteoprotegerin actively promotes aneurysm phenotype within the aortic wall

The specific aims of the study were to:

a) Assess relationship between aortic concentration of OPG and the presence of aneurysm

b) Define possible mechanism(s) by which OPG may be functionally active in the promotion of aneurysm development

c) Modulate aortic expression of OPG and assess the effect on aneurysm development

Serum OPG was correlated with aneurysm growth rate in 146 men with small AAA followed by ultrasound for 3 years (R=0.20; P=0.04), and a demonstrated predictor of aneurysm expansion on multiple-regression analysis (P=0.02; coefficient 1.33, SE 0.51) in a model consisting of patient age, diabetic status, smoking history, initial aortic diameter, serum cholesterol, and C-reactive protein. Western analysis showed 3-fold, 8-fold, and 12-fold greater OPG concentrations in human AAA biopsies compared to age and gender-matched atherosclerotic narrowed aorta (AOD; 1.4±0.1 ng/mg tissue vs 0.5±0.1 ng/mg tissue; P=0.002), post-mortem non-diseased abdominal aorta (PAA; 1.4±0.1 ng/mg tissue vs 0.2±0.1 ng/mg tissue; P<0.001), and non-diseased thoracic aorta (TA; 1.4±0.1 ng/mg tissue vs 0.1±0.06 ng/mg tissue; P<0.001), respectively. Resident vascular smooth muscle cells (VSMC) and infiltrating macrophages were identified as primary sources for OPG within the aneurysmal aortic media. The association between aortic expression of OPG and the presence of AAA was confirmed in an animal model of experimental aneurysm formation, in which levels of OPG protein were 4-fold greater in aneurysmal aortic tissue compared to non-aneurysmal tissue. Furthermore, aortic tissue levels of OPG in this model correlated strongly with vessel diameter.

Healthy human aortic VSMC incubated with recombinant human OPG (0-20 ng rhOPG/10^5 cells/ml/24h) developed an aneurysmal phenotype defined by dose-dependent impaired cell proliferation (P<0.001), increased apoptosis (P<0.01), decreased interleukin (IL)-6 expression (P<0.001), and increased matrix metalloproteinase (MMP)-9 activity (P=0.01). Gene expression in OPG-treated

VSMC reflected these results exhibiting downregulation of genes associated with cell growth and survival, and upregulation of genes that negatively regulate cell growth and promote cell death.

Incubation of human monocytic cells with OPG (0-20 ng rhOPG/10^5 cells/ml/24h) resulted in up to a 2-fold dose-dependent increase in IL-6 production in lipopolysaccharide (LPS)-activated cells (P=0.005). In addition, OPG (1 ng/10^5 cells/ml/24h) acted to induce a 2-fold increase in MMP-9 expression (P<0.001), with a 1.5-fold increase in MMP-2 production (P=0.01) in resting human monocytic cells.

Treatment of human AAA tissue in culture with the angiotensin II receptor blocker, Irbesartan, and the peroxisome proliferator-activated receptor gamma (PPARγ) ligands, Pioglitazone and Rosiglitazone, inhibited OPG production by up to 50%, as well as reducing inflammatory cytokine, and proteolytic enzyme production. The effects produced by thiazolidinedione treatment on aneurysm tissue *ex vivo* were reproduced *in vivo*. Both aortic expression of OPG and MMP activity within aortic tissue from a mouse model of experimental aneurysm formation were down-regulated significantly with Pioglitazone medication.

This study demonstrates for the first time the association of OPG with AAA and identifies a possible key role for the protein in the promotion of an aneurysmal phenotype within the normal aortic wall. The ability of existing medication to limit this action potentially opens a therapeutic pathway through which to limit aneurysm expansion in humans by targeting arterial expression of OPG.

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Abbreviations

AAA	Abdominal Aortic Aneurysm
AAMø	Aortic Aneurysm-derived Macrophage(s)
AASMC	Aneurysm Aortic Smooth Muscle Cell(s)
ACE	Angiotensin Converting Enzyme
AOD	Aortic Occlusive Disease
ApoE($^{-/-}$)	Apolipoprotein E Gene (homozygous deletion)
AT_1R	Angiotensin II Receptor Type 1
DMEM	Dulbecco's Modified Eagles Media
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ELISA	Enzyme-linked Immunoassay
FACS	Fluorescence-activated Cell Scanning
FBS	Foetal Bovine Serum
IFN	Interferon
IL	Interleukin
IRA	Infra-renal Aorta
LPS	Lipopolysaccharide
MMP	Matrix Metalloproteinase(s)
Mø	Macrophage(s)
NASMC	Normal Aortic Smooth Muscle Cell(s)
OPG	Osteoprotegerin
PAA	Post-mortem non-diseased Abdominal Aorta
PBM	Peripheral Blood Monocyte(s)
PPAR	Peroxisome Proliferator-activated Receptor
RANK	Receptor Activator of NFκB
RANKL (Rkl)	RANK Ligand
rhOPG	recombinant human Osteoprotegerin
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
SMC	Smooth Muscle Cell(s)
SRA	Supra-renal Aorta
TA	Thoracic Aorta
TNF	Tumour Necrosis Factor
VSMC	Vascular Smooth Muscle Cell(s)

Chapter 1

INTRODUCTION

An abdominal aortic aneurysm (AAA) is an abnormal focal dilation of the distal aorta, usually below the renal arteries. The condition affects a significant proportion of the elderly population, between five and ten percent of males and one percent of females over 60 years of age (Wilmink & Quick, 1998; Lederle *et al,* 1995) and currently ranks 13th among the leading causes of death in the Western world (Lederle *et al,* 1995). The only treatment option presently available is elective or emergency surgical repair, a procedure not suitable in all cases. The lack of non-invasive pharmacological treatment for the disease, especially at the initial stages of development, stems from an incomplete understanding of its pathogenesis. Despite extensive laboratory and clinical research, the precise mechanisms leading to aneurysm formation remain unclear.

The hallmark features of an aneurysmal aortic wall are degradation and fragmentation of the medial extracellular matrix (ECM) (Gandhi *et al,* 1994), and reduction in SMC density thought to occur via a process of programmed cell death known as apoptosis (Lopez-Candales *et al,* 1997). These processes are believed to be associated with the marked cellular inflammatory response also observed in the aneurysmal tissue (Brophy et al, 1991). The inflammatory infiltrate in AAA is composed of lymphocytes and macrophages (Pearce & Koch, 1996). These immune cells are thought to play an aetiological role through their ability to produce apoptosis-inducing proinflammatory cytokines (Henderson *et al,* 1999) and ECM-degrading proteolytic enzymes known as matrix metalloproteinases (MMP) (Lee *et al,* 1995).

Osteoprotegerin (OPG) is a newly identified member of the tumour necrosis factor receptor superfamily and is constitutively expressed within the normal artery wall (Simonet *et al,* 1997; Dhore *et al*, 2001), being produced by both vascular smooth muscle cells (Hofbauer et al, 2001; Fu et al, 2002; Zhang et al, 2002) and endothelial

cells (Malyankar *et al*, 2000; Collin-Osdoby *et al*, 2001). Expression of arterial OPG is upregulated under pathological conditions and associated with vascular disease (Dhore *et al*, 2001; Zhang *et al*, 2002; Golledge *et al*, 2004; Olesen *et al*, 2005). Studies investigating the biological action of OPG *in vitro* have demonstrated that its expression is positively regulated by cytokines (Palmqvist *et al*, 2001; Sakata *et al*, 2001; Zhang *et al*, 2002) associated with MMP activation and recruitment of inflammatory cells in AAA (Newman et al, 1994). Furthermore, OPG itself exhibits an ability to regulate proteolytic enzyme activity (Wittrant *et al*, 2002), and influence immune and inflammatory cell function (Yun *et al*, 2001).

The focus of this study is to understand whether OPG might be important in the development of AAA. Two hypotheses were proposed:

1. Expression of OPG is upregulated in the aneurysmal aorta
2. Osteoprotegerin actively promotes aneurysm phenotype within the aortic wall

Specifically, the aims of study were to:

a) Assess relationship between aortic concentration of OPG and the presence of aneurysm
b) Define possible mechanism(s) by which OPG may be functionally active in the promotion of aneurysm development
c) Modulate aortic expression of OPG and assess the effect on aneurysm development

Rupture of AAA is the end-stage, catastrophic failure of the aneurysmal aortic wall and is associated with a mortality rate of up to 95 percent (Johansson & Swedenborg, 1986). Presently, surgery is the only treatment option available but carries with it a mortality rate of up to five percent (Feinglass *et al*, 1995) and is usually reserved for repair of aneurysms showing high probability of rupture. What is required for the treatment of AAA, and essentially the basis of research in this area, is to understand the pathology of the disease enough so that non-surgical intervention aimed at

inhibiting small aneurysm progression can be developed. Elaboration on the involvement of OPG in AAA will determine its potential as a pharmacological target for the treatment of aneurysmal disease.

Chapter 2

LITERATURE REVIEW

2.1 AORTIC STRUCTURE IN HEALTH

The aorta is the largest elastic artery and main trunk of the systemic arterial system. It arises from the left ventricle of the heart and terminates, bifurcating to form the left and right iliac arteries, at the left side of the body of the fourth lumbar vertebra. The aortic trunk comprises the ascending aorta, aortic arch, and descending aorta, and is divided anatomically into the thoracic aorta (superior to the diaphragm) and the abdominal aorta (inferior to the diaphragm). The normal abdominal aorta gradually tapers from cephalad to caudad as it gives off visceral branches and, consequently, pulse pressure in the aorta increases from proximal to distal. Moreover, the abdominal aorta is stiffer than more proximal regions reflecting a reduced number of elastic lamellae distally (Dingemans *et al,* 2000). The maximum normal diameter of the aorta averages two centimetres in adults, however diameter may vary with respect to age, gender, race, and body size.

2.1.1 The Aortic Wall

The aortic wall consists of three concentric layers (Figure 2.1.). Innermost, the Tunica Intima consists of a monolayer of endothelial cells lying on top of a basement membrane. The endothelium possesses anticoagulant properties and is actively involved in the regulation of vascular tone. The tunica media, separated from the intima by a dense elastic membrane (Internal Elastic Lamina), is composed of numerous concentric layers of smooth muscle cells (SMC) and a network of fenestrated elastic lamellae interposed between the SMC layers. The elastic and cohesive properties of the media are important in preserving haemodynamic equilibrium, reducing arterial pressure during systole by compliance and maintaining pressure by recoil during diastole. Thin fibrils of collagen are associated with the elastic lamellae; however this protein plays a more prominent role within the

outermost layer of the artery wall, the Adventitia. The External Elastic Lamina separates the Media from the Tunica Adventitia. The Adventitia is a connective tissue sheath composed of blood vessels (vasa vasorum) and nerves embedded in a collagen-rich matrix with fibroblasts and adipose cells. Several investigators have observed that fibril diameter of adventitial collagen considerably exceeds that of collagen fibrils found in the media (Buck, 1987; Merrilees et al, 1987). This may reflect the high tensile strength of the Adventitia and the role it plays in maintaining arterial wall integrity (Buck, 1987). Structural support and acting as scaffold for sympathetic nerve endings and vasa vasorum not withstanding, the contribution of the adventitia to vascular function has by and large been disregarded. There is, however, increasing support for the adventitia as a mediator of vascular function (Gutterman, 1999; Rey *et al,* 2002).

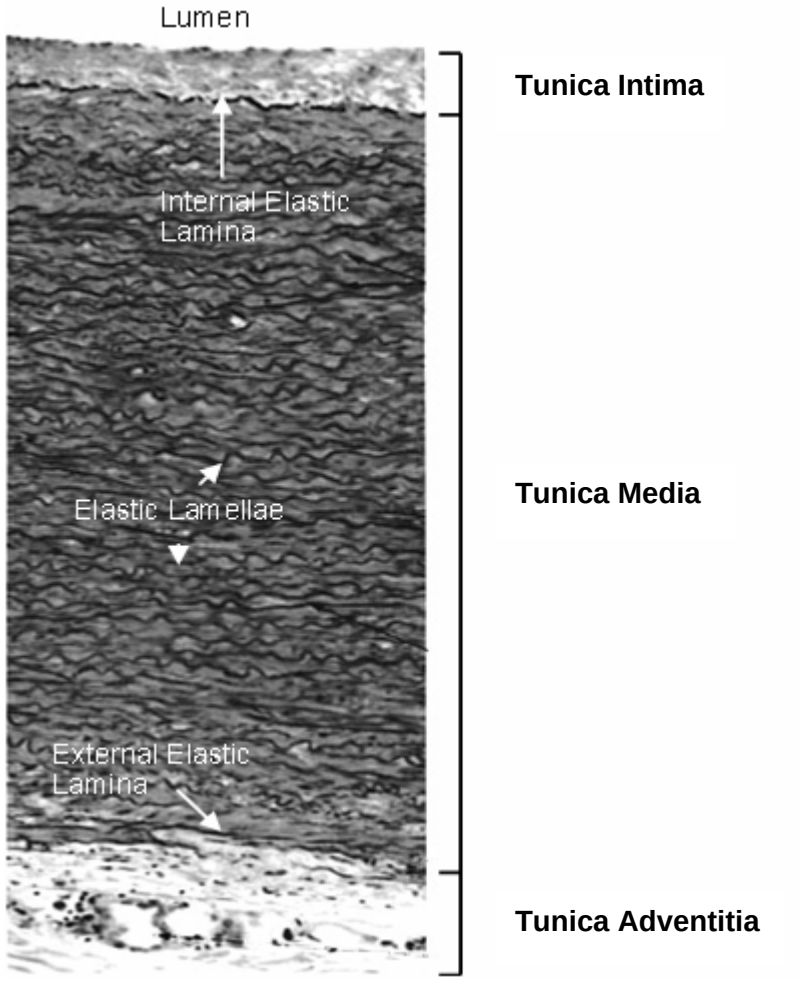


Figure 2.1. Cross-section through the normal aortic wall.

2.1.2. Aortic Extracellular Matrix: Tunica Media

2.1.2.1 Collagen

According to their structural characteristics, collagens have been divided into the 'fibril forming' and 'non-fibril forming' collagen subgroups (Miller & Gay, 1987). Of the former, fibrillar types I and III are the predominant collagens present in the vascular interstitial matrix (Rizzo *et al,* 1989). Type III collagen is often found in association with type I collagen in all soft tissues and is thought to participate in the tri-dimensional organization of type I collagen networks (Keene *et al,* 1987). These molecules assemble into cross-banded fibrils, seen particularly surrounding fibroblasts in the adventitial layer (Rizzo *et al,* 1989), and provide tensile strength to the vessel wall.

Collagen types IV, VI, and VIII are the non-fibrillar collagens present in normal vessel ECM (Miller & Gay, 1987). Within basement membranes beneath endothelial cells and surrounding medial SMC, collagen types IV and VIII form three-dimensional networks that serve as an anchoring substrate and assist in the formation of a permeability barrier (Dingemans *et al,* 2000; Timpl & Brown,1996). High molecular weight type VI collagen, formed via self-association and disulphide bonding, is commonly observed aggregated and situated between fibrils of type I and type III collagen (Katsuda *et al,* 1992).

2.1.2.2 Elastin

The elastic properties of the vasculature depend largely on the presence of elastin in the vessel wall. While collagens provide tensile strength, elastin assembled into elastic fibres provides distensibility and cohesiveness important in preserving haemodynamic equilibrium, reducing arterial pressure during systole by compliance and maintaining pressure by recoil during diastole thereby accommodating the pulsatile nature of blood flow. The aortic *tunica media* is composed of concentrically layered fenestrated elastic lamellae, between which resides a monolayer of SMC. Elastic lamellae begin to develop early in foetal life and rates of elastin synthesis in

blood vessels increase to a maximum in the perinatal period with synthesis rapidly decreasing soon thereafter (Bendeck & Langille, 1991). Mature cross-linked elastic fibres have an extremely slow rate of turnover exhibiting a half-life ranging between 40 and 70 years, so, despite vigorous synthesis in young growing vessels, newly synthesized elastin is virtually nonexistent in normal adult vessels (Davidson, 1987; Schapiro *et al,* 1991).

An insoluble amorphous core of elastin with a surrounding lattice of microfibrils primarily constitutes elastic fibres (Rosenbloom *et al,* 1993). Elastin is secreted from the cell (SMC) as a 72-kDa soluble precursor, tropoelastin. Within the extracellular matrix (ECM), tropoelastin is cross-linked by the action of the metalloenzyme lysyl oxidase (LOX) (Eyre *et al,* 1984; Rosenbloom *et al,* 1993), resulting in the irreversible polymerization of the monomer onto a pre-existing microfibrillar 'scaffold' comprised primarily of the glycoprotein, fibrillin (Reinhardt *et al,* 1995; Sakai *et al,* 1986). Cross-linked elastin molecules are organized in a random-coil configuration. It is this cross-linked random coiled structure of elastin that determines the capacity of the elastic network to stretch and recoil (Rosenbloom *et al,* 1993).

The *lamellar unit*, described by Wolinsky and Glagov (1967) is a basic element of the aortic media consisting of two elastic lamellae and intervening tissue. More precisely, the extracellular matrix separates a single central layer of SMC, the exclusive cellular component in the media, from the elastic lamellae on either side (Figure 2.2.). The number of lamellar units present in the normal aortic media ranges between 50 and 80 (Nakashima *et al,* 1990). Within the lamellar unit, SMC are surrounded by a basement membrane comprising laminin, collagen (type IV), proteoglycans (perlecan), and glycoprotein (entactin), and are embedded in an interstitial matrix of collagen type I and III, proteoglycan, and glycoprotein (fibronectin) (Dingemans *et al,* 2000). Consequently, relatively few direct contacts of SMC with solid elastic lamellae have been described. However, apart from constituting solid lamellae and fibres, elastin also forms microfibrillar 'streaks' that

Figure 2.2. Lamellar Unit. EL, elastic lamellae; SMC, smooth muscle cell. (Adapted from Dingemans *et al,* 2000)

protrude from the elastic lamellae and are observed in close association with SMC (Dingemans *et al,* 2000). Additionally, so-called oxytalan fibres, ubiquitous structural fibres containing fibrillin-1 and type IV collagen, are suspected to play a role in SMC-elastin contact by bridge-link between the cells and elastic lamellae (Davis, 1985). Interestingly, the glycoprotein fibronectin, which has been shown to play a role in the bridging of SMC to adjacent SMC (Clark & Glagov, 1985), may also be significant in the SMC-elastin linking process, its presence having been detected on the SMC-directed side of oxytalan fibres (Dingemans *et al,* 2000).

Aortic elastic lamellae are closely associated with collagen fibres types I, III, and V, orientated circumferentially and parallel to the main axis of the SMC (Dingemans *et al,* 2000). Observed in association with these fibres are the small dermatan sulfate proteoglycans biglycan and decorin (Reissen *et al,* 1994), thought to regulate fibrilogenesis and organization of collagen fibrils into larger fibres (Poole, 1986).

A major interstitial proteoglycan present in the aortic media is versican, a protein-polysaccharide complex covalently linked with the glycosaminoglycan (GAG)

chondroitin sulfate (Wight *et al*, 1991). Versican's role in the media is believed to be interaction with hyaluronan, a free existing GAG in the ECM, to form large hydrating (H_2O-binding) aggregates that fill the ECM space not occupied by the other fibrous proteins such as collagens and elastic fibres. These complexes create a reversibly compressive compartment and provide a swelling pressure within the ECM, offset by collagen and elastic fibres, which functions to maintain structural shape and sustain compression generated by pulsatile forces (Radhakrishnamurthy *et al*, 1993; Levesque *et al*, 1994).

2.1.3 Aortic Response to Physiological Change: Vascular Remodeling

Vascular remodeling contributes to physiological processes such as angiogenesis and blood vessel regression during foetal development and growth (Pepper, 2001). Defined as any enduring change in the size and/or composition of an adult blood vessel, remodeling not only allows blood vessels to adapt and heal but also underlies the pathogenesis of major cardiovascular diseases (Gibbons & Dzau, 1994). In non-disease conditions, remodeling is a homeostatic response to changes in blood flow and circumferential stretch to restore normal shear stress and wall tension, respectively (Langille, 1996). Remodeling of the vascular wall in response to changes in its haemodynamic environment may occur through a variety of structural adaptations resulting in vessel enlargement (outward remodeling) or diminution (inward remodeling). The direction and extent of remodeling is believed to be coordinated via endothelial production of growth factors and proteases (Herity *et al,* 1999).

Outward remodeling is largely dependent on shear-responsive production of nitric oxide by the vascular endothelium (Tronc *et al,* 1996). The pivotal role of nitric oxide stems from its ability to induce the activity of proteolytic enzymes (Sasaki *et al,* 1998), and to both inhibit the proliferation of, and promote apoptosis in, vascular SMC (Cooke & Dzau, 1997; Moran *et al,* 1997). Outward remodeling preserves the artery lumen, however, it may ultimately increase the propensity for atherosclerotic plaque destabilization and rupture (Collen, 1999). Also, dilation associated with aortic aneurysm may represent an extreme form of outward remodeling, thus

increased matrix degradation that enables outward remodeling may eventually result in weakening of the vessel wall.

In contrast, increased production of mitogenic and fibrogenic growth factors, (e.g. platelet-derived growth factor and transforming growth factor-β) within the artery wall, typically seen in association with low-flow states, may mediate inward remodeling by stimulating SMC proliferation and collagen deposition/cross-linking, with activation of proteolytic enzymes functioning to reorganize vessel structure (Mondy *et al*, 1997; Bassiouny *et al*, 1998).

2.1.3.1 Extracellular Proteolytic Systems

Remodeling of the arterial wall requires degradation of the ECM. Under normal conditions, elastin and collagen fibres resist spontaneous breakdown and can only be degraded by proteolytic enzyme activity. A variety of proteases have the potential to degrade the ECM, however two proteolytic systems in particular, the serine proteinase, fibrinolytic plasminogen/plasmin system, and the matrix metalloproteinase (MMP) system, are central to vascular remodeling (reviewed, Lijnen, 2001). The plasminogen/plasmin system can directly degrade only a limited number of ECM proteins (fibrin, fibronectin, and laminin) (Dano *et al*, 1985). Nevertheless, once generated, active plasmin can degrade several non-fibrillar proteins of the ECM and, more significantly, convert several latent proMMPs (zymogens) to active forms (Birkedal-Hansen, 1995; Alexander & Werb, 1989; He *et al*, 1989; Chapman & Stone, 1989). The MMP family is an ever-expanding class of endopeptidases currently consisting of more than 20 members, each having distinct but often overlapping substrate specificities, and together, possess enzymic activity against virtually all ECM components (Nagase, 1997).

(i) Plasminogen/Plasmin System

The serine proteases of the plasminogen/plasmin system have traditionally been considered as part of the haemostatic mechanism owing to the dissolution of fibrin clots by plasmin (fibrinolysis). However, over the last decade, an additional role for

this system in ECM proteolysis, cell migration, and tissue remodeling has become evident (Collen, 1999). The existence of two immunologically distinct physiologic plasminogen activators (PA), which convert the inactive zymogen, plasminogen, to the active protease, plasmin (Collen, 1999), accounts to some extent for these dual functions. Tissue-type plasminogen activator (tPA) is considered the primary fibrinolytic activator, with urokinase-type plasminogen activator (uPA) the primary cellular activator and involved primarily in ECM proteolysis (Bugge *et al,* 1996).

The expression of tPA *in vivo* is somewhat restricted. Endothelial cells are thought to be the primary source of endogenous tPA, the only protease of the haemostatic system that is continuously secreted from the endothelium in active form (Kristensen *et al*, 1984; Hrafnkelsdottir *et al*, 2004). Ellis and Whawell (1997) demonstrated the expression of tPA in vascular SMC upregulated in response to arterial injury, suggesting the potential influence of tPA in migration and/or phenotypic changes of these cells. Studies indicate that uPA is mainly produced by inflammatory cells (macrophages and leukocytes) (Reilly, 1996) consistent with its activity of mediating activation of plasminogen in tissues and ECM proteolysis (Bugge *et al,* 1996). Urokinase plasminogen activator possesses pleiotropic function with recent findings suggesting its critical involvement in cancer formation and metastasis (Choong & Nadesapillai, 2003; Duffy, 2004), and the pathogenesis of inflammation and immunity (Reviewed Mondino & Blasi, 2004).

The conversion of plasmin from plasminogen is regulated by specific physiological antagonists known as *plasminogen-activator inhibitors* (PAIs) (Pannekoek *et al,* 1986). Plasminogen-activator inhibitor types 1 and 2 (PAI-1 and PAI-2) belong to the superfamily of serine protease inhibitors, *serpins*, and are the principle inhibitors of the serine proteases tPA and uPA (Vassalli *et al,* 1991; Van Meijer & Pannekoek, 1995). Endothelium-derived PAI-1 is a multifunctional protein that rapidly forms inactive complexes with tPA and uPA, thereby preventing the conversion of plasminogen to plasmin. Undetected in serum of the healthy individual, PAI-2 is synthesized by the placenta during pregnancy and by monocyte/macrophages during chronic inflammation where it exhibits a high rate of uPA inactivation (Kruithof *et al,* 1995).

(ii) The Matrix Metalloproteinase (MMP) System

The MMPs constitute a family of proteinases that are part of a larger superfamily of zinc-dependent endopeptidases known as *metsincins* (Stocker *et al,* 1995). Matrix metalloproteinases are capable of degrading ECM and basement membrane components, thus contribute significantly to such processes as foetal development, tissue remodeling, tumour invasion, wound healing and angiogenesis (Matrisian, 1990; Parsons *et al,* 1997; Werb *et al,* 1999). In addition to their matrix degrading properties, studies have also demonstrated that MMPs have important roles in other biologic processes; most notably, activation of other MMPs (Knauper *et al,* 1996) and cytokines (TNFα) (Black *et al,* 1997), and the proteolysis of parent molecules to biologic proteins with separate and specific activities (e.g. angiostatin from plasminogen) (Cornelius *et al,* 1995).

Structurally, the multi-gene MMP family displays extensive sequence homology. Essentially, a pro-peptide domain (77-87 amino acids) that constitutes the amino-terminus of the enzyme follows a signal peptide (17-29 amino acids) required for secretion of the peptide from the cell (Nagase, 1999). The pro-peptide domain incorporates the 'cysteine switch', a unique conserved sequence with a cysteine residue that ligates the catalytic zinc to maintain the latency of the proenzyme form (Springman *et al,* 1990). The catalytic domain (~170 amino acids) contains the catalytic machinery including the zinc-binding site and a conserved methionine (Stocker *et al,* 1995). This domain contains additional zinc and calcium ions that maintain the three dimensional structure of MMPs, necessary for their stability and enzymic activity. The carboxy-terminal hemopexin-like domain (~210 amino acids) possesses four units of a four-stranded antiparallel beta sheet resulting in a four bladed propeller-like structure, and plays a central role in substrate specificity (Massova *et al,* 1998). The amino-terminal catalytic domain and the carboxy-terminal hemopexin domain are connected at the so-called *hinge-region* by a flexible proline-rich linker domain (Li *et al*, 1995). The MMP family is broadly classified into five major subclasses, *Collagenases*, *Gelatinases*, *Stromelysins*, *Membrane-type MMPs*, and '*Other*', based on substrate specificity and structures (Table 2.1.).

Table 2.1. MMP subclasses and their extracellular substrates

Subclass	Species	Substrate
Collagenases	MMP-1, MMP-8, MMP-13 MMP-18	Collagen Types I, II, III
Gelatinases	MMP-2, MMP-9	Denatured collagen; elastin; basement membrane
Stromelysins	MMP-3, MMP-10, MMP-11 MMP-19, MMP-20	Broad substrate specificity; release of matrix-bound growth factors; basement membrane
Membrane-type (MT-MMPs)	MMP-14, MMP-15, MMP-16 MMP-17	Pro-MMPs (activation)
Other: Matrilysin	MMP-7	Broad substrate specificity *in vitro*; insoluble elastin; basement membrane; proteoglycans (tenascin/versican)
Human Macrophage Metalloelastase	HME; MMP-12	Elastin; type IV collagen; Fibronectin; vitronectin; basement membrane

The *Collagenases* and *Gelatinases* act primarily to cleave native fibrillar collagen types I, II, and III (Welgus *et al*, 1990; Hasty *et al,* 1987; Frieje *et al,* 1994; Stolow *et al,* 1996), and degrade elastin, denatured collagen, and basement membrane components (Hibbs *et al,* 1987; Wilhelm *et al,* 1989), respectively. *Stromelysins* possess broad substrate specificity and function in the release of matrix-bound growth factors (Basset *et al,* 1990; Manes *et al,* 1997).

Membrane-type (MT)-MMPs are the more recently described members of the MMP family (Sato *et al,* 1994; Will & Hinzmann, 1995; Takino *et al,* 1995; Puente *et al,* 1996). They are cell membrane-associated proteases that function not only in matrix remodeling, but also in pericellular, proteolytic activation of pro-MMP (Knauper *et al,* 1996). Synthesized in their active form intracellularly, MT-MMPs possess a transmembrane domain that anchors the enzyme and facilitates local MMP activation (Murphy *et al,* 1999). The members of the fifth ('*Other*') subclass include *Matrilysin* (MMP-7) and *Human Macrophage Metalloelastase* (HME; MMP-12). Matrilysin is the smallest of the MMPs, lacking the hemopexin domain (Uria & Lopez-Otin,

2000), however possesses broad substrate specificity *in vitro*, degrades insoluble elastin, and most basement membrane components (Murphy *et al*, 1991). Its substrates also include two proteoglycans, tenascin and versican, the latter of which is also efficiently degraded in vivo (Halpert *et al,* 1996). On a molar basis, MMP-12 is the most active MMP against elastin (Shapiro & Senior, 1998). Nonetheless, MMP-12 possesses a broad substrate specificity being able to degrade type IV collagen, fibronectin, vitronectin, and basement membrane components (Chandler *et al*, 1996; Gronski *et al*, 1997). Recent findings also suggest that MMP-12 aids (basement membrane) transmigration of macrophages in inflammatory conditions (Vaalamo *et al,* 1999). Metalloproteinases 21, 22, 23, 24 (MT-MMP-5) and 25 (MT-MMP-6) have been described and included in the MMP family, however their respective substrates, and in some cases domain structure, remain to be determined.

Matrix metalloproteinases are expressed by a variety of cell types (parenchymal, connective tissue, inflammatory) (McDonnell *et al*, 1999). They are regulated at the transcriptional level by cytokines, growth factors, and cell-matrix interaction (Nagase, 1997; reviewed, Birkedal-Hansen *et al,* 1993). The enzymes are secreted as latent precursors (*zymogens*), most of which are proteolytically activated in the extracellular space by other proteases (plasmin, MMPs), or on the cell membrane via cell-associated MT-MMPs. Activation of the latent form involves removal of the pro-domain. This is dependent on disruption of the cysteine-zinc bond, which leads to autocatalytic cleavage to generate the fully activated MMP (Murphy *et al,* 1999).

The activation of MMPs and their proteolytic potential is tightly controlled, being regulated at multiple levels: gene transcription and synthesis of inactive zymogens, posttranslational activation of zymogens, and interactions of secreted MMPs with endogenous tissue inhibitors of metalloproteinases (TIMPs) (Brew *et al*, 2000). The MMP/TIMP ratio is critical for coordinating matrix production and degradation (Vincenti, 2001; Guedez *et al,* 1996). TIMPs form non-covalent 1:1 stoichiometric complexes with the active forms of MMPs as well as with some proMMPs, thereby inhibiting MMP activity or impairing the activation of proMMPs (Brew *et al*, 2000). To date, four distinct TIMP proteins have been identified, among which only TIMP-1, 2, and 3 have been characterized in vascular tissue (Fabunmi *et al,* 1996). TIMP-1

and 2 are secreted constitutively as soluble proteins (Galis *et al,* 1994) and TIMP-3 remains bound to the ECM (Leco *et al,* 1994). All three appear to be equally effective in binding to the catalytic site and inhibiting the active forms of collagenase, the gelatinases, and stromelysin (Cawston, 1996). TIMP-1 and 2 also interact with a separate carboxy-terminal site differentially on the two gelatinases (MMP-2 and 9); TIMP-1 binds and slows activation of the latent pro-form of MMP-9 (O'Connell *et al,* 1994) and TIMP-2 binds to and regulates the activation of proMMP-2 (Lemaitre *et al,* 2001).

Observations in normal and diseased human and experimental blood vessels indicate that both vascular and inflammatory cells (macrophages) produce MMPs, although the spectra of MMPs secreted basally or in response to stimuli are distinctive. The major cellular components of normal blood vessels, endothelial cells and SMC constitutively produce MMP-2, TIMP-1, and TIMP-2 *in vitro*. However, focally increased expression of several MMPs and presence of MMP activity is observed in diseased human arteries, and in association with arterial morphological changes in experimental models of vascular disease, demonstrates the contribution of MMPs to pathological vascular conditions characterized by arterial wall remodeling.

2.1.3.2 Vascular Cell (SMC) Apoptosis

Apoptosis of vascular cells is a prominent feature of blood vessel remodeling that occurs during normal development and under pathological conditions. Nevertheless, the role of vascular SMC apoptosis *per se* in determining the outcome of remodeling is uncertain, although the concept of 'cell suicide' may be considered a mechanism that counterbalances the effect of cell proliferation by mitotic division. This suicidal feature of apoptosis applies to embryonic development and morphogenesis as well as adult tissue turnover and provides a major mechanism by which tissues remove unwanted, aged, or damaged cells. Haemodynamic changes affect vessel wall tension and cell-matrix interactions, and it may be these factors that alter the survival characteristics of vascular cells. Vascular SMC apoptosis occurs when wall tension is diminished (Bayer *et al*, 1999), or enhanced (Lauth *et al*, 2000), or when the expression of matrix components (Jones *et al*, 1997) or MMPs (Baker *et al*, 1998) is

altered. Furthermore, although changes in pro-apoptotic proteins have been associated with flow-induced vascular remodeling (Kim *et al,* 2000), relatively little is known about the molecular mechanisms that regulate vascular cell viability under these circumstances.

Kerr and co-workers (1972) first described apoptosis 30 years ago as a specific type of cell death morphologically distinct from 'accidental cell death' (necrosis/oncosis) (Majno & Joris, 1995) (Table 2.2.). During oncosis, a depletion of intracellular adenosine triphosphate (ATP) is observed along with swelling and disruption of the cell membrane, releasing cytoplasmic contents into the extracellular space. In contrast, apoptosis takes place in cells with normal ATP levels and requires messenger RNA and protein synthesis (Reid, 1989). A cell undergoing apoptosis exhibits chromatin condensation and margination that result in a half-moon or horseshoe appearance of the nucleus (Majno & Joris, 1995).

Table 2.2. Feature differences between Apoptosis and Oncosis (Necrosis)

	Apoptosis	**Oncosis**
Death process	Programmed	Accidental
Triggers	External/Internal	External
Dying cells	Individual	Group
Cell body	Shrinking	Swelling
Cell membrane	Intact	Broken
Cytoskeleton	Collapse before death	Collapse after death
Mitochondria	Intact with cytochrome-c release and ATP synthesis	Broken without ATP synthesis
Nucleus	Shrinking and fragmented	Enlarged and broken
Chromatin	Condensing	Clumping
DNA	Internucleosomal fragmentation	Randomized fragmentation

Adapted from Geng *et al,* 2002

During later stages, nuclear fragmentation (*karyorrhexis*) becomes evident, cytoplasm progressively condenses, and one or more so-called *apoptotic bodies* are formed from each dying cell (Majno & Joris, 1995; Figure 2.3.). Cleavage of genomic DNA within internucleosomal DNA segments by a selectively activated endonuclease during apoptosis is the basis of the most commonly employed technique to detect apoptotic cell death (Enari *et al*, 1998). Separation of cellular DNA on agarose gel shows a characteristic ladder-like pattern of fragments with multiples of 200 base pairs in length (Wyllie, 1992).

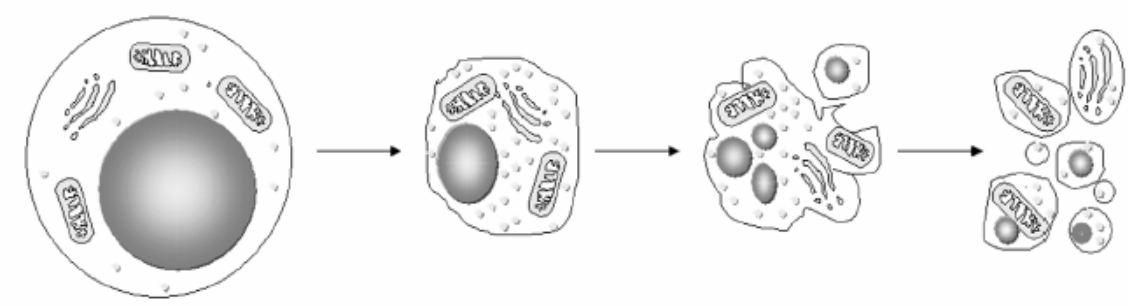


Figure 2.3. Morphological sequence of apoptosis: cell shrinkage in the presence of an intact cell membrane associated with cytoplasmic condensation, karyorrhexis, and formation of apoptotic bodies.

In light of its well-ordered cascade of cellular events, investigators often refer to apoptosis as *programmed* cell death. A key phenomenon of apoptotic cell death is the activation of a unique class of aspartate-specific proteases termed *Caspases* (Alnemri *et al,* 1996). All members of the caspase family, which now number 14, show similar substrate cleavage at an aspartyl residue and require processing of a zymogen to attain activity. Caspases can be categorized into three groups based on their functions (see Thornberry & Lazebnik, 1998): (1) those that have a limited role in apoptosis and participate mainly in cytokine activation and inflammation (caspases 1, 4, 5, and 11 to 14); (2) those that serve as initiators of apoptosis (caspases 2 and 8 to 10); and (3) those that act as effectors of apoptosis (caspases 3, 6, and 7).

Studies have shown that activation of caspases may take place either within 'death receptor' complexes of the cytoplasmic membrane, or by a mitochondrion-dependent mechanism within the cytosol (Smith K *et al,* 2003; Kluck *et al,* 1997; Figure 2.4.).

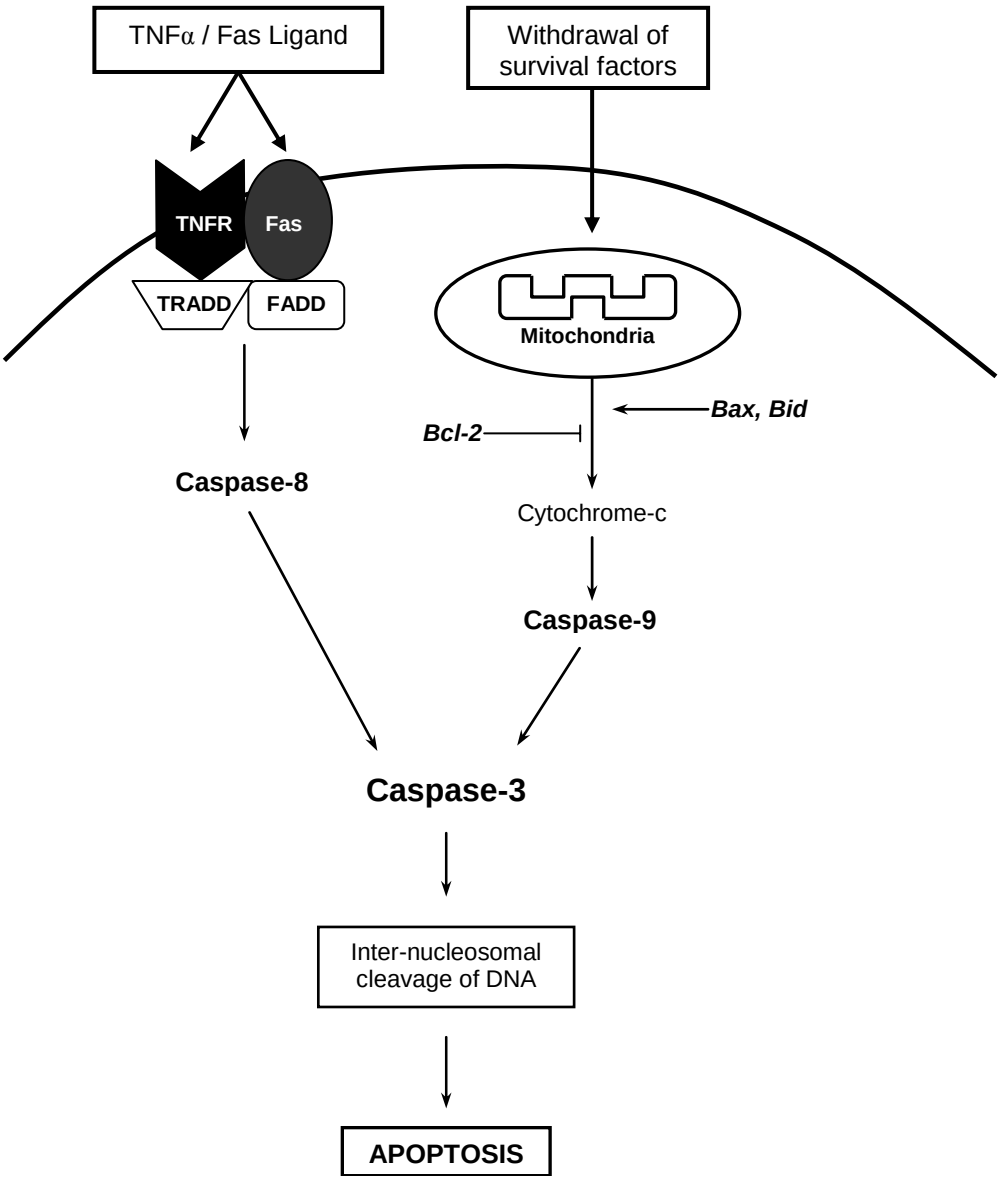


Figure 2.4. Schematic illustrating apoptosis pathway. Caspases are activated by a membrane receptor- or mitochondrion-dependent mechanism. Subsequent cleavage of multiple cellular target proteins results in apoptotic cell death. TRADD, TNF receptor-associated death domain; FADD, Fas-associated death domain.

The tumour necrosis factor (TNF) receptor superfamily presently consists of a group of more than 20 proteins with a wide range of different functions, including regulation of cell survival, differentiation, immune regulation, and control of gene expression (Daniel *et al*, 2001). Within the TNF receptor-related molecules, one subgroup is defined as the *death receptors*, as one of their main functions is to induce programmed cell death (Schultze-Osthoff *et al,* 1998). The binding of death-promoting proteins (TNF-α and Fas Ligand) to their cognate cell surface receptors recruits intracellular adaptor proteins (TRADD and FADD respectively) and caspase-8 (Cohen, 1997). The resultant protein complex, together with the receptors, forms the death-inducing signal complex (DISC) (Daniel *et al*, 2001).

The pivotal role of mitochondria in the regulation of apoptosis is becoming clearer (Kluck *et al,* 1997; Green & Reed, 1998). Translocation of cytochrome C from mitochondria into the cytosol is an early event in the apoptotic process, where it interacts with apoptotic protease-activating factor (Apaf)-1 to recruit and activate Caspase-9 (Li *et al*, 1997). Both caspase-9 and the death receptor-activated caspase-8 can activate the final executioner, caspase-3, which in turn causes the irreversible fragmentation and degradation of DNA (Slee *et al*, 1999).

Apoptosis via the mitochondrial pathway is regulated in part by a family of more than fifteen cytoplasmic proteins known as the Bcl-2 proteins (Adams & Cory, 1998), which fall into two subgroups based on their roles in apoptosis regulation. The first group comprises the anti-apoptotic proteins Bcl-2, Bcl-X, Mcl-1, Bcl-w, and A1, whilst the second, pro-apoptotic group, includes the Bax, Bak, Bad, Bik, Hrk, Bid, and Bcl-xs proteins. The process by which Bcl-2 inhibits apoptosis remains obscure, however numerous studies have examined the role of Blc-2 family proteins in controlling vascular cell viability. Bax expression is elevated in vascular SMC of human atherosclerotic plaques, where high frequencies of apoptosis are observed (Kockx *et al,* 1998). This is reflective of the situation in the neonate where vascular remodeling and regression are associated with upregulation of Bax expression in vascular SMCs (Kim *et al,* 2000). Under normal physiological conditions, the anti-apoptotic protein Bcl-X is abundantly expressed in SMC of the arterial media, but down regulated following insult with a time course that correlates with the early

wave of apoptotic cell death (Perlman *et al,* 1997). Collectively, these studies, together with acute ablation investigations (Pollman *et al,* 1998; Perlman H *et al*, 2000), demonstrate that endogenous levels of Bcl-2 and Bcl-X are essential for vascular SMC viability, and thus, stimuli that alter expression of these proteins could influence SMC survival in the vessel wall.

In addition to the Bcl-2 family proteins, evidence suggests that the transcription factor and tumour suppressor gene *p53* may also regulate the apoptosis of vascular cells (Ihling *et al,* 1998). This factor promotes apoptosis by functioning, at least in part, as a positive regulator of Bax expression (Miyashita & Reed, 1995) and a negative regulator of Bcl-2 expression (Miyashita *et al,* 1994). Moreover, p53 has been shown to promote vascular SMC apoptosis by increasing cell surface expression of the death ligand receptor Fas (Bennet *et al,* 1998). In an extensive analysis of the gene expression pattern during p53-mediated apoptosis (Polyak *et al,* 1997), seven of the 14 genes that were induced at least 10-fold by p53 were involved in cellular redox reactions. Since antioxidants reduced the extent of p53-mediated apoptosis, oxidative stress may constitute a significant intermediary step in apoptosis induced by p53 (Polyak *et al,* 1997).

2.1.3.3 Vasculopathology of Ageing

Structural, architectural, and compositional modifications take place in the vasculature with advancing age (reviewed, Bilato & Crow, 1996). Briefly, vessel diameter tends to increase, and thickening of intimal and medial layers is often observed. In the subendothelial space, blood-borne leukocytes and an increased amount of "activated" (fibrogenic) smooth muscle cells are present. Extracellular matrix accumulates and becomes particularly rich in glycosaminoglycans. Collagen content increases, while elastin fibres appear progressively disorganized, thinner, and frequently fragmented. In a study of age-related changes in the human aorta, Spina and co-workers (1983) found the amount of glycoprotein associated with elastin increased with age and the effect of this component was to stiffen the tissue. Matrix metalloproteinases (MMPs) are principally involved in the repair and maintenance process, which works to offset adverse age-related changes in the vessel wall.

Stromelysin-1 (MMP-3) may be particularly significant to arterial wall remodeling in both ageing and disease progression, as a result of its broad substrate specificity which includes fibronectin, type IV and V collagens, gelatin, laminins, elastin, and proteoglycan proteins (Nagase & Woessner, 1999). Indeed, Medley and co-workers (Medley *et al,* 2001) demonstrated that *MMP-3 genotype* is associated with large artery stiffness in older, but not young or middle-aged, healthy individuals.

These changes in the normal architecture of the vessel wall, that could be referred to as "the vasculopathology of ageing", are likely to be the consequence of adaptive mechanisms to maintain normal conditions of flow, mechanical stress and/or wall tension. They occur in everyone but not necessarily at the same rate, thus accounting for the differences seen in some people between chronological age and physiological age (Cheitlin, 2003). It is thought that cardiovascular structure and function change with time due to an "ageing process", and that this process, over time, alters the substrate upon which specific pathophysological disease mechanisms become superimposed (*vascular ageing*) (Lakatta & Levy, 2003). Vascular disease occurring at younger ages is attributable not only to exaggerated traditional risk factors, but also to accelerated, risk factor-mediated, ageing of the vascular wall.

Age-dependent stiffening of the artery wall is due largely to cyclic mechanical stress-mediated structural modification, resulting in a decrease in wall elasticity (Kingwell *et al,* 2001). The effect of cyclic stress is to gradually reorganize crystalline structure and cause it to fracture at a load previously sustainable. By middle age, the human aorta has undergone more than one billion cycles of expansion and contraction, and the fatiguing effects of cyclic stress lead to progressive disorganization of elastin fibres and the transfer of load to collagen fibres (Nichols & O'Rourke, 1999). This process is visible microscopically as a fracturing and loss of regularity in the elastic layers of the tunica media. The elastic fibre network develops longitudinal fissures, transverse breaks, and fragmentation. In addition, the elastin-collagen ratio is reduced reflecting fibrous remodeling (Nichols & O'Rourke, 1999). Collagen is approximately 1000-fold stiffer than elastin and the gradual loss of elastin is inevitably accompanied by a reduction in vascular compliance. Loss of compliance leads to a rise in pulse pressure and an increase in circumferential stress in the

arterial wall. Vascular smooth muscle cells respond to mechanical stretch by synthesizing collagen (Leung *et al,* 1977), and this, together with cellular hypertrophy, results in the thickening of the vessel wall and a further decrease in compliance. Subsequently, a feedback loop is established that tends to maintain higher levels of blood pressure.

Vascular ageing is also characterized by endothelial cell dysfunction (Cernadas *et al*, 1998). Endothelium-dependent relaxation declines with increasing age (Tschudi *et al*, 1996). Although the underlying cellular and molecular mechanisms associated with age related endothelial dysfunction have not been clarified, they might involve changes in expression and/or activity of eNOS (Cernadas *et al*, 1998), increased breakdown of NO due to an augmented production of superoxide anions (Gryglewski *et al,* 1986), or a gradual loss of antioxidant capacity (Azhar *et al,* 1995) which normally provides cellular protection against reactive oxygen species (endothelial injury).

Nonenzymic glycation of the vascular ECM and the accumulation of advance glycation end-products (AGEs) within the vessel wall (McVeigh *et al*, 1999) contributes significantly to arterial stiffening and vascular ageing. All proteins are prone to AGE formation and, once formed, AGEs cannot be removed from proteins unless the protein is removed (Reiser, 1998). As such, AGEs accumulate in all tissues with age, however this accumulation is most pronounced in tissues with long-lived proteins such as collagen and elastin (Verzijl *et al*, 2000). Glycation affects the interactions of collagen with cells and other matrix components, but the most damaging effects are caused by the formation of glucose-mediated intermolecular cross-links. These cross-links decrease the critical flexibility and permeability of tissues and reduce turnover (Paul & Bailey, 1996). Biochemical analysis of human aorta revealed the fluorescent AGE, pentosidine, as the primary glycation cross-link responsible for aortic stiffening (Sims *et al,* 1996). Tomizawa and co-workers (1993) demonstrated that increased glycation of elastin is associated with calcium deposit in the aorta. A major age-associated change in arterial wall composition is the accumulation of calcium within the media (Niederhoffer *et al,* 1997). As calcium deposition is primarily associated with elastic fibres, calcium accumulation may be

responsible for the progressive destruction of elastic network integrity observed with ageing, and a major cause of an age-linked pressure-independent increase in arterial stiffness (Niederhoffer *et al,* 1997).

2.2 THE ANEURYSMAL AORTA

2.2.1 Clinical Background

2.2.1.1 Definition

A *true* aneurysm is a localized, abnormal dilation of an artery or vein facilitated by a congenital or an acquired weakness in the vessel wall. Clinically, the more commonly affected arteries are (in order) the aorta, iliac, popliteal, and femoral (Thompson & Bell, 2000). In 1991, the Society of Vascular Surgery and the International Society for Cardiovascular Surgery published a consensus (Johnston *et al*, 1991) defining 'aneurysm' as – "*a permanent localized dilation of an artery having at least 50 percent increase in diameter compared with the expected normal diameter of the artery, or diameter of the segment proximal to the dilation*". Moreover, an abdominal aortic aneurysm (AAA) is usually diagnosed when the aortic diameter exceeds 3.0cm.

2.2.1.2 Prevalence

Screening studies for AAA indicate prevalence for the disease in 5-10 percent of older males (Wilmink & Quick, 1998; Lederle *et al*, 2000). In men, the process appears to begin when aged approximately 50 years and reaches peak incidence when aged approximately 80 years. The disease is five times more common in men than women, in whom the onset of the disease is delayed and appears to begin after the age of 60 (Lederle *et al,* 2001).

Over the last five decades, there has been a progressive increase in the number of deaths from aortic aneurysm (Hafez *et al,* 2001). Associated with a mortality rate of

75-95 percent (Johansson & Swedenborg, 1986; Budd *et al,* 1989; Brown & Powel, 1999), rupture of AAA is the end-stage, catastrophic failure of the diseased aortic wall and is responsible for 1-2 percent of all annual deaths in the United Kingdom (Hafez *et al,* 2001), and currently ranks 13th among the leading causes of death in the Western world (Patel *et al,* 1995).

2.2.1.3 Risk Factors

Abdominal aortic aneurysm is associated with multiple factors. The Aneurysm Detection and Management (ADAM) screening programme conducted by Lederle and co-workers (Lederle *et al*, 2000) is the largest study to date aimed at understanding the prevalence and both positive and negative risk factors for AAA. The risk factors established as being most strongly associated with AAA are male sex and a history of smoking. Other positive associations with AAA include advancing age, family history, and hypertension. Female sex, black race, and diabetes mellitus have been established as the principle negative associations for the disease (Table 2.3.).

Table 2.3. Risk factors for abdominal aortic aneurysms

Risk Factor	Odds Ratio*	95% CI†
Increased Risk		
Smoking history	5.60	4.2-7.3
Family history	1.94	1.6-2.4
Advancing age	1.71	1.5-1.8
Hypertension	1.23	1.1-1.3
Decreased Risk		
Diabetes Mellitus	0.52	0.4-0.7
Female sex	0.18	0.1-0.7

*Odds ratio indicates relative risk compared to patients without that risk factor
†Confidence Interval
(adapted from Lederle *et al*, 2000)

The excess prevalence associated with smoking accounted for approximately 75% of all AAAs measuring 4cm or greater in the total study cohort. This supports the hypothesis that formation and progression of AAA is significantly smoking-related. Wilmik and co-workers (1999) determined that current smokers are 7.5 times more likely and former smokers three times more likely to have an AAA than non-smokers. In a study examining the relationship between smoking and the progression of existing aneurysms, MacSweeney and colleagues (1994) concluded that cessation of smoking could potentially lower the growth rate of small AAAs.

Prior to the ADAM study, many epidemiological studies on AAA reporting potential risk factors comprised low population sample sizes (<5000) resulting in too few cases for multivariate analysis. The ADAM risk factor data was obtained from a cohort of over 100,000 and has subsequently called into question some long-held beliefs regarding risk factor associations and AAA development. A presumed association between AAA and atherosclerosis (arterial occlusive disease, AOD), for example, was not confirmed by the ADAM findings, a conclusion reached also by Blanchard and co-workers (2000). In addition, data obtained in both the Blanchard and ADAM studies highlighted a somewhat counterintuitive negative risk association between diabetes and AAA. The apparent protective effect afforded by diabetes against the formation of AAA remains unexplained. However, the fact that diabetes patients are two to three times more likely to develop arterial occlusive disease (AOD) (Beckman *et al,* 2002) precludes the commonly held notion that aberrant remodeling of the artery wall associated with the advanced stages of AOD is causal for AAA development.

2.2.1.4 Natural History

The majority of AAA are asymptomatic and diagnosis is often made only after aneurysm rupture or following detection during unrelated medical examinations. Aneurysm size (diameter) is the most important determinant of likelihood of rupture (Thompson, 1996; Brady *et al,* 2001). This was confirmed in a recent surveillance study that monitored 1743 patients for changes in AAA diameter by ultrasonography over a mean follow-up period of 1.9 years (Brady *et al,* 2004).

A mean aneurysm growth rate of 2.6 mm (95% CI, -1.0 to 6.1 mm/year) per annum was reported and baseline diameter strongly associated with growth, suggesting that expansion accelerates as the aneurysm enlarges.

The risk of aneurysm rupture is difficult to estimate due to the lack of controlled studies addressing the natural history of AAA. The United Kingdom Small Aneurysm Trial (Powell *et al,* 2002) in which 1090 patients with AAA of 40 to 55 mm in diameter were studied, demonstrated that the risk of aneurysm rupture in these patients, followed by surveillance and intervention (if diameter exceeded 55 mm or patient developed symptoms) was approximately one percent per annum. Additionally, it was established that there is no long-term difference in mean survival as a consequence of early surgery and repair of small aneurysms, advocating the use of 'watchful waiting' until the threshold diameter of 55 mm is reached (Powell *et al,* 2002). For AAA of 50 to 60 mm, the annual risk of rupture is estimated as five percent increasing exponentially for larger aneurysms (Thompson, 1996). Subsequently, the widespread consensus is that surgical intervention is warranted if an aneurysm diameter exceeds 50 mm and no confounding factors are present that could significantly raise the risk of repair. The option of surgery is based on an analysis of the risk of aneurysm rupture compared with the mortality rate associated with the surgical procedure.

2.2.1.5 Management Options

Surgical repair of AAA is performed using either open or endovascular surgery. Open repair involves a full laparotomy, aortic clamping, and prosthetic graft insertion. In multi-center studies, this option is associated with a mortality rate of around 5% (Finlayson *et al,* 1999) and significant other morbidities such as myocardial infarction, respiratory complications, and renal failure. The more recently developed and less invasive procedure of endoluminal AAA repair requires placement of a stent graft within the aorta via small groin incision. Studies over the last ten years suggest this procedure may be associated with lower mortality and morbidity, although this has not yet been demonstrated in randomized controlled trials. Moreover, the long-term outcome of endovascular repair has been questioned

due to the later development of small connections between the general circulation and aneurysm sac (endoleak) in up to 20% of patients (Matsumura *et al*, 2003). Neither open nor endovascular surgery is suitable for all patients with AAA. Open surgery is inappropriate in patients with severe cardio-respiratory impairment due to their high mortality from the procedure. Whole endoluminal surgery is unsuitable for up to 30% of AAA due to the shape of the aneurysm (Matsumura *et al*, 2003). As mentioned above, the present decision regarding AAA management is a complex evaluation of the calculated risk of aneurysm rupture and the outcome of open or endoluminal surgery.

Investigations carried out *in vitro*, involving medication association studies, and randomized controlled trials to investigate prospective pharmacological therapies for AAA, have highlighted the potential of statins, angiotensin II inhibitors, and cyclooxygenase inhibitors in reducing the production of proteinases and cytokines in human AAA tissue (Franklin *et al*, 1999a; Franklin *et al*, 1999b; Wilson *et al*, 2005). Unfortunately, clinical studies examining the role of these medications in slowing AAA progression have been limited (Mosorin et al, 2001; Vammen et al, 2001). The largest and most comprehensive study to date examined the effect of propraholol in a randomized controlled trial of 548 patients with small AAA (30-50 mm) followed for a mean of 2.5 years (PATI, 2002), however, no effect on AAA growth, intervention rate, or mortality could be demonstrated. Moreover, almost 50% of patients receiving propranolol had to discontinue the medication reporting reduced health-related quality of life. It is clear that a deeper understanding of AAA pathogenesis is required, particularly at early stages of progression, to develop medical treatment effective at preventing or at least slowing the aneurysm expansion process.

2.2.2 Pathogenesis of Abdominal Aortic Aneurysms

Despite extensive research aimed at characterizing AAA in humans, vectors responsible for initiating aneurysmal formation remain elusive. Exact determination of the influence of any aetiological agent has proven difficult due to the lack of a reliable model of spontaneous aneurysm formation. The majority of human studies rely on the analysis of established aneurysmal tissue at the end stage of a complex

pathological process. Molecular and cellular mechanisms observed at this delay may not reflect the mechanisms involved in initiation of the disease, making it difficult to separate primary aetiological factors from secondary degenerative effects. Nevertheless, it is clear that the pathogenesis of AAA involves the complex interaction of a variety of factors over time, which weaken the aortic wall and ultimately leads to rupture. Our present understanding of the disease supports roles for the following processes in its development (reviewed, MacSweeny *et al*, 1994; Wassef *et al*, 2001; Alexander, 2003):

1) Ageing
2) Degeneration of aortic wall connective tissue
3) Inflammation
4) Inherited extracellular matrix disorders

While it is clear that weakening of the aortic media by disruption of structural proteins (collagen and elastin) is a crucial factor in the pathogenesis of aneurysms, controversy surrounds the mechanisms involved and their relative importance. Several observations must be considered, including the familial basis of aneurysms, the association with smoking, atherosclerosis, and the role of inflammatory response (Figure 2.5.).

2.2.2.1 Altered Matrix Biology

Alteration in content and/or quality of ECM proteins in the aortic wall would contribute significantly to the development and progression of AAA. For example, reduction in elastin content (or elastin/collagen ratio) of the artery wall may be important in aneurysmal dilation, while changes in collagen structure may predispose the vessel to aneurysm rupture (MacSweeny *et al*, 1994; Wills *et al*, 1996). Change in the properties of other ECM proteins with structural and constructional roles may also have bearing on AAA development.

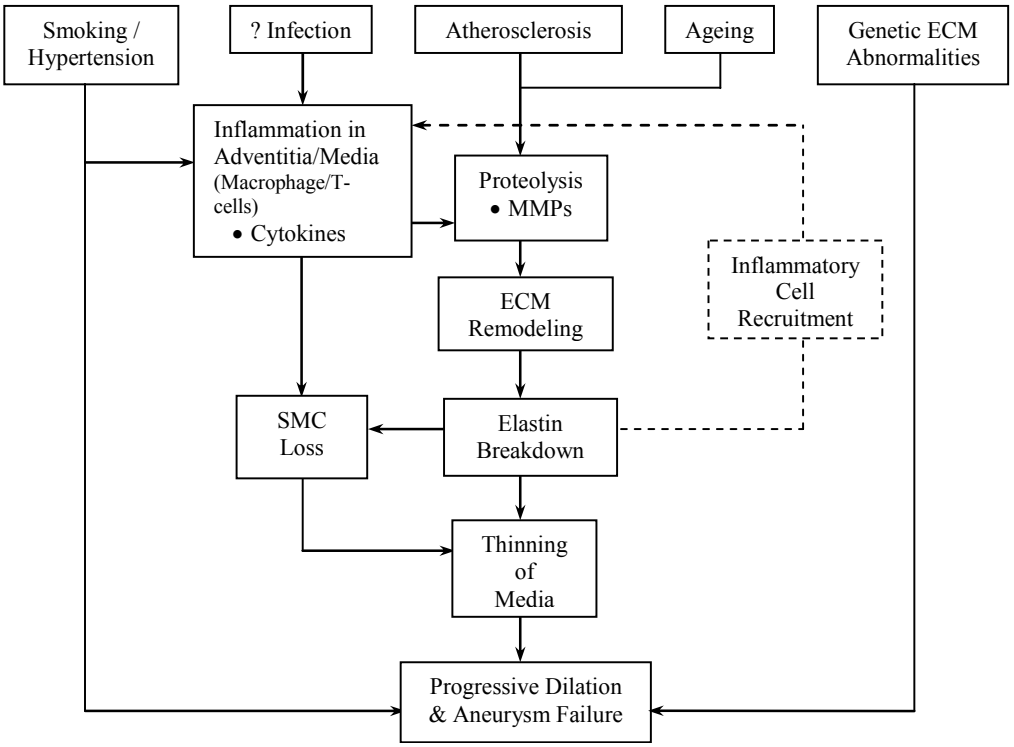


Figure 2.5. Schematic illustrating factors potentially involved in the pathogenesis of aortic aneurysmal disease.

(i) Elastin

Elastin contains lysine-derived cross-links that connect individual polypeptide chains into a rubber-like network (Rosenbloom *et al,* 1993). Carmo and co-workers (2002) reported a 90% decrease in the cross-links *desmosine* and *isodesmosine* with a corresponding depletion of elastin in AAA tissue, which is supported by several other studies incorporating cross-link analysis (Campa *et al,* 1987; Baxter *et al,* 1992; Gandhi *et al,* 1994). Although elastinolysis (elastin breakdown) appears to be fundamental in aneurysm formation (Baxter *et al*, 1994), studies have demonstrated an increase in the absolute mass of elastin in AAA tissue (Baxter *et al*, 1994; Minion *et al*, 1994). However, the observations from the latter were expressed relatively to total protein content and based on the debatable finding that synthesis and accumulation of collagen and other insoluble proteins is increased throughout the diseased aorta (Baxter *et al*, 1994). Consequently, a dilutional decrease in the concentration of elastin is observed and a significant imbalance is perceived between

collagen and elastin, which results not from the loss of elastin but rather from the increase in other major structural matrix proteins.

Mature cross-linked elastin is remarkable for its longevity and it is upheld that there is no appreciable synthesis of mature elastin in adult life (Davidson, 1987). It has been demonstrated, however, that several pathological stimuli may evoke elastogenesis in vivo (Isik *et al,* 1994; Nikkari *et al,* 1994; Forough *et al*, 1998). A recent study (Krettek *et al*, 2003) hypothesizes that dysregulated *de novo* synthesis of elastin accompanies aneurysm formation, despite its frequent association with elastin degeneration. It was shown that compared with normal aortic tissue, human AAA tissue expressed 5-fold higher levels of the elastin precursor *tropoelastin*. Additionally, levels of the cross-link desmosine were 9-fold lower when compared with normal tissue. The results suggest that elastogenesis in the aneurysmal aorta is an on-going process, but ineffective cross-linkage results in the failure of mature elastin production. An unexpected finding in the same study was the identification of macrophages as a novel source of tropoelastin. This is interesting when one takes into account the pathological role that these inflammatory cells are purported to play in elastin degradation and promotion of AAA.

There is suggestion that a critical period may exist when elastin is laid down during foetal development of the aorta and large arteries, and any aberration in synthesis at this juncture cannot be subsequently rectified. Using a rat model to illustrate this notion, Berry and co-workers (1973) briefly retarded growth when cellular propagation in the developing aorta was rapid (day 15 of foetal life), and induced persistent changes in the chemical composition of the aorta, including a reduction in the total content of elastin. Moreover, proponents of the 'foetal origins' hypothesis have suggested that cardiovascular health in later life is associated with nutrition *in utero* and early childhood (Barker, 1995). It has been proposed that impaired elastin synthesis in the aorta during the neonatal period is the initiating event in the pathogenesis of hypertension (Martyn & Greenwald, 1997). Vitamin D has been shown to inhibit elastin synthesis in cultured SMC (Pierce *et al*, 1992), with the hypothesis expanded to suggest that excess exposure to vitamin D during gestation may impair elastogenesis, resulting in predisposition to aneurysmal disease later in

life (Norman *et al*, 1995). To this end, Norman and co-workers (2002) have shown that in rats, increased vitamin D exposure during vital periods of development can result in changes in aortic structure and function. The changes reported include a significant reduction in elastin concentration of the abdominal aorta (cross-link analysis), an overall reduction in the number of elastic lamellae in the distal thoracic aorta, and impaired contractile properties of the mid-thoracic aorta. The mechanisms underlying this association may be via effects on elastin expression, maturation, or catabolism, or a combination of these. The effect of exposure to vitamin D at discrete periods of development in humans has yet to be demonstrated.

(ii) Collagen

Unlike elastin, collagen continues to be synthesized throughout the human lifespan and collagen content of tissues reflects the net effects of synthesis and degradation. Conflicting data ultimately highlights the fact that collagen metabolism in aneurysmal tissue is aberrant, with some investigators reporting an increase (Rizzo *et al*, 1989) while others observed a reduction (Minion *et al*, 1994) or no change (Baxter *et al,* 1992; Gandhi *et al,* 1994).

a. Collagen Synthesis

A classic method for assessing total collagen content of arterial tissue is to test for the collagen-specific amino acid *4-hydroxyproline*. Carmo and co-workers (2002) found the content of this and the additional collagen amino acid, 5-hydroxylysine, to be significantly reduced by 50% and 60% respectively in human AAA specimens compared to nonaneurysmal controls. Their assessment of collagen cross-links *pyridinoline* and *deoxypyridinoline* in the same tissue however, demonstrated a significant increase in the former of approximately 350%, and in the latter of more than 100%. In the absence of concomitant collagen increase, it was concluded that cross-linkage of established collagen fibres in the aneurysmal aortic wall persisted, while neosynthesis of collagen was in some way defective. The reported increase in collagen cross-links is supported by evidence that collagen molecules continue to form cross-links throughout their lifespan (Watanabe *et al,* 1996). The relative

excess in pyridinoline and deoxypyridinoline levels in this case may be explained by over-representation on aged collagen.

b. Collagen Metabolism

The two main interstitial collagens in the aortic wall are collagen type I and collagen type III, (Rizzo *et al,* 1989). Metabolism of type III collagen can be evaluated by assessing levels of *N-terminal propeptide of type III collagen* (PIIINP), an indicator of turnover (synthesis/degradation) (Treska & Topolcan, 2000). Treska and Topolcan (2000) demonstrated that metabolism of type III collagen is increased in individuals with AAA, finding significant increases in both tissue and serum levels of PIIINP. The increase in PIIINP tissue concentration was significant in patients with increasing AAA diameter thus confirming that type III collagen turnover is associated with remodeling of the aortic wall. However, a correlation between this and plasma levels of PIIINP could not be established. Biochemical studies conducted by Bode and co-workers (2000) assessed the processing of type III collagen and the proportion of type III pN-collagen (type III collagen that has retained the N-terminal propeptide domain PIIINP) present in AAA. The results indicated that the insoluble matrices of AAA contained at least 12 times more incompletely processed type III pN-collagen than the nonaneurysmal control specimens. Additionally, the soluble extracts of AAA tended to contain more non-processed type III pN-collagen than free, properly cleaved PIIINP. Thus, although turnover of type III collagen is increased in the aneurysmal aorta, synthesis and maturation of the protein may be delayed, supporting conclusions by Carmo *et al,* (2002), the consequences of which could lead to impaired tensile strength and predispose the vessel wall to rupture. Moreover, retardation in cross-link formation could render the incompletely processed fibril more susceptible to proteolytic attack.

The carboxy terminal propeptide of type I collagen (PICP) is cleaved from the procollagen molecule during synthesis and detected levels are regarded as an index of collagen neo-synthesis (Nakamura *et al*, 2000). Studies in both tissue and plasma samples have consistently shown either no significant difference (Norman *et al*, 1995) or a significantly lower concentration (Nakamura *et al*, 2000; Sumner *et al*,

1970) of PICP in AAA when compared with controls. A correlation between PICP levels (tissue or plasma) and aneurysm diameter has not been defined, although a slight negative association has been reported (Nakamura *et al*, 2000). These findings may justify observations of decreased total collagen content observed by others (Sumner *et al*, 1970) and support the conclusion that a low biosynthesis rate of type I collagen exists in the aneurysmal aorta. Collectively, it appears that the lack of, or at least reduced, collagen neo-synthesis (type I) coupled with aberrant metabolism of fibrillar proteins contributing to tensile integrity (type III collagen) constitute the fundamental elements leading to failure of the aneurysmal wall.

In contrast, Baxter and co-workers (1994), examining matrix protein changes in the proximal, nonaneurysmal section of aortas with infrarenal AAA, showed that collagen was increased in AAA when compared with normal aorta or aorta with AOD. Moreover, the same matrix alterations found in AAA appeared throughout the aorta, differing only in magnitude in the aneurysmal and nonaneurysmal segments. After compensating for 'normal' atherosclerotic and age-related increases in collagen content, the quantity of collagen in the nonaneurysmal segments in aortas with AAA remained significantly higher than that observed in normal or aortas with AOD. A correlation between increasing aortic circumference and increased collagen content was established, however whether this is suggestive of augmented matrix production playing a role in AAA formation or simply a compensatory response to increased wall stress that accompanies an increase in diameter, was not clarified. Although inconsistent with the above observations of decreased collagen content in AAA tissue, these findings do support the conclusions of other investigators (Ward, 1992; Goodall *et al*, 2001) that aneurysmal disease is systemic in nature, and any biological process initiating localized aneurysm formation might be manifest in the entire vascular tree.

(iii) Other ECM Components

The focus of other studies has been directed toward the biology of other ECM structural components and their role in AAA development. Proteoglycans are major artery wall matrix proteins that provide structural support and mediate cell-matrix

interactions. Of particular importance are the so-called 'small leucine-rich proteoglycans', *biglycan* and *decorin*. These two glycosaminoglycans are usually found in close association with collagen types I and III and are thought to play a central role in collagen assembly and stabilization of collagen fibre structure (Riessen *et al*, 1994; Theiszien & Rosenquist, 1994). Tamarina and co-workers (1998) quantified mRNA levels for both biglycan and decorin in AAA tissue and compared them with levels expressed in the normal aortic wall. They found no statistically significant difference in the expression of decorin between aneurysm samples and control, however, reported a 15-fold decrease in biglycan mRNA expression in AAA compared to non-dilated aorta. Whether the low level of biglycan expression in AAA is consequent to, or basis of, altered collagen biology observed in AAA remains ambiguous. Nevertheless, based on their findings, the authors concluded that proteoglycans have an important role in the aetiology of AAA, and that suppression of biglycan mRNA levels may signify changes in vital regulatory elements specific for AAA on a molecular level.

The association of proteoglycans with AAA is supported further by observations of compositional and specific structural modifications at the glycosaminoglycan level. A study in 1999 by Theocharis and co-workers characterized glycosaminoglycans (GAG) present in AAA as compared with those present in normal abdominal aorta. They identified the GAGs hyaluronan, chondroitin sulfate, dermatan sulfate, and heparan sulfate in both tissues, however, composition and structure of GAGs in AAA was altered. The overall GAG content in AAA was approximated 60% lower than in nonaneurysmal tissue, with a relative 90% decrease in heparan sulfate, and a 65% and 73% decrease in chondroitin sulfate and hyaluronan respectively. Only an 8% decrease in dermatan sulfate was observed when compared with control. Structural changes in disaccharide composition of GAGs corresponded mainly to significant decreases of heparan sulfate-derived N-sulfated disaccharides, chondroitin sulfate-derived 6-sulfated disaccharide and dermatan sulfate-derived disulfated disaccharides. These findings indicate that the development of AAA may be related to dramatic quantitative and structural modifications at the GAG level, which may be characteristic of destruction of arterial wall architecture and further functional inadequacies of the tissue.

2.2.2.2 Proteolytic Degradation of the Aortic Media

Accumulating evidence suggests that AAA development is associated with increased local production of MMPs. Coupled with an imbalance between these enzymes and their naturally occurring inhibitors (TIMPs), this results in the uncontrolled ECM destruction, particularly fragmentation and thinning of the elastic media, within the aortic wall. Metalloproteinases most associated with elastinolytic activity have been the focus of investigation (reviewed, Wassef *et al*, 2001; Alexander, 2003). Of these, the gelatinases, MMP-2 and MMP-9, and the human macrophage metalloelastase, MMP-12, have received particularly attention.

(i) MMP-2

Early investigation by Davis and co-workers (1998) into the expression of MMP-2 in AAA, AOD, and non-diseased tissue, demonstrated significantly greater levels of MMP-2 mRNA and protein in AAA than in AOD or controls. Moreover, the same study showed that the MMP-2 proenzyme binds to the interstitial matrix with high affinity and is activated locally with expression of the enzyme modulated through paracrine regulation. Inhibition of MMP-2 activity with the MMP-inhibitor *Marimastat* and the preservation of medial elastin in an organ culture model of AAA (Treharne *et al*, 1999) support the association of MMP-2 with AAA formation. Additional supportive data has come from culture studies that demonstrated MMP-2 production by aortic smooth muscle cells derived from patients with AAA was significantly greater than the (constitutive) levels secreted by control cells (Crowther *et al*, 2000a). *In vivo*, MMP-2 is produced as an inactive zymogen that is activated predominantly by the cell membrane-bound enzyme MT-MMP-1 (Nollendorfs *et al*, 2001). Membrane type (MT)-MMP-1 binds the inactive pro-MMP2/TIMP-2 complex at the cell surface and cleaves the pro-domain from MMP-2, thereby activating and localizing the enzyme at the cell membrane (Knauper *et al,* 1996). Crowther and co-workers (2000b) demonstrated the presence of MT-MMP-1 within the media of arterial tissue, which indicates a powerful pathway for the activation of MMP-2 in the aortic wall. Furthermore, the authors localized the MMP-2-TIMP-2-

MT-MMP-1 system to the medial layer of the artery wall, providing support that this system may play an aetiological role in the pathogenesis of AAA.

In light of the proposed systemic nature of aneurysmal disease, a recent study examined MMP-2 expression and matrix structure in venous tissue from individuals with AAA (Goodall *et al*, 2001). It was shown that inferior mesenteric vein (IMV) tissue of AAA patients had a significantly higher MMP-2/TIMP-2 ratio than tissue from age-matched controls. Histologically, a visible reduction in the quantity and integrity of elastin in IMV tissue from AAA patients was observed compared to the control group, with relative proportions of elastin in the medial layer comprising 19% and 27% respectively. The identification of MMP-2 expression in vascular tissue remote from the AAA supports both a systemic tendency toward increased proteolysis within the vasculature of AAA sufferers, and a primary role of MMP-2 in aneurysm formation. Investigations assessing the biochemical properties of human AAA have demonstrated that MMP-2 is the principal MMP in small aneurysms (40 - 55 mm), indicating a potential key role in the initial stages of aneurysm (Freestone T *et al*, 1995). Studies in which mice deficient in the expression of MMP-2 were subject to AAA induction by abluminal application of calcium chloride ($CaCl_2$) (Gertz *et al*, 1988) provide additional evidence to support the involvement of MMP-2 in the initiation of AAA (Longo *et al,* 2002). Despite infusion of competent macrophages, the source of other proteolytic enzymes, targeted deletion of MMP-2 protected mice from $CaCl_2$-induced aneurysm development.

There are, however, conflicting reports in the literature regarding the contribution of MMP-2 to AAA. Elmore and co-workers (1999) concluded that MMP-2 was not a crucial element in the development of AAA following their observation that MMP-2 mRNA expression was not significant in AAA tissue compared to AOD controls. A slight but significant increase in the MMP-2 inhibitor, TIMP-2, in AAA samples supported their conclusion. This study did, however, show a significant increase in MMP-9 activity in AAA tissue, which is congruent with other studies that propose a principal role for MMP-9 in AAA (Elmore *et al*, 1999; Pyo *et al,* 2000). Similar findings have been reported more recently in a study examining the immunohistochemical expression of MMP-2 and MMP-9 in symptomatic,

asymptomatic, and ruptured AAA (Papalambros *et al,* 2003). Matrix metalloproteinase-9 activity was shown to be significantly higher in aneurysms greater than 60 mm in diameter; however, no relation between aneurysm size and MMP-2 activity was demonstrated.

The inconsistency with other studies supporting the association of MMP-2 with aneurysm formation may arise from the AAA specimens themselves. The majority of AAA tissue taken for investigation is done so at the time of surgical repair, a late stage in the progression of AAA. In keeping with the observed presence of MMP-2 in small aneurysms and its suggested contribution to initiation of the disease, it is feasible that the enzyme is down regulated once AAA progression has reached a certain point. Moreover, the primary sources of MMP-2 in the artery wall are mesenchymal cells (SMC and fibroblasts) (Davis *et al,* 1998). A principal feature of advanced AAA is the rarefaction of medial SMC, and presumably an associated decrease in MMP-2 production.

(ii) MMP-9

The involvement of MMP-9 in the development of AAA has warranted significant interest, as it is the most abundant elastinolytic MMP in AAA tissue and secreted in high levels from AAA explants *in vitro* (Thompson *et al*, 1995). Expression of MMP-9 also correlates with increasing aneurysm diameter (McMillan *et al*, 1997; Papalambros *et al,* 2003), and is elevated in the circulation of individuals with AAA (McMillan & Pearce, 1999). The inability of MMP-9 to cause local matrix degradation in the MMP-2 deficient murine model described above, supports the hypothesis proposed by Freestone *et al* (1995) that MMP-9 is primarily associated with aneurysm expansion at a later stage (>5.5 cm), and thus progression, of AAA. The presence of inflammatory cells, including macrophages, has been associated with aneurysm augmentation in both experimental models and humans (Freestone *et al*, 1995; Pyo *et al,* 2000). Macrophages are the primary source of MMP-9 in the aneurysmal wall and although mesenchymal cells do not express MMP-9 under normal conditions, vascular SMC production of the enzyme can be induced by a proinflammatory milieu, as occurs in AAA tissue (Gurjar *et al,* 2001). The

significance of inflammatory-derived MMP-9 in AAA development is highlighted by investigations utilizing models for AAA in which MMP-9 expression has been ablated (Longo *et al,* 2002; Pyo *et al,* 2000; Thompson *et al*, 1995). In these studies, the progression of AAA was inhibited despite the fact that inflammation was not affected by the lack of this MMP. Allaire and co-workers (1998) provided additional evidence using a rat model of AAA, showing that local over-expression of TIMP-1 prevented aortic aneurysm degeneration via inhibition of MMP-9 activation. This finding was later corroborated in a study by Silence and co-workers (2002) in which inactivation of the TIMP-1 gene in ApoE$^{-/-}$:TIMP-1$^{-/-}$ mice resulted in the enhanced AAA progression.

(iii) MMP-12

Human macrophage elastase (HME; MMP-12) is considered by some to play an important role in aneurysm development. It has been shown that MMP-12 is present in human AAA, localized to aneurysm-infiltrating macrophages, and more significantly, unlike other MMPs, specifically associates with elastin fibre fragments in the aneurysm wall (Curci *et al*, 1998). Curci and co-workers (1998) were able to obtain human aortic wall specimens that encompassed the longitudinal zone between the normal caliber aorta and the proximal aspect of aneurysmal dilation, representative of a region in which artery wall degeneration might be observed in its earliest stages. The interface of the normal and dilated aorta exhibited evidence of early elastin fragmentation, with MMP-12 prominently localized to the majority of medial elastic fibres. Additionally, the same study demonstrated that MMP-12 remained bound to the elastin substrate in regions where on-going elastin degradation appeared most active. Finding no association of MMP-12 with intact elastin fibres in normal aorta, AOD tissue, or the proximal (undilated) zone in AAA, the authors concluded that localization of MMP-12 to residual elastic fibres in the degenerating artery wall reflects a particularly high binding affinity of the enzyme for elastin in selected regions of AAA tissue, and as such, MMP-12 may have a unique role in the evolution of aneurysmal degeneration. Metalloproteinase-12 appears to play a pivotal role in macrophage-mediated elastic fibre degradation *in vitro* (Shipley *et al*, 1996), and further examination of MMP-12 expression and activity in human AAA

in a more recent study confirmed its functional state at the site of AAA formation (Annabi *et al,* 2002). Data from animal models of AAA addressing the role of MMP-12 in aneurysm development is somewhat inconsistent with findings in human AAA tissue. Pyo and co-workers (2000) demonstrated that isolated genetic deficiency of MMP-12 (MMP-12$^{-/-}$) in mice did not have a significant influence on elastic fibre degradation or aneurysm development. The fact that in the same studies aneurysm formation was substantially reduced in MMP-9$^{-/-}$:MMP-12$^{-/-}$ mice, suggests that expression of MMP-9 is critical for AAA formation in this model. This, however, does not preclude the possible contribution of MMP-12 activity in further elastin degradation and expansion of the aneurysm wall. The fact that the authors concluded that MMP-12 was not essential for AAA formation in their model, highlights both issues of reproducibility of human pathology in an animal model, and the current lack of an accurate model of human AAA.

2.2.2.3 Aberrant Remodeling and AAA

Historically, atherosclerosis has been considered to be the main aetiological factor in AAA formation, perhaps due to the not infrequent finding of atherosclerotic changes within aneurysms and the fact that the lower abdominal aorta is the site at which atherosclerosis first develops and becomes common by middle age. This view appears paradoxical as atherosclerosis is characterized by the tendency to accumulate lipid, SMC, and extracellular matrix within the intimal layer of the vessel wall (Libby P, 2001). Gradual plaque development narrows the lumen with complications arising from compromised distal blood flow or plaque rupture. Smooth muscle cell proliferation and the accumulation of intimal ECM are prevalent without medial destruction. In AAA, there is progressive degeneration of the arterial media and SMC loss. Moreover, histological examination of the aneurysmal wall reveals chronic adventitial and medial inflammatory infiltrate that distinguishes AAA from the purely atherosclerotic aorta in which inflammatory cells are primarily localized within the subendothelial space and associated with plaque. Why atherosclerosis leads to occlusive disease (AOD) and narrowing of the arterial lumen in some individuals, but to aneurysmal disease and arterial dilation in others remains unclear.

In 2001, a study by Xu and co-workers assessed atherosclerotic plaque deposition and aortic wall responses in the abdominal aorta in relation to the development of aneurysmal and occlusive disease. The authors examined morphological differences at five standard locations in the infrarenal aorta, prone to both AAA and AOD, and concluded that associated with changes in arterial diameter, local differences in plaque-artery wall interaction and remodeling responses may exist, which are modified by long term alterations in plaque composition, progression, and regression. For instance, arterial enlargement (with concomitant medial thinning) may occur in compensation for plaque encroachment of the lumen, thus preventing occlusion (Libby P, 2001). It is suggested that in situations where there is plaque erosion or regression, reduction in total wall thickness would result in significant increases in wall stress (haemodynamic) and promote progressive aneurysmal dilation (Masawa *et al*, 1994). Consequently, if a plaque cycles between progression and erosion, the media may degenerate at such a rate that the weakened wall cannot sufficiently sustain wall stress and lead to aneurysm formation. Alternatively, a more stable plaque would allow sufficient time for plaque fibrosis and establishment of atheroma. This finding has to some extent been supported by studies investigating cholesterol-lowering therapy in hyperlipidaemic cynomoglus monkeys. Zarinss and co-workers (1990) observed that a proportion of these animals developed aneurysmal dilation of the abdominal aorta upon cessation of the atherogenic diet. They concluded also that aneurysm expansion was a result of plaque regression and degeneration of the atherosclerotic artery wall.

Nevertheless, the association between atherosclerosis and aneurysm formation has been questioned and has expanded into a multifactorial causation for the disease (Reed *et al*, 1992). It has become clear that atherosclerosis is not the sole cause, not least due to the fact that many individuals who develop AOD of the aorta never form an aneurysm. The most striking evidence in support of this comes from population studies that increasingly demonstrate the infrequency of AAA development in individuals with diabetes mellitus (Lederle *et al*, 2000; Blanchard *et al*, 2000; Brady *et al*, 2004). Diabetes increases the incidence and accelerates the clinical manifestations of atherosclerosis (reviewed, Beckman *et al*, 2002). However, a reduced incidence of AAA observed in patients with diabetes compared to the non-

diabetic population (Lederle *et al*, 2000; Blanchard *et al*, 2000) suggests diabetes as a negative risk factor for AAA. The apparent protective effect afforded by diabetes against the formation of AAA remains unexplained. However, the fact that patients with diabetes are two to three times more likely to develop AOD (Beckman *et al*, 2002) precludes the commonly held notion that aberrant remodeling of the artery wall associated with the advanced stages of AOD is causal for AAA development. Furthermore, the anatomical dichotomy between AAA and AOD suggests fundamental differences in pathobiology. Studies examining differential gene expression in human AAA and AOD, while detecting genes common to both, more importantly demonstrate that the expression of several genes remains unique to each disease (Armstrong *et al*, 2002; Tung *et al*, 2001). These data indicate a similarity in genetic expression for both AAA and AOD, with altered expression of particular genes playing a role in disease differentiation. This is supported somewhat by increasing differences in matrix biochemistry observed between AAA and AOD tissue. A number of MMPs and inhibitors of MMP activity (TIMPS) are present in both the aneurysmal and occlusive artery wall. The ultimate manifestation of either pathology appears to be associated with the relative expression and activation of these molecules (Table 2.4.).

Table 2.4. Differences in MMP expression/activity in AAA and AOD

MMP/TIMP	System	Profile	Reference
MMP-2	Human aortic tissue	Significantly higher levels in AAA vs AOD	Davis *et al,* 1998
	SMC culture	Significantly higher levels secreted from AAA-derived cells vs AOD cells	Crowther *et al,* 2000; Goodall *et al,* 2002
MMP-3	MMP-3$^{-/-}$ mice	Reduced AAA vs enhanced AOD	Silence *et al,* 2001
	Human aortic tissue	Significantly higher levels in AAA vs AOD	Carrell *et al,* 2002
MMP-9	Human aortic tissue	No significant difference AAA vs AOD	Davis *et al,* 1998
MMP-12	Human aortic tissue	Significantly higher levels in AAA vs AOD	Curci *et al,* 1998
TIMP-1	TIMP-1$^{-/-}$ mice	Enhanced AAA vs reduced AOD	Silence *et al,* 2002

As previously discussed, the plasmin/plasminogen system and its activators (tPA and uPA) regulate the activation of latent forms of MMP (He *et al*, 1989; Chapman & Stone, 1989). As such, differential expression of system components within the aortic wall could potentially influence MMP profile and vessel phenotype. Schneiderman and co-workers (1992) reported that expression of the type-1 inhibitor of both tPA and uPA (PAI-1) is increased in human AOD. Although expression of tPA has been detected at similar levels in both occlusive and aneurysmal aorta, expression of uPA appears to be greater in dilated artery (Shireman *et al*, 1997). The latter observation is supported by animal studies conducted by Carmeliet and co-workers (1997), and more recently Deng *et al* (2003), which demonstrates that uPA deficiency in $uPA^{-/-}$ mice is protective against media destruction and aneurysm formation.

2.2.2.4 Inflammation and AAA

The association of inflammation with AAA was described over three decades ago (Walker *et al*, 1972). The *inflammatory* aneurysm was initially regarded as a clinical and pathological entity distinct from the atherosclerotic or *degenerative* aneurysm. Moreover, present evidence suggests inflammation is an integral part of the pathology of all AAA. The factors instigating this inflammation remain unclear but include atherosclerosis, infection, and autoimmune response (see Figure 2.5).

The presence of inflammatory cells (macrophages and T-lymphocytes) within the media and adventitia of the dilated artery has been demonstrated in both humans and experimental models (Anidjar *et al*, 1992; Newman *et al*, 1994; Freestone *et al*, 1995). The significance of inflammatory cells in the pathogenesis of AAA is likely as a source of proteolytic enzymes, cytokines, and SMC apoptotic factors.

(i) Proinflammatory Cytokines

Newman and co-workers (1994) demonstrated that levels of the proinflammatory cytokines, tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) products of activated macrophages and T-cells, were significantly elevated in AAA

tissue compared with control tissue. These findings are validated by *in vitro* data showing significantly greater secretion of TNF-α and IL-1β by aneurysmal aortic tissue than controls in organ culture (Newman *et al,* 1994), and by the finding that both are present in the circulation of individuals with AAA at higher levels than nonaneurysmal control groups (Juvonen *et al*, 1997). Both TNF-α and IL-1β have been implicated in the induction of MMPs, the down-regulation of TIMPs, and the stimulation of prostaglandin E_2 (PGE_2) synthesis (Dinarello, 1991; Martel-Pelletier *et al*, 1991). PGE_2, synthesized at high concentration in the AAA wall (Holmes *et al*, 1997) inhibits DNA synthesis and proliferation of normal aortic SMC and induces apoptosis in SMC derived from aneurysmal aorta (Walton *et al*, 1999). Additionally, serum levels of IL-6 are shown to be elevated in patients with AAA (Juvonen *et al*, 1997), and a very recent investigation has identified TNF-α and IL-6 at higher levels in the walls of ruptured AAA than in tissue from asymptomatic aneurysms (Treska *et al*, 2002). However, whether the presence of either is causative of, or in response to, matrix degradation was not clarified.

The T-cell lymphocyte family includes the type-1 (T_H1) helper T-cells and the type-2 (T_H2) helper T-cells, which secrete characteristic, non-overlapping sets of cytokines (Mosmann *et al*, 1997). Recently it was demonstrated that the type-2 (T_H2) helper T-cells, and subsequent T_H2-immune response, has predominance in human AAA (Schönbeck *et al*, 2002). Cytokines of the T_H2 subset (IL-4, IL-6, IL-9, IL-10, and IL-13) augment collagenolytic and elastinolytic activities in cell types associated with AAA. Interleukin-4 and IL-10, for example, have been demonstrated to evoke the expression of interstitial collagenases MMP-1 and MMP-3 in monocytes and human aortic SMC (Sasaguri *et al*, 1998; Chizzolini *et al*, 2000). Aside from their ability to degrade matrix proteins directly, these MMPs can potentially stimulate further proteolytic activity by activating the proenzymes of other MMPs within aneurysmal wall (Knauper *et al*, 1996). Schönbeck and co-workers (2002) determined that AAA tissue lacked the receptor for interferon gamma (INF-γ) (a member of the T_H1 subgroup) despite the presence of cytokine within the aneurysmal wall. Interferon γ potently antagonizes the induction of MMP expression by inflammatory cell-derived cytokines IL-1β and TNF-α (Schönbeck *et al*, 2002);

hence impaired signaling of IFN-γ in AAA may potentially heighten over-expression of matrix degrading enzymes.

(ii) Inflammatory-cell Recruitment

Investigation into the chemotactic activity for inflammatory recruitment in aneurysmal tissues has demonstrated that human AAA contain different proteins known to be chemotactic for mononuclear phagocytes, such as monocyte chemotactic protein 1 (MCP-1) and IL-8 (Koch *et al*, 1993). Elastin-derived peptides (EDPs), released during the degradation of elastin, have been implicated in the localization of the inflammatory response to the outer aortic wall in AAA, as they are potent chemoattractants for leukocytes, and appear to promote increased production of MMPs by resident mesenchymal (SMC, fibroblasts) cells in the artery wall (Cohen *et al*, 1991). Hance and co-workers (2002) established that human AAA tissues contain soluble peptides capable of specifically directing the migration of mononuclear phagocytes, and that this chemotactic activity is attributable to peptides released during the process of elastin breakdown. Moreover, the authors demonstrated that ligand-receptor interactions with the cell-surface receptor known as the *67-kDa elastin-binding protein* (EBP) are likely to play a significant role in mediating inflammatory cell recruitment within AAA tissue.

(iii) Angiotensin II

Angiotensin II is involved in the inflammatory response through the induction of several proinflammatory mediators such as adhesion molecules, chemokines, and cytokines (Ruiz-Ortega *et al*, 2001). It has been shown that angiotensin II increases MCP-1, the main chemokine for monocyte/macrophages, in vascular SMC and endothelial cells, and IL-6 and TNF-α gene expression in macrophages (Chen *et al*, 1998; Nakamura *et al*, 1999; Defawe *et al*, 2004). An investigation by Nishijo and co-workers (1998) demonstrated that overproduction of angiotensin II in transgenic hypertensive rats stimulated aneurysm development, a finding later corroborated by Daugherty and co-workers (2000) in their angiotensin II infusion mouse model for atherosclerosis. The latter study observed a significant increase in adventitial

macrophage infiltrate and a medial fracture with pronounced vessel dilation. Angiotensin II is generated from its precursor (angiotensin I) by *angiotensin-converting enzyme* (ACE) and *chymase* (Nishimoto *et al*, 2002), and ACE-positive cells (macrophages) in the intima and media, and chymase-positive cells (mast cells) in the media and adventitia of the aneurysmal aorta have been detected at higher levels than nonaneurysmal aortas (Nishimoto *et al*, 2002). Subsequent treatment of elastase-induced inflammatory cell-mediated AAA in rats with ACE-inhibitors resulted in suppression of aneurysm formation (Liao *et al*, 2001). In this study, ACE inhibition attenuated degradation of medial elastin without diminishing mononuclear inflammation, suggesting that ACE-inhibitors might act to suppress inflammatory cell activation and possibly MMP activity (Pyo *et al*, 2000). Interestingly, earlier work by Huang and co-workers (1998) showed that treatment of inbred Brown Norway rats with ACE-inhibitors protected them against spontaneous rupture of the arterial internal elastic lamina, to which these animals are particularly prone.

Collectively, these data indicate that angiotensin II activates an inflammatory response that contributes to the progression of vascular damage. Despite the use of angiotensin II-inhibitors in clinical practice, however, the exact mechanism by which angiotensin II acts remains uncertain.

(iv) Hypoxia-induced Inflammation

The abdominal aorta is not fortified with the usual adventitial blood-supplying vasa vasorum. Instead, it relies on the diffusion of nutritional components and oxygen from the circulation through the lamina interna (Dobrin *et al*, 1984). A prominent anatomical feature of AAA is the presence of a laminated intraluminal thrombus. It has been shown that intraluminal thrombus attenuates the diffusion of oxygen from the lumen to the AAA wall (Vorp *et al*, 1998) and that vascular tissue responds to hypoxic conditions by initiating a series of events that lead to inflammation (Satta *et al*, 1996) and weakening of the aneurysmal wall (Vorp *et al*, 2001). It has been demonstrated *in vitro* that macrophages exposed to anoxic conditions exhibit enhanced bioreactivity (Albina *et al*, 1995). As such, intraluminal thrombus-induced hypoxia in the AAA wall could potentially augment proteolytic enzyme production

by adventitia and media-infiltrating inflammatory cells. Moreover, Adolph and co-workers (1997) have observed the presence of macrophages and neutrophils within the intraluminal thrombus, thus it is possible that regions of the AAA wall adjacent to thick layers of intraluminal thrombus are exposed also to proteolytic enzymes leeching from the thrombus mass.

2.2.2.5 Smooth Muscle Cell Apoptosis in AAA

Degeneration of the aneurysmal artery wall is associated with degradation and fragmentation of the medial ECM as well as a significant reduction in SMC density thought to occur via apoptosis (see section 2.1.4.2.). Vascular SMC are the predominant cell type of the elastic media and thereby make substantial contributions to the elastic lamellar architecture of the artery wall, both directly and indirectly through their production of collagen, elastin, and other matrix proteins (Ross & Klebanoff, 1971; Rusmussen *et al*, 1995). Because vascular SMC-mediated repair and resynthesis of structural matrix proteins might balance proteolysis induced by other (inflammatory) cell types, the fate and function of vascular of SMCs likely has an important influence on the progression of aneurysm degeneration (He & Roach, 1994).

In 1997, Lopez-Candales and co-workers (1997) disassociated the observed rarefaction of medial SMC from the concept of necrosis, describing cytoplasmic and nuclear features characteristic of apoptosis (see Table 2.2) in association with SMC production of the apoptosis-mediator p53. A number of different mechanisms for medial SMC apoptosis have been postulated (Pollman *et al*, 1996; Bennet *et al*, 1995). By far the most convincing to date is that of inflammatory cell-mediated apoptosis. *In vitro* studies in which SMC were co-cultured with monocyte/macrophages demonstrated an inhibition of proliferation in, and synthesis of ECM proteins by, SMC in the presence of these inflammatory cells (Proudfoot *et al*, 1999). Inflammatory cytokines known to induce SMC death *in vitro*, including IL-1β and TNF-α (Geng *et al*, 1996) are also produced in aneurysm tissues. Henderson and co-workers (1999) have shown that the cytotoxic mediators, *perforin* (cell membrane damage) and *Fas* (death receptor), are expressed in AAA tissue by T

lymphocytes and SMC respectively. Elevated local concentrations of these mediators might have a deleterious effect on medial SMC. The finding that SMCs in AAA express class II histocompatibility antigens (Kosierkiewicz *et al*, 1995) supports the possibility that medial SMCs may also be the target of cell-mediated immune responses leading to apoptotic cell death.

Additionally, SMC apoptosis in AAA occurs in a tissue environment where little if any elastin exists. It may be speculated that the association of medial SMCs with the intact elastic fibre may provide a measure of resistance to apoptotic stimuli that, in the absence of an elastin-rich ECM, might otherwise induce (programmed) cell death (Meredith *et al*, 1993). Finally, AAA-derived SMC exhibit a distinct morphologic appearance in culture, a diminished proliferative capacity compared to SMC from non-diseased tissue, and a limited life-span *in vitro* (Liao *et al*, 2000). Liao and colleagues (2000) concluded that these differences reflect an intrinsic alteration in SMC growth capacity independent of age alone, and that tissue-specific processes leading to accelerated replicative senescence may therefore contribute to the selective medial SMC depletion observed in AAA.

2.3 OSTEOPROTEGERIN

2.3.1 Characterization

Osteoprotegerin (OPG) is a soluble member of the tumor necrosis factor receptor (TNFR) superfamily and was initially identified as the decoy receptor for receptor activator of nuclear factor kappa B (RANK) ligand (RANKL). RANKL, also a TNF receptor family member, is an osteoclast-activating factor that is critical to osteoclast development and function. The interaction of osteoblast-derived RANKL with the RANK receptor on haemopoietic osteoclast precursors and mature osteoclasts represents the essential stage in bone remodeling through osteoclastogenesis and/or activation (Nakagawa *et al,* 1998; Kong *et al,* 1999). Osteoprotegerin functions as an antagonistic endogenous receptor which binds to RANKL through competition with the RANK receptor. The OPG/RANKL interaction counterbalances the stimulating

couple RANKL/RANK in that after binding to RANKL, OPG inhibits osteoclast maturation and activation and blocks the bone resorption process (Yasuda *et al,* 1998a).

Osteoprotegerin was characterized independently by several groups. Based on sequence homology, Simonet *et al* (1997) identified OPG as a novel TNFR superfamily member (TNFSFIIb) during a sequencing project of rat intestinal cDNA. Also at this time, Tsuda and co-workers (1997) purified a heparin-binding glycoprotein from conditioned medium of human lung fibroblast culture that inhibited osteoclast formation and termed it *osteoclastogenesis inhibitory factor* (OCIF). They subsequently cloned the human OCIF cDNA and showed it to be identical to OPG (Yasuda *et al,* 1998a). Osteoprotegerin was also identified by Tan and co-workers (1997) by screening various cell line libraries and Kwon *et al* (1998) by screening an expressed sequence tag database for homologous regions for the cysteine-rich motifs of the TNFR, and was named *TNF receptor-related molecule-1* (TR1). Finally, Yun and colleagues (1998) cloned OPG from a follicular dendritic cell (FDC)-like cell line FDC-1 and named it *FDC receptor-1* (FDCR-1). Today, *Osteoprotegerin* ('protector of bone') is the recognized standard nomenclature.

2.3.2 Gene Organization and Protein Structure

2.3.2.1 The OPG Gene

The genes for mouse (Simonet *et al,* 1997; Mizuno *et al,* 1998), rat (Simonet *et al,* 1997), and human OPG (Simonet *et al,* 1997; Mizuno *et al,* 1998; Yasuda *et al,* 1998; Morinaga *et al,* 1998) have been characterized. The human OPG gene, localized to chromosome 8q23–24 (Simonet *et al,* 1997; Kwon *et al,* 1998), is a single copy gene containing five exons distributed over 29 kb (Morinaga *et al,* 1998; Mizuno *et al,* 1998; Yamaguchi *et al,* 1998). The gene has at least three transcription initiation sites: one major transcription-initiation site located 67 nucleotides up-stream of the ATG codon and two minor transcription-initiation sites at positions 646 and 667, suggesting the presence of two promoters (Morinaga *et al,* 1998). Exon 1 encodes ten amino acid residues of the signal peptide, exon 2 encodes the first two cysteine-rich domains and

73% of the third, while exon 3 encodes the remainder of the third and the fourth cysteine-rich domain (Morinaga *et al,* 1998). Coding of more than one cysteine-rich motif by a single exon has been previously reported for human nerve growth factor receptor, but not for other TNFR members (Morinaga *et al,* 1998). Exon 4 encodes the majority of the first death domain homologous region (DDH), whereas exon 5 encodes the remainder of the first and the second DDH, and contains the translation-termination codon (Morinaga *et al,* 1998). The most abundant OPG mRNA species has a size between 2.2 and 3.0 kb (Simonet *et al,* 1997; Tan *et al,* 1997; Yasuda *et al,* 1998; Kwon *et al,* 1998). Variability is due to the alternative use of transcription-initiation sites (Morinaga *et al,* 1998). Two additional OPG mRNA species, a 6.5–6.6 kb mRNA and a 4.2–4.4 kb mRNA are present in some tissues and are usually less abundant (Yasuda *et al,* 1998a).

2.3.2.2 The OPG Protein

A high degree of evolutionary conservation has resulted in approximately 85 percent homology between human and mouse OPG protein; human OPG and rat OPG are 94 percent identical (Simonet *et al,* 1997). Osteoprotegerin is synthesized as a monomer and assembled as a homodimer within the cell, and then secreted mainly as a disulfide-linked homodimer into the extracellular compartment. However, monomeric OPG is produced by limited proteolysis in plasma (Tomoyasu *et al,* 1998) and can be detected in small amounts present in cell culture medium (Simonet *et al,* 1997). Non-reducing SDS-PAGE analysis of hepatic transgenic OPG in mice (Min *et al,* 2000) further suggests that the glycoprotein may exist as a trimer. The OPG monomer migrates at 55–62 kDa under reducing conditions and at 40 kDa following removal of the four or five potential N-glycosylation sites (Simonet *et al,* 1997; Tsuda *et al,* 1997; Kwon *et al,* 1998), while the OPG homodimer migrates at 110–120 kDa under non-reducing conditions (Simonet *et al,* 1997; Tsuda *et al,* 1997). The 21 amino acid signal peptide in both forms of OPG are cleaved during post-translational processing, giving rise to a mature protein of 380 amino acids (Tomoyasu *et al,* 1998).

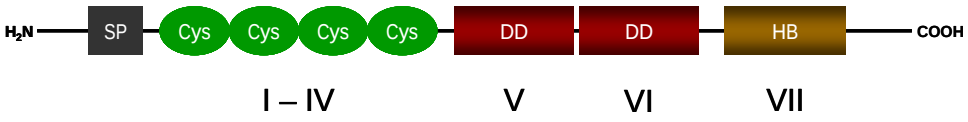
Members of the tumor necrosis factor (TNF) family of ligands and their receptors have pleiotropic biologic functions, many critically involved in regulation of cell growth, as well as immune response (Locksley *et al,* 2001; Grewal & Flavell, 1998; Younes & Kadin, 2003). Tumor necrosis factor receptors are characterized by the presence of 40-amino-acid, cysteine-rich repeats in the extracellular domains (Locksley *et al,* 2001). The intracellular domains of these receptors share no sequence homology, accounting for their diverse biologic functions. 'Death domain' sequences in the cytoplasmic tail of TNF-family receptors class them as *death receptors*. The amino (N)-terminal domains (I-V) of OPG contain the highly conserved cysteine-rich motifs found in other TNFR family proteins, and consist of four to six cysteine pairs that form intra-chain disulfide bridges (Tan *et al,* 1997; Figure 2.6.). Integrity of all four cysteine-rich motifs at the N-terminus is necessary and sufficient for bioactivity of OPG, whereas truncation of the carboxy (C)-terminal portion to residue 194 does not alter it (Yamaguchi *et al,* 1998). The C-terminal end shares no homology with any other TNFR superfamily members except for two death domain homologous (DDH) tandem regions (V & VI).

Osteoprotegerin is a unique TNFR superfamily member in that it lacks the usual hydrophobic membrane-spanning sequence (Yasuda *et al,* 1998a; Simonet *et al,* 1997). This feature is consistent with a role as a soluble antagonistic decoy receptor. Despite this, OPG can exist in a membrane-bound form. The presence of membrane-bound OPG on the surface of transfected kidney cells and a non-transfected dendritic cell line (Yun *et al,* 1998), of osteoclasts isolated from mouse long bones (Woo *et al,* 2002), and in multiple myeloma cells (Standal *et al,* 2002), has been reported.

Osteoprotegerin possesses a highly basic heparin-binding domain (Yamaguchi *et al,* 1998) which can facilitate binding to cell surface heparin, and transmembrane proteoglycans comprising heparan sulfate side chains. Not bound trans-membranously, the functions of the DDH regions in OPG have not been well characterized; however, they possess high homology to the death receptors Fas and TNFR-1 (Yasuda *et al,* 1998a), and *in vitro* studies in which the transmembrane domain of Fas had been inserted between domains IV and V, suggest that the DDH regions in the OPG molecule are active in mediating apoptotic signals (Yamaguchi *et*

al, 1998). Intermolecular disulfide linkage occurs at cysteine-400 within the heparin-binding domain (VII) to facilitate dimerization of the OPG molecule (Yamaguchi *et al,* 1998). Dimerization of OPG is not, however, necessary for biological activity (Tomoyasu *et al,* 1998). In humans, OPG is present in virtually all tissue systems (including bone) (Yasuda *et al,* 1998a), with its expression and production modulated by an extensive range of cytokines, peptides, hormones, and pharmacological agents (Table 2.5a. & 2.5b.).

A.



B.

```
Signal peptide (cleaved) MNKLLCCALVFLDISIKWTTQ   (21)

I                        ETFPPKYLHYDEETSHQLLCDKCPPGTYLKQHCTAKWKTVCAP   (64)
II                       CPDHYYTDSWHTSDECLYCSPVCKELQYVKQECNRTHNRVC   (105)
   TNFR
   Domains
III                      ECKEGRYLEIEFCLKHRSCPPGFGWQAGTPERNTVC   (142)
IV                       KRCPDGFFSNETSSKAPCRKHTNCSVFGLLLTQKGNATHDNICSGNSESTQK   (194)

V                        CGIDVTLCEEAFFRFAVPTKFTPNWLSVLVDNLPGTKVNAE
                         SVERIKRQHSSQEQTFQLLKLWKHQNKDQDIVKKIIQDIDL
   Death
   Domains
VI                       CENSVQRHIGHANLTFEQLRSLMESLPGKKVGAEDIEKTIK
                         ACKPSDQILKLLSLWRIKNGDQDTLKGLMHALKH

VII                      SKTYHFPKTVTQSLKKTIRFLHSFTMYKLYQKLFLEMIGNQVQSVKISCL   401)
                                                                    ↑
                                                                  Dimer
```

Figure 2.6. (A) Structure of the OPG protein. NH_2 indicates amino-terminus; COOH, carboxy-terminus; SP, signal peptide; Cys, cysteine-rich motifs; DD, death domains; HB, heparin binding and dimerization domain. (B) Amino acid sequence motifs. (------- indicates predicted disulfide linkages)

Table 2.5a. Upregulation of OPG

Factor	Cellular Source	Reference
Angiotensin II	Aortic SMC	Zhang et al, 2002
Bisphosphonates	Osteoblast	Onyia et al, 2004
BMP-2	Osteoblast	Hofbauer et al, 1998
Basic FGF	Aortic SMC	Zhang et al, 2002
Calcium	Fibroblast; Marrow stromal cell	Yasuda et al, 1998a Bergh et al, 2004
CD40L	Follicular dendritic cell	Yun et al, 1998
1α,25-$(OH)_2D_3$	Osteoblast	Hofbauer et al, 1998
17b-Estradiol	Osteoblast	Hofbauer et al, 1999b
IL-1α	Osteoblast; Vascular endothelial cell	Vidal et al, 1998a Collin-Osdoby et al, 2001
IL-1β	Osteoblast; Fibroblast; Aortic SMC; Vascular endothelial cell, Periodontal cell	Hofbauer et al, 1998 Kwon et al, 1998 Zhang et al, 2002 Sakata et al, 2002 Zannettino et al, 2005
IL-6	Osteoblast	Palmqvist et al, 2002
hLIF	Osteoblast	Palmqvist et al, 2002
Leptin	Marrow stromal cell; Osteoblast	Burguera et al, 2001 Holloway et al, 2002
Nitric Oxide	Marrow stromal cel	Fan et al, 2004
OPN	Vascular endothelial cell	Malyankar et al, 2000 Pritzker et al, 2004
OSM	Osteoblast	Palmqvist et al, 2002
PDGF	Aortic SMC	Zhang et al, 2002
TGFβ	Marrow stromal cell; Osteoblast	Murakami et al, 1998 Takai et al, 1998
TNFα	Osteoblast, Fibroblast; Aortic SMC; Vascular endothelial cell	Hofbauer et al, 1998 Brandström et al, 1998a Kwon et al, 1998 Collin-Osdoby et al, 2001 Zhang et al, 2002 Zannettino et al, 2005
TNFβ	Osteoblast	Brandström et al, 1998a

Abbreviations: **SMC**, smooth muscle cell; **BMP**, bone morphogenic protein; **FGF**, fibroblast growth factor; **IL**, interleukin; **hLIF**, human leukaemia inhibitory factor; **OPN**, osteopontin; **OSM**, oncostatin M; **PDGF**, platelet-derived growth factor; **TGF**, transforming growth factor; **TNF**, tumor necrosis factor

Table 2.5b. Downregulation of OPG

Factor	Cellular Source	Reference
Basic FGF	Marrow stromal cell; Osteoblast	Nakagawa et al, 1999 Mukohyama et al, 2000
Cyclosprorin A	Coronary SMC	Hofbauer et al, 2001
Dexamethasone	Marrow stromal cell; Osteoblast	Vidal et al, 1998b Hofbauer et al, 1999c Murakami et al, 1998 Ishii et al, 2004
1α,25-$(OH)_2D_3$	Marrow stromal cell; Osteoblast	Horwood et al, 1998 Murakami et al, 1998
Thiazolidinedione	Aortic SMC	Fu et al, 2002
Glucocorticoid	Osteoblast	Murakami et al, 1998 Vidal et al, 1998b Hofbauer et al, 1999c
IL-1α	Osteoblast	Murakami et al, 1998 Nakagawa et al, 1999 Tanabe et al, 2005
Insulin	Vascular SMC	Olesen et al, 2005
OPN	Marrow stromal cell	Ishii et al, 2004
PGE_2	Marrow stromal cell; Osteoblast	Murakami et al, 1998 Brandström et al, 1998b Yasuda et al, 1998b
PTH	Marrow stromal cell; Osteoblast	Murakami et al, 1998 Takai et al, 1998 Horwood et al, 1998 Lee & Lorenzo, 1999 Kanzawa et al, 2000

Abbreviations: **FGF**, fibroblast growth factor; **SMC**, smooth muscle cell; **IL**, interleukin; **OPN**, osteopontin; **PGE**, prostaglandin; **PTH**, parathyroid hormone

2.3.3 OPG and the Vascular System

2.3.3.1 The Skeletal-Vascular Link

Osteoporosis and vascular disease frequently coexist. Thus, it has been hypothesized that these disorders negatively affect bone metabolism and vascular integrity through identical direct and indirect pathways (Schoppet *et al*, 2002). Since the discovery of OPG and the OPG/RANK/RANKL system, and as far as the biological action of OPG is concerned, the primary area of investigation over the last decade has centred on bone biology and diseases characterized by excessive bone resorption. Extensive research has been conducted investigating the OPG/RANK/RANKL system and its role in mediating conditions such as metabolic bone disease (*postmenopausal* and *glucocorticoid-induced osteoporosis, hyperparathyroidism, Sporadic Paget's Disease*), malignant bone disease (*multiple myeloma, osteolytic bone metastases*), and inherited skeletal disorders (*Familial* and *Juvenile Paget's Disease*), and is extensively reviewed (Hofbauer, 1999a; Hofbauer & Schoppet, 2004).

Investigation of immune-mediated bone diseases such as rheumatoid arthritis and periodontal infection has produced evidence further linking OPG (and its cognate ligand, RANKL) with immune and inflammatory processes (reviewed, Drugarin *et al,* 2003; Hofbauer & Schoppet, 2004). Expression of OPG has been observed in cells of the immune system including T lymphocytes, B lymphocytes, and dendritic cells (Yun *et al,* 1998; Choi *et al,* 2001). Studies using OPG-deficient ($OPG^{-/-}$) mice suggest that OPG influences the development and function of the immune system with the protein shown to be critically involved in B cell maturation and the generation of efficient antibody responses, and, bone marrow-derived dendritic cells are more potent in stimulating allogeneic T cells in $OPG^{-/-}$ mice as compared with dendritic cells from wild-type mice (Yun *et al,* 2001). Immune and inflammatory-mediated mechanisms are involved in promoting vascular disease (Ross, 1999) and in the development and progression of AAA (see section 2.2.2.4.).

In addition to osteoporosis, a further consequence of OPG gene ablation in mice was the development of arterial calcification of the aorta and renal arteries (Bucay *et al,*

1998). Concurrent over-expression of transgenic OPG resulted in the rescue of these mice from vascular mineralization (Min *et al,* 2000). A link between bone resorption and arterial wall mineralization under the control of OPG has been further supported in warfarin or high dose vitamin D-treated rats. In these animals, vascular calcification induced by treatment was prevented with administration of exogenous OPG (Price *et al,* 2001). These observations suggest that OPG (and RANKL) may represent a paracrine link between osteoporosis and vascular calcification. The phenotype of these two systems in $OPG^{-/-}$ mice is reflective of the high clinical prevalence of arterial calcification and cardiovascular disease in post-menopausal women and elderly people with osteoporosis (Hak *et al,* 2000), although, vascular mineralization in this patient population typically occurs in the tunica intima adjacent to atherosclerotic plaques (Hak *et al,* 2000). Thus, the concept that metabolic bone diseases and vascular diseases such as arterial calcification share common pathways (Sattler *et al,* 2004) is supported; nevertheless the ability of OPG to block vascular calcification in humans remains to be determined.

2.3.3.2 Association of OPG with Vascular Disease

Osteoprotegerin is expressed in the normal artery wall (Dhore *et al,* 2001). A number of *in vitro* studies have confirmed VSMC (Hofbauer et al, 2001; Fu et al, 2002; Zhang et al, 2002) and endothelial cells (Malyankar *et al,* 2000; Collin-Osdoby *et al,* 2001) as resident cellular sources of OPG within the artery wall. Treatment of VSMC with growth factor such as PDGF, basic fibroblast growth factor (bFGF), Angiotensin II, TNF-α, and IL-β induces high levels of OPG mRNA expression (Zhang *et al,* 2002). The consequence of this within the artery wall *in vivo* is not known. In endothelial cells, OPG is upregulated upon ligation of $\alpha_v\beta_3$ integrin by osteopontin (Malyankar *et al,* 2000) and purportedly acts as an autocrine endothelial cell survival factor, which suggests a (protective) role for OPG in the maintaining integrity of the arterial luminal surface.

Low level, constitutively expressed arterial OPG (Simonet *et al,* 1997) has been shown to be upregulated following balloon angioplasty (Zhang *et al,* 2002), a technique employed to induce vascular disease by injury in experimental animal

models. Immunohistochemical analyses have identified OPG in human atherosclerotic vessels localized at the margins of mineralized lamellar bone-like structures (Dhore *et al,* 2001; Golledge *et al,* 2004). Atherosclerotic plaque destabilization and degradation of arterial media in aneurysmal disease is attributed in part to the aberrant activity of proteolytic enzymes. Upregulation in the expression of MMP-2 and MMP-9 by OPG *in vitro* has been documented (Wittrant *et al,* 2002). These observations present a less favourable view of OPG and its association with vascular disease. This notion is further supported by findings that OPG is upregulated in unstable human carotid atherosclerosis. Golledge *et al,* (2004) demonstrated that OPG was present at higher levels in, and secreted in greater amounts from, human symptomatic carotid plaques compared with asymptomatic, more stable lesions.

Serum analyses have provided additional evidence for the involvement of OPG in human vascular disease. Elevated levels of serum OPG are positively correlated with the onset and severity of atherosclerosis (Jono et al, 2002; Schoppet et al, 2003; Kiechl et al, 2004) and overall cardiovascular mortality (Browner et al, 2001). A recent study, conducted in haemodialysis patients to examine the association of serum OPG concentration with aortic calcification (Nitta *et al,* 2004) showed that levels of OPG were significantly greater in serum of patients with a higher aortic calcification index (ACI; as determined by computer tomography scans) than in those with lower ACI. Multiple regression analyses indicated that serum OPG levels were independently associated with the severity of aortic calcification (Nitta *et al,* 2004). Medial artery calcification is a prominent feature associated with complications of diabetes, and it is established that the occurrence of medial calcification in Type 2 diabetes is a strong independent predictor of cardiovascular mortality (Niskanen *et al,* 1994; Lehto *et al,* 1996). A very recent investigation comparing the level of OPG protein in arterial tissue from diabetic and non-diabetic subjects demostrated a higher content of OPG in the aortic media from diabetic patients (Olesen *et al,* 2005). This observation supports those of an earlier study in a cohort of postmenopausal women aged over 65 years, in which OPG serum levels were 30% higher in women with diabetes mellitus than those without. Sub-analysis identified women within the highest OPG serum quintile as having a four-fold

elevated relative risk of cardiovascular mortality (Browner et al, 2001). Olesen *et al* (2005) concluded that increased OPG concentrations in the diabetic artery wall may be part of generalized matrix alterations, putatively related to the development of vascular calcification.

In view of the phenotype of OPG-deficient mice (osteoporosis and arterial calcification), elevated OPG serum levels have been interpreted as an insufficient counter-regulatory mechanism to prevent further bone loss and vascular lesions. Data from human tissue and epidemiological studies (reviewed, Hofbauer & Schoppet, 2004) suggest an alternative view; it is possible that RANKL- and RANK-dependent pathways are protective in the vascular system, and that elevated OPG levels indicate antagonism of these pathways during the evolution of vascular disease.

Genetic diagnostics is a field of investigation increasingly utilized in the study of disease aetiology. Alteration in human genes is currently believed to reveal the cause of individual susceptibility to disease. The most common type of genetic diversity is single-nucleotide polymorphism (SNP). The use of SNP-detection technology has provided further insight into the role of OPG within the vasculature and its association with vascular disease. In 2002, Brandström and co-workers used gene sequence analysis to identify a novel base pair substitution from a thymidine (T) to a cytosine (C) in the promoter region of the human OPG gene in apparently healthy subjects. The T/C transition was located at position 950, 129 base pairs upstream of the TATA-box according to the published sequence by Morinaga *et al* (1998). The SNP was reported to be significantly associated with cardiovascular morphology and function and linked with vascular structural changes indicative of early atherosclerosis in the carotid artery. Subsequent investigation in hypertensive individuals by the same group also related the presence of the C allele in the in the promoter region of the OPG gene with intima-media thickness (IMT) of the common carotid artery (Brandström *et al,* 2004). Carotid artery IMT is considered a marker of early atherosclerosis (Allen *et al,* 1997), related to the prevalence of overt carotid atherosclerotic plaques (Mack *et al,* 2000), and a powerful predictor of atherosclerotic events, such as stroke and myocardial infarction (Bots *et al,* 1997).

DNA sequencing in a recent study investigating OPG polymorphisms in males with coronary artery disease (CAD) identified the T/C substitution at position 950 within the promoter of the OPG gene described by Brandström *et al* (2002) and correlated serum OPG levels with the presence of a C allele at that position (Soufi *et al,* 2004). In the same study, nucleotide substitutions in the promoter region were additionally revealed at positions 149 (T/C), 163 (A/G), 209 (G/A), and 245 (T/G), and furthermore at exon 1 position1181 (G/C) and intron 4 position 6890 (A/C). Interestingly, single polymorphisms were not associated with CAD, however linkage of polymorphisms 950 and 1181 revealed that haplotypes were overrepresented in subjects with CAD with an increased risk of CAD in carriers of genotypes 950 TC/1181 GC and 950 CC/1181 CC (Soufi *et al,* 2004).

2.4 SUMMARY

Aneurysmal disease of the human abdominal aorta (AAA) is an inflammatory disorder characterized by local connective tissue degradation leading to progressive vessel wall dilation and rupture. The condition occurs in a large proportion of the older generation with a considerable degree of morbidity and mortality. Although risk factors leading to AAA later in life are not yet completely defined, smoking, age, and familial predisposition remain the positive associations with the disease, while female gender and diabetes are negatively associated.

Human AAA is commonly associated with atherosclerosis, however, it is clear now that AAA aetiology is distinctly separate than that of the occlusive disease. Two histopathological features discriminate AAA from aortic atherosclerosis:

1. Medial attenuation with loss of elastic lamellae and smooth muscle cell (SMC) death (apoptosis)
2. Intense inflammatory response within the adventitia and media

Proteolytic disruption of the elastic media is considered to be an important cause of aortic dilation. Degeneration of the media is attributed to enzymic degradation of the

structural extracellular matrix consequent to activation of inflammatory cell (macrophage)-derived matrix metalloproteinases. Increased destruction and decreased production of matrix proteins resulting from protease activity and SMC loss respectively, may act in concert to promote the aneurysmal remodeling process. Nevertheless, mechanisms responsible for aneurysmal formation and progression remain poorly defined. There is no proven treatment to limit the growth of AAA. Aneurysm diameter is generally used to assess timing of surgical repair based on *watchful-waiting* following detection. What is needed in the treatment of AAA is to understand the pathology of the disease enough so that pharmacological intervention aimed at halting its formation, or at least progression, can be developed.

There is increasing evidence that the glycoprotein OPG is actively associated with the presence and development of vascular disease. Establishing the role of OPG in human AAA may identify a basis for development of pharmacological therapy to slow or halt aneurysm expansion, providing active, non-invasive management of small aneurysm progression, and, more importantly, a treatment option for those unsuitable for surgical repair.

Chapter 3

GENERAL MATERIALS AND METHODS

3.1 HUMAN TISSUE STUDIES

Informed consent was obtained for tissue and blood collection in accordance with protocols approved by the ethical bodies of The Townsville Hospital, Australia, James Cook University, Australia, and The University of Western Australia (see Appendix 4 for Ethics Approvals).

3.1.1 Preparation and Storage of Human Serum

Whole blood samples were collected from participants of the Western Australia Abdominal Aortic Aneurysm Screening study (Jamrozik *et al*, 2000) and at surgery from patients undergoing elective surgical repair of abdominal aortic aneurysm (AAA) at The Townsville Hospital. Blood samples were allowed to clot at room temperature and serum obtained by centrifugation at 3,400 x *g* for 10 minutes. The serum was removed carefully by pipette and transferred to clean microfuge tubes and stored at -80°C in preparation for protein estimation and enzyme-linked immunoassay (see section 3.2.3.).

3.1.2 Collection of Vascular Tissue

Biopsies were obtained from the aneurysm or atherosclerotic aorta opposite the inferior mesenteric artery from male age-matched patients undergoing elective surgical repair of AAA or aortic occlusive disease (AOD) bypass. Biopsies of thoracic aorta (nonaneurysmal control aorta) obtained from male age-matched patients undergoing coronary artery bypass graft. Samples were transferred immediately following excision to a sterile specimen pot containing chilled DMEM (Appendix 3) and immediately brought to the laboratory. Additional nonaneurysmal abdominal aortic tissue was collected post-mortem from age-matched male subjects.

3.1.3 Preparation and Storage of Biopsies

All procedures were performed in a laminar flow hood under sterile conditions. Biopsies were rinsed in chilled DMEM to clear away excess blood. Excess fatty tissue and any residual thrombus were removed. Each AAA sample was bisected; one half transferred immediately to a clean specimen pot containing 10% formalin fixative (ProSciTech) in preparation for paraffin blocking (section 3.1.4.(i)), with the remaining tissue was divided into small pieces (5 x 2 x 2 mm) and each piece transferred to a separate clean cryovial (Cellstar) containing 1 ml DMEM plus 10% dimethyl sulfoxide (DMSO; BDH Chemicals). The cryovials were then placed into a *Mr Frosty*® cryo-freeze container (Nalgene) containing isopropanol and stored overnight at -70°C before transfer to liquid nitrogen in preparation for protein extraction, western analysis, and zymography (sections 3.1.5.-3.1.7.). Selected AAA biopsies were taken for studies *ex vivo*. In these cases, biopsies were dissected into full-thickness 10 x 10 mm explants and placed directly into tissue culture (section 3.2.2.). The AOD and saphenous vein biopsies were dissected into as many equal sized pieces as permitted and cryopreseved as described.

3.1.4 Histology

(i) Tissue and Slide Preparation

Tissue fixed in 10% formalin was prepared for paraffin wax embedding in the Citadel 1000 automatic tissue processor (Shandon), in which it was subjected under vacuum to a series of graded alcohols, xylene, and liquid paraffin in a preset 17-hour cycle. The tissue was blocked on edge in paraffin wax either *in toto* or cut in transverse section/s, depending on its size, and microtome sectioned at 5 μm. Cut sections were floated in a heated water bath (40°C) to facilitate adherence to *SuperFrost*®*Plus* microscope slides (Menzel-Glaser) which were then heat-fixed in a 60°C oven prior to storage at room temperature.

(ii) Haematoxylin and Eosin Stain

Histological morphology of biopsies was examined using a standard haematoxylin and eosin (H&E) stain. After heating slides at 60°C for 30 minutes, sections were dewaxed in two changes of xylene (2 x 2 minutes) before transfer to two changes of 100% alcohol (2 x 2 minutes). Rehydration was facilitated by a 2-minute wash in running tap water prior to staining of the slides in Mayer's Haematoxylin (ProSciTech) for 4 minutes, and followed by further washing in running tap water (2 minutes). Sections were differentiated with a 30-second dip in acid alcohol (Appendix 3), a 2-minute wash in running tap water, and another 30-second dip in Scott's blueing solution (Appendix 3). The slides were washed extensively (5-10 minutes) in running tap water, then stained in eosin (1 minute) before being transferred directly (dehydrated) to four changes of 100% alcohol (4 x 1 minute). Two changes of xylene (2 x 2 minutes) cleared the sections which were then mounted with coverslips and DePex cement (ProSciTech) in preparation for examination by light microscopy.

(iii) Immunohistochemistry

Immunostaining of formalin-fixed 5 μm paraffin sections was performed using either the mouse or rabbit DAKO EnVision™Plus HRP system (DakoCytomation). The primary antibody and horseradish peroxide-labelled polymer (secondary immunoglobulin) were used as per kit instructions. Sections were dewaxed and rehydrated (section 3.1.4.(ii)) and maintained in 1 x phosphate-buffered saline (PBS; Amresco). Subsequent procedures were performed in a humidity chamber to prevent drying out of sections. A 5-minute peroxidase-block preincubation prevented endogenous enzyme activity within the tissue sections that might present a false-positive reaction. If required, antigenic determinants masked by formalin-fixation and paraffin embedding, may be exposed by employing an epitope-retrieval (unmasking) step at this point (Chapter 4, section 4.2.). Following three washes in PBS (3 x 5 minutes), sections were incubated in 10% normal serum (serum species same as secondary immunoglobulin) to block any non-specific binding of antibodies. The serum was drained (not washed) after 10 minutes and the relevant primary

antibody applied and incubated for 30 minutes at room temperature. Sections were washed thee times in PBS (3 x 5 minutes) and the horseradish peroxide-labelled polymer (secondary immunoglobulin) applied and sections incubated for 30 minutes at room temperature. Following three washes in PBS (3 x 5 minutes), staining was completed by a 5-10 minute incubation with 3,3'-diaminobenzidine (DAB+) substrate-chromogen which results in a brown-coloured precipitate at the antigen site. Sections were rinsed in distilled water to stop the DAB+ reaction, counterstained with Mayer's Haematoxylin, rehydrated, cleared, and mounted (section 3.1.4.(ii)) in preparation for examination by light microscopy.

3.1.5 Tissue Protein Extraction and Quantification

Vascular tissue samples (see section 3.1.3.) were rinsed in PBS, dried, and weighed. Each sample was then minced manually with a scalpel blade before being pulverized under liquid nitrogen using a mortar and pestle. The powdered tissue was quickly transferred to a clean microfuge tube, combined with chilled extraction buffer (Appendix 3), and homogenized manually with a microfuge pestle. The homogenate was subjected to several liquid nitrogen freeze/thaw cycles for maximum cell rupture and tissue breakdown. Following a 1-hour incubation at 4°C and extensive vortexing, the homogenate was centrifuged at 18,000 x *g* for 30 minutes using an ultra-centrifuge (Sigma 3-18k) prechilled to 4°C. The resultant supernatant was collected and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Supernatants were stored at -20°C.

3.1.6 Western Blot Analysis

Initial analyses employed commercially available, precast 12% SDS gels (Gradipore), however, these gels allowed no flexibility for troubleshooting if so required and ultimately gels made in-house were used (Appendix 3). Commercially available (Bio-Rad Laboratories) gel moulds, polyacrylamide gel electrophoresis (PAGE) and protein transfer apparatus were used as per manufacturers instructions.

(i) Protein Separation and Transfer

Extracted proteins (see section 3.1.5.) were separated according to molecular weight using SDS PAGE. A volume of extracted protein sample appropriate for desired protein quantity (20-40 μg) was combined with loading buffer (Appendix 3), loaded onto a gel, and run in 1x SDS buffer (Appendix 3) at 100 volts for 1 hr at room temperature. During this time, PVDF membrane (Immun-Blot™; Bio-Rad Laboratories) briefly pre-wet in 70% methanol, and pieces of blotting paper, all cut to the size of the gel, were soaked in transfer buffer (Appendix 3) and allowed to equilibrate at 4°C. Following protein separation, the gel was sandwiched with the PVD membrane and blotting paper and the protein transferred from the gel to the membrane in transfer buffer at 30 volts overnight at 4°C.

(ii) Protein Detection and Visualization

Target protein was detected via immunolabeling. Following protein transfer, the PVD membrane was washed briefly in tris(hydroxymeththyl)aminomethane (Tris)-buffered saline (TBS, Amresco; 0.3 M NaCl) containing 0.1% Tween 20 (Amresco) (TBST) then incubated overnight at 4°C in a 5% skim milk powder (Diplomat) TBST blocking solution. The block solution was removed and the membrane then incubated for 1 hour at room temperature with a primary antibody to the target protein diluted in fresh blocking solution. The membrane was then washed in three changes of TBST (3 x 10 minutes) before incubation with a horseradish peroxidase (HRP)-labelled secondary immunoglobulin in fresh blocking solution for 1 hour at room temperature. After three washes in TBST (3 x 10 minutes), the membrane was incubated with the chemiluminescence substrate StrepTactin (Bio-Rad Laboratories), diluted 1:10,000 in TBST, for 15 minutes at room temperature. This was followed by two changes of TBST (2 x 10 minutes) then transfer to TBS. Visualization of the target protein was achieved using a commercially available enhanced chemiluminescence kit (ECL *Advance*™; Amersham Biosciences) with bands corresponding to the target identified on a ChemiDoc™ imaging system (Bio-Rad Laboratories) supported by QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories).

3.1.7 Gelatin Zymography

A volume of extracted protein sample appropriate for desired protein quantity (5-10 μg) was combined with loading buffer (Appendix 3) and separated using 200 volts at 4°C on a 9% acrylamide-SDS gel impregnated with 1 mg/ml gelatin (Sigma). Upon disappearance of the dye-front, the gel was washed in two changes (2 x 15 minutes) of 2.5% (v/v) Triton X-100 (Amresco) in distilled water, then incubated 4-6 hours at 37°C in 50 mM Tris (pH 8) containing 5 mM $CaCl_2$. Bands were visualized in a 10% ethanol-10% acetic acid solution after staining with 0.125% Coomassie Blue (ICN Biochemicals Inc), with enzyme activity semi-quantified by densitometric analysis using the ChemiDoc™ imaging system (Bio-Rad Laboratories) and QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories).

3.2 *IN VITRO* STUDIES

3.2.1 Cell Culture

(i) Human Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMC) were isolated from 'healthy' abdominal aorta without evidence of advanced atherosclerosis, AAA biopsies, and biopsies of saphenous vein by combined collagenase and elastase digestion (enzymic dissociation). All procedures were performed in a laminar flow hood under sterile conditions. Tissue samples were rinsed in chilled DMEM to clear away excess blood. Excess fatty tissue and any residual thrombus removed. The tissue was cut into square pieces approximately 10 mm^2, transferred to 30 mm culture dish (Nunc), and incubated in 5 ml sterile collagenase (Worthington) (5 mg/ml serum-free DMEM) at 37°C in an atmosphere of 5% CO_2 for 1 hour. Following this, the tunica media was separated from the adventitia, the adventitia discarded, and the media torn into small strips using watchmaker forceps. The strips were transferred to a clean, small, sealable flask (Nunc) and fresh collagenase added to just cover the tissue. The flask was incubated at 37°C in an agitating water bath for 1 hour. The collagenase was

then carefully removed and replaced with sterile elastase (Worthington) (1 μg/ml serum-free DMEM) and the flask incubated in the water bath for 1 hour, after which fresh collagenase (at least equal to volume of elastase) was added. Clumping of cells was prevented via gentle up and down pipetting of the solution every 15 minutes. Dissociation of the tissue into a single cell suspension was assessed by viewing a drop of the solution on a slide under a microscope. When ready, the suspension was collected into a clean centrifuge tube and spun at 180-200 *g* for 10 minutes. The supernatant was discarded and the cell pellet resuspended in fresh DMEM containing 10% foetal bovine serum (FBS; Gibco). The resuspended cells were then plated and incubated at 37°C in an atmosphere of 5% CO_2 with medium refreshed every 2-3 days until the cells reach confluency. At this point, the primary culture was passaged and/or frozen down for storage in liquid nitrogen (see sections 3.2.1.(iv) and 3.2.1.(v).).

(ii) Monocytic THP-1 Cells

The human leukaemic cell-line, THP-1, was kindly provided by the Tumour and Immunology Laboratory of the Queensland Institute of Medical Research, Brisbane, Australia. THP-1 are non-adherent, 'floating' cells and were maintained in RPMI (JRH Biosciences) containing 10% FBS (Appendix 3) at 37°C in an atmosphere of 5% CO_2. Cell passage and storage were performed as described (see sections 3.2.1.(iv) and 3.2.1.(v).).

(iii) Human Aortic Macrophages and Peripheral Blood Mononuclear Cells

All procedures were performed in a laminar flow hood under sterile conditions. Human AAA tissue was cut into pieces approximately 5 mm^2 and incubated in 10 ml sterile collagenase (5 mg/ml serum-free DMEM) at 37°C in an atmosphere of 5% CO_2 for 2 hours. Following this, the tissue was teased apart using watchmaker forceps and transferred to a clean, small, sealable flask with sterile elastase (1 μg/ml serum-free DMEM) and an equal volume of collagenase and was incubated at 37°C in an agitating water bath for 1 hour with frequent up and down pipetting. Mononuclear cells were obtained by allowing the enzymically digested slurry to

stand for 1 hour before the supernatant was collected and subjected to Ficoll-Hypaque (Amersham) gradient centrifugation at 500 x *g* for 20 minutes. Macrophages (Mø) were isolated following their adherence to the surface of a culture dish after incubation at 37°C for 30 minutes. Cell-type was verified using FACS analysis (see section 2.2.4.) with the macrophage-specific marker CD-71 (clone Ber-T9, DakoCytomation). The isolated macrophages were maintained in RPMI containing 10% FCS at 37°C in an atmosphere of 5% CO_2. Peripheral blood mononuclear cells from human blood samples were also obtained using Ficoll-Hypaque gradient centrifugation and maintained in RPMI containing 10% FCS at 37°C in an atmosphere of 5% CO_2.

(iv) Cell Passaging

All procedures were performed in a laminar flow hood under sterile conditions. Passaging was performed when cells, whether adherent or floating, reached confluence. For adherent cells (VSMC/Mø), Trypsin-EDTA (0.25% trypsin, 1mM EDTA-4Na; Gibco) enzyme was added just to cover the monolayer surface and incubated at 37°C in an atmosphere of 5% CO_2 for 2-5 minutes. Culture medium containing 10% FBS was then added to neutralize the enzyme activity and the cells resuspended via gentle up and down pipetting. The suspension was transferred to a clean centrifuge tube and centrifuged at 180-200 *g* for 10 minutes. The supernatant was discarded and the cell pellet resuspended in a total volume of fresh DMEM containing 10% FBS to be distributed between the desired number of new plates/flasks.

(v) Cell Storage

Cells that were actively proliferating were optimal for freezing. All procedures were performed in a laminar flow hood under sterile conditions. Adherent cells were trypsinized as per cell passaging. The cell suspension (or non-adherent cell culture) was transferred to a clean centrifuge tube and spun at 180-200 *g* for 10 minutes. The supernatant was removed and the cell pellet diluted into a final volume of DMEM containing 20% FBS to be distributed in 1ml aliquots between the desired number of

cryovials. A cell count was performed using a haemocytometer to ensure that each cryovial received a minimum of 1 x 10^6 cells. DMSO at a final concentration of 10% was added to each vial, which was then placed into a *Mr Frosty*® freezing chamber containing isopropanol, stored overnight at -70°C, then transferred to liquid nitrogen for storage.

(vi) Trypan Blue Exclusion Test of Cell Viability

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a non-viable cell will have a blue cytoplasm.

An aliquot of cell suspension being tested for viability was centrifuged for five (5) minutes at 100 × *g* and supernatant discarded. The cell pellet was resuspended in 1 ml PBS and then mixed 1:1 with 0.4% trypan blue and the mixture incubated approximately three (3) minutes at room temperature. Longer incubation periods will lead to cell death and reduced viability counts.

The original aliquot volume depends on the approximate number of cells present. The aliquot should contain a convenient number of cells to count in a hemacytometer when suspended in 1 ml PBS and then diluted again by mixing with 0.4% trypan blue (e.g., 5 × 10^5 cells/ml).

A drop of the trypan blue/cell mixture was applied to a hemacytometer and the number of unstained (viable) and stained (nonviable) cells determined separately. The total number of viable cells per ml of aliquot was obtained by multiplying the total number of viable cells by two (2) (the dilution factor for trypan blue). The total number of cells per ml of aliquot was obtained by doubling the total number of viable and nonviable cells.

The percentage of viable cells was thus calculated:

$$\text{Viable Cells (\%)} = \frac{\text{Total Number Viable Cells per ml Aliquot}}{\text{Total Number Cells per ml Aliquot}} \text{ X } 100$$

(vii) Cell Culture Immunocytochemistry

Ten (10) mm round microscope slide coverslips (ProSciTech) were placed in each well of a 24-well cell culture plate (Nunc) prior to the addition of cells. Cultures were maintained in relevant culture medium containing 10% foetal bovine serum and incubated at 37°C in an atmosphere of 5% CO_2 until cells had attached to the coverslips and reached desired confluency. At this point, the culture medium was removed, the cells washed in three changes of PBS (squirt bottle), then fixed in ice-cold methanol for 10 minutes at room temperature prior to blocking with 10% normal serum (species of secondary immunoglobulin). The serum was drained (not washed) after 10 minutes and the relevant primary antibody applied and incubated for 30 minutes at room temperature. The coverslips were then washed thee times in PBS and the horseradish peroxide-labelled polymer (secondary immunoglobulin) applied and sections incubated for 30 minutes at room temperature. Following three washes in PBS, staining was completed by a 5-10 minute incubation with 3,3'-diaminobenzidine (DAB+) substrate-chromogen which results in a brown-coloured precipitate at the antigen site. Sections were rinsed in distilled water to stop the DAB+ reaction, counterstained with Mayer's Haematoxylin, rehydrated, cleared, and mounted (see section 3.1.4.(ii)) in preparation for examination by light microscopy.

3.2.2 Explant Culture

All procedures were performed in a laminar flow hood under sterile conditions. Biopsies collected from patients undergoing AAA repair (section 3.1.2.) were immediately dissected into full-thickness 10 x 10 mm explants and maintained under experimental conditions in DMEM containing 10% FBS, at 37°C in an atmosphere

of 5% CO_2 for the duration of the experimental period. Explant viability over this period was confirmed using histology (see section 3.1.4.(ii)), and assessment of tissue production of adenosine 5'-triphosphate (ATP) (Chapter 4, section 4.10.).

3.2.3 Enzyme-linked Immunoassay

Cytokine concentrations present in cell and explant culture supernatants (and human serum; see section 3.1.1.) were assessed with the commercially available *DuoSet*® Enzyme-linked Immunoassay (ELISA) system (R&D Systems). The kit facilitates the development of sandwich (capture-detection antibody) immunoassays that were used as per the manufacturer's instructions. Colorimetric analysis (optical density (OD) of samples) was performed at 450 nm using a *Sunrise*™ microplate (96-well) reader (TECAN) supported by *XREAD Plus*® version 4.30.

3.2.4 Fluorescence-activated Cell Scanning

Fluorescence-activated Cell Scanning (FACS) analysis of cell samples was performed on a *FACScan*® bench top flow cytometer (Becton Dickinson) equipped with an Argon laser (excitation wavelength 488 nm) and supported by CellQuest ™ Pro Software (Becton Dickinson). The *FACScan*® can measure five parameters comprising forward light scatter (FSC), side light scatter (SSC), and pulse height and width of three fluorescence channels, FL1 (emission wavelength 515-545 nm), FL2 (564-606 nm), and FL3 (650 nm).

(i) Cell Fixation

Experimental cell cultures (adherent cells trypsinized) were transferred to 1 ml microfuge tubes and centrifuged at 200 x *g* for 5 minutes. The supernatant was gently tipped off and the cell pellet resuspended in 200 µl PBS containing 0.5% bovine serum albumin (BSA; Serologicals, Fr V protease-free). A further 200 µl of 4% formaldehyde (ProSciTech) was added and the suspension mixed well, after which it was allowed to stand in the dark at room temperature for 20 min. The cells were then centrifuged at 200 x *g* for 5 minutes and the pellet gently washed in two changes (2 x

200 µl) of ice-cold PBS/0.5% BSA. The cells were again pelleted at 200 x *g* for 5 minutes and the supernatant gently removed in preparation for the labeling of target epitopes (sections 3.2.4.(ii) and (iii)).

(ii) Cell-surface Staining

Following fixation (see section 3.2.4.(i)) pelleted cells were resuspend in 25 µl of PBS/0.5% BSA containing 2 µl of primary antibody to the target protein, and incubated in the dark at room temperature for 30 min. The cells were then centrifuged at 200 x *g* for 5 minutes and the pellet gently washed in two changes (2 x 200 µl) of ice-cold PBS/0.5% BSA. The cells were again pelleted at 200 x *g* for 5 minutes, the supernatant gently removed, and the pellet resuspended in 25 µl of PBS/0.5% BSA containing a fluorescent-labeled secondary immunoglobulin and incubated a further 30 minutes in the dark at room temperature. Following two washes with 200 µl of PBS/0.5% BSA, the cells were again pelleted at 200 x *g* for 5 minutes, the supernatant gently removed, and the pellet resuspended in 200 µl of PBS/0.5% BSA and transferred to FACS tubes in preparation for analysis.

(iii) Intracellular Staining

Following fixation (see section 3.2.4.(i)) pelleted cells were permeabilized by rigorous vortexing in ice-cold methanol (~500 µl/10^6 cells) and incubated at 4°C for 10 minutes. Following two washes in PBS/1.0% BSA (2 x 200 µl), the cells were incubated in 25 µl PBS/1.0% BSA containing 2 µl of primary antibody to the target protein at room temperature for 30 minutes. The cells were then centrifuged at 200 x *g* for 5 minutes and the pellet gently washed in two changes (2 x 200 µl) of ice-cold PBS/1.0% BSA. The cells were again pelleted at 200 x *g* for 5 minutes, the supernatant gently removed, and the pellet resuspended in 25 µl of PBS/1.0% BSA containing a fluorescent-labeled secondary immunoglobulin and incubated a further 30 minutes at room temperature. Following two washes with 200 µl of PBS/1.0% BSA, the cells were again pelleted at 200 x *g* for 5 minutes, the supernatant gently removed, and the pellet resuspended in 200 µl of PBS/1.0% BSA and transferred to FACS tubes in preparation for analysis.

3.2.5 Assessment of Cell Proliferation

Under sterile conditions, cell cultures were pulsed with 1.25 μCi/ml tritiated (^{3}H)-thymidine (Amersham Pharmacia Biotech) four (4) hours prior to completion of the experimental period and cell harvest. Cells were lysed and transferred to a glass-fibre filter with the aid of an automatic *Filtermat-196*™ cell harvester (Packard). The filter, allowed to dry overnight, was then immersed in scintillant (PerkinElmer) and cell lysate radioactivity (^{3}H-thymidine incorporation) assessed with the *Top Count*™ microplate scintillation counter (Packard) as an indicator of cell proliferation.

3.2.6 Assessment of Cell Apoptosis

(i) Plasma Membrane Asymmetry (Annexin V Labeling)

Experimental cell cultures were trypsinized, transferred to a clean 5 ml FACS tubes, and centrifuged at 200 x *g* for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml culture medium. The suspension was centrifuged at 200 x *g* for 5 minutes, the supernatant discarded, and the cell pellet resuspended in 500 μl PBS (1x). This wash step was repeated, with the final cell pellet resuspended in 500 μl HEPES binding buffer (Appendix 3). Following centrifugation at 200 x *g* for 5 minutes, the supernatant was gently tipped off and the cell pellet resuspended in the 50-100 μl binding buffer remaining in the tube. To this, 5 μl of fluorescent-tagged phospholipid-binding protein Annexin V (BD Biosciences) was added together with 10 μl of the cationic dye propidium iodide (Sigma) diluted in PBS to 50 μg/ml, and the suspension incubated in the dark for 15 minutes at room temperature. A further 500 μl binding buffer was added and the solution mixed gently in preparation for FACS analysis of cell membrane incorporation of Annexin V.

(ii) DNA Fragmentation

Experimental cell cultures were trypsinized, transferred to a clean centrifuge tube, and spun at 200 *g* for 10 minutes. The supernatant was discarded and the cell pellet resuspended in lysis buffer (supplied with diagnostic kit; see below) and incubated

on ice for 30 minutes. Following this, the cells were centrifuged at 1,500 *g* for 10 minutes at 4°C. The lysate was then frozen at -20°C for a minimum of 18 hours. Quantitation of apoptotic cells was performed by DNA affinity-mediated capture of free nucleosomes with a commercially available ELISA (Oncogene Research Products) as per the manufacturer's instructions.

3.2.7 Extraction of Cellular mRNA

(i) Purification

Messenger (m)RNA was obtained from cell samples using the commercially available *RNeasy Mini*® extraction system (QIAGEN) as per the manufacturer's instructions. Purification of mRNA samples was performed by adding 0.1 extraction volume of 3M sodium acetate (pH 5.2) followed by addition of 2.5 extraction volume of ice-cold absolute ethanol. The solution was then centrifuged at 16,000 *g* for 10 minutes at 4°C. The supernatant was quickly and carefully removed by pipette (retained in a clean microfuge tube for further purification) and replaced with 1 ml 70% ethanol (room temperature) diluted in 0.1% DEPC (Sigma)-treated water. The pellet was resuspended by inverting the tub 3-4 times before further centrifugation at 16,000 *g* for 10 minutes at 4°C. The supernatant was removed and retained, and the microfuge tube placed in a sterile laminar flow hood to allow the purified mRNA pellet to air-dry. When completely desiccated, the tube was sealed and the mRNA sample stored at -20°C.

(ii) Assessment of Purification, Yield, and Stability

Purity of extracted RNA was determined by spectrometry and indicated by the absorbance ratio 260 nm:280 nm. Acceptable 260/280 ratios fall in the range of 1.8 to 2.2. Ratios below 1.8 indicate possible protein contamination. Ratios above 2.2 indicate presence of degraded RNA. A 2 µl aliquot of RNA eluate was dilted to a final volume of 100 µl with RNAse-free H_2O and analysed on an *UVmini-1240*™ spectrometer (Shimadzu) prior to sample desiccation.

Sample yield was calculated with the formula:

$$\frac{A_{260} \times 50 \text{ (dilution factor)} \times 40^{*}}{\text{Eluate Volume}} = \frac{\mu g/ml}{ml} = \ldots\ldots \mu g \text{ RNA}$$

* known standard: A_{260} of 1.0 = [RNA] of 40 µg/ml

Stability of the extracted RNA was determined by electrophoresis. Samples were loaded onto a 1.2% Agarose gel (Appendix 3) containing 10 mg/ml ethidium bromide (Sigma) and run at 80V for 40 minutes at room temperature. Ribosomal RNA bands were visualized under UV with the *ChemiDoc*™ imaging system (Bio-Rad Laboratories) supported by *QuantityOne*™ 1-D Analysis Software (Bio-Rad Laboratories).

3.3 MOUSE MODEL OF AAA

3.3.1 Animals

Male C57BL/6 ApoE$^{-/-}$ mice were obtained from commercial colonies (Animal Resources Centre, Western Australia) and housed in a purpose-built mouse facility at James Cook University, Townsville, Australia. Animal surgery and supply of aortic tissue was provided by Dr Bradford Cullen as approved by the James Cook University Animal Ethics sub-committee (Appendix 4). Water and normal laboratory diet were available *ad libitum*.

3.3.2 Aneurysm Formation

Two-month old ApoE$^{-/-}$ mice maintained on normal diet were fitted with osmotic minpumps (Alzet Corporation) that delivered angiotensin II at a dosage rate of 1.0 µg/kg/min over 28 days. Pumps were placed subcutaneously in the dorsal midline (interscapular space) under ketamine and xylazine anaesthesia. At three months of

age, the mice were sacrificed by lethal anaesthesia, perfused with PBS via cannulas placed in the left ventricle and atria, and the aorta harvested.

3.3.3 Assessment of Aneurysm Development

The aorta was harvested at sacrifice from arch to iliac bifurcation and the specimen photographed with a *Nikon Fujix*™ digital camera. The digital images were then transferred to computer and maximum aortic diameter assessed with image analysis software (Scion). Aortas were sectioned at the site of maximal dilatation (usually suprarenal region) and specimens obtained for histology, immunohistochemistry (IHC), western analysis and zymography (sections 3.1.4.-3.1.7.).

3.4 STATISTICS

3.4.1 Human Studies

Serum OPG concentration and aneurysm growth rate association was assessed using Spearman's correlation coefficient. Multiple regression analysis was performed with aneurysm growth rate as the dependent variable and patient age, diabetic status, smoking history, initial aortic diameter, and serum cholesterol, OPG, HDL, LDL and C-reactive protein as the independent variables. The concentration of OPG in 40 aortic biopsies is expressed as Mean ± standard error (SEM) and compared between AAA and AOD, PAA, or TA using Mann-Whitney U-test. VSMC and THP-1 function in three repeat experiments is expressed as Mean ± SEM and compared statistically using the Kruskal-Wallis test. Where appropriate, Bonferroni adjustment was applied to post-hoc Mann-Whitney multiple comparisons to prevent type I error inflation. Significance was assumed at a P value < 0.05. Statistical data related to gene analysis was provided by the Australian Genome Research Facility Inc. Significance of signal-detection (detection P-value) and change-in-signal (change P-value) was determined using the Wilcoxon's Signed Rank test for nonparametric pair-wise comparison.

3.4.2 Animal Model

Statistically significant difference in frequency of aortic aneurysm formation between experimental groups was determined using the Fisher's exact test. The concentration of OPG and activity of MMP-9 within aortic tissue, each expressed as Relative Density Units (RDU), was correlated with aortic diameter and compared between aneurysmal and nonaneurysmal or treated and non-treated aortic specimens using Mann-Whitney U-test.

Chapter 4

FEASIBILITY STUDIES AND PROTOCOL OPTIMIZATION

4.1 INTRODUCTION

The work presented in this thesis employed a range of investigative techniques. The following chapter presents data from preliminary studies confirming experimental feasibility and validity of *in vitro* and *in vivo* methods used in the primary study.

4.2 R&D DuoSet® ELISA for OPG and IL-6

A significant proportion of work involved assaying of OPG and IL-6 concentrations in both human serum (Chapter 3, section 3.1.1.) and supernatant from cell/tissue culture (Chapter 3, sections 3.2.1. and 3.2.2.). The commercially available R&D DuoSet® ELISA system was used for quantification of each cytokine (Chapter 3, section 3.2.3.). Protocol required preparation of standards and antibodies for target protein capture, and involved a multiple-step procedure in execution. Assay reproducibility is a primary issue and the commercial ELISAs were assessed at both the intra- and inter-assay level.

4.2.1 Study Design

Generation of standard curves in seven (7) separate trials was used to assess reproducibility of the ELISA. The curves were generated using materials and reagents supplied with the kit as per manufacture's instructions. In a single trial (1 plate), the Mean (*M*) and Standard Deviation (*SD*) of duplicate standard curve concentration points (n=7) were obtained from colorimetric analysis. The Means and Standard Deviations were averaged and the intra-assay coefficient of variance for each plate (expressed as %) calculated:

$$\frac{(SD1+ \ldots\ldots +SD7)/7}{(M1+ \ldots\ldots +M7)/7} \times 100$$

The trials were conducted in three runs; two runs of two plates and a third run of three plates. The *M* and *SD* of duplicate standard curve concentration points of a single plate in a run were averaged with the *M* and *SD* of duplicate standard curve concentration points of other plates of the same run, and the inter-assay coefficient of variance (expressed as %) calculated:

$$\frac{(SD\ (Plate\ 1\ M1 + Plate\ 2\ M1) + \ldots\ldots + SD\ (Plate\ 1\ M7 + Plate\ 2\ M7))/7}{(M\ (Plate\ 1\ M1 + Plate\ 2\ M1) + \ldots\ldots + M\ (Plate\ 1\ M7 + Plate\ 2\ M7))/7} \times 100$$

4.2.2 Results and Conclusion

Results of individual plates are presented in Appendix 1. The combined results are presented graphically in Figure 4.1. Intra-assay reproducibility was particularly robust with the coefficient of variance between repeats within the same trial falling between 0.92 and 6.91%, with an average of 4.08%. Reproducibility between plates (inter-assay co-efficient of variance) remained within the upper limit of acceptance averaging 8.79%.

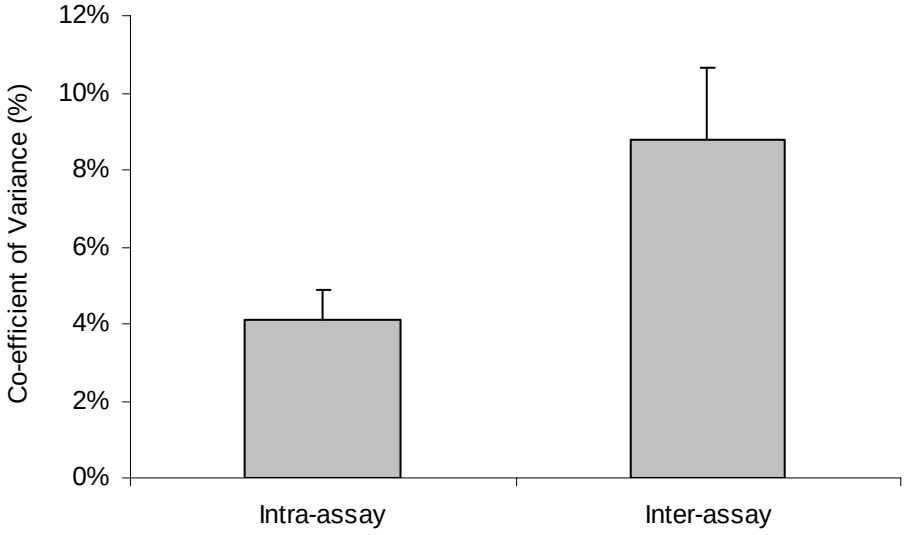


Figure 4.1. Assessment of reproducibility in the R&D *DuoSe*t® OPG ELISA as evaluated by the coefficient of variance between repeats (intra-assay) and individual plates (inter-assay).

4.3 ANTIGEN EPITOPE RETRIEVAL FOR OPG IMMUNOSTAINING

Formalin or other aldehyde fixation involved in paraffin embedded tissue immunohistochemistry can lead to cross-linkage of protein in tissue specimens that masks target antigenic sites and potentially leading to weak or false negative staining. Antigen epitope retrieval is designed to break protein cross-links in paraffin sections resulting in epitope unmasking and enhanced immunostain intensity. Requirement for epitope retrieval in the immunodetection of OPG in formalin-fixed, paraffin sections of human AAA tissue was investigated.

4.3.1 Study Design

Rehydrated 5 μm serial paraffin wax sections of AAA tissue (Chapter 3, section 3.1.4.(i)) were either taken straight to PBS (n=3), or subjected to an epitope retrieval process prior to immunostaining (n=3). Sections selected for epitope retrieval were boiled 3 x 5 minutes in citrate buffer (Appendix 1) using a 600 W microwave oven, after which the slides were cooled under slow-running tap water for 10 minutes before being transferred to PBS. Endogenous peroxidase activity and non-specific binding in all sections were blocked with 1% hydrogen peroxide (5 minutes) and 10% normal swine serum (10 minutes), respectively. Immunostaining on both the epitope retrieval-treated sections and sections not treated was carried out with a mouse monoclonal antibody to OPG (clone 98A1071, Imgenex) at 1:1000, using the DAKO EnVision™Plus HRP system as described (Chapter 3, section 3.1.4.(iii)). A negative control consisted of sections incubated in the absence of the primary antibody. To assess the efficiency of epitope retrieval, measurements were taken at three (3) sites of darkest staining and a background (BG) region for each stained section. The mean optical density (MOD) minus BG for each area was calculated and the results averaged to give an average MOD for each slide. Measurements were performed using Scion Image for Win95/98 and Windows NT, version ALPHA 4.0.3.2 (Scion Corporation), downloaded from the National Institute of Health website (www.nih.com)

4.3.2 Results and Conclusion

Pre-treatment of sections for epitope retrieval did not significantly improve the level of immunodetection of OPG compared with sections that did not undergo the epitope retrieval procedure (Figure 4.2. and 4.3.). Moreover, epitope retrieval actively contributed to increased levels of background and non-specific staining in some sections. Therefore, the decision was made to omit the epitope retrieval step from subsequent OPG immunostaining.

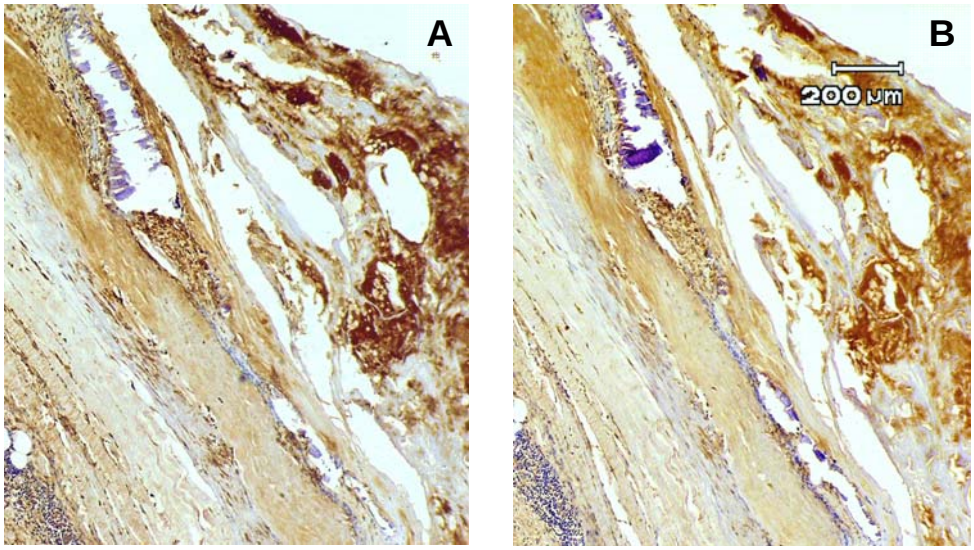


Figure 4.2. The effect of antigen epitope retrieval (A) compared with no epitope retrieval (B) on the immunodetection of OPG (brown stain) in human AAA tissue.

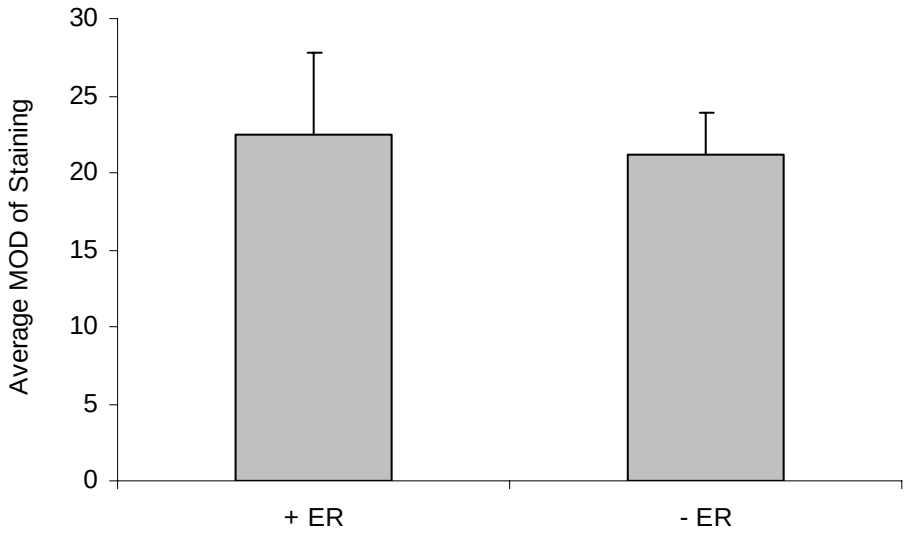


Figure 4.3. Mean optical density (MOD) of immunostain in paraffin sections pre-treated for antigen epitope retrieval (+ER) compared with paraffin section not processed for epitope retrieval (-ER).

4.4 WESTERN BLOT ANALYSIS FOR OPG

Western-blot analysis involves a complex protocol in which proteins are extracted from cells/tissue, separated electrophoretically according to molecular weight on a gel medium, and then transferred to a second membrane for immuno-visualization and quantification using image analysis. The aim of this study was three-fold:

1. To optimize the efficiency of the protein extraction and the overall western blot technique
2. To compare the performance of two potential anti-OPG markers
3. To evaluate non-specific and/or cross-reactive staining of polyclonal secondary immunoglobulins (DakoCytomation)

4.4.1 Study Design

The protein extraction and western blot transfer procedures (Chapter 3, sections 3.1.5. and 3.1.6.) were assessed using tissue obtained from human saphenous vein

(Chapter 3, section 3.1.2.), relatively normal vascular sample that provided abundant amounts of the target protein, alpha (α)-actin, a common molecule present in arterial and venous VSMC. A monoclonal (mouse) primary antibody (1:500; Dako-Cytomation) to α-actin was used to detect and visualize the protein. Biopsies of human AAA (Chapter 3, section 3.1.2.) were used to compare staining efficiency of a rabbit polyclonal antibody (R&D) and a mouse monoclonal antibody (clone 98A1071, Imgenex) to OPG to determine which antibody was more suitable for detection and visualization of the protein in this tissue. Non-specific and cross-reactive staining of anti-rabbit (DakoCytomation) and anti-mouse (DakoCytomation) secondary immunoglobulins, respectively, was evaluated by omitting the primary antibody incubation from the staining procedure (Chapter 3, section 3.1.6.(ii)).

4.4.2 Results and Conclusion

Both the protein extraction and western blot protocols were validated with the successful isolation and detection of VSMC α-Actin from human venous tissue (Figure 4.4.).

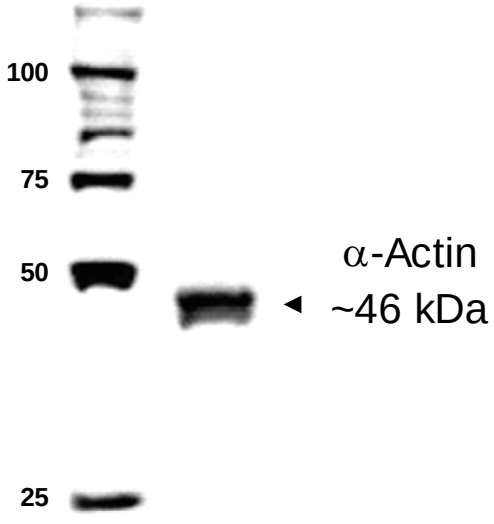


Figure 4.4. Validation of protein extraction and western blot protocols with the isolation, detection, and visualization of α-Actin from human venous tissue.

The detection of OPG in human AAA tissue using both polyclonal and monoclonal probes, and secondary antibody specificity, was subsequently demonstrated using the validated protocols (Figure 4.5.). The mouse monoclonal OPG antibody was selected for use in future western analyses. A cleaner visualization of the blot was achieved when using this antibody as compared to the 'smear' appearance seen with the rabbit polyclone.

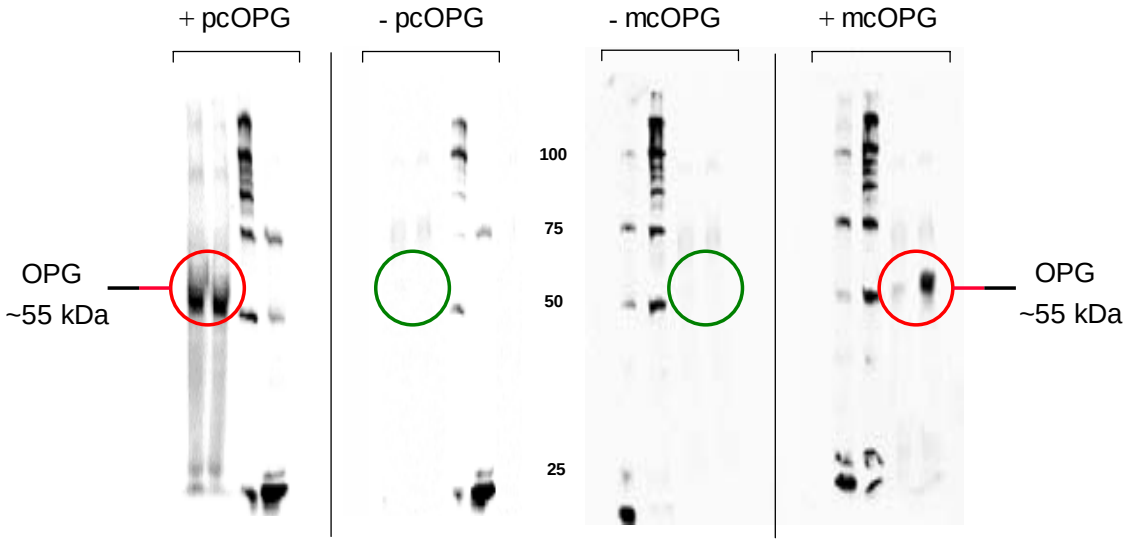


Figure 4.5. Western blot comparing the use of a polyclonal (pc) primary antibody with that of a monoclonal (mc) antibody in the detection of OPG in human AAA tissue. Validation of primary antibody with the absence of non-specific/cross-reactive staining by respective secondary immunoglobulins (green circles).

4.5 ISOLATION AND CULTURE OF VSMC

The majority of proposed *in vitro* studies required the successful isolation and culture of VSMC from non-diseased, aneurysmal, and occluded human aorta. The aim of this study was to assess the efficiency of the enzymic cell extraction method, to confirm that cultures were clean and not contaminated with monocyte/macrophages that can inhibit VSMC growth, and to demonstrate that viable cultures could be maintained.

4.5.1 Study Design

Human aortic VSMC were enzymically dispersed as described (Chapter 3, section 3.2.1.), plated and allowed to grow to confluency. Cell phenotype was confirmed microscopically by the characteristic 'hill and valley' morphology of plated VSMC. Additionally, enzymically dispersed cells were transferred to a 24-well cell culture plate (Nunc) with 10 mm round microscope slide coverslips placed in each well in preparation for assessment using immunohistochemistry (Chapter 3, section 3.2.1.(vi)). Immunostaining of adhered cells was performed using the VSMC-specific marker, α-actin (1:100; DakoCytomation), and the macrophage-specific marker, CD68 (1:100; DakoCytomation) to assess the relative proportion of phenotypes present after successive passages. The ratio of CD68-positive cells to α-actin-positive cells after passage was determined using high-power field microscopy.

4.5.2 Results and Conclusion

Vascular SMC from healthy aortic tissue were successfully isolated using the enzymic dispersion method and maintained in viable cell culture undergoing consistent passage. The aortic tissue was obtained from donors between 39 and 48 years of age. As such, atherosclerotic changes in the tissue would be in development. Immunostain of the primary culture with α-actin and CD68 antibodies demonstrated a co-culture of VSMC and macrophages (foam cells). High-power field cell counts produced a low CD68:α-actin ratio with approximately 1.4 macrophages for every VSMC (Figure 4.6.). Propagation of CD68 positive cells was not evident upon successive subculture and resulted in a macrophage-free, predominantly α-actin positive, cell culture by the third passage (Figure 4.6 and 4.7.).

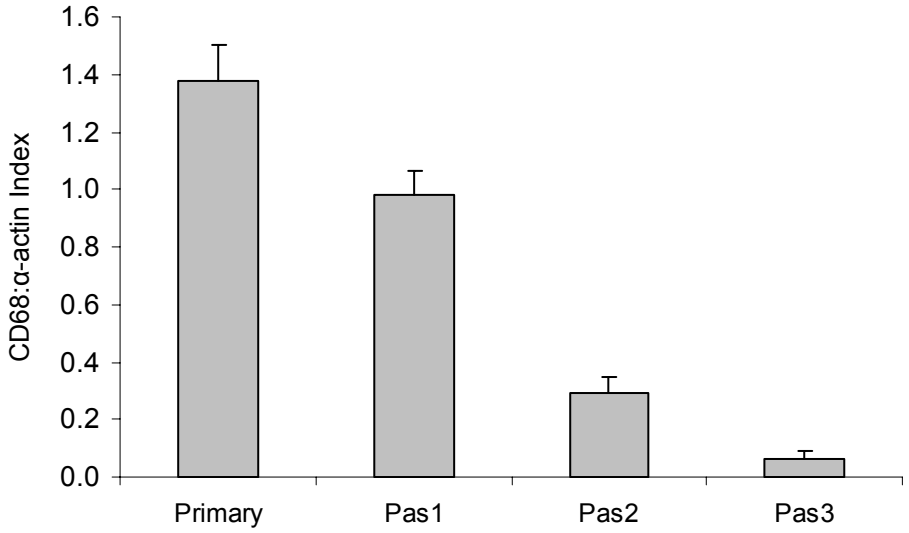


Figure 4.6. Ratio of CD68-positive cells (macrophages) to α-actin-positive cells (VSMC) at primary culture through to third passage determined by high-power field microscopy. Data expressed as mean±SEM of triplicate cultures.

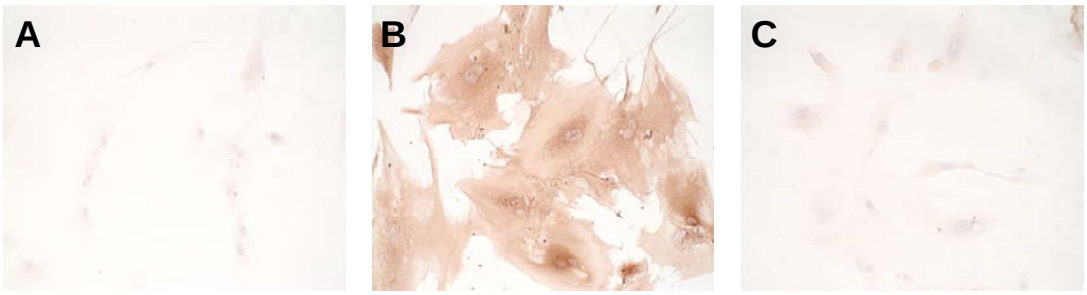


Figure 4.7. Immunocytochemical validation of pure human aortic VSMC culture isolated by enzymatic extraction. (A) Negative control. (B)VSMC phenotype identified by the cytoskeletal protein α-Actin. (C) Non-contamination of the culture by monocyte/macrophages at passage three confirmed by absence of staining for CD68.

4.6 MACROPHAGE ISOLATION FROM AAA TISSUE

Obtaining macrophages from human AAA biopsies for study *in vitro* required a viable extraction method. The aim of this study was to evaluate tissue macrophage isolation using FACS analysis.

4.6.1 Study Design

A cell suspension was obtained from human AAA tissue homogenate and the mononuclear cell population isolated by Ficoll-Hypaque gradient centrifugation as described (Chapter 3, section 3.2.1.(iii)). Sample of these cell were prepared for cell-surface staining and FACS analysis (Chapter 3, section 3.2.4(i) and (ii)) using a monoclonal antibody to the macrophage-specific surface marker, CD71 (Ber-T9; DakoCytomation) and an IgG negative control (X0931, DakoCytomation).

4.6.2 Results and Conclusion

Detection of the cell surface marker CD71 by FACS analysis confirmed the presence of macrophages in the mononuclear cell population isolated from human AAA tissue homogenate (Figure 4.8.) and validated the use of Ficoll-Hypaque gradient centrifugation in the extraction protocol. Separation of macrophages from the total mononuclear cell population for culture studies was achieved by exploiting their capacity to adhere to the surface of a plastic culture flask following incubation at 37ºC in an atmosphere of 5% CO_2 for 30 minutes.

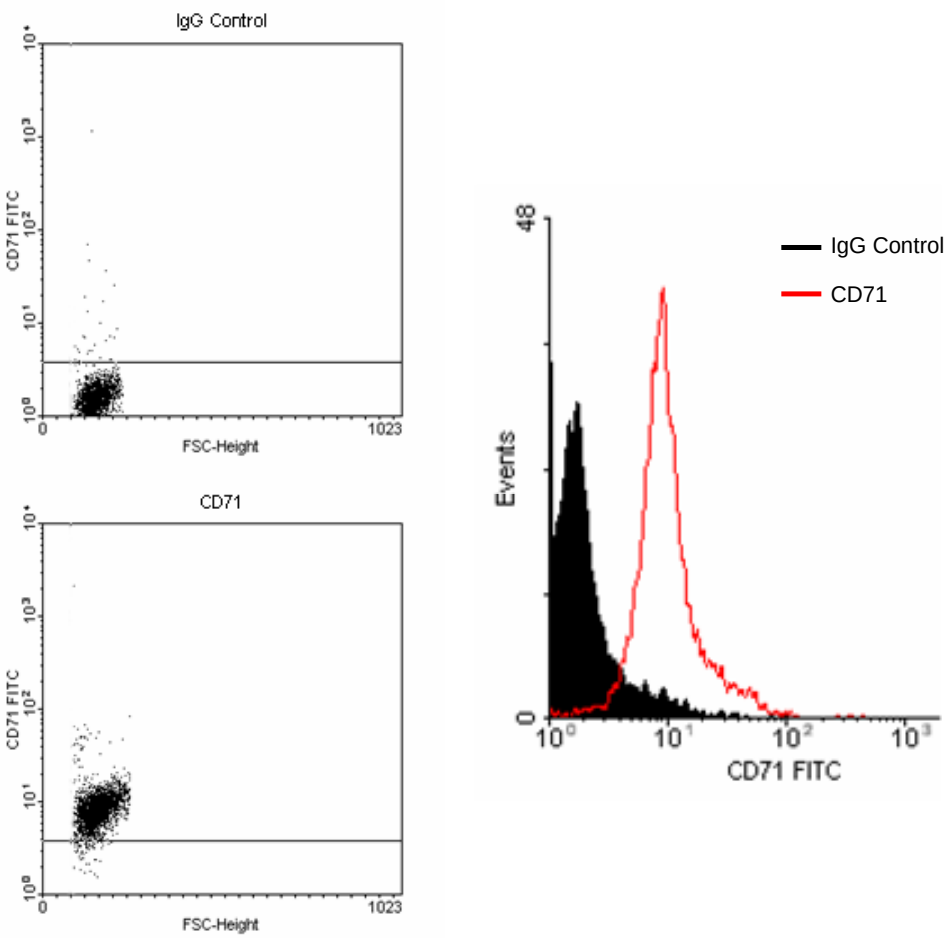


Figure 4.8. FACS detection of the macrophage-specific cell surface marker CD71. Comparison with negative (IgG) control.

4.7 DETERMINATION OF VSMC PROLIFERATION

The aim of this study was to assess the use of tritiated (^{3}H)-thymidine incorporation (DNA synthesis) in the assessment of VSMC proliferation, and to evaluate the suitability of foetal bovine serum (FBS) as the sole stimulator of proliferation.

4.7.1 Study Design

Vascular SMC were isolated from both non-diseased (NASMC) and aneurysmal (AASMC) human aorta as described (Chapter 3, section 3.2.1.(i)) and transferred to 96-well culture plates (Nunc) at a seeding density of 1x10^4 cells per well, and

incubated in DMEM containing 10% FBS at 37°C in an atmosphere of 5% CO_2 for 24 hours. The cells were then incubated for a further 24 hours in serum-free DMEM, after which experimental medium containing 0, 0.5, 1, 5, 10, or 20% FBS was substituted. Cells were incubated for 24 and 48 hours. Four (4) hours prior to termination of the experimental period, cultures were pulsed with ^{3}H-thymidine in preparation for the assessment of cell proliferation (Chapter 3, section 3.2.5.).

4.7.2 Results and Conclusion

The ^{3}H-thymidine-incorporation assay was robust and clearly reflected the ability of FBS to stimulate proliferation in NASMC in a dose-dependant manner. Maximal stimulation of growth in these cells occurred in the presence of 10% FBS over 24 hours. At the same parameters, AASMC not surprisingly exhibited a marked inability to carry out DNA synthesis compared to NASMC (225±40 cpm vs 11300±937 cpm; P<0.001; Figure 4.9.). The rate of DNA synthesis was significantly reduced after 48 hours (Figure 4.10). The reduced rate of DNA synthesis after 24 hours reflects cell confluency reached at this time. Subsequent proliferation assays involving NASMC were conducted over 24 hours, using 10% FBS as a positive control for proliferation.

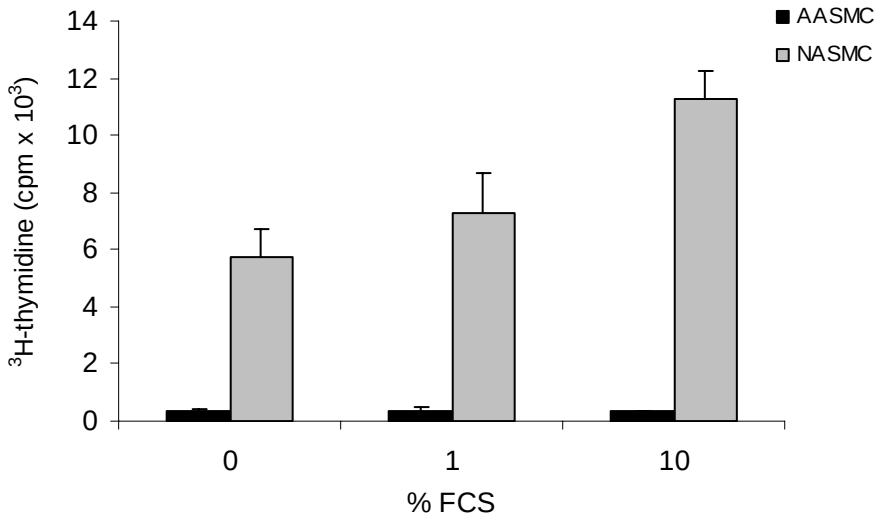


Figure 4.9. ^{3}H-thymidine-incorporation (DNA synthesis in VSMC isolated from nondiseased (NASMC) and aneurysmal (AASMC) human aorta over 24 hours. Data expressed as mean±SEM of triplicate cultures.

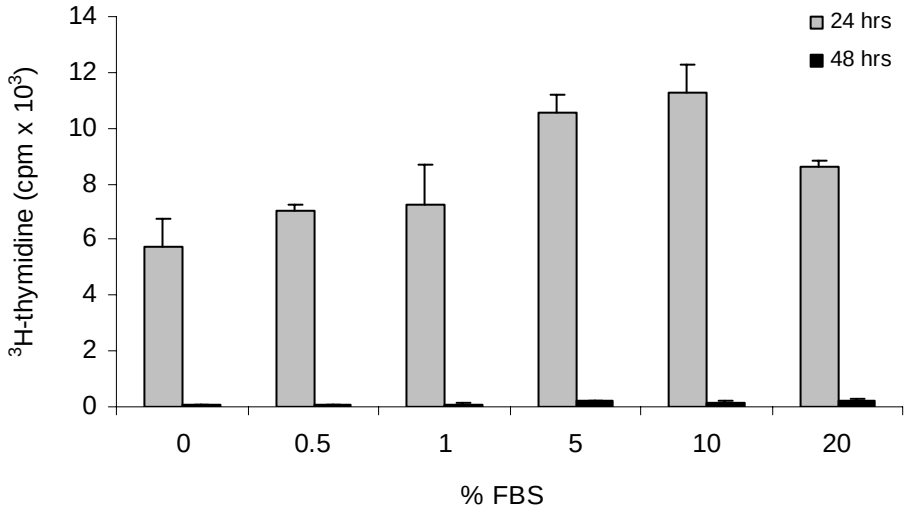


Figure 4.10. The rate of DNA synthesis (cell proliferation) in NASMC exposed to increasing concentration of FBS over 24 and 48 hours. Data expressed as mean±SEM of triplicate cultures.

4.8 LPS-ACTIVATION OF THP-1 CELLS

Blood-borne monocytes attach to and infiltrate the atherosclerotic artery wall, upon which they become activated and differentiate into macrophages. Experimentally, monocytes can be activated by exposure to lipopolysaccharide (LPS). The aim of this study was to determine both concentration and exposure time of LPS to be used in the activation of cultures of the monocytic cell-line THP-1.

4.8.1 Study Design

THP-1 cells were transferred to 96-well culture plates (Nunc) at a seeding density of $1x10^5$ cells per well, and incubated in RPMI containing 10% FBS at 37°C in an atmosphere of 5% CO_2, and in the presence of 0, 0.5, 1, 5, 10, or 20 μg LPS per 10^5 cells. Cultures were incubated over 24 and 48 hours, after which supernatants were harvested and assayed for levels of the proinflammatory cytokine interleukin (IL)-6 (Chapter 3, section 3.2.3.).

4.8.2 Results and Conclusion

Culture of THP-1 cells in the presence of LPS resulted in their dose-dependant activation as determined by cellular production and secretion of IL-6 (Figure 4.11.). Maximal stimulation of IL-6 production was observed at an LPS concentration of 10 μg per 10^5 cells. At this concentration, levels of IL-6 detected in culture supernatant after 48 hours were 8-fold higher than levels detected after 24 hours. Subsequent studies incorporating maximally activated THP-1 cells, maintained experimental cultures over 48 hours in the presence of 10 μg LPS per 10^5 cells.

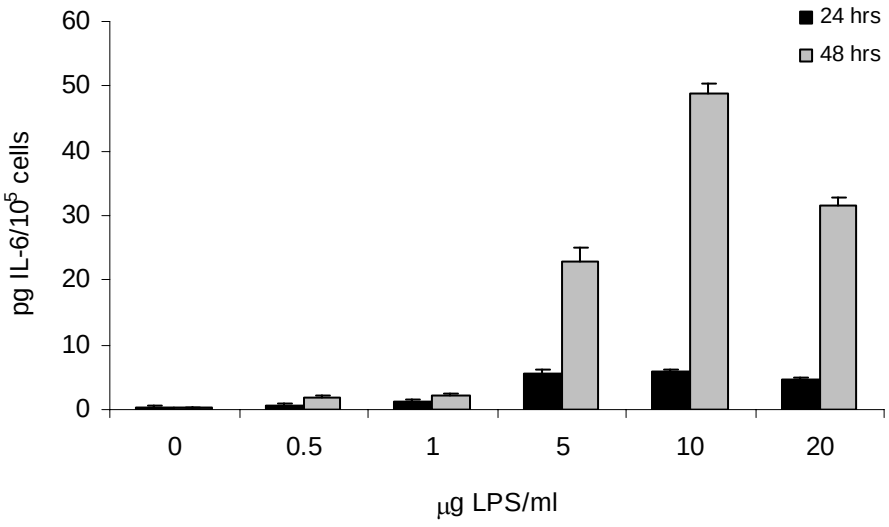


Figure 4.11. Dose-dependant secretion of IL-6 from THP-1 cells in response to activation by increasing concentration of LPS over 24 and 48 hours. Data expressed as mean±SEM of triplicate cultures.

4.9 EXTRACTION OF VSMC RNA

Gene expression studies required the extraction/purification of cellular RNA, a procedure facilitated by a commercially available RNA extraction kit. The aim of this study was to assess the efficiency and reproducibility of the extraction procedure.

4.9.1 Study Design

Although proposed gene studies would involve human aortic VSMC, this preliminary assessment of the RNA extraction/purification protocol was carried out using the monocytic cell-line THP-1 to preserve VSMC stocks. THP-1 cells were distributed to six 1 ml microfuge tubes at a density of $1.0x10^6$ cells per ml and pelleted by centrifugation at 180-200 *g* for 10 minutes. Each cell pellet was prepared for and underwent RNA extraction and assessment as described (see Chapter 3, section 3.2.7.).

4.9.2 Results and Conclusion

Spectrometric analysis of each sample determined an acceptable average RNA yield of 11.24 μg per 10^6 cells (Table 3.1.). Overall sample purity was high with a 260/280 ratio (0.25/0.13) of 1.9. Agarose gel electrophoresis demonstrated stability of the RNA samples after 14 days and storage at -20ºC (Figure 4.12.).

Table 4.1. Spectrometric analysis of extracted THP-1 RNA

Spec (260nm)	[μg/ml]	Vol (μl)	μgRNA
0.179	358	30	10.74
0.193	386	30	11.58
0.189	378	30	11.34
0.159	318	30	9.54
0.184	368	30	11.04
0.220	440	30	13.2

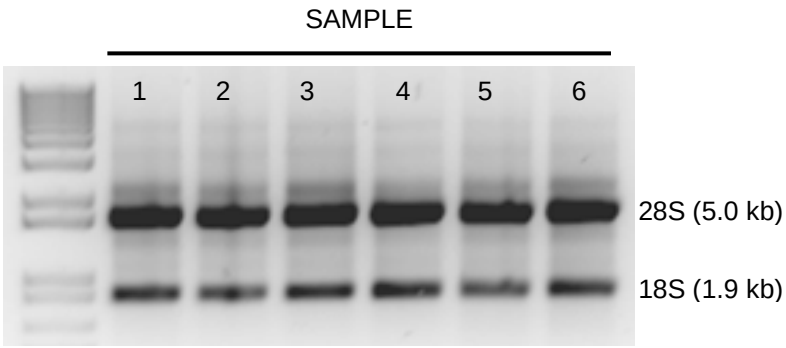


Figure 4.12. Stability of ribosomal RNA extracted from THP-1 cultures (n=6) demonstrated with agarose gel electrophoresis following storage at -20ºC for 14 days.

4.10 EXPLANT CULTURE OF HUMAN AAA TISSUE

The study of human AAA tissue *ex vivo* was performed to assess the effect of medication on cytokine production within the aneurysm wall. This required maintenance of biopsy samples in explant culture over an experimental period of up to six days either in the presence or absence of treatment. The aim of this study was first to assess the viability of explants over this period, and second, to determine whether observed effects of treatment were genuine and not due to cytotoxicity of the medication.

4.10.1 Study Design

Explants were derived from the same AAA biopsy. A sample was taken immediately to liquid nitrogen storage and the remainder prepared for tissue culture (Chapter 3, section 3.2.2.). Explants were incubated in the absence (control) or presence of the angiotensin II-receptor blocker, Irbesartan (1 mg/ml). On completion of the experimental period, tissue levels of ATP were determined in, and compared between, the incubated control, Irbesartan-treated explants, and the third sample frozen pre-culture, using a commercially available bioluminescent somatic cell assay (Sigma) as per the manufacturer's instructions.

4.10.2 Results and Conclusion

Viability of untreated (n=6) and treated (n=6) explants over six days was assessed and confirmed by comparison of tissue ATP with specimens frozen pre-culture (n=6). No significant difference in tissue ATP levels between samples was detected ($P>0.5$; Figure 4.13.), demonstrating both the survival of explants in culture, and non-toxicity of the experimental dosage of Irbesartan.

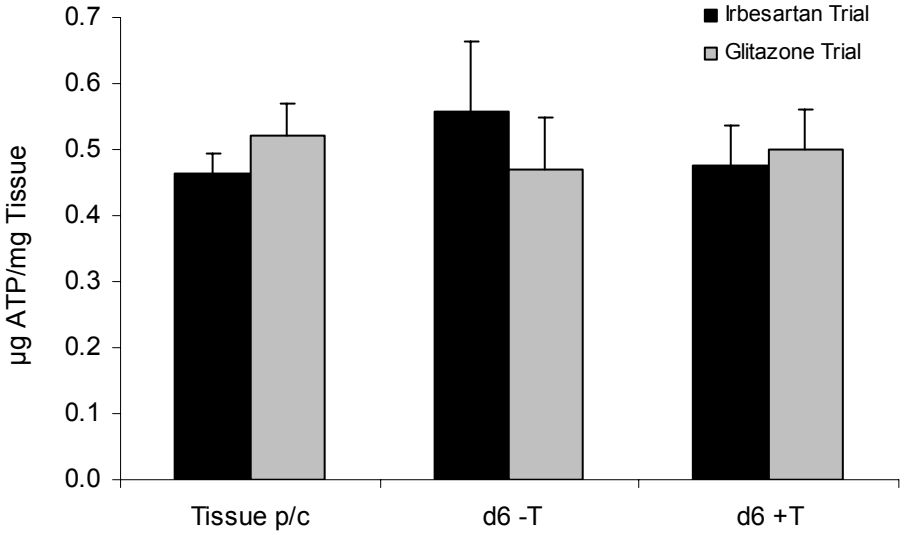


Figure 4.13. Comparison of tissue ATP concentration between explants pre-culture (Tissue p/c; n=6) and explants incubated over six days in the absence (d6 –T; n=6) or presence (d6 +T; n=6) of treatment. Data expressed as mean±SEM.

4.11 ANIMAL MODEL FOR AAA

Several animal models for AAA have been developed. In mice, methods of inducing aneurysm formation vary and include the local intraluminal perfusion of elastase, perivascular application of calcium chloride ($CaCl_2$), and the infusion of angiotensin II in the ApoE lipoprotein gene knockout ($ApoE^{-/-}$) mouse. The latter develops spontaneous hyperlipidaemia and when infused subcutaneously with angiotensin II, produces focal aneurysms primarily of the supra-renal aorta. This model is favourable, as physical manipulation of the aorta required in other models is not needed. The aim of this study was to confirm the suitability and use of the model for investigation of AAA.

4.11.1 Study Design

Aortic aneurysms were induced with angiotensin II infusion in six male $ApoE^{-/-}$ mice and the procedure assessed by macroscopic examination of the aorta, and the

correlation of the proteolytic enzyme (MMP-9) activity within the aortic wall with aortic diameter (see Chapter 3, sections 3.3.2. and 3.3.3.).

4.11.2 Results and Conclusion

Focal dilatation in response to angiotensin II infusion was observed primarily in the abdominal aorta above the renal arteries, but also demonstrated in the descending thoracic aorta. Aneurysms were not demonstrated within the infra-renal aorta. An aneurysm in this model was defined as a supra-renal or thoracic aorta diameter two or more times greater than the infra-renal diameter in that mouse. Using this definition, aneurysms were detected in four of the six aortas examined (Figure 4.14.).

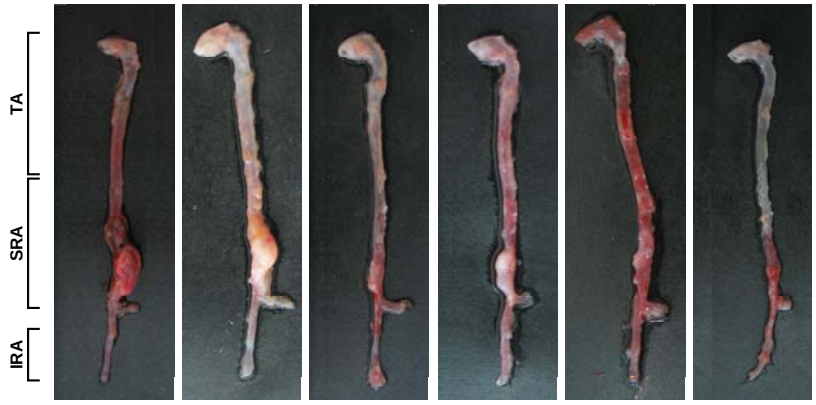


Figure 4.14. Aneurysms detected in the suprarenal aorta (SRA) of angiotensin II-infused ApoE$^{-/-}$ mice. (TA, thoracic aorta; IRA, infrarenal aorta)

Morphometric analysis on 12 aortic segments on two separate occasions demonstrated an inter-assessment reproducibility correlation of 1.0 (95% confidence intervals 0.99-1.0). The level of MMP-9 activity measured within the suprarenal and thoracic aorta correlated very strongly with the maximal aortic diameter at that site (r=0.91; Figure 4.15.). This result is in keeping with the known involvement of MMP-9 in human AAA pathology, and together with the relatively high incidence of aneurysm formation observed in this study, validates the ApoE$^{-/-}$/angiotensin II model for investigation of AAA.

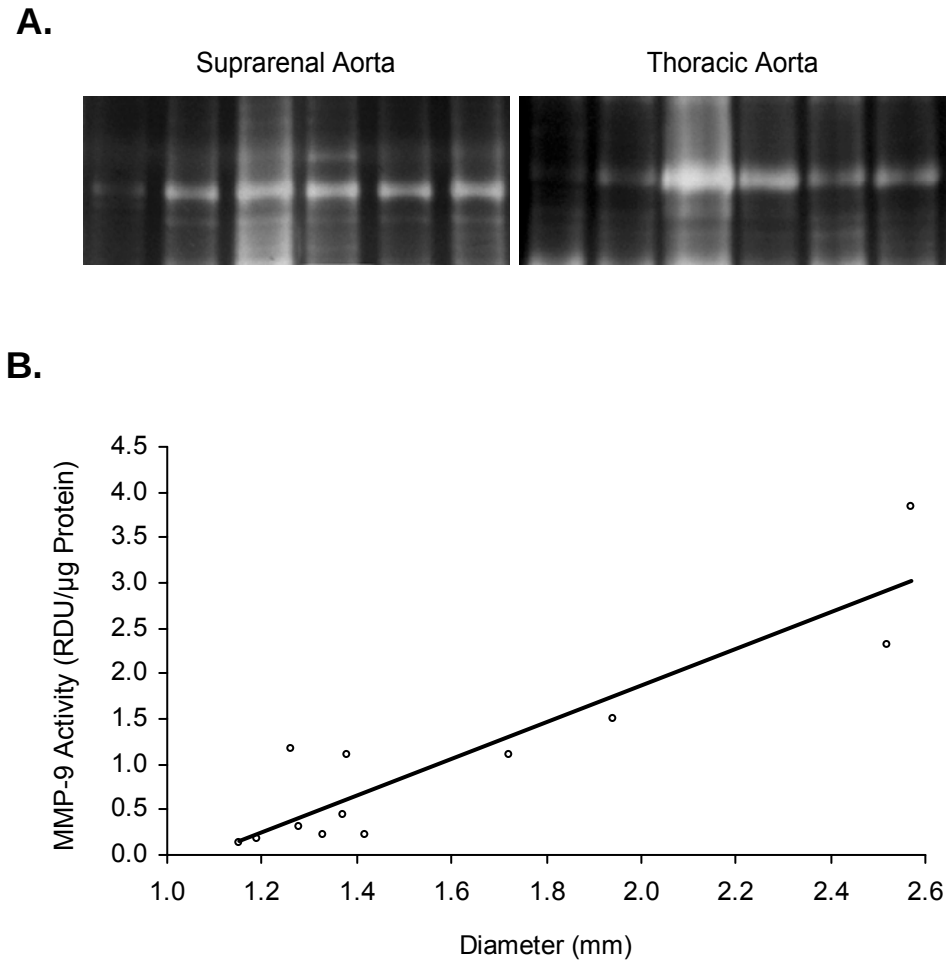


Figure 4.15. (A) Zymographic detection of MMP-9 activity in suprarenal and thoracic aortic segments from angiotensin II-infused ApoE$^{-/-}$ mice. (B) Correlation between MMP-9 activity and increasing aortic diameter.

4.12 DETERMINATION OF THE DOSE-RANGE OF rhOPG FOR *IN VITRO* STUDIES

Previous *in vitro* studies examining the biological effect of OPG used concentrations ranging between 1 and 5000 ng/ml. The aims of this study were to:

1. Quantify the level of OPG protein per mg tissue present in AAA tissue
2. Approximate an average cell (VSMC) number present in 1.0 mg AAA tissue

The estimation of the concentration of OPG to which cells within the human aortic aneurysm are exposed would enable determination of an approximate concentration range of OPG, which corresponded closely to *in vivo* levels, for use in functional studies *in vitro*.

4.12.1 Study Design

The amount of OPG present in AAA biopsies was quantified using western analysis (Chapter 3, sections 3.1.5. and 3.1.6.) and a standard curve generated with known quantities of recombinant human OPG (ImmunoKontact). Additionally, OPG levels produced *in vitro* by cells isolated from AAA were determined by ELISA (see Chapter 5, section 5.2.3.(ii).). Vascular smooth muscle cell number per mg AAA tissue was estimated by haemocytometer count of enzymically dispersed medial VSMC (see Chapter 3, section 3.2.1.(i)).

4.12.2 Results and Conclusion

The concentration of OPG in AAA samples was approximately 1.3±0.1 ng per mg tissue as determined by western analysis (Table 4.2.). The number of VSMC isolated from 1.0 mg AAA tissue averaged 4.6±0.4 x 10^5 cells (Table 4.3.). Theoretically therefore, approximately 0.3 ng OPG per 10^5 VSMC is present within the aneurysm wall.

Table 4.2. Average quantity of OPG (ng) per milligram of AAA tissue

AAA Sample	μgProtein/mgTissue	ngOPG/μgProtein	**ngOPG/mgTissue**
1	33.93	0.034	1.17
2	38.69	0.042	1.62
3	47.06	0.027	1.25
4	32.49	0.035	1.14
Av			**1.29**
SD			0.22
SEM			0.11

Table 4.3. Average number of VSMC per milligram AAA tissue

AAA Sample	Tissue Weight (mg)	Cell Number ($x10^7$)	**Cells ($x10^5$) per mg Tunica Media**
1	65	3.13	4.81
2	109	5.58	5.12
3	178	6.02	3.38
4	83	4.23	5.09
Av			**4.60**
SD			0.83
SEM			0.41

The concentration of OPG secreted *in vitro* by VSMC and macrophages isolated from AAA tissue measured 0.27 and 0.11 ng per 10^5 cells per ml per 24 hours, respectively (see Chapter 5, section 5.2.3.(ii).). From this, normal human VSMC and monocytes were exposed to an *in vitro* concentration range of 0 to 20 ng rhOPG per 10^5 cells per ml. The high-end of the range was chosen taking into account the probable accumulation of OPG within the aneurysmal aortic wall associated with *de novo* synthesis by VSMC and inflammatory cells over years of aneurysm development.

Chapter 5

OSTEOPROTEGERIN AND THE PRESENCE OF AORTIC ANEURYSM

5.1 INTRODUCTION

There is evidence that suggests the involvement of OPG in vascular disease. Epidemiological studies have shown that serum levels of OPG positively correlate with the onset and severity of atherosclerotic artery disease (Schoppet *et al,* 2003; Keichl *et al,* 2004), and are associated with increased cardiovascular mortality (Browner *et al,* 2001). Increased expression of OPG has been demonstrated in the walls of rat aorta following balloon angioplasty (Zhang *et al*, 2002) and in atherosclerotic human vessels (Dhore *et al*, 2001). *In vitro* studies using human aortic smooth muscle cells confirmed that OPG mRNA is highly expressed in these cells and that growth factors such as PGDF, β-FGF, Angiotensin II, TNFα, and IL-1β induce OPG expression (Zhang *et al*, 2002). Finally, OPG modulates processes fundamental to the pathogenesis of AAA, such as immune and inflammatory cell function (Yun *et al,* 2001) and proteolytic enzyme expression and activity (Wittrant *et al*, 2002).

In view of the association between OPG and atherosclerosis and the influence of the cytokine on cellular mechanisms underlying aneurysm expansion, the data provided in this chapter investigates a potential association between arterial expression of OPG and the presence of aortic aneurysm. The relationship between serum and aortic concentration of OPG and aneurysm growth in human AAA was examined as well as the relative contribution of VSMC and macrophages to OPG concentration within the aneurysmal wall. The direct involvement of OPG with AAA development *in vivo* is further investigated in a mouse model of the disease.

5.2 EXPERIMENTAL METHODS

5.2.1 Relationship between serum levels of OPG and AAA

Serum samples were obtained as described (Chapter 3, section 3.1.1.) from 146 male patients with small aortic aneurysms (30-50 mm) and 20 male patients undergoing aneurysm repair (>50 mm). The former cohort was followed up with regular ultrasound scans for a minimum of three years. The concentration of OPG was determined in each sample using ELISA (Chapter 3, section 3.2.3.) and correlated with (abdominal) aortic diameter, and, rate of expansion.

5.2.2 Comparison of OPG levels in aneurysmal versus non-aneurysmal aortic tissue

Ten (10) AAA biopsies, 10 AOD biopsies, 5 post-mortem nonaneurysmal abdominal aortic biopsies (PAA), and 10 thoracic aorta (TA) biopsies were obtained from male patients aged 70±6, 67±7, 63±7, and 65±6 years, respectively (see Chapter 3, section 3.1.2.). Immunostaining of paraffin sections from formalin-fixed biopsies was performed using a mouse monoclonal antibody to human OPG (clone 98A1071, Imgenex; 1:1000) (Chapter 3, section 3.1.4.(iii)). Extracted tissue proteins were separated using SDS gel electrophoresis and western analyses (Chapter 3, sections 3.1.5. and 3.1.6.) carried out using mouse monoclonal antibody to human OPG (Imgenex) at 1.5 μg/ml. Bands corresponding to OPG (55 kDa) were analyzed as described (Chapter 3, section 3.1.6(ii)). Quantification of OPG was performed by comparison of band densities with a standard curve of known quantities of recombinant human OPG (ImmunoKontact).

5.2.3 Secretion of OPG by vascular and inflammatory cells within the aneurysm wall

Immunostaining of serial paraffin sections from formalin-fixed AAA tissue was performed using a mouse monoclonal antibody to human smooth muscle α-actin (DakoCytomation; 1:100) and OPG (Imgenex; 1:1000) (Chapter 3, section

3.1.4.(iii)). Vascular SMC and macrophages were isolated from four (4) AAA biopsies (Chapter 3, sections 3.2.1.(i). and 3.2.1.(iii)) and maintained in separate cell culture for 48 hours. Production of OPG by these cells was then assessed by quantifying supernatant levels of the protein by ELISA (Chapter 3, section 3.2.3.) and comparing them with levels secreted from VSMC isolated from healthy aorta, peripheral blood monocytes, resting THP-1 cells, or LPS-activated THP-1 cells. Upon removal of the supernatant, both aneurysm-derived VSMC and macrophages were fixed and pelleted in preparation for analysis of intracellular OPG by FACS (Chapter 3, section 3.2.4.) using a mouse monoclonal antibody to human OPG (clone 98A1071, Imgenex) and an IgG negative control (X0931, DakoCytomation). Detection was facilitated by the use of a secondary FITC-labeled anti-mouse IgG (F2272, Sigma).

5.2.4 Expression of aortic OPG in experimental AAA

Male ApoE$^{-/-}$ mice (n=12) were infused subcutaneously with Angiotensin II and the aorta from each animal harvested after 28 days (see Chapter 3, sections 3.3.2. and 3.3.3.). Outcome was assessed by measurement of aortic diameter (Chapter 3, section 3.3.3.) and western analysis of aortic OPG concentration. Extracted tissue proteins were separated using SDS gel electrophoresis and western analyses (Chapter 3, sections 3.1.5. and 3.1.6.) carried out using goat polyclonal antibody to human OPG (R&D) at 0.2 μg/ml and rabbit anti-goat secondary immunoglobulin (1:5000; DakoCytomation). Bands corresponding to OPG (55 kDa) were analyzed as described (Chapter 3, section 3.1.6(ii)) and expressed as relative density units (RDU) per μg protein.

5.3 RESULTS

5.3.1 Serum levels of OPG correlate with aneurysm growth rate

Sixty percent of aortic aneurysms measured 30-40 mm resulting in an observed mean growth rate of 1.2±0.8 mm per year, slower than previously reported by other

investigators. No significant correlation between serum OPG concentration and abdominal aortic diameter was observed (Figure 5.1.A.; r=0.017.). A weak correlation between serum OPG and aneurysm growth rate was demonstrated (Figure 5.1.B.; r=0.20; *P*=0.04). In 34 patients with rapidly expanding aneurysms (>5mm over a 36 month period) mean serum OPG at the onset of surveillance was 1.1±0.1 compared with 0.9±0.03 ng/ml in 112 patients with slower growing aneurysms (*P*=0.04, unpaired t-test). Moreover, serum OPG concentration remained a predictor of aneurysm expansion upon multiple-regression analysis (*P*=0.02; coefficient 1.33, SE 0.51) in a model consisting of patient age, diabetic status, smoking history, initial aortic diameter, serum cholesterol, HDL, LDL and C-reactive protein.

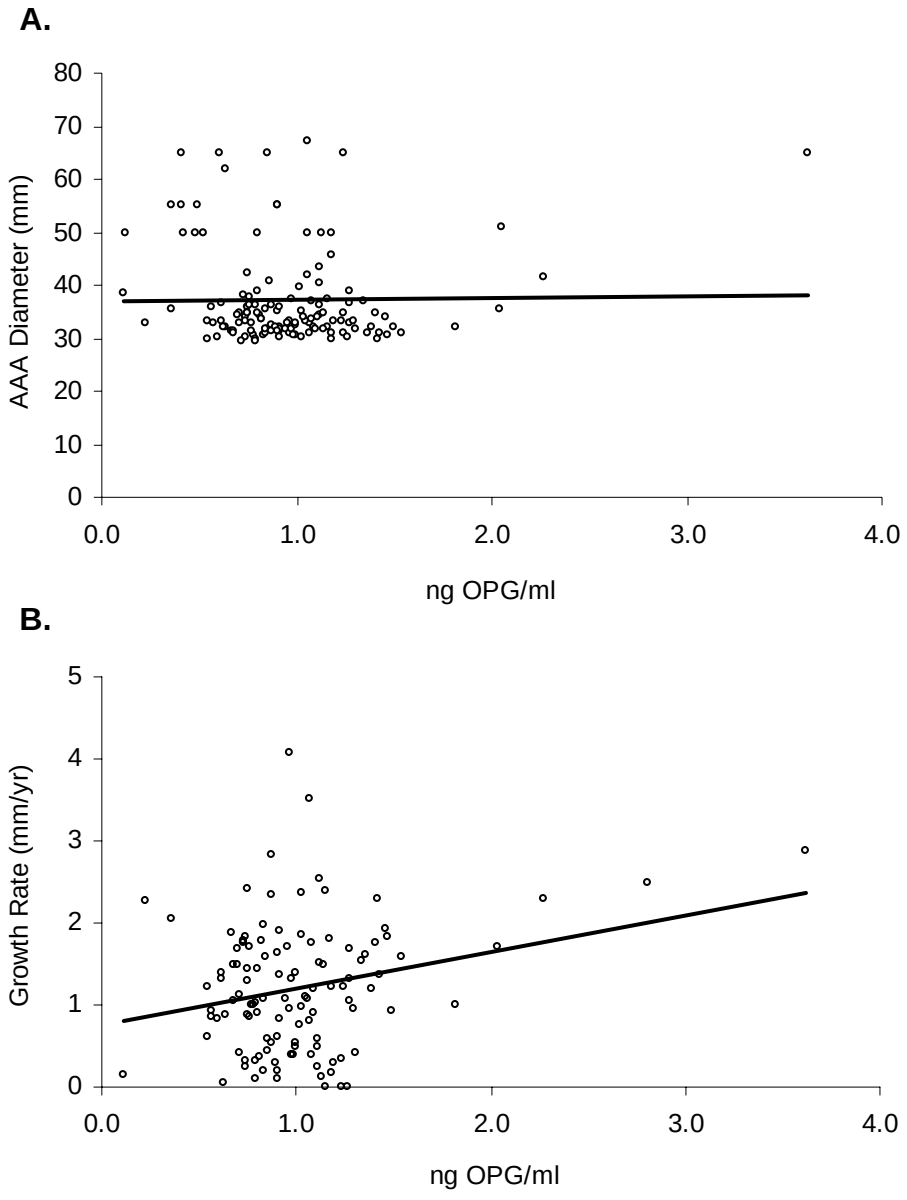


Figure 5.1. Correlation in AAA patients between serum OPG concentration, aortic diameter (A), and aneurysm growth rate (B).

5.3.2 OPG is upregulated in human aneurysmal aorta compared with non-aneurysmal aorta.

Immunodetection of OPG in healthy aorta was minimal (Figure 5.2.A.) compared to the dense staining observed within AAA biopsies (Figure 5.2.B.). Expression of OPG was demonstrated in both aneurysmal and atherosclerotic-narrowed aorta. The presence of OPG in AAA tissue spanned all three arterial layers (Figure 5.2.C.). In contrast, the presence of OPG in biopsies of occlusive aorta was limited primarily to that of the intima and associated atheroma (Figure 5.2.D.). Protein analysis demonstrated no significant difference in OPG concentration between control thoracic and control abdominal aortic biopsies (Figure 5.3.A. and 5.3.B.; P=0.57).

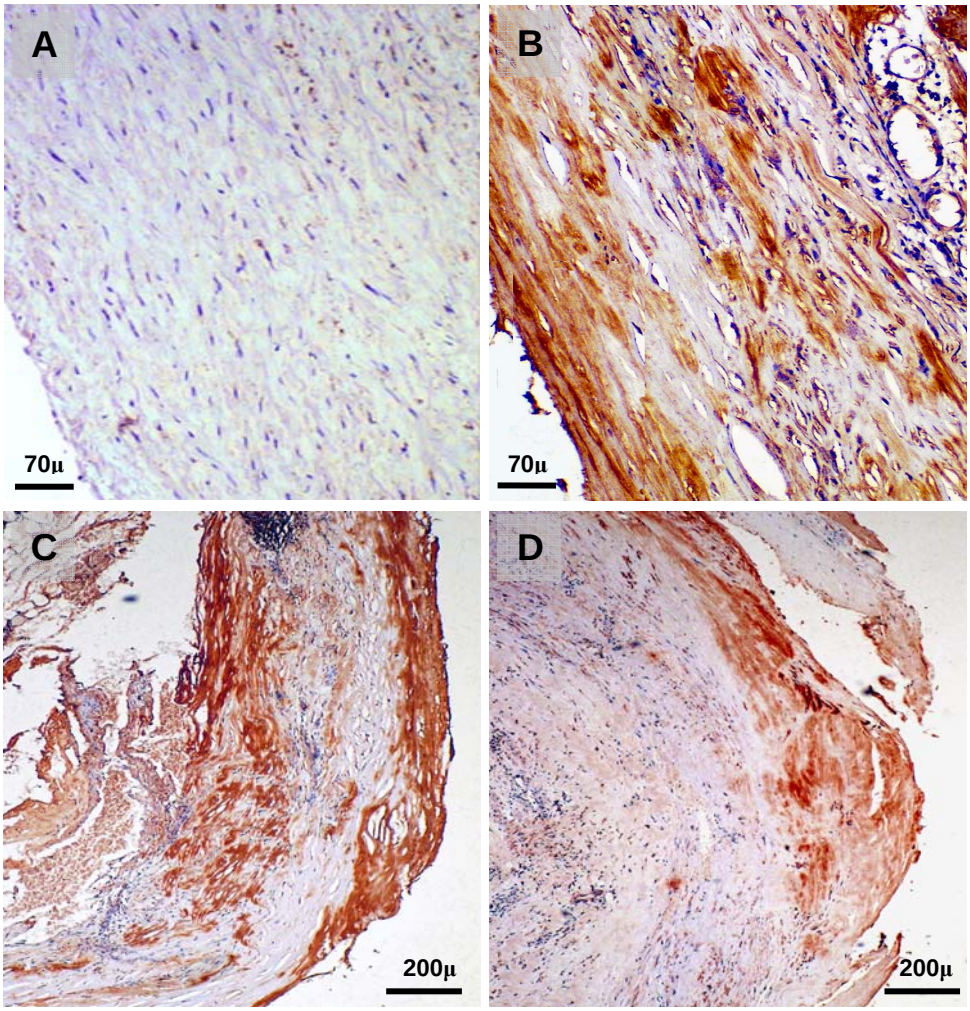


Figure 5.2. Immuno-detection of OPG (brown stain) in healthy human aorta (A) and human aneurysmal aorta (B). OPG localized in the intima and throughout the fragmented media of AAA tissue (C) but primarily restricted to the intima in specimens from AOD (D).

Compared to age-matched TA or PAA, OPG detected in AOD biopsies measured 0.5±0.1 ng/mg tissue versus 0.1±0.06 and 0.2±0.1 ng/mg tissue respectively (*P*=0.02, *P*=0.07). The concentration of OPG in age-matched AAA biopsies was significantly greater: 3-fold higher than in AOD (1.4±0.1 vs 0.5±0.1 ng/mg tissue; *P*=0.002), and, 8 to 12-fold higher than in PAA or TA tissue respectively (*P*<0.001).

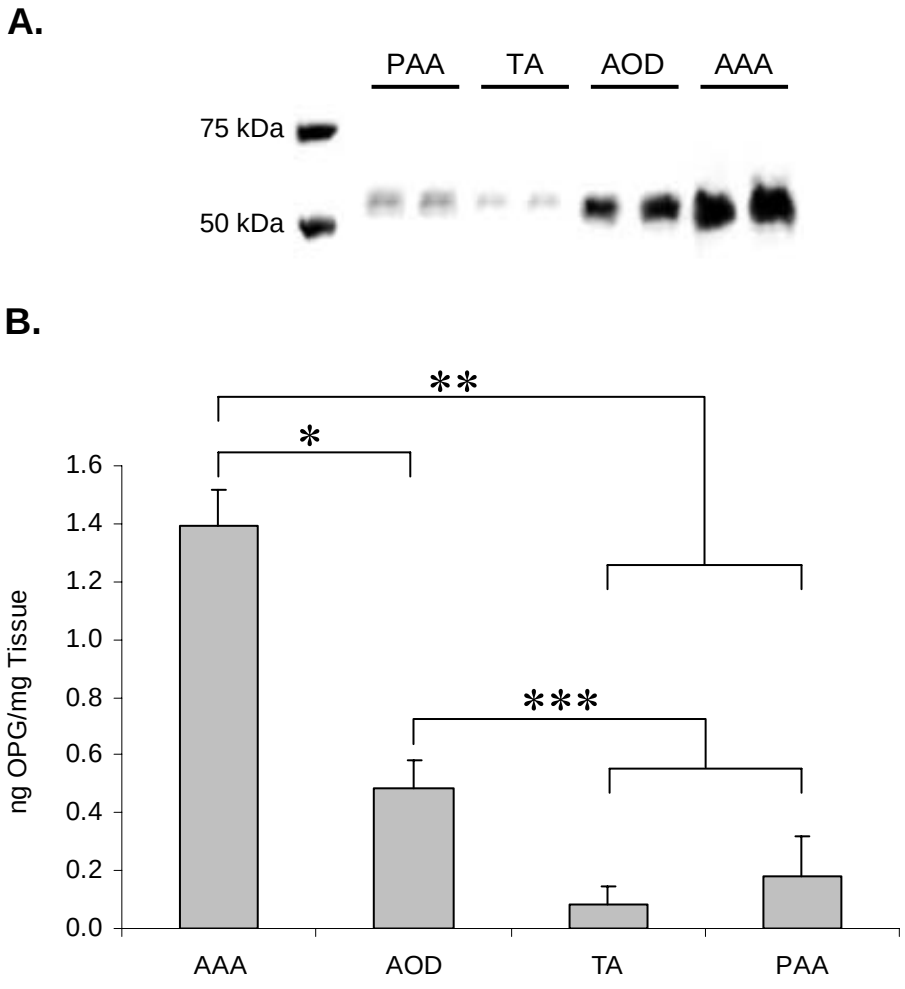


Figure 5.3. (**A**) Upregulation of OPG in AAA compared with aortic occlusive disease (AOD), and, thoracic aorta (TA) and post-mortem abdominal aorta (PAA) control tissue confirmed with western analysis (B). Data expressed as mean±SEM; n=10 (PAA n=5); **P*=0.002, ***P*<0.001, ****P*<0.05.

5.3.3 OPG is secreted at high levels by medial smooth muscle cells and inflammatory cells within the human aortic aneurysm wall.

Serial-staining of AAA tissue for smooth muscle-specific α-actin (Figure 5.4.A.) and OPG (Figure 5.4.B.) co-localized OPG with medial VSMC. Vascular SMC isolated from AAA tissue secreted significantly higher levels of OPG *in vitro* than VSMC from healthy abdominal aorta, measuring 267±20 pg/10^5 cells/ml/24h and 17±5 pg/10^5 cells/ml/24h respectively (Figure 5.5.; P=0.001).

Macrophages isolated from AAA tissue (AAMø) secreted levels of OPG similar to those observed from LPS-activated THP-1 cells (aTHP-1) (107±10 pg/10^5 cells/ml/24h and 98±6 pg/10^5 cells/ml/24h, respectively), and significantly higher levels compared to resting THP-1 cells (rTHP-1) or peripheral blood monocyte (PBM) controls (107±10 pg/10^5 cells/ml/24h versus 0.43±0.02 pg/10^5 cells/ml/24h; P<0.001). The presence of intracellular OPG in both VSMC and macrophages derived from human AAA was confirmed with FACS analysis (Figure 5.6. and 5.7.).

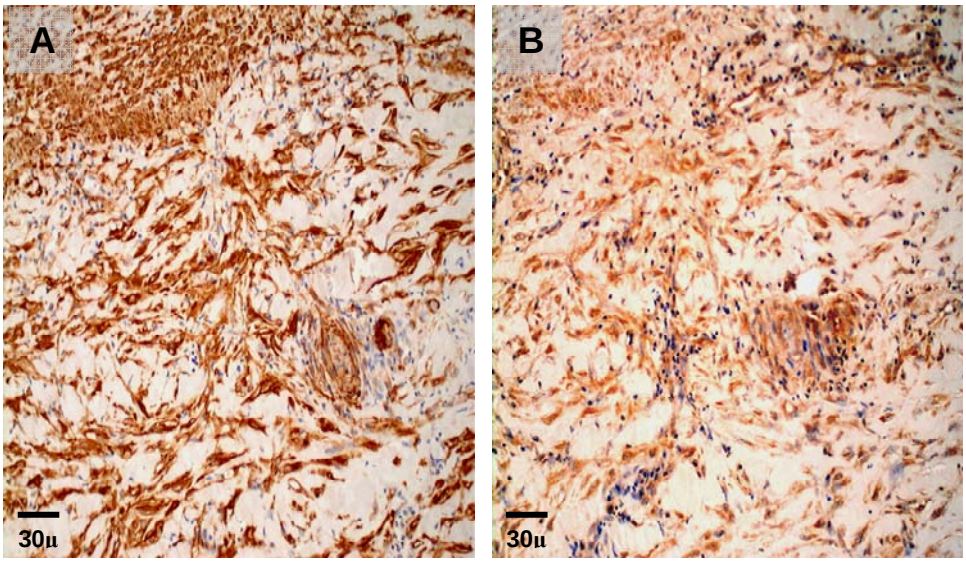


Figure 5.4. Immunostain for SMC α-actin (A) and OPG (B) in serial sections of human AAA tissue demonstrating primarily medial VSMC-associated OPG expression.

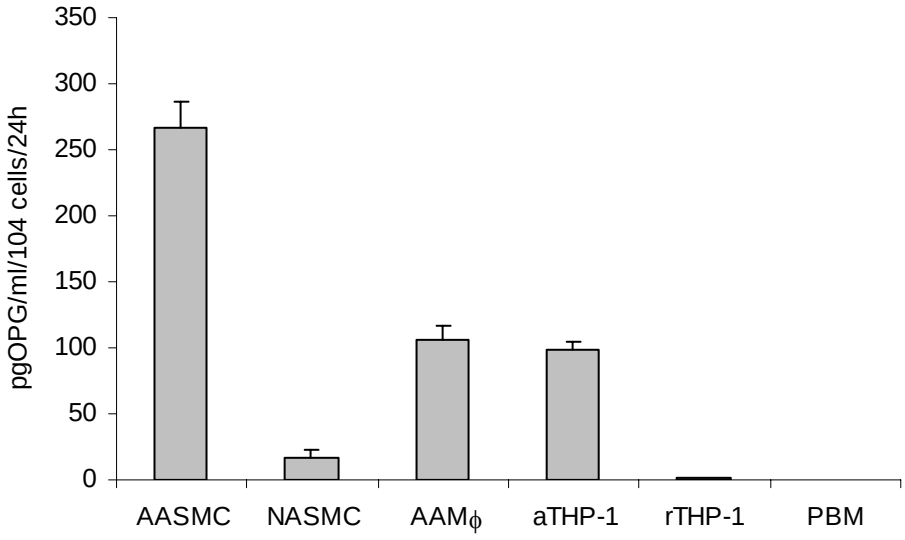


Figure 5.5. Comparison of OPG secretion by aneurysm-derived smooth muscle cells (AASMC), smooth muscle cells from healthy aorta (NASMC), aneurysm-derived macrophages (AAMø), LPS-activated THP-1 cells (aTHP-1), resting THP-1 cells (rTHP-1), and peripheral blood monocytes (PBM).

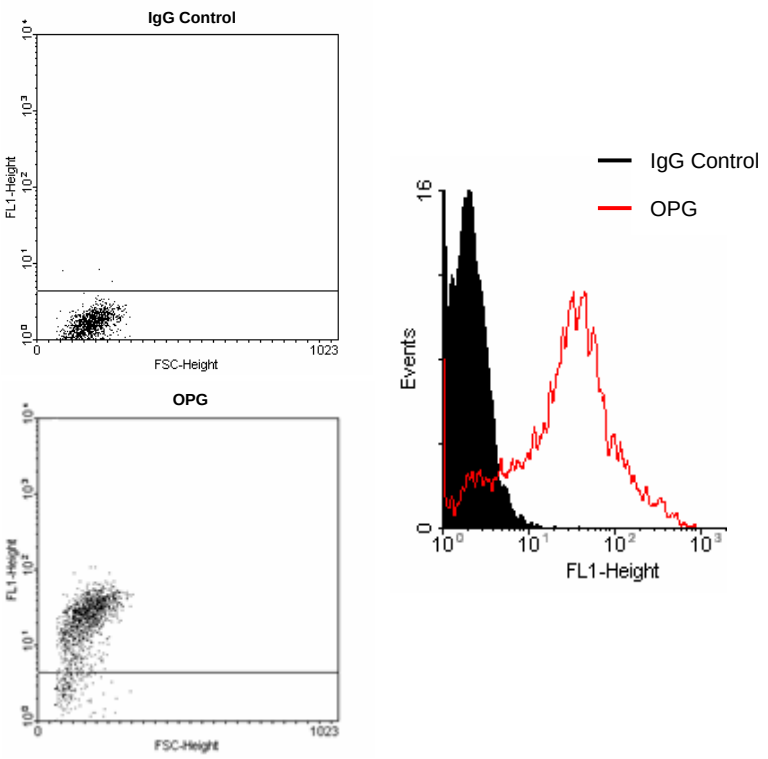


Figure 5.6. FACS detection of intracellular OPG in AAA-derived VSMC. Comparison with negative (IgG) control.

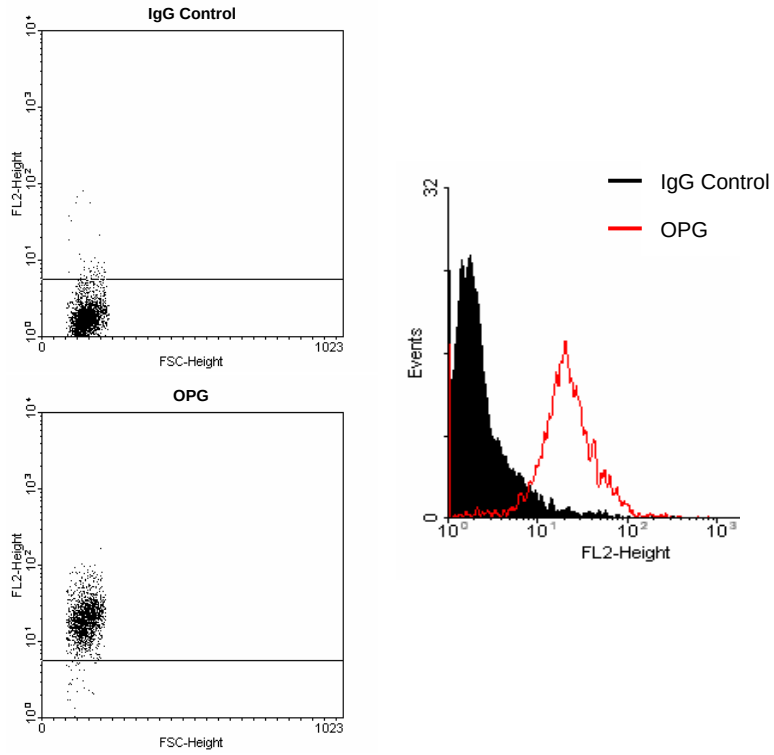


Figure 5.7. FACS detection of intracellular OPG in AAA-derived macrophages. Comparison with negative (IgG) control.

5.3.4 OPG concentration is higher in aneurysmal aorta compared to non-aneurysmal aorta and correlates with aortic diameter in a mouse model of AAA.

Maximum aortic diameter was measured in the suprarenal and infrarenal aorta, and, by definition (see Chapter 4, section 4.10), aortic aneurysms were identified in six of the 12 mice. Western analysis confirmed an upregulation of aortic OPG associated with aneurysm formation in the suprarenal aorta of Angiotensin II-infused ApoE$^{-/-}$ mice compared to non-aneurysmal aorta (1.4±0.1 versus 0.6±0.08 RDU/μg protein, respectively; P<0.01; Figure 5.8.A). Aortic OPG concentration correlated well with aortic diameter (R=0.86; Figure 5.8.B.).

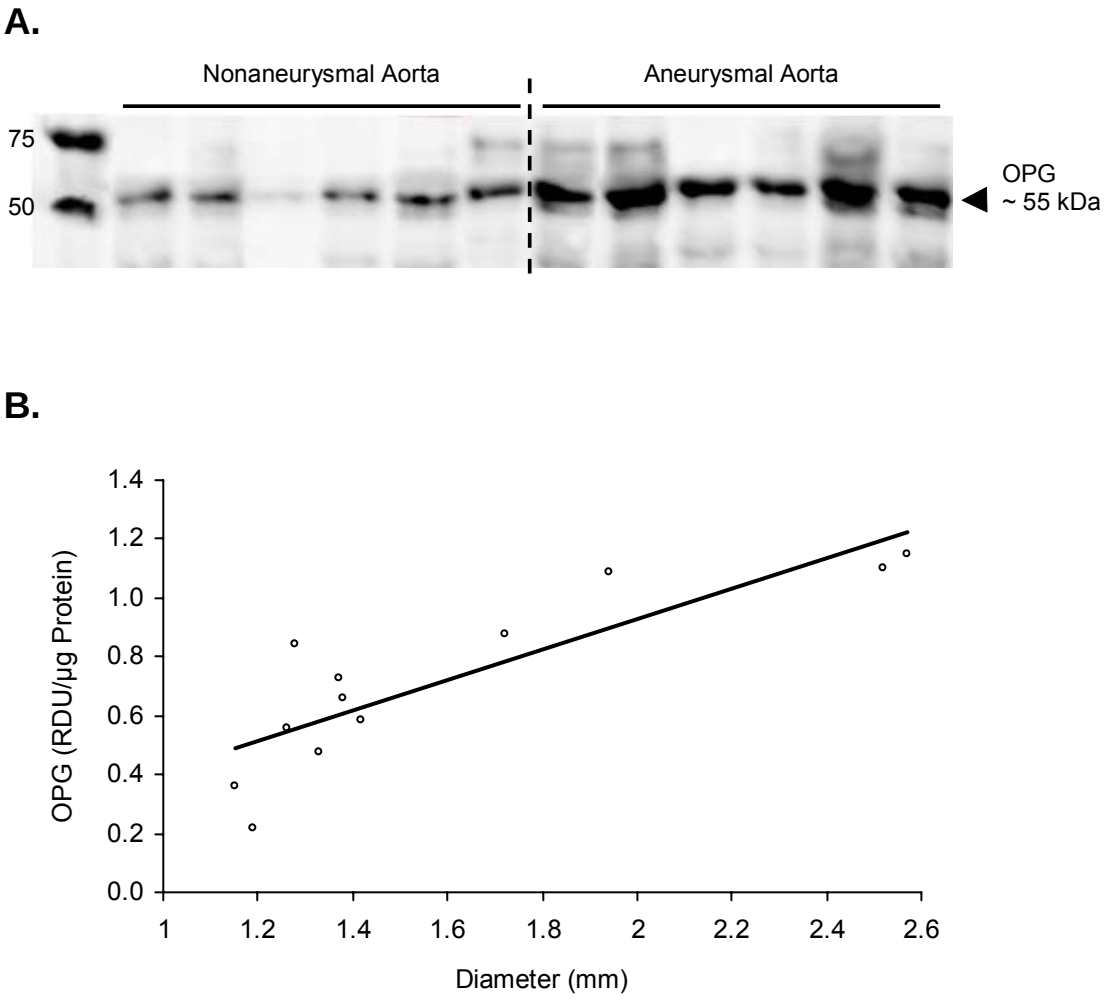


Figure 5.8. (A) Upregulation of aortic OPG associated with aneurysm formation in the suprarenal and thoracic aorta of angiotensin II-infused ApoE$^{-/-}$ mice. (B) A positive correlation between aortic concentration of OPG and increased diameter.

5.4 DISCUSSION

The above findings support an association between OPG expression and aneurysm development in the human abdominal aorta. A positive correlation between serum concentration of OPG in patients with AAA and aneurysm growth rate was demonstrated. Despite a cohort of 146 patients, the observed range of OPG serum concentrations was small (0.1 to 3.6 ng/ml) which resulted in a correlation coefficient associating serum OPG with aneurysm growth rate of only 0.2. However, allowing for other known determinants of AAA expansion on multiple regression analysis, the association between serum OPG concentration and aneurysm expansion remained significant. Interestingly, serum OPG has been acknowledged as a risk factor for the progression of atherosclerosis and onset of cardiovascular disease (Keichl *et al,* 2004). Nevertheless, analysis of a larger cohort is required to confirm the association of serum OPG concentration with AAA progression.

More convincing data of OPG upregulation in human AAA was demonstrated by assessing tissue levels of the protein. Expression of OPG was assessed in biopsies of four types of human aortic tissue: AAA; atherosclerotic abdominal (AOD) non-diseased post-mortem abdominal (PAA); non-diseased thoracic (TA). The highest levels of OPG were found in AAA, up to12-fold higher than levels observed in control tissue (PAA/TA) and 3-fold higher than seen in AOD samples. This finding was supported by observations in the Angiotensin II-infused $ApoE^{-/-}$ mouse. In this experimental model of AAA, elevated tissue concentrations of OPG were detected in aneurysmal aorta compared to nonaneurysmal aorta, and a correlation between higher aortic OPG concentration and increased aortic diameter was demonstrated. No correlation could be shown in this model between serum OPG and aortic diameter. This supports the human data and likely reflects a disassociation between tissue and serum OPG concentration.

Immunodetection of OPG in AAA biopsies localized the protein primarily within the fragmented arterial media. This region comprises a reduced VSMC population together with a dense infiltration of inflammatory cells, including monocyte/macrophages. It was demonstrated in this study that both AAA-derived

VSMC and macrophages contribute to OPG production. VSMC isolated from AAA biopsies secreted 16-fold higher levels of OPG than VSMC isolated from healthy abdominal aorta. Macrophages isolated from AAA tissue secreted significantly greater levels of OPG *in vitro* compared to human peripheral blood monocytes, and reflected quantities of OPG produced by LPS-stimulated THP-1 cells *in vitro*. The level of OPG secreted by AAA-derived macrophages per 10^5 cells per ml was less in direct comparison with aneurysm VSMC. However, the difference in cell number between these two populations at late-stage aneurysm should be taken into account. With a decreased VSMC density typically observed within biopsies taken at surgery, it is probable that the majority of OPG present at this stage was derived from inflammatory cells.

Osteoprotegerin is known to be upregulated by pro-inflammatory cytokines such as TNF-α (Zannettino *et al*, 2005). It is possible that the observed high concentration of this protein simply represents an expected result of the inflammatory process central to atherosclerosis. As such, the functional consequence of elevated concentrations of OPG within the aortic wall, if any, needs to be clarified. Data on the biological action of OPG on the two main cell-types important in AAA progression, VSMC and macrophages, is presented in Chapter 6.

Chapter 6

BIOLOGICAL ACTION OF OPG IN AAA PATHOGENESIS

6.1 INTRODUCTION

The loss of resident VSMC, and the dense adventio-medial infiltration of inflammatory cells, such as macrophages, which are the source of proteolytic enzymes responsible for degradation and fragmentation of the aortic media are the two pathological hallmarks of medial degeneration in AAA. Within the aortic wall both VSMC and inflammatory cells may be responsible for secretion of OPG. For example, activated T lymphocytes have been shown to express substantial quantities of OPG *in* vitro (Choi *et al*, 2001). Similarly human aortic smooth muscle cells express mRNA for OPG and synthesis can be induced by cytokines implicated in AAA (Zhang *et al,* 2002).

The data presented in the previous chapter demonstrated that the presence of AAA is associated with an increase in expression of aortic OPG. Further, production of the cytokine within the (aneurysmal) aortic wall was attributed largely, but not solely, to resident medial VSMC and infiltrating macrophages. In work described in this chapter, the functional significance of increased aortic OPG in relation to aneurysm development is assessed by examining the effect of OPG on human VSMC and monocyte biology *in vitro*.

6.2 EXPERIMENTAL METHODS

6.2.1 OPG and proliferation of normal human aortic VSMC

Vascular smooth muscle cells isolated from healthy human abdominal aorta (Chapter 3, section 3.2.1.(i)) were transferred to triplicate 96-well culture plates at a seeding density of $5x10^5$ cells per ml per well in a final volume of 200 µl per well. The cells were plated in DMEM containing 10% FBS and allowed to attach before being growth arrested in DMEM without FBS. The serum-free medium was replaced after 24 hours with experimental medium containing 0, 1, or 10% FBS, and recombinant human OPG concentrations of 0, 1, 5, 10, or 20 ng per10^5 cells per ml and cultures incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. Cells were pulsed with ^{3}H-thymidine four hours prior to harvest and DNA synthesis assessed as described (see Chapter 3, section 3.2.5.).

6.2.2 OPG and apoptosis in normal human aortic VSMC

Vascular smooth muscle cells isolated from healthy human abdominal aorta (Chapter 3, section 3.2.1.(i)) were transferred to triplicate 24-well culture plates at a seeding density of $1x10^5$ cells per ml per well in final volume of 1.0 ml per well. The cells were plated in DMEM containing 10% FBS and allowed to attach before being growth arrested in DMEM without FBS. The serum-free medium was replaced after 24 hours with experimental medium containing 10% FBS and rhOPG concentrations of 0, 0.5, 1, 5, or 10 ng per10^5 cells per ml and cultures incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. Apoptosis was induced in positive control cultures with 400 U per ml each of TNFα and IFNγ. Incubations were terminated at four (4), eight (8), and 12 hours, and the harvested cells assessed using FACS analysis of annexin V/propidium iodide staining, an indicator for early-stage apoptosis (see Chapter 3, section 3.2.6.(i)). DNA fragmentation (late-stage apoptosis) was examined in cells incubated 24 hours in DMEM containing 0, 10, or 20% FBS and rhOPG concentrations of 0, 0.5, 1, 5, or 10 ng per10^5 cells per ml using an oligonucleotide ELISA as described (Chapter 3, section 3.2.6.(ii)). Serum deprivation over the 24-hour experimental period served as apoptosis-positive control.

6.2.3 Effect of OPG on IL-6 production and gelatinase activity in normal human aortic VSMC

Cell culture supernatants were retained from the above studies (section 6.2.2) and were assayed for both IL-6 concentration and MMP-9 activity as previously described (Chapter 3, sections 3.2.3. and 3.1.7. respectively).

6.2.4 Effect of OPG on IL-6 production and gelatinase activity in human monocytic cells

THP-1 cells were transferred to triplicate 24-well culture plates at a seeding density of $1x10^5$ cells per ml per well in final volume of 1.0 ml per well. The cells were plated in RPMI containing 10% FCS and incubated with increasing concentrations (0, 1, 5, 10, 20 ng/ml) of rhOPG at 37°C in an atmosphere of 5% CO_2. During incubation, cells either remained resting or were activated with 1.0 or 10 μg LPS/ml. The culture medium was collected after 48 hours, centrifuged (200 g), and the supernatant assayed for both IL-6 concentration and MMP-9 activity as described (Chapter 3, sections 3.2.3. and 3.1.7. respectively).

6.3 RESULTS

6.3.1 Recombinant human OPG inhibits proliferation in normal human aortic VSMC

Cells incubated in the absence of FBS exhibited a very low rate of DNA synthesis over 24 hours. No significant change in this basal rate was observed in the presence of rhOPG concentration (Figure 6.1). The addition of 1% FBS to quiescent cells stimulated growth activity and increased DNA synthesis 2-fold compared to cells incubated in the absence of FBS, raising levels of ^{3}H-thymidine incorporation from 3400 cpm to 7500 cpm. The presence of rhOPG in these cultures again had no significant stimulatory or inhibitory effect on DNA synthesis. Incubation of cells with 10% FBS stimulated ^{3}H-thymidine incorporation markedly and induced a 5-fold increase in DNA synthesis compared with the basal rate exhibited by cells incubated in the absence of FBS (16300 cpm versus 3400 cpm). However, treatment of these

cultures with increasing concentrations of rhOPG decreased DNA synthesis dose-dependently (P<0.01; Figure 6.1). The rate of ^{3}H-thymidine incorporation over 24 hours was halved with a dose of only 1 ng rhOPG per 10^5 cells per ml. A higher dose of 20 ng rhOPG per 10^5 cells per ml resulted in a 5-fold reduction in DNA synthesis compared to cells not treated with rhOPG (3100 cpm versus 16300 cpm), equivalent to the basal rate of DNA synthesis (3400 cpm) exhibited by quiescent cells.

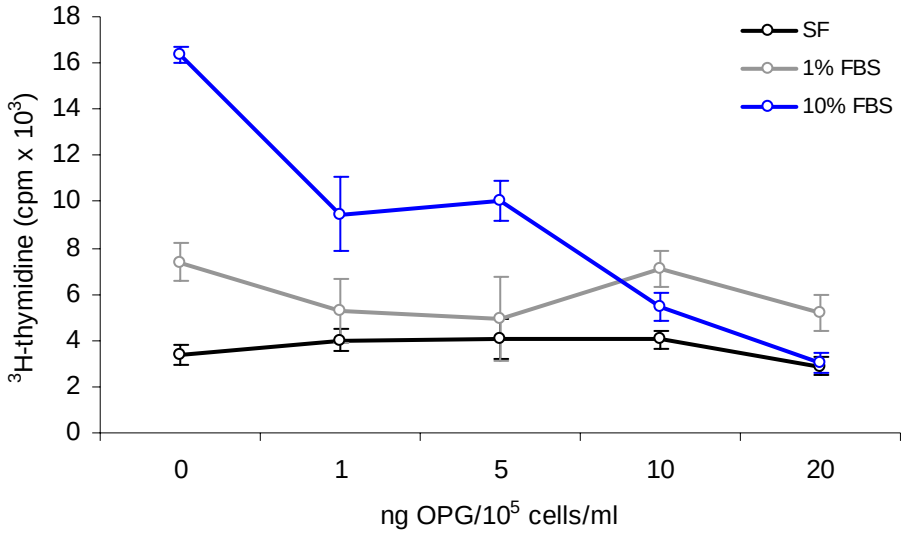


Figure 6.1. Proliferation in healthy human abdominal aortic VSMC induced by 10% FBS, dose-dependently inhibited by rhOPG over 24 hours. Data expressed as mean±SEM of triplicate cultures. SF, serum-free.

6.3.2 Recombinant human OPG promotes apoptosis in normal human aortic VSMC

Annexin V/propidium iodide staining of VSMC and assessment by FACS demonstrated dose-dependent induction of apoptosis in the cells after four hours incubation with rhOPG (0 to 10 ng/10^5 cells/ml/24h). The level of apoptosis in these cells was not statistically significant when compared with either the experimental or apoptosis-positive control cells (Figure 6.2.A. and 6.2.B). However, significant cell apoptosis was observed after 8 hours incubation, and maximal with 10 ng/10^5

cells/ml/24h rhOPG with the percentage of apoptotic cells more than 2-fold higher than untreated cells (P=0.02; Figure 6.2.C. and 6.2.D.). No significant difference in annexin V staining was observed between the control and treatment groups at termination of the 24-hour experimental period.

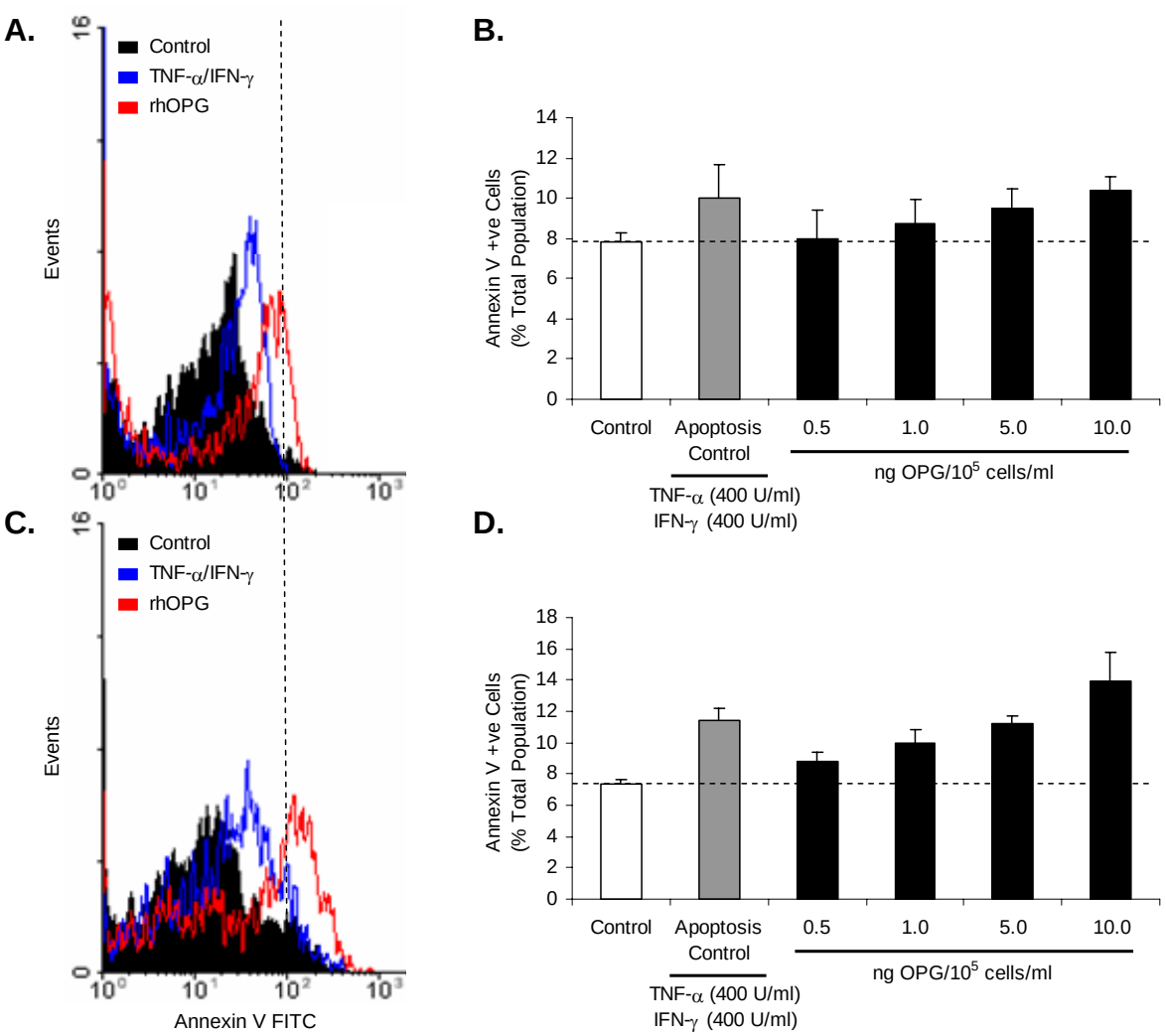


Figure 6.2. Apoptosis in healthy human aortic VSMC induced by rhOPG at 4 hours (A & B) and 8 hours (C & D). Annexin V staining after 4 hours (A) and 8 hours (C) demonstrated in cells treated with 10 ng rhOPG/10^5 cells/ml vs control (untreated) cells. Data presented as mean±SEM of triplicate cultures.

Analysis of VSMC lysate for apoptosis (DNA degradation) at 24 hours demonstrated 3-fold higher levels of nucleosomal fragmentation in the positive control (untreated, serum-deprived) cells compared to untreated cells incubated in the presence of either 10% or 20% FBS (1.09 nucleosome U/ml versus 0.38 nucleosome U/ml; $P<0.001$; Figure 6.3.). The degree of cell apoptosis detected in serum-free culture remained unchanged in the presence of increasing concentrations of rhOPG. Cells cultured in the presence of 20% FBS (negative control) exhibited some degree of nucleosomal fragmentation in the presence of rhOPG, however statistical significance could not be demonstrated. In contrast, significant nucleosomal fragmentation was detected in lysate from cells incubated in 10% serum-enriched medium treated with rhOPG at 1, 5, and 10 ng per 10^5 cells per ml ($P=0.02$). The degree of DNA degradation at the latter concentrations was maximal and mirrored levels observed in the serum-deprived control cultures (Figure 6.3.).

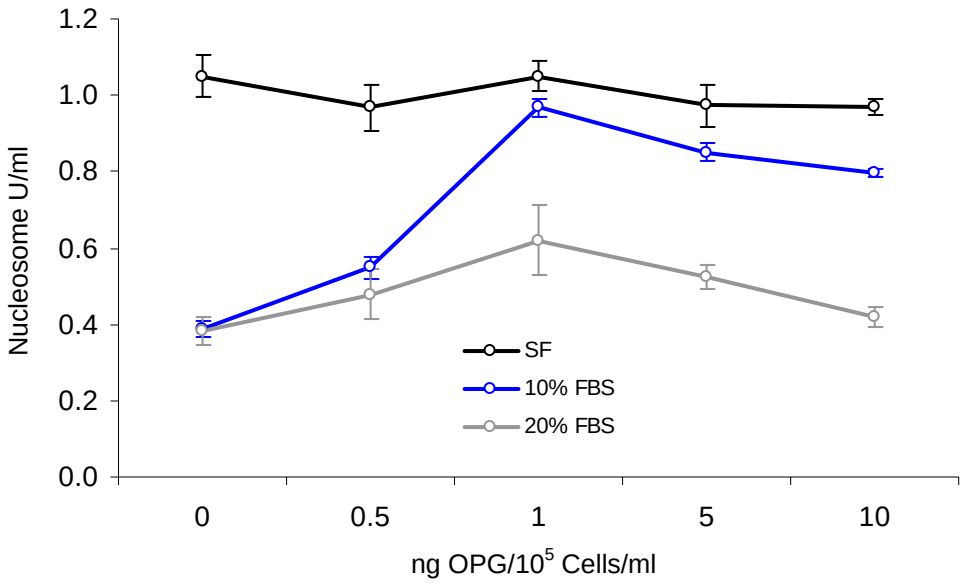


Figure 6.3. DNA fragmentation as a measure of apoptosis induced in normal human aortic VSMC by increasing concentrations of rhOPG over 24 hours. Data expressed as mean±SEM of triplicate cultures. SF, serum-free; FBS, foetal bovine serum.

6.3.3 Recombinant human OPG inhibits IL-6 production and augments MMP-9 activity in normal human VSMC

Incubation of healthy human abdominal aortic VSMC with rhOPG concentrations of 0, 0.5, 1, 5, 10 , and 20 ng/10^5 cells/ml/24h) dose-dependently decreased IL-6 production in these cells (457±7, 367±42, 302±49, 220±9, 211±14, 229±19 pg IL-6 per ml, respectively; P<0.001; Figure 6.4.A.). Conversely, analysis of gelatinase activity in culture supernatant from the same cells demonstrated a dose-dependent upregulation of MMP-9 proform (92kD) activity in these cells in the presence of rhOPG (0.9±0.08, 1.2±0.1, 2.4±0.1, 4.0±0.07, 5.2±0.04 RDU, respectively; P=0.01; Figure 6.4.B.). OPG had no significant influence on MMP-2 expression in VSMC (data not shown).

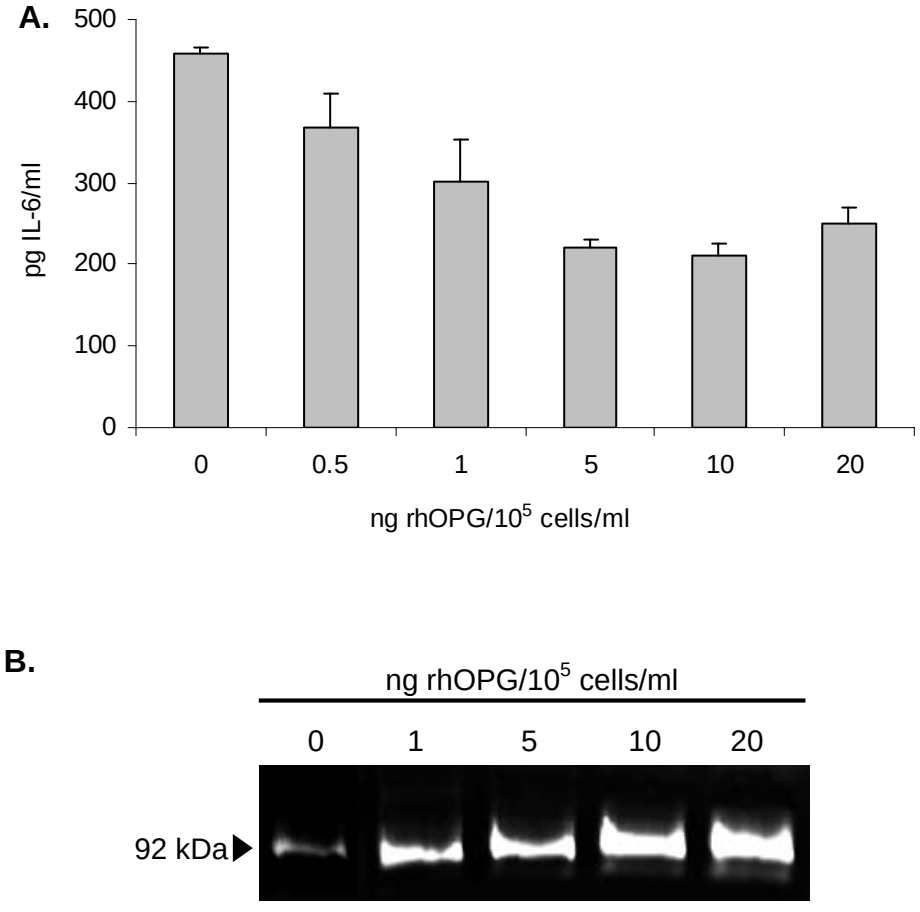


Figure 6.4. (A) rhOPG-induced reduction in IL-6 production by normal human aortic VSMC over 24h. Data is expressed as mean±SEM of triplicate cultures. (B) Representative zymographic gel demonstrating dose-dependent up-regulation in activity of MMP-9 (92 kDa) by rhOPG in normal human aortic VSMC.

6.3.4 Recombinant human OPG stimulates IL-6 production and MMP-9 activity in THP-1 cells.

Incubation of resting THP-1 cells with rhOPG resulted in no significant change (either stimulatory or inhibitory) in IL-6 production. Activation of cells with LPS induced significant production of IL-6 in these cells compared with non-activated cells, with supernatant levels of the cytokine measuring 40 and 85-fold higher in the presence of 1.0 and 10 μg LPS per ml respectively. The treatment of activated THP-1 cells with rhOPG produced a further, dose-dependent increase in IL-6 production (Figure 6.5.).

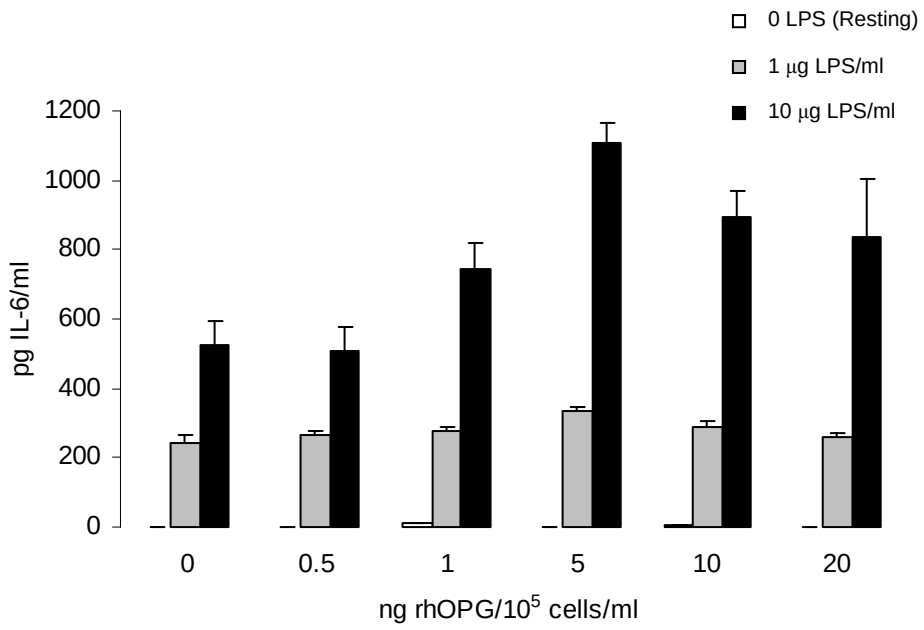


Figure 6.5. Dose-dependent effect of rhOPG-treatment on IL-6 production in resting and LPS-activated THP-1 cells over 48 hours. Data is expressed as mean±SEM of triplicate cultures.

Supernatant IL-6 concentration from cultures activated with 1.0 μg LPS per ml was increased by up to 1.5-fold in the presence of rhOPG (5 ng/10^5 cells/ml) (P=0.01). Similarly, exposure of THP-1 cells to 10 μg LPS per ml and resulted in a two-fold stimulation of IL-6 production upon treatment with rhOPG (P=0.005; Figure 6.5.). Equimolar co-incubation of THP-1 cells with OPG and its physiological ligand, RANKL (5 ng per10^5 cells per ml), inhibited this action of OPG keeping production of IL-6 by activated THP-1 cell at control levels (Figure 6.6.).

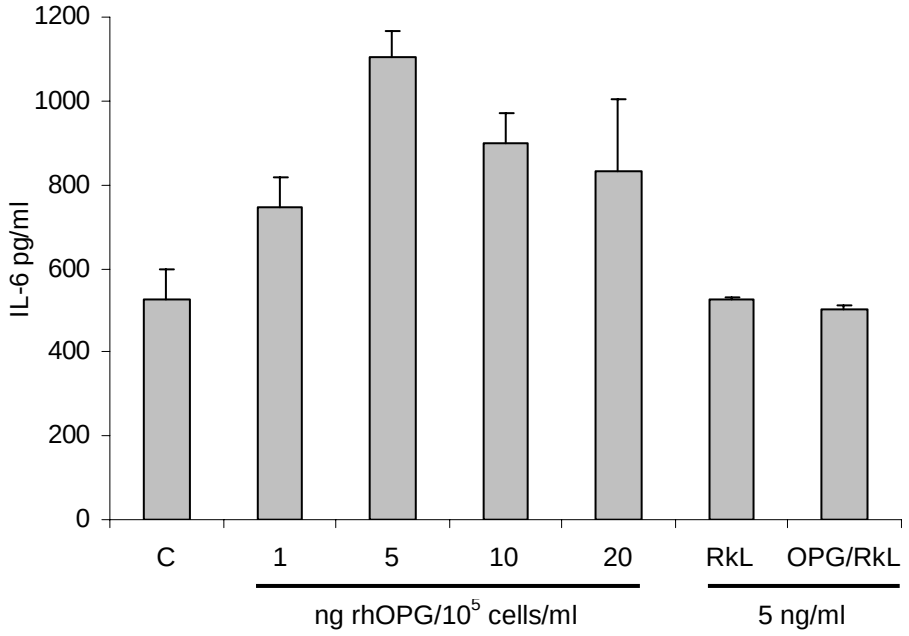


Figure 6.6. IL-6 production in LPS-activated (10 μg/ml) THP-1 cells in the presence of rhOPG over 48h. Negation of the effect of OPG by co-incubation with RANKL (RkL). Data is expressed as mean±SEM of triplicate cultures.

Incubation of resting THP-1 cells with rhOPG also induced MMP-9 activity in these cells. Unlike in VSMC, this effect was not dose-dependent. A biphasic action of OPG was again observed with stimulation of activity in treated cells significant only in the presence of 1.0 ng per 10^5 cells per ml rhOPG (Figure 6.7.). MMP-2 activity induced in THP-1 cells by rhOPG was increased by over 30% compared to control cells (1.1±0.02 RDU versus 0.7±0.01 RDU; *P*=0.01). Expression of the 92kDa proform and 83kDa active form of MMP-9 was concurrently upregulated (0.4±0.05 RDU versus 0.2±0.01 RDU; *P*=0.03; and, 0.9±0.03 RDU versus 0.7±0.03 RDU; *P*=0.01, respectively; Figure 6.7.).

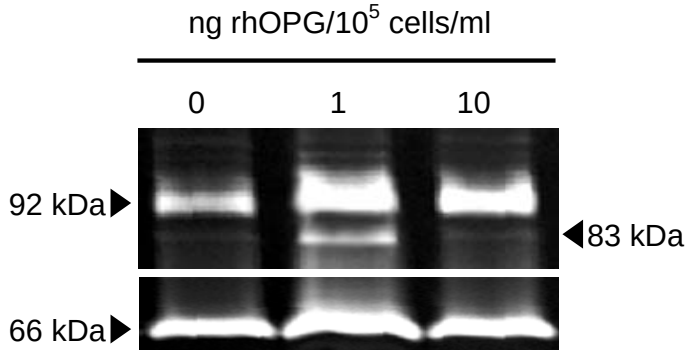


Figure 6.7. Representative zymographic gel demonstrating rhOPG-induced up-regulation of MMP-9 proform (92 kDa), active MMP-9 (83 kDa), and MMP-2 (66 kDa) in LPS (10 μg/ml)-activated THP-1 cells.

6.4 DISCUSSION

The key findings presented in this chapter are:

1. OPG negatively regulates the growth of human aortic VSMC *in vitro*
2. OPG promotes apoptosis of human aortic VSMC *in vitro*
3. OPG inhibits the production of IL-6 by human aortic VSMC *in vitro*
4. OPG stimulates IL-6 production in the human monocytic cell-line THP-1
5. OPG is a positive regulator of MMP-9 activity in both human aortic VSMC and THP-1 cells *in vitro*

Previous studies have demonstrated that OPG influences cell survival in a variable way depending upon the cell type and conditions in which experiments are performed. For example, OPG promotes osteoclast apoptosis (Shiotani *et al,* 2002), but protects prostate cancer and myeloma cell lines from TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Shipman & Croucher, 2003). The direct effect of OPG on VSMC, however, has not previously been investigated. Results of the present study show that incubation of isolated human aortic normal VSMC with increasing concentrations of rhOPG results in a dose-dependant reduction in DNA synthesis, while exposure of these cells to 0.5-1.0 ng rhOPG per ml induces apoptosis. In light of these findings, it is tempting to speculate with regard to aneurysm formation, that under pathological conditions Angiotensin II-mediated upregulation of OPG expression in medial VSMC exerts autocrine regulation in these cells leading to the diminution of VSMC typically observed in AAA.

Extensive lymphocytic (T-cells/B-cells/dendritic cells) and monocytic infiltrate within the aortic media and adventitia supports the notion that AAA formation is an (auto)immune response (Gregory *et al,* 1996; Hirose *et al,* 1997; Curci & Thompson, 2004) in which these cells generate high concentrations of proinflammatory cytokines and metalloproteinases (MMP) believed to be central in degradation of the aortic wall (Alexander, 2004). Previous studies have shown that antigen-presenting dendritic cells may be responsible for eliminating activated T lymphocytes at the end of primary immune responses via expression of the apoptosis-inducing molecule

TRAIL (Yu *et al,* 2002). The involvement of OPG in mediating an immune response in AAA is supported in part by the identification of OPG as a 'decoy' receptor that can bind TRAIL and inhibit its action (Shipman & Croucher, 2003). By conferring a survival advantage upon activated T lymphocytes, OPG would be promoting uninhibited expression of proinflammatory mediators within the aneurysm wall. Additionally, OPG may act upon monocyte/macrophages *in vivo* to promote the production of proinflammatory mediators in these cells. It is demonstrated here that incubation of activated (LPS-stimulated) human monocytic THP-1 cells with rhOPG results in a sensitive dose-dependant increase in the secretion of interleukin-6, a cytokine particularly implicated in the pathogenesis of AAA by way of its ability to induce MMP expression and increase matrix turnover (Galis *et al,* 1994). The present study demonstrated that rhOPG (1.0 ng per 105 cells per ml) induced a 30% increase in MMP-2 (66kDa) activity and a concurrent increase in the 92kDa proform and 83kDa active form of MMP-9 by 12% and 9% respectively compared to untreated cells.

The ability of OPG to induce aneurysmal phenotype in normal VSMC by stimulating MMP-9 activity, and limiting proliferation and cell survival, together with a potential role in the promotion and maintenance of an immune/inflammatory response within the aortic wall suggests a proactive role of the cytokine in the formation and progression of AAA. To addresses how OPG might be effecting these outcomes *in vivo*, signalling pathways and molecular mechanisms associated with change exerted by OPG *in vitro* were investigated and the data presented in the following chapter.

Chapter 7

MECHANISMS OF OPG-INDUCED ANEURYSMAL PHENOTYPE IN HUMAN ABDOMINAL AORTIC VSMC

7.1 INTRODUCTION

Vascular smooth muscle cells (VSMC) are the predominant cell type of the normal elastic media and make substantial contribution, both directly and indirectly, to the elastic lamellar architecture of the aortic wall through production of elastin, collagen, and other matrix proteins. Histological examination of human AAA tissue consistently demonstrates a paucity of VSMC within the aortic media, and it is now widely accepted that programmed cell death (apoptosis) of VSMC has aetiological significance to aneurysmal disease (Lopez-Candales *et al,* 1997; Henderson *et al,* 1999).

Data presented in preceding chapters has demonstrated that increased OPG expression is associated with the presence of aortic aneurysm *in vivo* (Chapter 5), and that its presence can influence VSMC survival *in vitro* by both the suppression of cell proliferation and the promotion of apoptosis (Chapter 6), thus identifying it as a potential element in the development of AAA. In this chapter, molecular mechanisms by which OPG acts to induce aneurysmal phenotype in aortic medial VSMC is examined using gene expression analysis.

7.2 EXPERIMENTAL METHODS

7.2.1 Preparation of control and OPG-treated VSMC

Vascular smooth muscle cells isolated from healthy human abdominal aorta (Chapter 3, section 3.2.1.(i)) were transferred to four (4) T-75 (40 ml) culture flasks at a seeding density of $2.0x10^6$ cells per flask in final volume of 20 ml. The cells were cultured in DMEM containing 10% FBS and allowed to reach 100% confluency before being growth arrested in DMEM without FBS. Cultures were divided into two batches, each comprising a control (untreated) and a treated flask. The serum-free medium was replaced in each flask after 24 hours with experimental medium containing 10% FBS only (control) or 10% FBS plus 5 ng per10^5 cells per ml recombinant human OPG, and incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. At harvest, cells from each flask were trypsinized, pelleted, and processed immediately for mRNA extraction and purification (see Chapter 3, section 3.2.7.).

7.2.2 Gene Expression

Gene analysis was contracted to the Australian Genome Research Facility (AGRF) Ltd, at the Walter and Elisa Hall Institute of Medical Research, Melbourne, Victoria, Australia. Purified mRNA from control and rhOPG-treated VSMC (see above) was supplied to the AGRF, from which cDNA was synthesized and comparative analysis of gene expression performed using the Affymetrix® *GeneChip*® automated hybridization and scanning DNA microarray system. This platform hybridizes denatured (single-strand), biotin-labeled cDNA with 25-mer oligonucleotides, or *probes*, which are bound to the array chip surface and designed to be complementary to a reference (sample) sequence. Several probes represent a single gene. Biotinylated nucleotides are incorporated into the sample cDNA during an *in vitro* transcription reaction, and a streptavidin-phycoerythin dye is used to bind the biotin such that hybridization to probes provides signal intensity data for quantitative measure of the relative abundance of a transcript. A single Affymetrix® *Human 133 Plus 2.0 Array* gene chip was used for each of the control

and rhOPG-treated mRNA samples, and allowed interrogation of approximately 45,000 genes.

7.3 RESULTS

7.3.1 Yield and Purity of VSMC mRNA

Spectrometric analysis of mRNA extracted from control cells of each batch determined yields of 7.82 μg and 8.16 μg (Table 7.1.). Yields from rhOPG-treated cells were slightly lower at 6.34 μg and 6.82 μg mRNA per 10^5 cells, however all samples were stable with distinct ribosomal RNA bands indicating non-degradation (Figure 7.1.). Control and treated samples from each batch were combined to obtain the minimum of 10 μg mRNA required for gene expression analysis. Overall purity of combined samples was high with a 260/280 ratio of 2.2 for both control and rhOPG-treated samples (Figure 7.2.).

Table 7.1. Total RNA yield from control and rhOPG-treated VSMC

	SPEC (260nm)	[μg/ml]	VOL (μl)	**μgRNA**
BATCH #1				
Control	1.303	52.13	150	7.82
rhOPG	1.057	42.27	150	6.34
BATCH #2				
Control	1.360	54.40	150	8.16
rhOPG	1.137	45.47	150	6.82
TOTAL				
Control				**16.08**
rhOPG				**13.16**

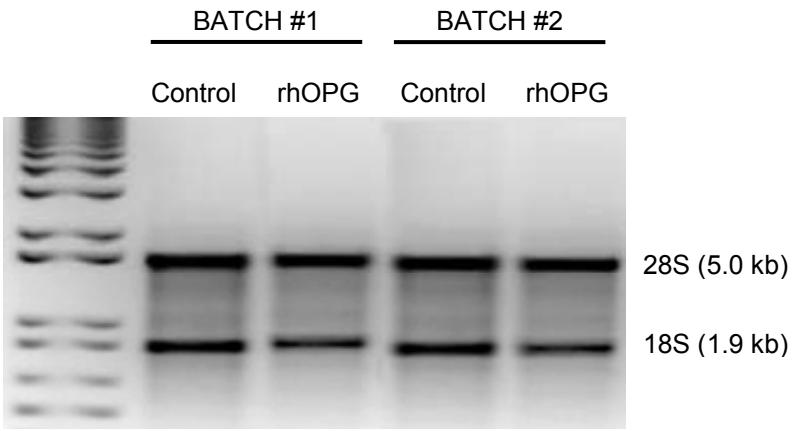


Figure 7.1. Stability of extracted mRNA confirmed with agarose gel electrophoresis and visualization of ribosomal RNA bands.

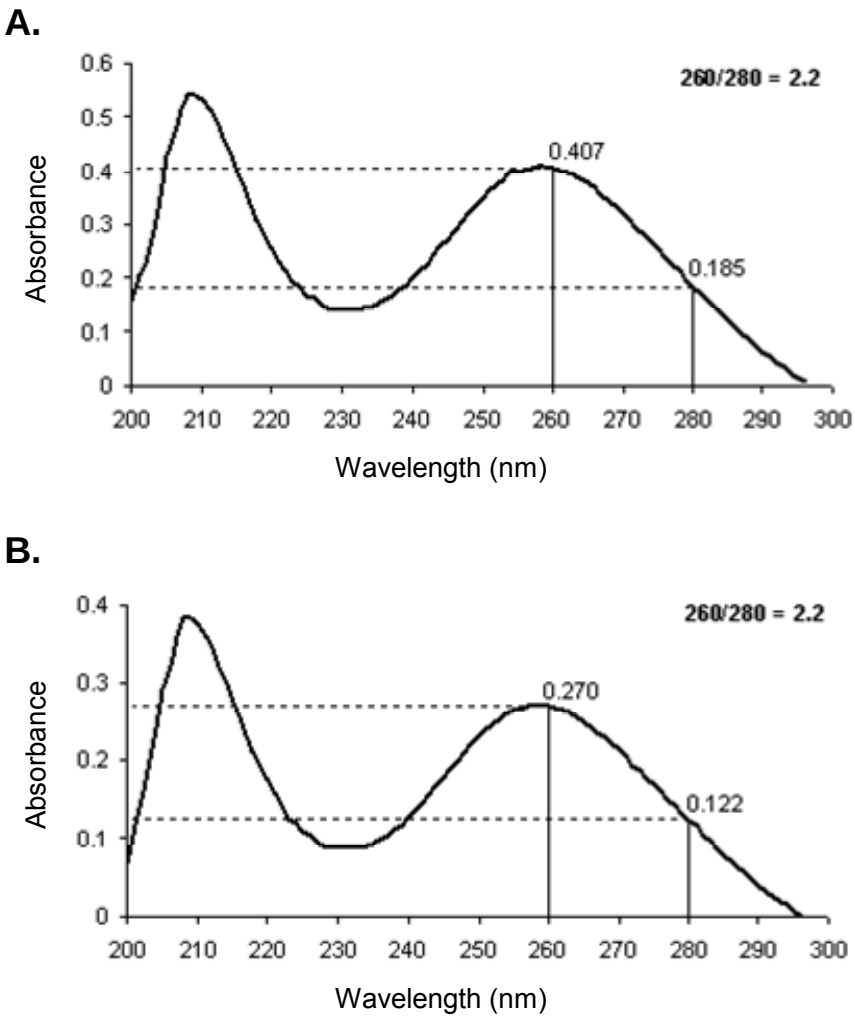


Figure 7.2. Purity of extracted mRNA from control (untreated) VSMC (A) and rhOPG-treated VSMC (B) as determined by spectrometric analysis.

7.3.2 OPG regulates expression of genes governing VSMC growth and survival

Approximately 18,000 sequences (40%) hybridized at completion of the analysis. Several hundred genes exhibited either increased or decreased expression when compared between the control and recombinant human OPG-treated group. From these, a total of 51 were directly related to the *in vitro* effects of OPG on human aortic VSMC observed in Chapter 6 (see Appendix 2; Tables 7.2 and 7.3).

Table 7.2. OPG-induced change in expression of genes associated with cell-cycle regulation and survival in healthy human aortic VSMC.

GENE	CELL ACTIVITY	EXPRESSION*
14-3-3	Survival/Proliferation	
AIF	Apoptosis	
ALG2	Apoptosis	
Bad	Apoptosis	
Bax	Apoptosis	
Bcl-x	Survival	
Calcineurin B, type I	Apoptosis	
Cdc37	Proliferation	
CDK4	Proliferation	
CKS1	Proliferation	
DAPK3	Apoptosis	
DAXX	Apoptosis	
EGR-1	Survival/Proliferation	
ENT1	Proliferation	
Farnesyl-protein transferase	Proliferation	
FKHRL1	Apoptosis/Growth Inhibition	
GADD45B	Apoptosis/Growth Inhibition	
I-FLICE isoform 4	Survival	
IRAK1	Apoptosis	
JAK1	Proliferation	
MEK3	Apoptosis	
PAK-1	Proliferation	
PGE2 receptor, EP3 subtype	Apoptosis	
PIK3CA	Survival	
RGS4	Growth Inhibition	
RHEB2	Proliferation	
SHC	Proliferation	
STAT6	Proliferation	
TLR4	Proliferation	
TNFRSF21	Apoptosis	
TR3beta	Apoptosis/Growth Inhibition	

*Compared to non OPG-treated (control) cells

Thirty-one genes associated with survival, growth, and matrix production were significantly downregulated in VSMC treated with recombinant human OPG compared with untreated VSMC. An average fold-decrease of four was observed in gene expression, with changes ranging between 1.4 and 12-fold. Nineteen genes, associated with the inhibition of cell growth and promotion of cell death, were significantly upregulated in recombinant human OPG-treated VSMC compared to the untreated control cells. In this instance, change in gene expression ranged between 1.5 and 7-fold, with a 3-fold average.

Table 7.3. OPG-induced change in expression of genes associated with growth and extracellular matrix synthesis in healthy human aortic VSMC.

GENE	CELL ACTIVITY	EXPRESSION*
ARC20	Actin Polymerization	
Biglycan	Proliferation	
Calmodulin 2	Apoptosis	
CCT	Actin/Tubulin Synthesis	
Collagen VI-α1	ECM Structural Protein	
Collagen VIII-α1	ECM Structural Protein	
CST3	Protease Inhibition	
Elastin	ECM Structural Protein	
EMILIN	Elastogenesis/Adhesion	
Fibrillin 1	Microfibril Synthesis	
Fibulin 5	Elastogenesis	
HSP2 (Perlecan)	Growth Inhibition	
LOX	ECM Cross-linkage	
MAP4	Mitosis	
Plectin	Cytoskeletal Stabilization	
Prolyl 4-hydroxylase	Collagen Synthesis	
SPARC	Growth inhibition	
TIMP-3	Protease Inhibition	
Tubulin β	Microtubule Synthesis	
Versican 5	Survival/Growth	

*Compared to non OPG-treated (control) cells

7.4 DISCUSSION

The most significant outcome of this gene expression study is the validation of the negative action of rhOPG upon the survival of normal human aortic VSMC in *vitro*, described in Chapter 6. An emphasis upon OPG-induced downregulation of genes related to cell-cycle maintenance and cell growth was demonstrated, together with the upregulation of genes associated with inhibition of cell growth and apoptosis. The results further suggest that although both effects potentially contribute to an OPG-induced aneurysmal phenotype in aortic VSMC *in vivo*, the downregulation of genes linked to cell proliferation and growth maintenance (Table 7.2) takes precedence. Cell cycle transitions are controlled by the action of the cyclin-dependent kinases (CDKs) and their activating subunits, the cyclins (Macleod *et al*, 1995; Jacks and Weinberg, 1996; Collins *et al*, 1997). Arterial VSMC are normally quiescent, proliferate at low indices (<0.05%), and stay in the G0/G1 phase of the cell cycle (Gordon *et al*, 1990). After vascular injury, VSMC are stimulated to divide in response to mitogens, and they exit the G1 phase and enter the S phase. Data presented in the previous chapter demonstrated that OPG suppresses VSMC production of IL-6, a potent mitogen for these cells, which, *in vivo*, may partly account for the absence of VSMC proliferation in AAA. In addition, the present gene analysis revealed a decrease of approximately 3-fold in the expression of cyclin-dependent kinase 4 (CDK4) mRNA in OPG-treated VSMC compared with untreated cells. Cyclin-dependent kinase 4 acts during the G1/S transition and is required for cell cycle progression through this period (Sherr, 1994; 1996). The effect of OPG to downregulate CDK4 gene expression in VSMC provides direct mechanistic evidence for OPG's inhibitory action on VSMC proliferation.

On average, OPG affected a greater number of genes associated with cell survival and produced higher fold-decreases in gene expression compared with genes it upregulated and were responsible for inhibition of cell growth and death. An explanation for this might be found in the experimental plan. VSMC were incubated in the presence of rhOPG over 24 hours prior to their harvest and RNA extraction. The 24-hour experimental period was chosen due to the fact that the anti-proliferative and pro-apoptotic effects of rhOPG in these cells remained active and

demonstrable at this time-point. However, apoptosis in VSMC induced by OPG was detected after only four hours incubation (Chapter 6). The transcription and expression of genes involved in this process would have been upregulated prior to this point and maintained for varying intervals thereafter. It is reasonable that by 24 hours, an analysis of gene expression would identify only those genes whose expression had be prolonged, or associated with late-stage apoptosis. Consequently, analysis of gene expression at an earlier stage of incubation may have given a greater magnitude of change between control and OPG-treated cells. Further studies should include several experimental time-points to confirm this.

A second important conclusion arising from DNA analysis is that apoptosis induced by rhOPG manifested strongly through the mitochondrial apoptotic pathway. Bcl-2 family members are characterised as either pro-apoptotic (Bax, Bad, Bid) or anti-apoptotic (Bcl-2, Bcl-x). Activation of pro-apoptotic Bcl-2 family members causes their translocation to mitochondria where they interact with anti-apoptotic members that are components of the mitochondrial membrane. This interaction causes changes in voltage-dependent mitochondrial channels and releases mitochondrial mediators of apoptosis. The treatment of normal human aortic VSMC with rhOPG induced the upregulation in gene expression of pro-apoptotic Bcl-2 family members. This, together with concomitant downregulation of inhibitors of apoptosis (I-FLICE; Bcl-x) may account for the balance shift favouring cell-death.

The ability of rhOPG to suppress IL-6 production and increase gelatinase activity in normal human aortic VSMC was previously demonstrated (Chapter 5). However, DNA analysis in cells treated with rhOPG over 24 hours failed to detect any significant change in IL-6, MMP-9, or MMP-2 gene expression. Early abatement of expression following induction might again explain these unexpected observations and additional study of gene expression at earlier time points, 1 to 4 hours post treatment for example, would be useful.

Nonetheless, downregulation of tissue-inhibitor of metalloproteinase (TIMP)-3 transcription by 2-fold in rhOPG-treated VSMC was observed. TIMP-3 regulates the activity of most MMPs and their proforms (Yu *et al,* 2000), and, unlike other

TIMPS, it is strongly bound to the extracellular matrix reflecting its involvement in cellular regulation of MMP activity. Additionally, TIMP-3 is involved in the stabilization of the vessel wall in areas of inflammation and repair (Yu *et al,* 2000). In light of a tenuous equilibrium between MMPs and their inhibitors within the aortic wall, the sustained decrease in TIMP-3 gene expression at 24 hours could be of potential importance in relation to MMP activity and aneurysm progression *in vivo*.

The role of calcium (Ca^{2+}) changes in the commitment to apoptosis has been appreciated for more than two decades. Release of cytochrome c from mitochondria and subsequent activation of the apoptotic cascade is reliant upon mitochondrial uptake of endoplasmic reticulum-derived Ca^{2+}, a process regulated by members of the Bcl-2 family of proteins (Wang *et al,* 1999). Normal human aortic VSMC incubated with rhOPG exhibited increased gene expression for the EP3 subtype of the prostaglandin E_2 receptor. EP3 receptor signals are associated with increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) primarily through the inhibition of adenyl cyclase activity (Gerlo *et al,* 2004). Intriguingly, OPG transcription is regulated via activation of adenyl cyclase–dependent (cAMP/PKA) pathways. Adenyl cyclase activation increases cAMP, decreases $[Ca^{2+}]i$, and suppresses OPG mRNA expression (Takami *et al,* 2000). Elevated cAMP decreases net $[Ca^{2+}]i$ by enhancing Ca^{2+} extrusion to the extracellular space and Ca^{2+} sequestration to the $[Ca^{2+}]i$ store James *et al*, 1989). A striking 6-fold upregulation in expression of the EP3 receptor gene by rhOPG in this study is highly suggestive of a novel feedback mechanism by which OPG positively affects not only Ca^{2+}- associated cell dysfunction, but also its own gene expression.

Finally, a number of VSMC genes downregulated by rhOPG were those of proteins associated with extracellular matrix and its synthesis (collagen, elastin, fibrillin-1, prolyl-4-hydroxylase, LOX), cell replication (MAP4, Tubulin, CCT) and the cytoskeletal ultrastructure of the cell itself (ARC20, Plectin). The decrease in expression of these genes *in vitro* is significant when one considers the impact of such an effect by OPG *in vivo* on the progression of aortic aneurysm. Cell-cycle dysfunction and apoptosis notwithstanding, degeneration of the aortic media would

be exacerbated by the inability of VSMC to effectively contribute to extracellular matrix regeneration.

The results presented in this chapter describe a genetic basis upon which OPG may act to induce the pro-aneurysmal phenotype of aortic VSMC described in Chapter 5. Together with the proinflammatory effects of OPG on human monocytic cells (Chapter 5) and the strong association of OPG with the presence of aortic aneurysm *in vivo* (Chapter 4), the data presented here support a significant functional role for OPG in the development of human AAA. As such, would targeting OPG production in the aneurysmal aorta be of clinical relevance in the search for non-invasive treatment of AAA? Work carried out to determine this aspect is outlined in the following section.

Chapter 8

MODULATION OF OPG IN THE ANEURYSMAL AORTA

8.1 INTRODUCTION

At present there is no proven treatment that will limit the growth of small aneurysms. Surgical repair, the only option for the prevention of aneurysm rupture, is not suitable in every case of AAA. Consequently, a need exists for a pharmacological alternative that can inhibit AAA formation and/or restrict the expansion of small aneurysms, thereby reducing the number of patients ultimately requiring intervention. Taken together, the findings presented in previous experimental chapters (Chapters 5, 6, and 7) strongly propose the functional importance of OPG in aortic aneurysm development. As such, targeting the expression of OPG within the aortic wall may be of significant therapeutic value.

Angiotensin II is pivotal in the development and progression of vascular disease and instrumental in promoting aneurysm development in animal models of atherosclerosis (Daugherty *et al,* 2000). Importantly, the expression of OPG has previously been shown to be upregulated by Angiotensin II in human aortic VSMC (Zhang *et al,* 2002).

Thiazolidinediones (*troglitazone*, *ciglitazone*, *rosiglitazone*, *pioglitazone*) are a group of insulin sensitizing drugs widely used for treating Type 2 diabetes (Martens *et al*, 2002). Peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear transcription factor, has been identified as the thiazolidinedione target receptor (Martens *et al*, 2002). *In vitro* evidence suggests that in conjunction with the suppression of inflammatory cytokine and proteolytic enzyme production (Tao *et al*, 2003), activation of PPARγ may inhibit OPG expression in normal human aortic VSMC (Fu *et al,* 2002).

The effect of angiotensin II blockade and PPARγ activation on OPG production in human aneurysm tissue *ex vivo*, and, the effect of PPARγ ligand therapy on aortic OPG expression *in vivo* in a murine model of AAA were examined in studies described in this chapter.

8.2 EXPERIMENTAL METHODS

8.2.1 Effect of AT_1R Blockade on OPG Production in AAA Tissue

Six (6) full-thickness human AAA biopsies were bisected with each 1 cm^2 specimen stabilized in DMEM containing 10% FBS for 24 hours at 37ºC in an atmosphere of 5% CO_2 before being maintained in explant culture for six (6) days under the same conditions (see Chapter 3, sections 3.1.3. and 3.2.2.). During this period, one explant (half) was incubated in the presence of the AT_1R-blocker Irbesartan while the second half of the biopsy remained untreated and served as control. Biopsies from patients receiving angiotensin II blockers or ACE inhibitors were excluded. An Irbesartan concentration of 1.0 mg per ml was based on safe serum levels measured in patients receiving this medication (Ruilope, 1997). Culture supernatant was collected and refreshed at 48-hour intervals. Assessment of explant viability and assay of culture supernatant concentration of OPG was performed as described (Chapter 3, section 3.2.3. and Chapter 4, section 4.9.). Supernatant concentration of IL-6 and tissue levels of MMP-9 and MMP-2 activity was measured as indicators of Irbesartan action (Chapter 3, sections 3.1.7. and 3.2.3.).

8.2.2 Effect of PPARγ Activation on OPG Production in AAA Tissue

Six (6) full-thickness human AAA biopsies were divided into three 1 cm^2 specimens and each stabilized in DMEM containing 10% FBS for 24 hours at 37ºC in an atmosphere of 5% CO_2 before being maintained in explant culture for six (6) days under the same conditions (see Chapter 3, sections 3.1.3. and 3.2.2.). During this period, one explant was incubated in the presence of the PPARγ ligand Pioglitazone, the second in the presence of the PPARγ ligand Rosiglitazone, while the third biopsy

specimen remained untreated and served as control. A concentration of 5 μM was used for treatment based on thiazolidinedione serum levels in healthy subjects detected during pharmacokinetic studies (Budde *et al,* 2003). Culture supernatant was collected and refreshed at 48-hour intervals. Assessment of explant viability and assay of culture supernatant concentration of OPG was performed as described (Chapter 3, section 3.2.3. and Chapter 4, section 4.9.). Supernatant concentration of IL-6 and tissue levels of MMP-9 and MMP-2 activity was measured as indicators of PPARγ activity (Chapter 3, sections 3.1.7. and 3.2.3.). Biopsies from patients receiving statins or angiotensin II inhibitors were excluded due to the influence of these medications on cytokine and protease production (Cipollone *et al,* 2004).

8.2.3 Effect of Activation on Aortic Expression of PPARγ in Experimental AAA

Two groups of male ApoE$^{-/-}$ mice were randomized between pioglitazone (drinking water, 20mg/kg/day; n=12) and placebo (n=12) two weeks prior to subcutaneous administration of angiotensin II for 28 days, after which, the aorta from each animal was harvested (see Chapter 3, sections 3.3.2. and 3.3.3.). The effect of pioglitazone on aortic expression of PPARγ was assessed by western analysis and compared with a third group of control male ApoE$^{-/-}$ mice (n=6) receiving neither angiotensin II nor pioglitazone. Extracted tissue proteins were separated using SDS gel electrophoresis and PPARγ detected using rabbit primary antisera (sc-7196, Santa Cruz Biotechnology; 0.4 μg/ml) and goat anti-rabbit secondary immunoglobulin (1:5000; DakoCytomation) (Chapter 3, sections 3.1.5. and 3.1.6.). Bands corresponding to PPARγ (~60 kDa) were analyzed as described (Chapter 3, section 3.1.6(ii)) and expressed as relative density units (RDU) per μg protein.

8.2.4 Effect of PPARγ Activation on Expression of Aortic OPG in Experimental AAA

The effect of PPARγ-ligation on aortic OPG concentration was assessed by western blot analysis. Aortic tissue was obtained from the two experimental (angiotensin II-infused) groups described above (section 8.2.3(i)). Aortic tissue levels of MMP-9

and MMP-2 were taken as indicators of PPARγ activity and determined using zymography (Chapter 3, sections 3.1.7.). Extracted tissue proteins were separated using SDS gel electrophoresis and OPG detected using goat polyclonal antibody to human OPG (R&D) at 0.2 μg/ml and rabbit anti-goat secondary immunoglobulin (1:5000; DakoCytomation) (see Chapter 3, sections 3.1.5. and 3.1.6.). Bands corresponding to OPG (55 kDa) were analyzed as described (Chapter 3, section 3.1.6(ii)) and expressed as relative density units (RDU) per μg protein.

8.3 RESULTS

8.3.1 AT_1R Blockade Suppresses OPG Production in AAA Tissue

Ex vivo administration of Irbesartan at 1.0 mg per ml had no significant effect on either MMP-9 or MMP-2 activity in human AAA tissue (Figure 8.1.A.). In contrast, IL-6 secretion from explants incubated in the presence of the AT_1R-blocker was significantly reduced in a time-dependent manner compared with untreated explants (P=0.02; Figure 8.1.B.). A significant effect of AT_1R blockade on OPG production in AAA tissue was also observed. Supernatant levels of OPG were time-dependently decreased from 350 pg per mg tissue to 160 pg per mg tissue over the six-day incubation ($P<0.001$; Figure 8.2.A.). Explant production of OPG was halved by day four of culture. Comparison between Irbesartan-treated and untreated explants by western analysis at day six confirmed the AT_1R-blocker effect of inhibiting OPG expression in AAA tissue (Figure 8.2.B.).

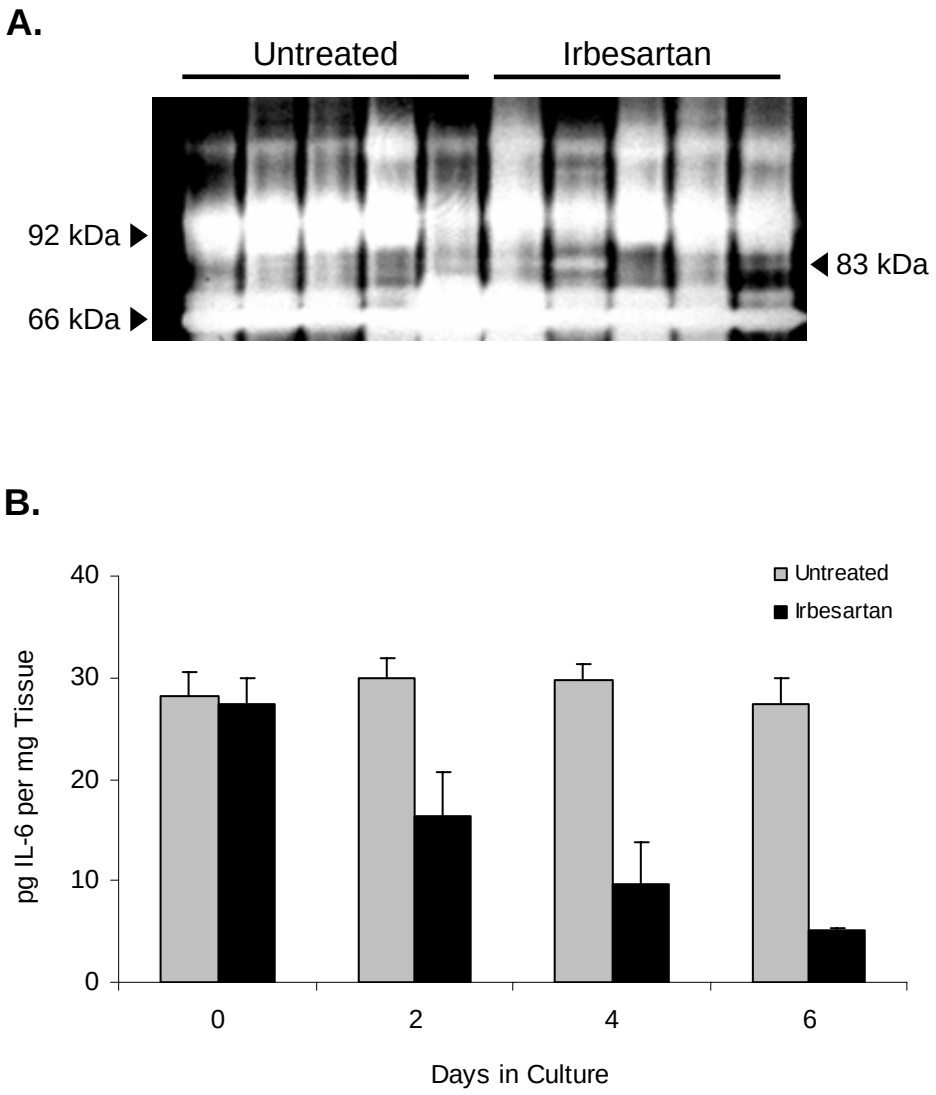


Figure 8.1. (A) Representative zymogram of MMP-2 and MMP-9 activity in human AAA explants, with no significant change in activity of either MMP in the presence of the AT_1R blocker Irbesartan (1 mg/ml) after 6 days. (B) Time-dependent decrease in IL-6 secretion by AAA explants treated with Irbesartan (1 mg/ml; black bar) over 6 days. Data expressed as mean±SEM of six explant culture trials.

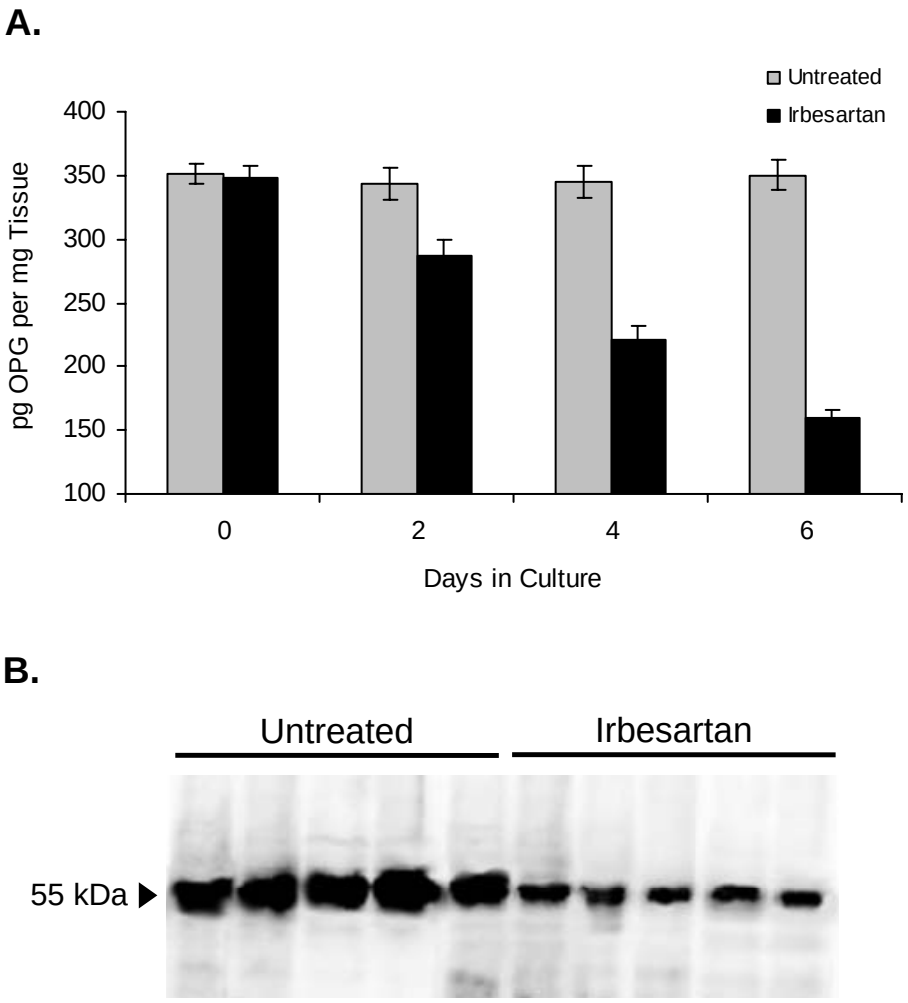


Figure 8.2. (A) OPG secretion by AAA explants suppressed by AT_1R blocker Irbesartan (1 mg/ml; black bar) over 6 days. Data expressed as mean±SEM of six explant culture trials. (B) Representative western blot showing Irbesartan-induced reduction in OPG concentration within explant tissue

8.3.2 PPARγ Activation Downregulates OPG Production in AAA Tissue

Interleukin-6 secretion from explants exposed to either pioglitazone or rosiglitazone was reduced by 15% compared to control tissue, with supernatant levels measuring 17.5±0.4, 17.8±0.2, and 20.2±0.4 pg IL-6 per mg tissue per 24 hours respectively (P<0.01; Figure 8.3.A.). Ligation of PPARγ had little effect on MMP-2 activity in explant tissue after 6 days. In contrast, tissue MMP-9 activity in treated samples was significantly reduced. The effect was most marked in explants incubated with pioglitazone in which MMP-9 activity was suppressed 3-fold compared to activity in untreated explants (0.4±0.01 versus 1.2±0.04 relative density units (RDU) per μg protein, respectively; P=0.001; Figure 8.3.B.).

Treatment with either rosiglitazone or pioglitazone resulted in a significant decrease in OPG secretion from AAA explant tissue (p<0.001; Figure 8.4.A.). The amount of OPG secreted in a 24-hour period in the presence of either rosiglitazone or pioglitazone was decreased by 60% compared to levels measured in untreated explant (125±16 versus 315±19 pg OPG per mg tissue, and, 134±17 versus 315±19 pg OPG per mg tissue, respectively). The inhibition of OPG production in AAA tissue by PPARγ activation was confirmed by western analysis of explants at day 6 (Figure 8.4.B.).

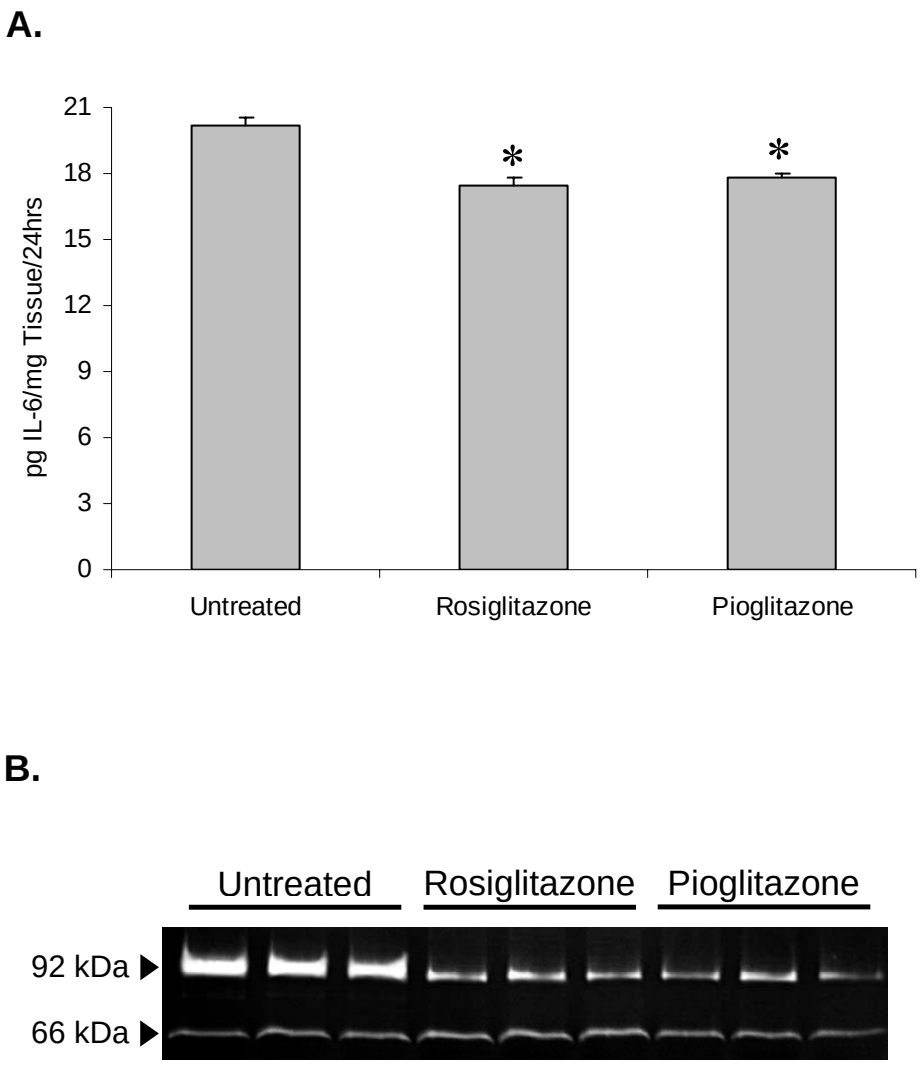


Figure 8.3. (A) Suppression of IL-6 secretion from human AAA explants in the presence of PPARγ activators rosiglitazone and pioglitazone (5 μM). Data expressed as mean±SEM of six explant culture trials; *****P<0.01; t-test. (B) Representative zymogram illustrating PPARγ activator-induced downregulation of tissue MMP-9 (92 kDa) production. MMP-2 (66 kDa) remained unchanged.

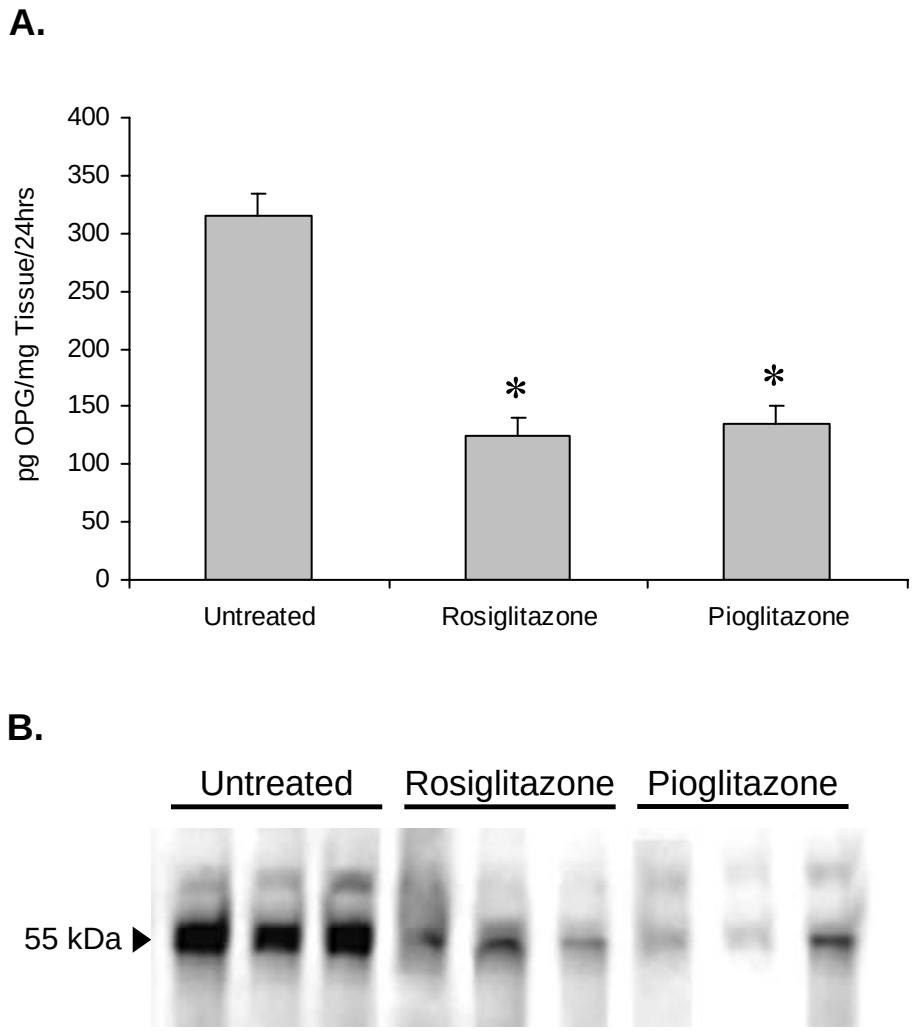


Figure 8.4. Suppression of OPG secretion from human AAA explants in the presence of synthetic PPARγ activators rosiglitazone and pioglitazone (5 μM). Data expressed as mean±SEM of six explant culture trials; **P*<0.001; *t*-test. (B) Representative western blot demonstrating a corresponding downregulation of tissue OPG protein induced by PPARγ activation.

8.3.3 Pioglitazone Increases PPARγ within the Aorta of Angiotensin II-infused Mice

Two (2) mice out of 12 in the non-medicated angiotensin II-infused group expired prior to completion of the infusion period, one at day 10 and the other at day 11. Three fatalities were documented in the pioglitazone group occurring at day seven, 16, and 18. The concentration of PPARγ in aortic tissue from angiotensin II-infused untreated animals did not differ significantly from levels determined in aortae from unmanipulated control mice (P=0.3, t-test; Fig. 9). Conversely, concentration of PPARγ was detected up to 5-fold higher in aortic tissue from angiotensin II-infused animals medicated with pioglitazone, compared with control and angiotensin II infusion alone (3.4±0.1 versus 0.7±0.1 RDU/μg protein, P<0.001; t-test; Figure 8.5.).

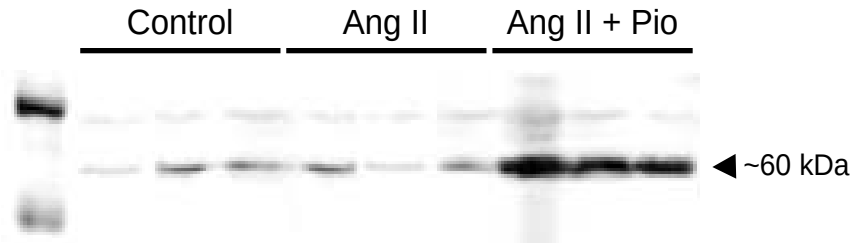
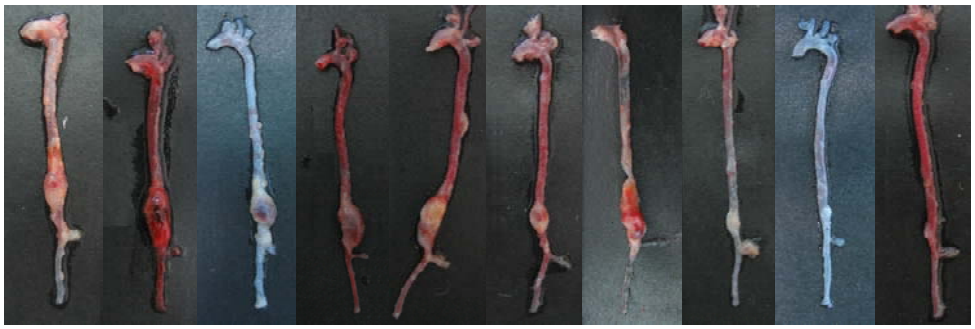


Figure 8.5. Representative western blot showing Pioglitazone-induced upregulation of aortic PPARγ in angiotensin II-infused (Ang II + Pio) mice. Comparison with unmanipulated (Control) and angiotensin II-infused alone (Ang II) mice.

8.3.4 PPARγ-Activator Therapy *In Vivo* Decreases OPG Expression in the Experimental Aneurysml Aorta

Aneurysm (defined in Chapter 3, section 3.10) of the supra-renal aorta (SRA) was identified in eight (8) of the 10 angiotensin II-infused untreated mice (Figure 8.6.A.). Aneurysm formation in mice receiving pioglitazone therapy was reduced to more than half, five (5) out of nine (9) animals identified with aortic dilations of the SRA (Figure 8.6.B.). The reduction in incidence of aneurysm formation in the treated group was not statistically significant when compared with the untreated group (P=0.07).

A.



B.

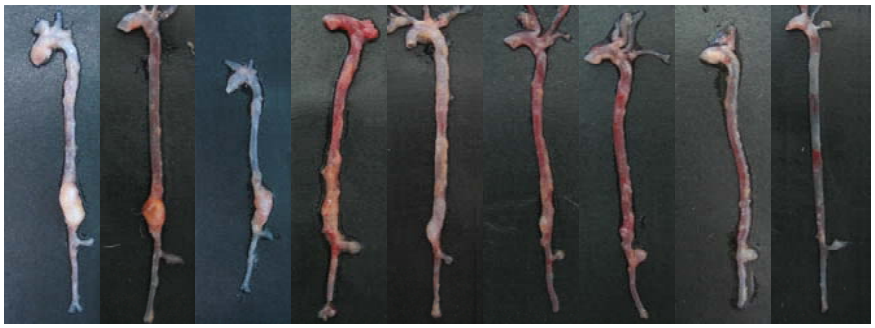


Figure 8.6. (A) Aneurysm formed primarily of the SRA in angiotensin II-infused (control) mice after 28 days. (B) Aneurysm formation after 28 days in mice pre-treated with pioglitazone (20mg/kg/day) 14-days immediately prior to infusion of angiotensin II.

Pioglitazone had no significant effect on the level of aortic MMP-2 activity compared with levels in aorta from untreated mice (2.2±0.4 versus 2.3±0.2 RDU pr µg protein, *P*=0.42). In contrast, a significant decrease in both the 92 kDa proform and the 83 kDa active form of MMP-9 was demonstrated (1.1±0.1 versus 2.3±0.6 RDU per µg protein (*P*=0.01), and, 0.8±0.03 versus 1.27±0.3 RDU/µg protein *P*=0.02), respectively; Figure 8.7.A.). The mean level of OPG detected in aortic tissue of pioglitazone-treated mice was 0.9±0.05 RDU per µg protein. This was significantly less than OPG concentration in aortae from non-treated animals (1.7±0.2 RDU per µg protein, *P*=0.002; Figure 8.7.B).

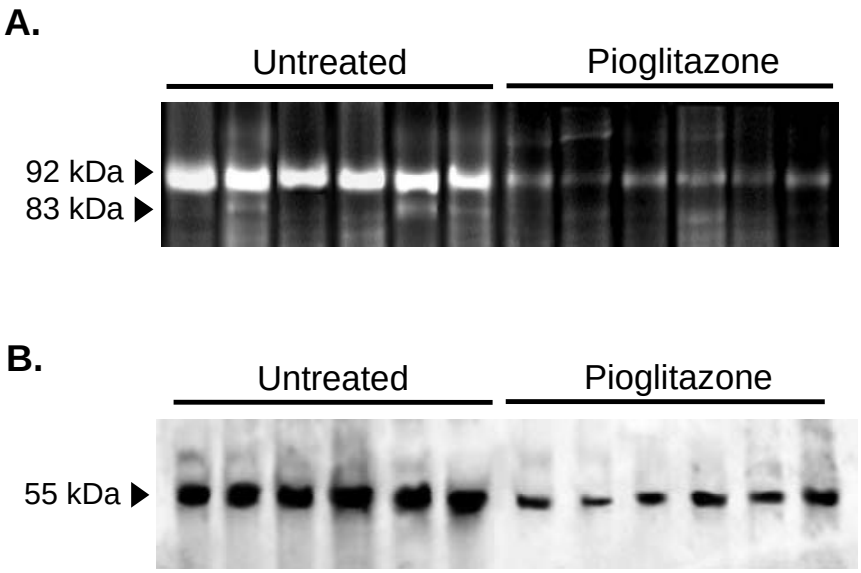


Figure 8.7. (A) Reduced MMP-9 (92 kDa) production and activity (83 kDa) within the SRA of pioglitazone-treated (20mg/kg/day) angiotensin II-infused mice compared to untreated, angiotensin II-infused controls. (B) Angiotensin II-induced upregulation of OPG in SRA (untreated) decreased in mice pre-treated with pioglitazone.

8.4 DISCUSSION

Angiotensin II plays a crucial role in the development and progression of arterial disease as a mediator of inflammation (Tham *et al,* 2002). In this capacity it is instrumental in the pathogenesis of AAA (Nishimoto *et al,* 2002; Deng *et al,* 2003). Most cardiovascular effects of angiotensin II are mediated via the type-1 isoform of the angiotensin II receptor, AT_1R. Expression of AT_1R in VSMC is upregulated in atherosclerotic lesions (Schieffer *et al,* 2000) reflecting the contribution of angiotensin II within the vessel wall to the progression of atherosclerosis and vascular disease.

The final stage in classical angiotensin II synthesis involves the conversion of angiotensin I to angiotensin II catalyzed by angiotensin-converting enzyme (ACE). Initially, clinical disruption of this pathway centred on the use of ACE-inhibitors. However, subsequent understanding that angiotensin II can be formed by alternative non-ACE pathways (chymase, tPA, cathepsin G, tonin, trypsin, tissue kallikrein; reviewed, Belova 2000) recognize that ACE inhibitors may not provide total

prevention of angiotensin II generation. Consequently, angiotensin II-receptor (AT_1R) antagonists were developed. ACE inhibitors interfere with the formation of angiotensin II, whereas AT_1R antagonists inhibit the actions of angiotensin II at the receptor site and thus exert more specific and complete blockade of the angiotensin pathway. Studies carried out examining the effect of angiotensin II blockade on human AAA tissue-production of OPG *ex vivo* are presented in this chapter. Treatment of AAA explants with the AT_1R antagonist Irbesartan resulted in a time-dependent inhibition of OPG secretion from AAA tissue, almost 2.5-fold over the six-day incubation. This finding supports a role for angiotensin II in the induction of aortic OPG synthesis *in vivo* and highlights the potential for AT_1R blockade in slowing aneurysm expansion through targeting of OPG production.

Angiotensin II promotes vascular injury through nuclear factor-kappa B (NFκB)-mediated induction of pro-inflammatory genes (Ross 1999; Tham *et al,* 2002), and the downregulation of the anti-inflammatory nuclear receptor transcription factors, PPAR (Tham *et al,* 2002). A number of genes are regulated by PPAR through ligand-dependent transcriptional activation and repression (Kliewer *et al,* 1992). The PPAR gamma isoform (PPARγ) is purported to play an important role in inflammation and immune response associated with vascular disease. Evidence links PPARγ with the downregulation of inflammatory cytokines, tumour necrosis factor-α, interleukin (IL)-1β, and IL-6, in monocytes and macrophages (Jiang *et al,* 1998). Markers of macrophage activation, such as inducible nitric-oxide synthase and MMP-9 are also suppressed by PPARγ in monocytes (Colville-Nash *et al,* 1998; Marx N *et al,* 1998b; Ricote *et al,* 1999), and in human VSMC (Marx *et al,* 1998a). These observations support the present finding that treatment of human AAA tissue *ex vivo* with PPARγ ligand (pioglitazone/rosiglitazone) decreases both IL-6 secretion and tissue MMP activity. This study further demonstrated that administration of PPARγ ligand (pioglitazone) prior to, then concomitant with, angiotensin II infusion in an *in vivo* model of AAA, limited any inhibitory effect of angiotensin II on expression of the transcription factor (Tham *et al,* 2002), and in fact resulted in its upregulation within the aortic wall. In support of this, thiazolidinedione-activation of PPARγ has previously been shown to inhibit angiotensin II-induced pro-

atherosclerotic processes in VSMC by suppressing AT_1R expression (Sugawara *et al,* 2001; Sugawara *et al,* 2003).

The inhibition of OPG gene expression in human aortic smooth muscle cells following activation of PPARγ *in vitro* (Fu *et al,* 2002) is of particular relevance to the present study; a 2.5-fold decrease in OPG production in PPARγ ligand-medicated AAA explants supports the concept of OPG expression as a novel PPARγ target gene. The present study provides *in vivo* evidence with a mouse model to support the demonstrated effect of thiazolidinedione treatment in downregulating OPG in human AAA tissue *ex vivo*. High levels of aortic OPG associated with angiotensin II-induced aneurysm formation in this model were, together with MMP activity, significantly reduced in mice that received pioglitazone therapy prior to the infusion of angiotensin II, compared with mice without pre-treatment. Associated with this, ongoing pioglitazone treatment appeared to reduce the incidence of aneurysm formation induced by angiotensin II infusion. However, small numbers of animals per group (n=12) further decreased by fatalities that occurred in experimental and control groups may have contributed to the statistical non-significance in aneurysm frequency between the groups. Further studies should include larger group numbers to conclusively determine any effect of PPARγ ligation on aneurysm development in this model.

Chapter 9

GENERAL DISCUSSION

Abdominal aortic aneurysm (AAA) is a significant health problem affecting an estimated ten percent of men and one percent of women over 60 years of age. Unlike coronary artery, cerebrovascular, and peripheral vascular disease, AAA usually remains asymptomatic and undetected. Without intervention, the aneurysmal aorta slowly increases in size. At diameters equal to or greater than 60 mm the risk of aneurysm rupture, the end-stage, catastrophic failure of the aortic wall, is estimated as 10% per year and associated with a mortality rate of up to 95 percent (Johansson and Swedenborg, 1986; Budd *et al*, 1989).

Thorough clinical examination may identify the presence of AAA. However, the majority are impalpable, particularly in the obese patient. Because of this, imaging modalities such as ultrasound scanning are the mainstay of diagnosis. With the development of imaging technology, incidence of AAA has seemingly increased in several countries over the last ten to fifteen years. For example, the number of AAA detected within the Danish population over a thirteen year period rose from 7.1 per 100,000 population in 1977 to 25.8 per 100,000 in 1990 (Eickhoff, 1993). A corresponding increase in the incidence of ruptured AAA and of age-standardized mortality rules out a simple correlation with imaging advancement and indicates a real increase in the incidence of AAA. The number of deaths in the United States between 1979 and 1991 resulting from ruptured AAA increased by almost 20 percent; in 1991, 16,696 deaths were attributed to aortic aneurysm, AAA accounting for 52 percent (Gillum, 1995). In England, AAA rupture is responsible for 1.5 percent of total mortality in males over 55 years of age, amounting to 8000 deaths per year (Thompson, 2003). Mortality due to AAA in Australia rose from 2.9 per 100,000 in 1968 to 4.1 per 100,000 in 1997 in which 1,505 Australian deaths were attributed to aortic aneurysms (Lake & McCaul, 2001).

Overall mortality from this condition is unlikely to change in the absence of more comprehensive diagnostic screening. Two randomized controlled trials, the United Kingdom Small Aneurysm Trial and the United States-based Aneurysm Detection and Management Trial, have shown that a policy of early elective surgery for small AAA (40 to 55 mm in diameter) does not improve survival rate of AAA sufferers (Uksapt, 1998; Lederle *et al*, 2002; Uksatp, 2002). These trials and follow-up of patients with AAA detected in the United Kingdom Multicenter Aneurysm Screening Study (Ashton *et al*, 2002) suggest that surveillance until the AAA diameter exceeded 55 mm (approximately 3 times the normal aortic diameter) is safe and associated with a very low rate of AAA rupture, around one percent per annum.

One outstanding issue of management is how frequently surveillance should be offered to patients with AAA less than 55 mm in diameter. Unfortunately, population-based screening for AAA has met with limited enthusiasm. Two reasons for this are immediately clear: 1) most AAA detected in screening programs are too small to warrant repair, and, 2) there are no known forms of treatment for patients with small asymptomatic AAA. Elective surgery, with its own mortality rate of around 5 percent (Feinglass *et al*, 1995; Uksatp, 1998) is the only treatment option presently available and usually reserved for repair of aneurysms showing high probability of rupture. There is a need to develop medication that can slow or halt aortic destruction. Such pharmacological therapy could be used in patients with small aneurysms to reduce the number requiring surgical treatment, in those unsuitable for surgical repair, and in combination with surgical treatments to reduce the need for further intervention. The lack of therapeutic options and our inability to predict the natural history of AAA reflect our limited understanding of the clinical and biologic factors that influence aneurysmal degeneration. Fundamental knowledge of the pathophysiology of AAA therefore remains an important gap in the basic science of vascular disease, and understanding the mechanisms that underlie this condition is a critical goal of vascular research.

There is much evidence that cytokine production within the vasculature is altered in patients with AAA. For example, patients with AAA have increased plasma levels of IL-1b, IL-6 and TNFα compared to controls (Juvonen *et al,* 1997), with

concentrations of IL-6 correlated with AAA size (Treska *et al,* 2000). A range of locally active cytokines is thought to play a role in promoting aortic wall destruction. There is evolving evidence that angiotensin II participates in the initiation and propagation of AAA (Reviewed, Daugherty & Cassis, 2004a). Animal studies have consistently demonstrated the ability of angiotensin II to promote the formation of AAA (Daugherty & Cassis, 2004b), although the mechanisms of this effect have not been defined. Over recent years, a relationship between the angiotensin II-associated cytokine osteoprotegerin (OPG) and vascular structure, function, and pathology has become increasingly evident (Brandström *et al,* 2002; Golledge *et al,* 2004; Hofbauer & Schoppet, 2004). The focus of this thesis was to understand whether OPG might be important in the development of human AAA. The aims of the study were to:

1. Define a relationship between aortic concentration of OPG and the presence of aneurysm (Chapter 5)
2. Identify possible mechanism(s) by which OPG may be functionally active in the promotion of aneurysm development (Chapter 6 and 7)
3. Examine what effect altering OPG levels within the aneurysm wall would have on AAA development with emphasis on assessing OPG as a potential therapeutic (pharmacological) target for non-invasive treatment of AAA (Chapter 8)

Human and experimental animal model data presented in Chapter 5 provide strong evidence for aortic expression of OPG and its association with the presence and development of AAA. Expression of OPG was demonstrated within the wall of both nonaneurysmal and aneurysmal human aorta. The concentration of OPG detected in late-stage human AAA, however, was up to 12-fold higher compared to the thoracic, and post-mortem abdominal, nonaneurysmal aortic controls, linking elevated aortic tissue OPG with the presence of AAA in humans. Upregulation of aortic OPG in the angiotensin II-infused ApoE-/- mouse model of AAA not only confirmed the human tissue observations, but additionally supported a role for the protein in aneurysm development *in vivo*. A limiting factor with the *in vivo* model used in this study is the extent to which experimental aneurysms produced in mice represent clinical AAA in humans. There is no perfect animal model of AAA; investigation using an animal

model remains limited and relevance to the human condition will always be questioned. This study utilized a mouse model of AAA in which mice lacking the ApoE lipoprotein gene were infused subcutaneously with angiotensin II. Pathology in this model can vary and is not restricted to the true circumferential medial destruction seen in human AAA. For example, destruction of the internal elastic lamina and focal dissection of the aortic wall is frequently observed. However, it can be argued that this system is adequately representative for three main reasons:

1. Angiotensin II is acknowledged as being critically involved in human atherosclerosis and aneurysm formation;
2. Infusion of angiotensin II in these animals results in focal recruitment of inflammatory cells and elevated proteolytic enzyme activity within the vessel wall, two crucial features in human AAA pathogenesis;
3. Focal dilation of the aorta occurs without physical experimental manipulation of the vessel.

In examining the human cohort of 146 patients, no relationship between serum OPG and aneurysm diameter was evident, despite high concentrations of OPG measured within aortic tissue. This observation was unexpected, as studies over the last five years have consistently demonstrated that relative risk of cardiovascular mortality is increased three- to four-fold in patients with high serum OPG (Browner *et al,* 2001; Kiechl *et al,* 2004), and, correlation between elevated serum OPG and severity of artery disease (Jono *et al,* 2002; Schoppet *et al,* 2003; Rhee *et al,* 2005; Ziegler *et al,* 2005). Moreover, data from Ueland *et al*. (2004) demonstrates a positive association between elevated OPG levels and poor cardiovascular prognosis. As such, one might have anticipated a positive correlation between serum concentrations of OPG and aneurysm diameter. The fact that this could not be shown suggests that OPG serum level alone would not be a suitable indicator for aneurysm size. Although a larger study is needed, the finding in this study of a correlation, albeit a weak one, between serum OPG and aneurysm growth rate, may provide basis for the use of raised serum OPG concentration as a clinical marker for identifying faster expanding aneurysms in existing AAA patients undergoing management.

Abdominal aortic aneurysm is invariably associated with severe atherosclerotic damage of the aortic wall. As such, atherosclerosis has historically been considered the main aetiological factor in AAA formation (Reed *et al,* 1992); however this view has been increasingly challenged in recent years. Despite morphological differences that delineate AAA and aortic occlusive disease (AOD) at advanced stages, it remains uncertain as to why atherosclerosis leads to narrowing of the arterial lumen in some individuals and to arterial dilation in others, leaving the role of atherosclerosis in aortic aneurysm a matter of great debate (Davies, 1998; Michel, 2001). Clinical and basic research indicates that aneurysms arise through pathogenic mechanisms that differ, at least in part, from those responsible for AOD (Ghorpade & Baxter, 1996; Tilson, 1998; Xu *et al,* 2001; Carrell *et al,* 2002). For example, Defawe and colleagues (2003) showed that two physiological inhibitors of proteases (TIMP-2 and PAI-1) were expressed less in AAA than in occlusive disease, suggesting a significant role for protease inhibitors during the divergent evolution of the initial atherosclerotic plaque towards either AAA or AOD. Moreover, not all patients with atherosclerosis develop AAA; therefore, even if atherosclerosis does have a role in AAA pathogenesis, additional factors are likely to be involved in aneurysm development. In support of this and providing further evidence of disparity between AAA and AOD pathology, the observations made in the present study (Chapter 5) showed expression of OPG three-fold greater within the wall of AAA biopsies compared to age- and gender-matched biopsies of occluded aorta (AOD). As such, one might speculate that it is the level of expression of OPG within the aortic wall, both spatial and temporal, which plays a role in triggering aneurysm evolution.

A recent study by Olesen *et al* (2005) shows elevated OPG concentration within the aortic wall of individuals suffering diabetes mellitus, a patient group two to three times more likely to develop atherosclerosis and occlusive disease compared to patients without diabetes (Beckman *et al,* 2002), and is supported by an earlier report documenting serum levels of OPG are increased approximately 30 percent in sufferers of diabetes (Browner *et al,* 2001). This presents somewhat of a paradox as far as the findings of the present study were concerned. An important fact to consider at this point is that diabetes mellitus has been established as a negative risk factor for

AAA by several epidemiological studies (Lederle *et al,* 2000; Blanchard *et al,* 2000; Brady *et al,* 2004), with sufferers only half as likely to develop AAA (Lederle *et al,* 2000). Although the apparent protective effect afforded by diabetes against the formation of AAA remains unexplained, any and all of a number of biochemical alterations observed within the diabetic aorta may contribute. Of these, extensive calcification of the aortic media seen in diabetics (Chen & Moe, 2003) may confer the artery with a degree of resistance against aneurysm formation. It is speculated that increased expression of OPG observed within the diabetic aorta may be part of generalised matrix alterations, putatively related to the development of vascular calcifications (Olesen *et al,* 2005); however the situation remains complex. Earlier *in vivo* studies using OPG gene knockout mice resulted in osteoporosis with an unexpected corresponding phenotype of arterial calcification. This, in contrast, suggests that OPG may be involved in limiting the process of arterial calcification (Bucay *et al,* 1998; Price *et al,* 2001). Ultimately, both the physiological role and the effect of change in OPG expression within the vasculature remain obscure.

What, then, is the functional consequence of upregulated OPG expression within the aortic wall with respect to AAA? End-stage AAA exhibits many features of a chronic inflammatory disease, characterized by extensive inflammatory cell accumulation, consisting of T cells, B cells and monocyte/macrophages, throughout the media and adventitia of the vessel wall (Pearce & Koch, 1996; Henderson *et al,* 1999). Leukocyte recruitment into the aortic wall may be promoted by degradation of elastin (and other extracellular matrix components), an important histological feature of aneurysmal tissue (Baxter *et al,* 1992; Sakalihasan *et al,* 1993). Soluble peptide fragments are believed to act as chemotactic agents for infiltrating monocyte/ macrophages through interactions with the 67 kDa elastin-binding protein found on the surface of these cells (Hance *et al,* 2002). Prostaglandin derivates and proinflammatory cytokines produced by both the resident smooth muscle cells and the inflammatory cells themselves also contribute to the process (Koch *et al,* 1993; Newman *et al,* 1994; Walton *et al,* 1999; Hance *et al,* 2002).

In addition to inflammation and rarefaction of extracellular matrix, the aneurysmal aortic media also undergoes a reduction in the density of smooth muscle cells. These

resident cells participate in vascular wall homeostasis and remodeling through localised expression of various extracellular matrix proteins as well as proteases and their inhibitors (He & Roach, 1994). As such, the process of medial smooth muscle cell dysfunction and loss is regarded as a key event in the development of AAA in humans (Lopez-Candales, *et al,* 1997) and are discussed further in this chapter. Examination of human AAA-derived smooth muscle cells and macrophages using FACS and protein secretion analysis (Chapter 5) revealed both the presence of OPG within, and its secretion at high concentration from, these cells. These observations suggest that both cell-types contribute significantly to the increased level of this cytokine detected within the aneurysm wall.

The potential effect that supra-physiological levels of OPG may have on medial smooth muscle cells and monocyte/macrophages within the aortic wall was investigated (Chapter 6). *In vitro* exposure of normal human aortic smooth muscle cells and the human monocytic cell-line THP-1 to increasing concentration of OPG resulted in modulation of inflammatory cytokine (IL-6) production and metalloproteinase (MMP-2 and 9) activity in both cell-types. However, the negative action of OPG on smooth muscle cell growth and survival was most apparent. Indeed, gene expression studies in these cells (Chapter 7) clearly demonstrated OPG-induced downregulation of cell cycle proteins associated with proliferation, and upregulation of pro-apoptotic mitochondrial proteins associated with cell death. Moreover, genes associated with cytoskeletal machinery, cell morphology, and production of extracellular matrix proteins, were also negatively regulated. Therefore, upregulated and sustained expression of OPG within the aortic wall may potentially stimulate an aneurysmal phenotype in medial smooth muscle cells and infiltrating inflammatory cells, and promote a cumulative process that contributes to a breakdown in the homeostatic function of resident smooth muscle cells and degeneration of the aortic wall.

Mentioned earlier, a characteristic feature of late-stage AAA is chronic inflammation. The cause of the inflammatory process is unknown; possibilities include an infectious agent such as *Chlamydia pneumoniae* (Tambiah & Powell, 2002), or extracellular matrix-derived (fragmentation) peptides (Hance *et al,* 2002)

and autoimmune processes (Koch *et al,* 1990; Gregory *et al,* 1996). From this, one realizes that the 'chicken-or-the-egg' scenario complicates the understanding of AAA pathogenesis. Is it inflammation drawn from an external antigen that leads to increased proteolytic activity within the artery wall and breakdown of extracellular matrix and wall degeneration? Or is it the mechanical degeneration of the extracellular matrix, motivated by haemodynamic forces, and/or genetic predisposition, that triggers an immune/inflammatory response within the artery wall? In either case, it has been postulated that healthy medial smooth muscle cells play a protective role within the artery wall by exerting a paracrine effect on the adventitia, which participates in artery wall homeostasis against inflammation and proteolysis, and that it is the failure of this protective mechanism that leads to AAA formation (Allaire *et al,* 1998, 2002). *In vitro*, aortic smooth muscle cells have been shown to produce less monocyte chemotactic protein-1 (MCP-1), a major inflammatory mediator in abdominal aortic aneurysms, under cyclic stretching than with static culture, which lends support to the notion of a protective paracrine function of smooth muscle cells (Defawe *et al,* 2004).

Survival and normal function of smooth muscle cells within the media is thought to depend primarily on their association with intact elastic fibres (Meredith *et al*, 1993). The fragmentation of these elastic fibres, and the absence of an elastin-rich extracellular environment typically associated with degeneration of the aortic media, stimulates smooth muscle cell apoptosis (Meredith *et al*, 1993). It is thus conceivable that the development of smooth muscle cell death and medial infiltration of inflammatory cells represent early and late stages of aneurysm progression, respectively, linked by the breakdown of extracellular matrix proteins. Theoretically, at initial stages of aneurysm formation, as yet undefined repeated insult to the artery wall results in disruption of extracellular matrix structural proteins. Continual degradation of these proteins eventually compromises the physiology of resident medial smooth muscle cell and facilitates gradual invasion by inflammatory cells of the artery wall. Ultimately, a point is reached where the putative protective function of the medial smooth muscle cells in wall homeostasis is overwhelmed, resulting in the increase of inflammation and proteolytic activity and subsequent aneurysm progression.

In line with this theory, the present study provides *in vitro* evidence indicating the involvement of OPG in AAA development at both the initial (formation) stage, i.e. the ability of OPG to suppress proliferation and induce apoptosis in human aortic smooth muscle cells, and later progressive stage, i.e. the ability of OPG to stimulate inflammatory cytokine production and protease activity in human monocytic cells. Thus, *in vivo*, one might speculate that in response to injurious stimuli and/or disruption of extracellular matrix, medial smooth muscle cells over-express OPG, which, via autocrine action, negatively regulates their physiology resulting in depletion of the smooth muscle cell population and its protective effect against inflammation. Facilitated by this, inflammatory cells invade the media where OPG, derived from these cells, may again act via autocrine mechanisms to promote increased transmural inflammation, extracellular matrix degradation, and aneurysm growth (Figure 9.1.).

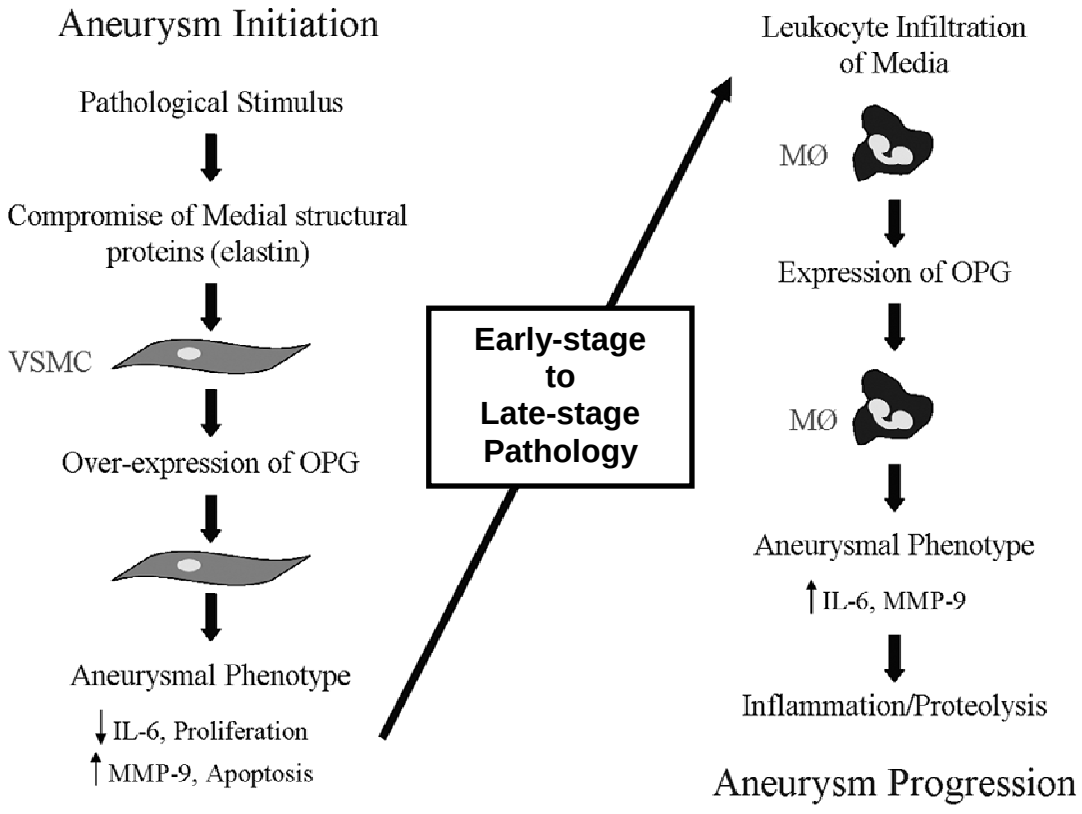


Figure 9.1. Postulated role of OPG in AAA pathogenesis and progression in humans. VSMC, vascular smooth muscle cell; OPG, osteoprotegerin; IL-6, interleukin-6; MMP-9, metalloproteinase-9; Mø, macrophage.

Findings of large randomized trials support conservative treatment for small aortic aneurysms. However, with continued aortic destruction, more than half of patients initially treated conservatively require surgery. Recent elucidation of the biological processes involved in aneurysm development and expansion has led to translational research investigating the use of novel medication aimed at retarding aneurysm growth. The most promising drugs have been those with either anti-inflammatory or anti-MMP properties, both of which have had some success in experimental models (Holmes *et al*, 1996; Boyle *et al,* 1998; Thompson & Baxter, 1999; Walton *et al*, 1999). Tetracyclines provide a potentially effective treatment. Doxycycline, a synthetic tetracycline derivative, was shown to prevent MMP-mediated aneurysmal growth in animal models (Curci *et al,* 1998b). Moreover, findings from a clinical study suggested that doxycycline treatment prevents aneurysm growth in humans (Baxter *et al,* 2002). The use of synthetic inhibitors of MMP activity, such as batimastat (BB-94), has also been shown to suppress the expansion of experimental abdominal aortic aneurysms (Bigatel *et al,* 1999; Moore *et al,* 1999).

Another attractive option of aneurysmal pharmacotherapy is to target the inflammatory response and interfere with the MMP pathway. Non-steroidal anti-inflammatory drugs, such as indometacin, are known to prevent development of abdominal aortic aneurysms in animal models (Holmes *et al*, 1996). The use of β-blocking agents (e.g. propranolol) seems to reduce the growth rate of large (>50 mm) aneurysms and even to lessen the size of experimental aneurysms (Gadowski *et al*, 1994; Slaiby *et al*, 1994), however, a randomized trial reported that propranolol does not have a significant effect on the growth rate of small aneurysms (PATI, 2002). Statins (hydroxymethylglutaryl coenzyme A reductase inhibitors), besides their cholesterol lowering effects, reduce the expression of various inflammatory molecules, including MMP (Bellosta *et al,* 1998). Nagashima *et* al (2002) reported that addition of cerivastatin to tissue organ cultures of abdominal aortic aneurysms has been shown to down-regulate the production of MMP-9, which suggests that members of the statins family could prevent elastolysis in patients with this disorder. Very recently, however, Defawe and colleagues (2005) demonstrated that MMP can alter matrix remodeling independently of their proteolytic function, which suggests

that the role of MMP might be more complex than mediation of a degradation process.

Angiotensin II plays a significant role in the development and progression of cardiovascular disease. Most cardiovascular effects of angiotensin II are mediated via the type-1 isoform of the angiotensin II receptor, AT_1R. Inhibitors of angiotensin II action, AT_1R-blockers, limit inflammation and atherosclerosis progression and are routinely employed clinically in the management of cardiovascular disorders. It is reasonable that these compounds may also be effective in slowing aneurysm progression. The present investigation supports both a correlative and functional relationship between angiotensin II, upregulated expression of OPG within the aortic wall, and aortic aneurysm pathobiology. Moreover, experimental modulation of aortic OPG expression via AT_1R regulation identifies the cytokine as a novel potential therapeutic target for the treatment of AAA. Exposure of human AAA explants to the AT_1R antagonist Irbesartan (Chapter 8) produced time-dependent inhibition of both OPG and IL-6 secretion into culture, as well as significant reduction in the production of OPG within explant tissue. It is important to note that many patients with AAA are already prescribed AT_1R blockers for the management of hypertension, and many inevitably require surgical repair of an aneurysm at risk of rupture despite medication. Nevertheless, it could be argued that AT_1R blockade is prolonging presentation of these patients at surgery by acting to limit (OPG-mediated) aneurysm expansion rate and growth. A randomized study would be necessary to confirm this. Unfortunately, trialling AT_1R antagonists for the treatment of AAA would be problematic, a potential study being confounded by concurrent use of these compounds for co-morbidities in this patient group.

Sugawara and co-workers (2001, 2003) may have provided an alternative. Their studies demonstrate that activation of peroxisome proliferator-activated receptor (PPAR)γ can suppress AT_1R expression at the level of transcription. Synthetic ligands (activators) of PPARγ comprise the Thiazolidinedione family of pharmacological compounds currently in clinical use for the treatment of diabetes (Martens *et al,* 2002). The present study identifies a protective function of PPARγ-activation against AAA formation and development, associated with regulation of

AT_1R and angiotensin II-induced expression of OPG within the aortic wall. Chapter 8 demonstrates the significant effect of the PPARγ-ligands pioglitazone and rosiglitazone in reducing OPG and IL-6 production, and MMP-9 activity in cultured human AAA explant, paralleling the action of AT_1R-blockade seen with Irbesartan treatment. The effect of PPARγ-activation on late-stage AAA tissue suggests potential benefit in regulating aneurysm progression. Pioglitazone therapy in an *in vivo* (angiotensin II-infused) experimental model of aneurysm progression (Chapter 8) resulted in significantly lower levels of both aortic OPG and MMP-9 compared with levels detected in aortas from untreated animals, mirroring the effect of thiazolidinedione treatment on human AAA explants. *In vitro* and animal data from other investigators supports PPARγ ligands in reducing inflammation and proteolytic enzyme production in atherosclerosis. Of most significance is the finding that activation of PPARγ also inhibits OPG gene expression in normal human aortic VSMC (Zhang *et al*, 2002). It is therefore postulated that within the (aneurysmal) aortic wall, ligation of PPARγ effects OPG production directly via suppression of gene transcription, and/or indirectly by acting negatively upon the angiotensin II pathway through downregulation of AT_1R expression. Unlike all the above mentioned medications suggested as a potential therapy for AAA, PPARγ ligands are rarely indicated in patients with AAA as the condition is rare in patients with diabetes and the medication has only recently been introduced into clinical practice. Consequently, these compounds have excellent potential to be investigated in large-scale human AAA trials.

As stated earlier in discussion, the goal of research into human AAA is to identify potential targets for pharmacological therapy to limit the requirement for invasive intervention. The body of work presented in this thesis describes for the first time an association between the presence and upregulation of OPG within the aortic wall and the development of aortic aneurysm. To summarize, upregulated expression of OPG is observed in human aortic aneurysm biopsies. Medial smooth muscle cells and inflammatory cells (macrophages) isolated from human AAA are primary sources of OPG production within the aneurysm wall. *In vitro*, at concentrations measured within human AAA, OPG induces development of an aneurysmal phenotype in normal human aortic smooth muscle cells by inhibiting proliferation and promoting

apoptosis and in human monocytic cells by stimulating proinflammatory cytokine and metalloproteinase production. Additionally, the study identifies angiotensin II as an important mediator of OPG synthesis *in vivo* and (over)expression within the aortic wall leading to aneurysm formation. The potential for AT_1R blockade in slowing aneurysm expansion through targeting of OPG production is highlighted; thiazolidinedione therapy and PPARγ-activation is postulated to reduce progressive aortic dilation in patients suffering AAA by directly suppressing OPG production in aortic VSMC and macrophages, in addition to inflammatory and proteolytic pathways mediated through AT_1R and angiotensin II.

Moving forward to clinical trials is ultimately the best assessment of any potential therapeutic intervention aimed at reducing invasive treatment of human AAA, as laboratory investigation remains restricted due to difficulty in modelling true AAA in animals and the limitations of using human AAA biopsies (concurrent medication, increasing use of endoluminal repair). Nevertheless, to fully assess the value of OPG as a therapeutic target in human AAA, several issues require further examination. Of particular importance are the following:

1. ***Confirmation of the concept that OPG is pro-aneurysmal***. It is the conclusion of this thesis that OPG is pathologically significant in the formation and progression of AAA and provides both *in vitro* and experimental *in vivo* (animal model) evidence to support this hypothesis. Despite this, the human data remains primarily correlative. Gene-knockout technology can further elucidate a functional role for OPG in promoting human AAA. For example, *in vivo* studies using the angiotensin II-infusion ($ApoE^{-/-}$ mouse) model of AAA would be performed with concomitant ablation of the OPG gene. Outcome would determine the cytokine's profile at least as far as its importance in angiotensin II-induced change within the aortic wall is concerned. Similar studies can be performed *in vitro* by employing techniques such as *siRNA* gene-knockdown to target OPG expression in the desired cell-type. At the very least, further understanding of the physiological role of OPG within the (normal) aorta can be gained from removing it from the system.

2. ***A broader understanding of the molecular mechanisms of OPG action***. A significant effect on human monocytic cell and aortic smooth muscle cell biology by OPG was demonstrated *in vitro*. Gene expression analysis in normal aortic smooth muscle cells exposed to OPG in culture identified genes for an extensive range of factors both positively and negatively regulated by OPG. Closer inspection of OPG's regulation of gene expression, with Real-Time (RT)-PCR for example, in smooth muscle cells and other cell-types significant in AAA pathobiology (e.g. monocyte/macrophages, T-cells, dendritic cells) would provide further information as to the cytokine's role within the vasculature and its contribution to the development of AAA. Furthermore, defining the molecular actions of OPG can potentially lead to the identification of additional pharmacological targets for AAA therapy.

3. ***Assessment of known OPG-regulating therapeutic agents in experimental AAA***. The present investigation has demonstrated that over-expression of aortic OPG in human AAA tissue and in an experimental model of AAA is ameliorated upon administration of an AT_1R antagonist or PPARγ ligand, the latter also a negative regulator of AT_1R function. In addition to their negative regulation of OPG expression and production within the (aneurysmal) aortic wall, these medications exhibit additional protective properties against aneurysm progression such as anti-inflammatory and anti-proteolytic actions. The potential benefit of these medications to sufferers of AAA needs to be pursued further both experimentally and clinically.

Appendix 1

REPRODUCIBILITY DATA OF DUOSET® OPG ELISA

1. INTRA-ASSAY CO-EFFICIENT OF VARIENCE

Plate 1

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.093	1.102	1.098	0.006
2000	0.669	0.645	0.657	0.017
1000	0.473	0.491	0.482	0.013
500	0.330	0.332	0.331	0.001
250	0.268	0.267	0.268	0.001
125	0.311	0.261	0.286	0.035
62.5	0.279	0.264	0.272	0.011
Mean			**0.485**	**0.012**
Intra Co Var			**2.48%**	

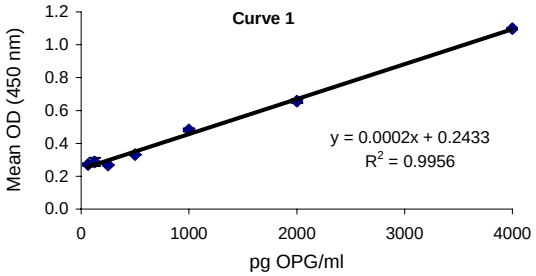


Plate 2

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.243	1.241	1.242	0.001
2000	0.638	0.717	0.678	0.056
1000	0.369	0.396	0.383	0.019
500	0.234	0.265	0.250	0.022
250	0.184	0.204	0.194	0.014
125	0.166	0.174	0.170	0.006
62.5	0.147	0.159	0.153	0.008
Mean			**0.438**	**0.018**
Intra Co Var			**4.12%**	

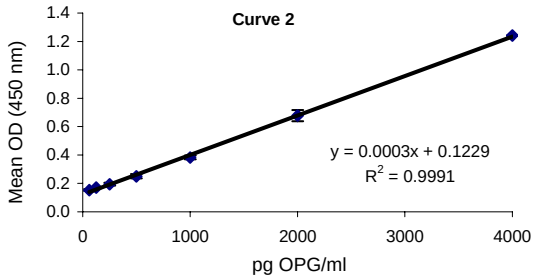


Plate 3

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.291	1.239	1.265	0.037
2000	0.604	0.635	0.620	0.022
1000	0.359	0.365	0.362	0.004
500	0.218	0.230	0.224	0.008
250	0.134	0.163	0.149	0.021
125	0.109	0.114	0.112	0.004
62.5	0.092	0.099	0.096	0.005
Mean			**0.404**	**0.014**
Intra Co Var			**3.55%**	

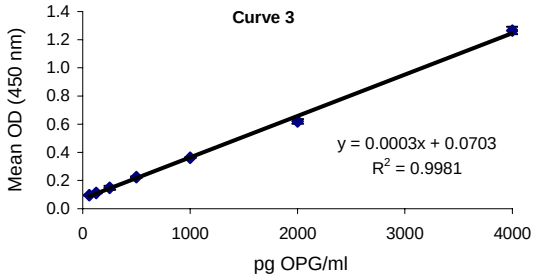


Plate 4

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.239	1.282	1.260	0.031
2000	0.600	0.719	0.660	0.084
1000	0.301	0.262	0.282	0.027
500	0.217	0.203	0.210	0.010
250	0.155	0.159	0.157	0.003
125	0.120	0.162	0.141	0.030
62.5	0.105	0.120	0.112	0.011
Mean			**0.403**	**0.028**
Intra Co Var			**6.91%**	

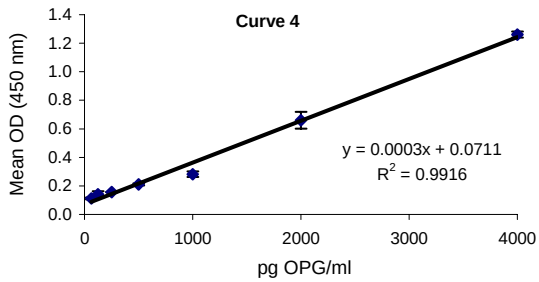


Plate 5

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.263	1.290	1.277	0.019
2000	0.598	0.669	0.634	0.050
1000	0.344	0.379	0.361	0.024
500	0.230	0.218	0.224	0.009
250	0.194	0.142	0.168	0.037
125	0.150	0.116	0.133	0.024
62.5	0.118	0.090	0.104	0.020
Mean			**0.414**	**0.026**
Intra Co Var			**6.29%**	

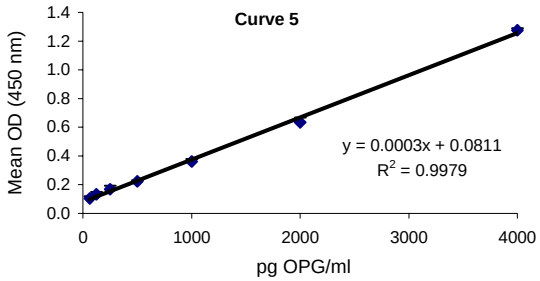


Plate 6

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.287	1.268	1.278	0.013
2000	0.743	0.739	0.741	0.003
1000	0.366	0.366	0.366	0.000
500	0.225	0.218	0.221	0.005
250	0.136	0.131	0.134	0.004
125	0.105	0.104	0.104	0.001
62.5	0.086	0.087	0.087	0.000
Mean			**0.419**	**0.004**
Intra Co Var			**0.92%**	

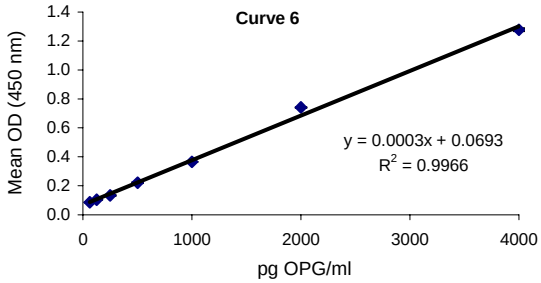
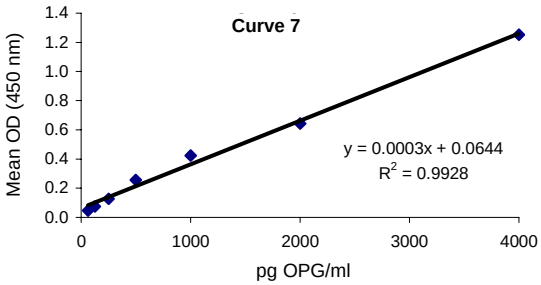


Plate 7

Standard (pg/ml)	Dup1 OD	Dup2 OD	Mean OD	Stand Dev
4000	1.230	1.274	1.252	0.031
2000	0.626	0.659	0.643	0.023
1000	0.435	0.411	0.423	0.017
500	0.290	0.224	0.257	0.047
250	0.128	0.126	0.127	0.001
125	0.074	0.074	0.074	0.000
62.5	0.049	0.046	0.048	0.002
Mean			**0.403**	**0.017**
Intra Co Var			**4.31%**	



2. INTER-ASSAY CO-EFFICIENT OF VARIENCE

2 Plate Analysis	Plate 1	Plate 2		
Standard (pg/ml)	Mean OD	Mean OD	Av Mean OD	Stand Dev
4000	1.098	1.242	1.170	0.102
2000	0.657	0.678	0.667	0.014
1000	0.482	0.383	0.432	0.070
500	0.331	0.250	0.290	0.058
250	0.268	0.194	0.231	0.052
125	0.286	0.170	0.228	0.082
62.5	0.272	0.153	0.212	0.084
Mean			**0.462**	**0.066**
Inter Co Var			**14.32%**	

2 Plate Analysis	Plate 3	Plate 4		
Standard (pg/ml)	Mean OD	Mean OD	Av Mean OD	Stand Dev
4000	1.2650	1.2604	1.263	0.003
2000	0.6195	0.6598	0.640	0.028
1000	0.3620	0.2815	0.322	0.057
500	0.2240	0.2104	0.217	0.010
250	0.1485	0.1567	0.153	0.006
125	0.1115	0.1411	0.126	0.021
62.5	0.0955	0.1124	0.104	0.012
Mean			**0.403**	**0.020**
Inter Co Var			**4.85%**	

3 Plate Analysis	Plate 5	Plate 6	Plate 7		
Standard (pg/ml)	Mean OD	Mean OD	Mean OD	Av Mean OD	Stand Dev
4000	1.277	1.278	1.252	1.269	0.014
2000	0.634	0.741	0.643	0.672	0.060
1000	0.361	0.366	0.423	0.383	0.034
500	0.224	0.221	0.257	0.234	0.020
250	0.168	0.134	0.127	0.143	0.022
125	0.133	0.104	0.074	0.104	0.029
62.5	0.104	0.087	0.048	0.080	0.029
Mean				**0.412**	**0.030**
Inter Co Var				**7.22%**	

Appendix 2

Regulation of Gene Expression in Healthy Human Abdominal Aortic VSMC by OPG

Genes downregulated by OPG and associated with cell survival and growth in normal human abdominal aortic VSMC

GENE	FOLD DECREASE	FUNCTION	REFERENCE
14-3-3 protein	2.8	A phosphoserine/ phosphothreonine-binding molecule that bind critical mediators of intracellular signalling pathways. Participates in regulation of cell proliferation and cell-cycle checkpoint control. Important role in cell survival signaling and preventing cell death. Antagonizes activity of proapoptotic proteins Bad and ASK1. Expression of 14-3-3 inhibitor peptides in cells sufficient to induce apoptosis.	Masters & Fu, 2001
Cdc28 protein kinase 1 (CKS1)	2.2	CKS1 promotes the transition of cells from G1 to S phase, progression through S phase, and entry into mitosis. CKS1 binds to the catalytic subunit of the cyclin dependent kinases and is essential for interaction of $p27^{Kip1}$ with the S-phase kinase-associated protein-ubiquitin ligase complex and $p27^{Kip1}$ degradation. $p27^{KIP1}$ belongs to the family of cell cycle regulators called cyclin-dependent kinase inhibitors (CDKI), which bind to "cyclin-CDK" complexes and cause cell cycle arrest in the G1 phase. $p27^{KIP1}$ is often known as a universal CDKI, since it interacts with all subtypes of "cyclin-CDK" complexes to inhibit cell cycle progression.The degradation of $p27^{Kip1}$ in the late G1 phase activates Cdk2-cyclin E and Cdk2-cyclin A complexes that, in turn, signal the cells to enter the S phase of the cell cycle.	Yu *et al,* 2005; Spruck *et al,* 2001
Cdc37	3.4	Required for association of the protein kinase Cdc28 with G1 and mitotic cyclins. An upstream regulatory element of key cell cycle kinases and essential for chomosome segregation and cytokinesis.	Gerber *et al,* 1995; Lange *et al,* 2002
Cyclin-dependent kinase 4 (CDK4)	2.6	Cell cycle progression from G1 to S phase is governed by CDK4 and the homologous CDK6 and CDK2. CDK4 and CDK6 are activated by D-type cyclins at early to mid-G1 phase, whereas CDK2 is activated by E- and A-type cyclins during late G1 and S phase, respectively.	Sherr & Roberts, 1999
p21-activated kinase (PAK)-1	5.6	Essential for Ras-induced upregulation of cyclin D1 during the G1 to S transition.	Granville *et al,* 2001
Phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA)	2.4	PIK3CA encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K). The serine/threonine kinase Akt plays a key role in suppression of apoptotic cell death by phosphorylation and suppression of the pro-apoptotic protein Bad. Activation of Akt is mediated by PI3K, stimulating phosphorylation of Akt by activating protein kinase B/Akt kinases (PDK-1 and PDK-2).	Jung *et al,* 2000

Genes downregulated by OPG and associated with cell survival and growth in normal human abdominal aortic VSMC

GENE	FOLD DECREASE	FUNCTION	REFERENCE
Equilibrative NBMPR-sensitive nucleoside transporter (ENT1)	3.4	Cell proliferation (DNA replication) involves high nucleic acid synthesis rates. Nucleosides from the extracellular milieu are the main source for nucleic acid synthesis. Nucleoside and nucleobase trans-membrane transporters play key roles in the uptake of precursors for nucleotide synthesis. Depending on the cell type, different equilibrative and concentrative transport activities may be coexpressed. Equilibrative uptake involves passive nucleoside transport mediated by members of the equilibrative nucleoside transporter (ENT) family (ENT1 and ENT2).	Soler *et al,* 2001
Farnesyl-protein transferase beta-subunit	4.2	The Ras proteins are low molecular weight GTP binding proteins that function in the regulation of the transduction of growth proliferative signals from the membrane to the nucleus. Ras does not function unless it is attached to the inside of the cell membrane. Post-translational modification of Ras is requisite for localization in the plasma membrane. The first and obligatory reaction in a series of Ras protein modifications is farnesylation, a reaction catalyzed by the farnesyl protein transferase enzyme.	Kouchi *et al,* 1999
Janus kinase 1 (a protein tyrosine kinase) (JAK1) Signal transducer and activator of transcription 6 (STAT6)	4.2 5.2	The JAKs and STATs transduce signals of. Activated JAKs tyrosine-phosphorylate the latent cytoplasmic STATs which homodimerize or heterodimerize and translocate to the nucleus and function in the transcription of factors leading to proliferation and differentiation. Maximal activation of STATs requires both tyrosine phosphorylation by JAKs and serine/threonine phosphorylation by mitogen-activated protein kinase.	Huang *et al,* 1999
Ras family small GTP binding protein RHEB2	2.0	The RHEB family of proteins play a role in the mTOR/S6K signaling pathway in mammalian cells. p70S6K plays a crucial role in cell proliferation, collagen expression, and cell cycle control.	Tabancay *et al,* 2003; Gabele *et al,* 2005
SHC (Src homology 2 domain-containing) transforming protein 1	2.4	SHC is a signaling adapter that couples activated growth factor receptors with signaling pathways. Isoform p46 and isoform p52, once phosphorylated, couple activated receptor kinases to Ras and PI3K via the recruitment of the GRB2/SOS complex and are implicated in the cytoplasmic propagation of mitogenic signals.	
Toll-like receptor 4 (TLR4)	3.6	TLR4-mediated activation of p44/p42 MAPK induces VSMC proliferation	Sasu *et al,* 2001
BCL2-like 1 (BCL2L1; Bcl-x)	8.6	A potent inhibitor of cell death by preventing the activation of caspases. Appears to regulate cell death by blocking the voltage-dependent anion channnel (VDAC) by binding to it and preventing the release of the caspase activator, cytochrome c, from the mitochondrial membrane. Sequence	Huang *et al,* 1998

Genes downregulated by OPG and associated with cell survival and growth in normal human abdominal aortic VSMC

GENE	FOLD DECREASE	FUNCTION	REFERENCE
		comparison of Bcl-2 family members has revealed four recurring motifs, commonly denoted Bcl-2 Homology domains (BH1 to BH4). The five most closely related mammalian homologues, Bcl-2, Bcl-x_L, Bcl-w, Bax and Bak, possess the BH1, BH2 and BH3 domains but only the first three, which inhibit apoptosis, also bear the N-terminal BH4 domain. BH1 and BH2 of Bcl-2 and Bcl-x_L are critical for heterodimerization with Bax and for promoting cell survival. In contrast, the small BH3 domain of the pro-apoptotic molecules appears to be essential for their interaction with Bcl-2 and its functional homologues and for acceleration of apoptosis. This domain is also found in several distantly related proteins that promote apoptosis: Bik/Nbk, Bid, Hrk, Bad and Bim. Recent structural studies on Bcl-x_L have revealed that its BH1, BH2 and BH3 domains form an elongated hydrophobic cleft which can bind the BH3-containing peptides of the death promoters thus preventing their action.	
I-FLICE isoform 4	3.4	Inhibitor of the apoptosis initiator FLICE (caspase-8).	Hu *et al,* 1997
Cystatin C (CST3)	2.4	Cysteine protease inhibitor normally expressed in VSMC is significantly reduced in both atherosclerotic and aneurysmal aortic lesions. Induced deficiency in ApoE-null mice increases elastic lamina degradation and aortic dilation.	Shi *et al,* 1999; Sukhova *et al,* 2005
Early growth response 1	2.4	Egr-1 is rapidly and transiently expressed in many different cell types in response to a variety of extracellular stimuli, including growth factors, cytokines and injurious stimuli and regulates the expression of several genes implicated in the pathogenesis of atherosclerosis (VSMC proliferation/migration) including TNFα, FGF-2, PDGF, TGFβ.	Santiago *et al,* 1999; McCaffrey *et al,* 2000

Genes upregulated by OPG and associated with growth inhibition and apoptosis in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
BCL2-associated X protein (Bax)	2.6	Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. In contrast to the initiation and degradation stages, the effector stage of apoptosis is subject to regulation by the family of Bcl-2 related proteins and includes both death agonists (Bax, Bak, Bcl-xS, Bad, Bik, and Hrk) and death antagonists (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1, Brag-1 and a1). Bax forms homodimers and also heterodimerizes with Bcl-2, Bcl-xL, Mcl-1, and a1. The cellular concentrations of death antagonists and agonists (ratio of antagonists to agonists) and the competitive dimerization between selective pairs of antagonists and agonists appear to determine a cell's susceptibility to apoptosis. Deregulation of cell death genes leading to overexpression of Bax would alter the Bcl-2:Bax ratio favouring apoptosis.	Chipuk *et al,* 2004
Bcl-2 binding component 6 (Bad)	3.5	Promotes cell death. Successfully competes for the binding to Bcl-x(L), Bcl-2 and Bcl-w, thereby affecting the level of heterodimerization of these proteins with Bax. Bad can reverse the death repressor activity of Bcl-x(L), but not that of Bcl-2. It is a Ca2+-sensitive pro-apoptotic Bcl-2 protein, which on activation translocates from cytosol to mitochondria to initiate cytochrome c release. Concurrent with Bad translocation, a Ca2+-sensitive increase in cellular calcineurin activity has been observed. It is suspected that increased cytosolic Ca2+ and calcineurin activation stimulates Bad translocation. Appears to act as a link between growth factor receptor signaling and the apoptotic pathways.	Huang & Strasser, 2000
Protein phosphatase 3 regulatory subunit B (calcineurin B, type I)	2.6	Regulatory subunit of calcineurin, confers calcium sensitivity. Calcineurin is a heterodimer of catalytic (A) and regulatory (B) subunits. It exhibits very low phosphatase activity alone, but binding of Ca2+/CaM to the A subunit and/or binding of Ca2+ to the B subunit induces noticeable activation. It is a Ca2+/calmodulin-dependent serine/threonine phosphatase that on activation predisposes to apoptosis. Constitutive high levels of activated calcineurin and subsequent dephosphorylation of Bad regulates mitochondria-mediated apoptosis.	Shou *et al,* 2004
Death-associated protein 6 (DAXX)	1.5	The Daxx protein (also known as the Fas-binding protein) is a component of nuclear promyelocytic leukemia protein (PML) oncogenic domains (PODS) and plays a role in apoptosis by enhancing Fas-mediated apoptosis through the JNK signal transduction pathway. Daxx activates the JNK kinase kinase ASK1. Fas activation induces Daxx to interact with ASK1, which consequently relieves an inhibitory intramolecular interaction between the amino- and carboxyl-termini of ASK1, activating its kinase activity. The Daxx-ASK1 connection completes a signaling pathway from a cell surface death receptor to kinase cascades that modulate nuclear transcription factors.	Chang *et al,* 1998

Genes upregulated by OPG and associated with growth inhibition and apoptosis in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
Death-associated protein kinase 3 (DAPK3)	3.6	Death-associated protein kinase (DAPK) is a Ca(2+)/calmodulin-regulated serine/threonine kinase which acts as a positive regulator of apoptosis. DAP kinase is a proapoptotic protein kinase with homology to ZIP kinase (ZIPK). ZIPK is present in PODs where it colocalizes with and binds to proapoptotic protein Daxx. ZIPK recruits Daxx to PODs via its catalytic activity and also binds and phosphorylates proapoptotic protein Par-4. Association of ZIPK with Daxx is enhanced by coexpression of Par-4. Activation of caspases and induction of apoptosis has also been observed in cells over expressing these proteins.	Shohat *et al,* 2002; Kawai *et al,* 2003
Forkhead box O3A (FOXO3A; FKHRL1)	2.8	Overexpression of the Forkhead transcription factors AFX, FKHR and FKHR-L1 causes growth suppression and apoptosis. Expression of AFX blocks cell-cycle progression at phase G1 dependent on the cell-cycle inhibitor p27kip1. The FoxO forkhead transcription factors FoxO4 (AFX), FoxO3a (FKHR.L1), and FoxO1a (FKHR) represent important physiological targets of phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) signaling. Overexpression or conditional activation of FoxO factors antagonizes many responses to constitutive PI3K/PKB activation including its effect on cellular proliferation. FoxO-induced cell cycle arrest is partially mediated by enhanced transcription and protein expression of the cyclin-dependent kinase inhibitor p27kip1. Additionally, a p27kip1-independent mechanism has been described that plays an important role in the antiproliferative effect of FoxO factors. Forced expression or conditional activation of FoxO factors leads to reduced protein expression of the D-type cyclins D1 and D2 and is associated with an impaired capacity of CDK4 to phosphorylate and inactivate the S-phase repressor pRb. Downregulation of D-type cyclins involves a transcriptional repression mechanism and does not require p27kip1 function. Ectopic expression of cyclin D1 can partially overcome FoxO factor-induced cell cycle arrest, demonstrating that downregulation of D-type cyclins represents a physiologically relevant mechanism of FoxO-induced cell cycle inhibition. The pro-apoptotic Bcl-2 family member, Bim, has been shown to be dramatically upregulated by FOXO transcription factors. Specific activation of FOXO3a alone was found to be sufficient to induce Bim expression, and could recapitulate all known elements of the apoptotic program normally induced by cytokine withdrawal.	Schmidt *et al,* 2002; Medema *et al*, 2000
Programmed cell death 8 (AIF)	1.5	Known as a mitochondrial effector of apoptotic cell death, apoptosis-inducing factor (AIF) functions in the release of apoptotic proteins such as cytochrome C and caspase-9. Extra-mitochondrial AIF induces nuclear chromatin condensation and large scale DNA fragmentation (in vitro). Induces phosphatidyl-serine exposure on outer leaflet of membrane bilayer (binding to which is the basis of Annexin V labeling for detection of apoptosis).	Granville *et al*, 2001

Genes upregulated by OPG and associated with growth inhibition and apoptosis in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
TR3beta	6.6	TR3 is an immediate-early response gene and an orphan member of the steroid-thyroid hormone-retinoid receptor superfamily of transcription factors. TR3 translocates from the nucleus to mitochondria to induce cytochrome c release and apoptosis. Characteristically anti-apoptotic, Bcl-2 promotes cell death by interacting with orphan nuclear receptor TR3. The interaction is mediated by the N-terminal loop region of Bcl-2 and is required for TR3 mitochondrial localization and apoptosis. TR3 binding induces a Bcl-2 conformational change that exposes its BH3 domain, resulting in conversion of Bcl-2 from anti- to pro-apoptotic. Overexpression of TR3 in VSMC inhibits DNA synthesis and promotes arrest of the cell cycle at G1 through the regulation of p27Kip1 and cyclin A.	Li H *et al*, 2000; Arkenbout *et al*, 2003; Lin *et al*, 2004
Growth arrest and DNA damage inducible protein beta (GADD45B)	2.5	The Gadd45 gene family (Gadd45α, Gadd45β, and Gadd45γ, MyD118, and CR6) encodes small, evolutionarily conserved proteins that are highly homologous to each other, are highly acidic, and are localized primarily within the cell nucleus. Gadd45 genes are rapidly induced by a wide spectrum of genotoxic agents as well as by terminal differentiation and apoptotic cytokines. They play pivotal roles in negative growth control, either dependent or independent of p53. All three Gadd45 proteins specifically interact with and inhibit the kinase activity of the Cdk1/CyclinB1 complex. Inhibition of the kinase activity of the Cdk1/cyclinB1 complex by Gadd45β involves physical dissociation of the complex. GADD45β is a positive mediator of apoptosis induced by certain cytokines and oncogenes. It is an immediate early response gene for TGFβ and an effector of TGFβ-induced apoptosis. The proximal Gadd45β promoter is activated by TGFβ through the action of Smad2, Smad3, and Smad4. Ectopic expression of GADD45β in vitro is sufficient to activate p38 and to trigger apoptotic cell death.	Yoo *et al*, 2003; Vairapandi *et al,* 2002; Balliet *et al,* 2001; Zhang *et al*, 1999
Death receptor 6 (DR6; TNFRSF21)	4.5	Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands. They play an important role in apoptosis and can activate a caspase cascade within seconds of ligand binding. Induction of apoptosis via this mechanism is therefore very rapid. Death receptors belong to the tumour necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis. Apoptosis is induced by TNF and Fas ligand through their death domain containing receptors, TNFR1 and Fas. Several novel death receptors including DR3, DR4, and DR5 have been identified. A new death domain containing receptor in the TNFR family has been cloned recently and termed death receptor 6 (DR6). Like TNFR1, DR6 interacts with death domain containing adapter molecule TRADD. Overexpression of DR6 activates NFκB and JNK and induces apoptosis.	Pan *et al,* 1998

Genes upregulated by OPG and associated with growth inhibition and apoptosis in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
Programmed cell death 6 (ALG2)	1.5	Calcium is actively involved in apoptosis. ALG2, an apoptosis-linked Ca(2+)-binding protein and a member of the calpain small subunit subfamily of Ca2+-binding proteins, was originally discovered as a pro-apoptotic protein in a genetic screen. It is considered a link between the known apoptotic effect of calcium and the molecular death machinery due to its high affinity Ca2+-binding property.	Lee *et al,* 2005
PGE_2 receptor, EP3 subtype	5.6	Prostaglandin E(2) produces a broad range of physiological and pharmacological actions in diverse tissues through specific receptors on plasma membranes for maintenance of local homeostasis in the body. PGE receptors are divided into four subtypes, EP1, EP2, EP3, and EP4, which have been identified and cloned. These EP receptors are members of the G-protein coupled receptor family. Among these subtypes, the EP3 receptor is unique in its ability to couple to multiple G proteins. EP3 receptor signals are primarily involved in inhibition of adenylyl cyclase via G(i) activation, and in Ca(2+)-mobilization through G(beta)(gamma) from G(i) resulting in an increase in intracellular calcium concentration and predisposition to cell death.	Gerlo *et al*, 2004 Shoj *et al*, 2004
Interleukin-1 receptor-associated kinase 1 (IRAK1)	3.0	The transcription factor NFkB is a key regulator of immune and stress. Different receptors are known to use distinct combinations of intracellular proteins to initiate NFkB activation; however, some signaling pathways converge downstream into a common pathway that leads to activation of the IkB kinase (IKK) complex and the phosphorylation and degradation of IkB (inhibitor of NFkB). Although several serine/threonine kinases have been suggested to activate IKK, the NFkB-inducing kinase (NIK) has been identified as the upstream kinase. Interleukin (IL)-1 is a major cytokine responsible for the induction of a number of proteins associated with inflammation. Many of these responses are activated by the rapid activation of the transcription factor NFkB following signal transduction by IL-1β bound to the type I IL-1 receptor. Activation of the type I IL-1 receptor leads to recruitment of IL-1 receptor-associated kinase (IRAK) to the receptor complex via its association with the IL-1 receptor accessory protein and an adaptor protein MyD88. Upon recruitment, IRAK is highly phosphorylated and subsequently dissociates from the receptor complex to interact with tumor necrosis factor receptor-associated factor (TRAF) 6, which in turn is involved in NIK and NFkB activation and the induction of target genes that are able to orchestrate or suppress the immune response. Signals triggered include the two MAP kinases often associated with apoptotic responses, p38MAPK and JNK.	Li *et al*, 2001; Yanagisawa *et al*, 2003
Mitogen-activated protein kinase kinase 3 (MAP2K3; MKK3; MEK3)	1.6	The p38 mitogen-activated protein kinase (p38MAPK) is activated in response to various stimuli, including cellular stress, inflammatory cytokines and cell surface receptors. The activation of p38MAPK is predominantly mediated by the two upstream MAPK kinases, MKK3 and MKK6. The stimulation of p38MAPK is often associated with apoptotic responses.	Edlund *et al*, 2003

Genes upregulated by OPG and associated with growth inhibition and apoptosis in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
Regulator of G-protein signalling 4 (RGS4)	2.5	Inhibits signal transduction by increasing the GTPase activity of G-protein alpha subunits thereby driving them into their inactive GDP-bound form. Activity on Gα is inhibited by phosphorylation of the G-protein. RGS4 inhibits MAPK and VEGF signaling resulting in inhibition of cell proliferation, migration, and invasion.	Albig & Schiemann, 2005

Genes downregulated by OPG and associated with cytoskeletal and ECM proteins in normal human abdominal aortic VSMC

GENE	FOLD DECREASE	FUNCTION	REFERENCE
Arp23 complex 20 kDa subunit (ARC20)	5.0	The Arp2/3 protein complex has been implicated in the control of actin polymerisation in cells. The complex plays a central role in the regulated assembly of actin-based structures. It nucleates formation of new actin filaments in response to upstream signaling events and simultaneously cross-links them into orthogonal networks. The complex consists of actin related proteins (Arp) 2 and 3 and five other polypeptides, which form a stable unit in vivo. In isolation the Arp2/3 complex has a very low actin nucleating activity. However, the addition of recombinant VCA domain protein to a mixture of G-actin, the Arp 2/3 complex and ATP leads to a dramatic stimulation of actin polymerization.	Dayel *et al,* 2001
Chaperonin containing TCP1 (CCT), subunit 2 (beta)	3.0	CCT expression is upregulated during cell growth especially from G(1)/S transition to early S phase and appears to play important roles for cell growth by assisting in the folding of actin, tubulin and mitotic spindle formation.	Miklos *et al,* 1994; Yokota *et al,* 1999
Microtubule-associated protein 4 (MAP4)	2.6	Microtubules are dynamic polymers of tubulin that are involved in many diverse functions, including spindle formation, vesicle and organelle transport, and cell motility. MAP4 is a microtubule-associated protein ubiquitously expressed in proliferating cells. The level of MAP4 phosphorylation has been shown to increase at the G2/M transition. MAP4 interacts directly with cyclin B-Cdc2, the major kinase responsible for mitosis-specific phosphorylation of structural proteins.	Chang *et al,* 2001
Tubulin, beta polypeptide	8.0	Microtubules are composed of subunits of a globular cytoplasmic protein known as tubulin. Each subunit is made of two slightly different but closely related simpler units called alpha-tubulin and beta-tubulin that are bound very tightly together to form heterodimers. In co-operation with other components of the cytoskeleton, namely with actin microfilaments and intermediate filaments, microtubules are involved in several basic cellular processes including segregation of genetic material and the formation of spindle fibers during cell division (mitosis). Irreversible elimination of microtubules causes cell death.	
Biglycan	4.0	An extracellular matrix glycoprotein that enhances VSMC proliferation and migration via Cdk2/p27-dependent pathways. Downregulated in AAA compared with AOD.	Shimizu-Hirota *et al,* 2004; Armstrong *et al,* 2002

Genes downregulated by OPG and associated with cytoskeletal and ECM proteins in normal human abdominal aortic VSMC

GENE	FOLD DECREASE	FUNCTION	REFERENCE
Collagen, type VI, alpha-1	6.6	Formed via self-association and disulphide bonding, type VI non-fibrillar collagen is commonly observed aggregated and situated between fibrils of type I and type II collagen, the two predominant fibrillar collagens present in vascular interstitial matrix. Downregulated in AAA compared with AOD.	Armstrong *et al,* 2002
Collagen, type VIII, alpha 1	2.8	Non-fibrillar type VIII collagen form a 3-dimensional network with type IV collagen that serves as an anchoring substrate, with increased expression in VSMC following arterial injury potentially contributing to vascular remodeling via promotion of cell migration.	Sibinga *et al,* 1997
Elastin	2.8	Major structural protein of aorta. Elastic fibres are comprised of 2 distinct components, a more abundant amorphous component (elastin) and the microfibrillar component. Elastin is composed largely of glycine, proline, and other hydrophobic residues and contains multiple lysine-derived cross-links, such as desmosomes, which link the individual polypeptide chains into a rubber-like network.	
Elastin microfibril interface located protein (EMILIN)	1.8	EMILIN is detected in elastic fibres, located at the interface between the amorphous core and the surrounding microfibrils, and may play a role in VSMC elastogenesis. EMILIN-1 is adhesive for cells and binds to elastin and fibulin 5, thus may regulate elastogenesis and vascular cell maintenance by stabilizing molecular interactions between elastic fibre components and by endowing elastic fibres with specific cell adhesion properties.	Zanetti *et al,* 2004
Fibrillin 1	5.4	Fibrillin is the major constitutive element of extracellular microfibrils and has widespread distribution in both elastic and non-elastic connective tissue.	
Fibulin 5	1.4	Fibulin 5 is an elastin-binding protein that is thought to play a role in elastogenesis. Fibulin 5 gene induction in vitro is correlated with a rapid increase of tropoelastin accumulation suggesting that Fibulin-5 plays a role in the accumulation of elastic fibres within matrices.	Tsuruga *et al,* 2004
Lysyl oxidase (LOX)	1.6	LOX is secreted by activated VSMC and fibroblasts and catalyzes a key step in the cross-linking and stabilization of collagen and elastin in the vascular wall.	Kagen & Li, 2003
Plectin	11.8	Interlinks intermediate filaments with microtubules and microfilaments and anchors intermediate filaments to desmosomes or hemi-desmosomes. May be involved not only in the cross-linking and stabilization of cytoskeletal intermediate filaments network, but also in the regulation of their dynamics. The phenotypes of EBS-MD (epidermolysis bullosa simplex-muscular dystrophy)	Osmanagic-Myers & Wiche, 2004

Genes downregulated by OPG and associated with cytoskeletal and ECM proteins in normal human abdominal aortic VSMC

GENE	FOLD DECREASE	FUNCTION	REFERENCE
		patients and of plectin-deficient mice both lend strong support to the concept that plectin is a stabilizer of cells against mechanical stress due to its linker and scaffolding functions. However, plectin-/- cell cultures from plectin-deficient mice provide new insight into plectin functions that go beyond this concept. Actin stress fibres of mutant cells are insensitive to extracellular stimuli activating rho, rac, and cdc42 GTPase signalling cascades, which control actin stress fibre (rho), lamellipodia (rac), and filopodia formation (cdc42). Plectin deficiency substantially diminishes the ability of cultured astroglial cells to undergo dibutyryl-cAMP-induced differentiation, a process accompanied by dramatic morphological changes, involving actin-based mechanisms mediated by rho, strongly support the idea that plectin also plays an essential role as regulator of cellular processes linked to actin filament dynamics.	
Procollagen-proline, 2-oxoglutarate 4-dioxygenase (prolyl 4-hydroxylase)	3.0	The key enzyme in the biosynthesis of collagens. The stability of the triple helical structure, and hence functionality, requires hydroxylation of specific proline residues within precursor polypeptide collagen chains by the enzyme prolyl 4-hydroxylase. In the absence of proline hydroxylation, the essential triple-helical conformation of collagen is thermally unstable.	
Versican V	4.4	A major extracellular arterial proteoglycan, Versican is a member of the family of large aggregating proteoglycans (also including aggrecan, brevican, and neurocan). The protein shows wide tissue distribution, which includes fibrous, articular, and elastic cartilages, as well as nervous, epidermal, arterial, and loose connective tissues. Versican V1 has been shown to enhance cell proliferation, and also induce cell transformation and protect cells from apoptosis via downregulation of proapoptotic Bad. Human AAA is characterized by decreased Versican concentration and specific downregulation of Versican isoform V(0).	Sheng *et al,* 2005; Theocharis *et al,* 2001
Tissue-inhibitor of metalloproteinase (TIMP)-3	2.0	TIMP-3 is an inhibitor of many MMPs, including MMP-2 and MMP-9 and their proenzymes, the stromelysins (MMP-3), matrilysins (MMP-7), and macrophage metalloelastase (MMP-12). Unlike other TIMPs, TIMP-3 is strongly bound to the extracellular matrix, possibly reflecting its involvement in cellular regulation of MMP activity.	Yu *et al,* 2000

Genes upregulated by OPG and associated with extracellular matrix proteins in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
Calmodulin 2	1.5	Overexpression of Calmodulin causes Ca(2+)-dependent apoptosis. As a primary calcium signal transducer, calmodulin (CaM) responds to cytosolic calcium fluxes by binding to and regulating the activity of target CaM-binding proteins (CaMBPs). More than 30 CaM-binding proteins have been identified, including enzymes such as kinases, phosphatases, and nitric-oxide synthase, as well as receptors, ion channels, G-proteins, and transcription factors. It has been shownthat CaM directly binds to the cytoplasmic death domain (DD) of apoptosis-inducing receptor, Fas, an interaction that is Ca(2+)-dependent.	Ahn *et al,* 2004
Secreted protein, acidic, cysteine-rich (SPARC) (osteonectin)	1.5	SPARC is a multifunctional secreted protein that regulates cell-cell and cell-matrix interactions, leading to alterations in cell adhesion, motility, and proliferation. SPARC inhibits human arterial smooth muscle cell proliferation stimulated by PDGF or by adhesion to monomeric type I collagen. Binding studies with SPARC and SPARC peptides indicate specific and saturable interaction with smooth muscle cells that involves the C-terminal Ca2+-binding region of the protein. SPARC arrests monomeric collagen-supported smooth muscle cell proliferation in the late G1-phase of the cell cycle independent of Cdk inhibitor levels. Cyclin-dependent kinase-2 activity, p107 and cyclin A levels and retinoblastoma protein phosphorylation are markedly reduced in response to the addition of exogenous SPARC and/or peptides derived from specific domains of SPARC *in vitro*.	Motamed *et al,* 2002
Heparan sulfate proteoglycan 2 (perlecan)	6.2	Perlecan, a large multidomain heparan sulfate proteoglycan, is essential for the assembly and maintenance of a functional basement membrane. Growth inhibition by the extracellular basement membrane is driven by perlecan compared with chondroitin sulfate–rich proteoglycans and other basement membrane proteins. Decreased SMC-derived perlecan production is associated with increased SMC replication rates after vascular injury. However, accumulation of perlecan in the later stages of injury repair is associated with the attenuation of neointimal SMC proliferation. The proliferation of most nontransformed cells is mediated through the cooperation between extracellular matrix (ECM)–integrin receptor interactions and growth factor signaling pathways. Focal adhesion kinase (FAK) integrates integrin and growth factor receptor signaling pathways and transduces such signals to the downstream ERK1/2 pathway, making FAK important for cell growth. Focal adhesion kinase-related nonkinase (FRNK) is a critical regulator of FAK activity, its expression is highly restricted to vascular SMC lineages, and its in vivo expression patterns are similar to those reported for perlecan and inversely correlated to SMC growth. Perlecan actively suppresses SMC proliferation via the up-regulation of FRNK, which mediates the SMC-specific growth-inhibitory effects of perlecan via active inhibition of FAK-induced, ERK1/2-dependent cell cycle progression. PTEN, first discovered as a potent tumor suppressor, is a dual-specificity lipid and protein phosphatase and a negative regulator of PI3K- and FAK-mediated signaling. PTEN directly antagonizes growth factor receptor– and integrin-stimulated signaling, thus promoting cell	Walker *et al,* 2003; Garl *et al,* 2004

Genes upregulated by OPG and associated with extracellular matrix proteins in normal human abdominal aortic VSMC

GENE	FOLD INCREASE	FUNCTION	REFERENCE
		cycle arrest, decreased cell migration, and apoptosis. VSMC growth rates during vascular development and after vascular injury are associated with decreased perlecan expression and PTEN inactivation. It is suspected that perlecan:SMC interactions upregulate PTEN phosphatase activity mediating perlecan-induced suppression of SMC growth.	

Appendix 3

BUFFERS, GELS, AND SOLUTIONS

ACID ALCOHOL

0.25% HCl in 1:1 v/v distilled H_2O/Ethanol

500ml dH_2O
500ml Ethanol
2.5ml HCl

AGAROSE (RNA) GEL (1.2%)

0.6 g Agarose
50 ml 1x TAE buffer
1 µl Ethidium Bromide (Sigma; 10 mg/ml)

- Agarose dissolved in TAE buffer using microwave and allowed to touch-cool before addition of ethidium bromide and pouring into gel mould apparatus.

COOMASSIE STAIN

0.1% w/v coomassie blue (R-250)
40% v/v methanol
10% v/v glacial acetic acid
Distilled water

- Dissolve dye powder in methanol first

CULTURE MEDIUM

RPMI 1640 MODIFIED

Per 500ml bottle (JRH Biosciences):
10 ml penicillin-streptomycin-glutamine solution (Gibco)

Per 50 ml working aliquots:
1.25 ml HEPES (JRH Biosciences)
5 ml FBS (Gibco; 10% final concentration)

DMEM

Per 1000 ml Dulbecco's Modified Eagles Media (Gibco):
4.5 g glucose
8 mM glutamine
2.5 mg amphotericin
20 mM HEPES
5 mM sodium pyruvate
5 M mercaptoethanol
50 mg^{-1} gentamicin
240 g $NaHCO_3$
10,000 units penicillin/streptomycin

Per 50 ml working aliquots:
5 ml FBS (Gibco; 10% final concentration)

DE-STAIN SOULTION

10% v/v methanol
10% v/v glacial acetic acid
Distilled water

EPITOPE RETRIEVAL (CITRATE) BUFFER

Solution A:
1.0 M Citric Acid (100x)

Solution B:
1.0 M Sodium Citrate (100x)

Per 100 ml working solution:
1.0 ml solution A
4.0 ml solution B
95.0 ml PBS
pH 5.5

HEPES BINDING BUFFER

10 mM HEPES/NaOH (ph 7.4)
140 mM NaCl
2.5 mM $CaCl_2$

SCOTTS BLUING SOLUTION

Per 1000 ml distilled water:
3.5g sodium bicarbonate
20g magnesium sulphate

SODIUM DODECYL SULFATE (SDS)-POLYACRYLAMIDE GEL

Stacking Gel

875 µl distilled water
375 µl 0.5 M Tris-HCl (pH 6.8)
225 µl 30% w/v acrylamide/bis (37.5:1)
15 µl 10% w/v SDS
1.5 µl TEMED
12.5 µl 10% w/v ammonium persulphate

Resolving Gel

	9%	10%	12%	15%
30% w/v Acrylamide/bis (37.5:1)	1.125 ml	1.250 ml	1.500 ml	1.875 ml
1.5 M Tris-HCl (pH 8.8)	937.5 µl	937.5 µl	937.5 µl	937.5 µl
distilled water	1.625 ml	1.500 ml	1.250 ml	875 µl
10% w/v SDS	37.5 µl	37.5 µl	37.5 µl	37.5 µl
TEMED	1.5 µl	1.5 µl	1.5 µl	1.5 µl
10% w/v ammonium persulfate	15 µl	15 µl	15 µl	15 µl

- **Zymography gel**: 3.982 mg gelatin added per ml 1.5 M Tris-HCl buffer

SDS RUNNING BUFFER

Stock (5x, 2000 ml):
20 g SDS
60 g Tris base
288 g Glycine

Working (1x):
200 ml 5x SDS
800 ml distilled water

TISSUE PROTEIN EXTRACTION BUFFER

1x PBS
1% Triton X-100
0.1% SDS
10 mM cacodylic acid
0.2% NaN_3

TRIS-ACETATE EDTA (TAE) BUFFER

Stock (50x, 1000 ml):
242 g Tris base
57 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

Working (1x):
0.04 M Tris-acetate
0.001 M EDTA

TRANSFER BUFFER (Western Blot)

Per 1000 ml:
200 ml 1x SDS (running) buffer
200 ml Methanol
600 ml distilled water

WESTERN BLOT CRACKING BUFFER (4x)

10% w/v SDS
300 mM Tris-HCl (pH 6.8)
50% v/v glycerol
0.01% w/v bromophenol blue
100 mM dithiothreitol (DTT; added fresh prior to loading of sample)

- **DTT stock**: 1M in 0.01 M Na-acetate; aliquots stored at -20°C

ZYMOGRAPHY LOADING BUFFER (4x)

10% w/v SDS
300 mM Tris-HCl (pH 6.8)
50% v/v glycerol
0.01% w/v bromophenol blue

Appendix 4

ETHICS APPROVALS

JAMES COOK UNIVERSITY
Townsville QLD 4811 AUSTRALIA
Tina Langford, Ethics Administrator, Research Office. Ph: (07)4781 4342, Fax: (07)4781 5521, Tina.Langford@jcu.edu.au

ETHICS REVIEW COMMITTEE

(Human Ethics Sub-Committee)

APPROVAL FOR RESEARCH OR TEACHING INVOLVING HUMAN SUBJECTS

PRINCIPAL INVESTIGATOR	*Associate Professor Jonathan Golledge*
CO-INVESTIGATOR	*Dr M McCann*
CO-INVESTIGATOR	*Professor A Lam*
CO-INVESTIGATOR	*Dr F Quigley*
CO-INVESTIGATOR	*Dr M Diger*
CO-INVESTIGATOR	*Dr I Virdi*
SCHOOL	*Medicine*
PROJECT TITLE	***Role of Bone Forming Proteins in Abdominal Aortic Aneurysm***

DATE	*28 August 2002 – 31 August 2004*	**CATEGORY**	*3*

This project has been allocated Ethics Approval Number with the **following provisos and reservations**: **H1464**

1. All subsequent records and correspondence relating to this project must refer to this number.
2. The Principal Investigator **must advise** the responsible Monitor appointed by the Ethics Review Committee:
   - periodically of the progress of the project;
   - when the project is completed or if suspended or prematurely terminated for any reason;
   - if serious or adverse effects on participants occur; and if any
   - unforeseen events occur that might affect continued ethical acceptability of the project.
3. In compliance with the National Health and Medical Research Council (NHMRC) ***"National Statement on Ethical Conduct in Research Involving Humans"*** you must provide an annual report detailing security of records and compliance with conditions of approval. The report should very briefly summarise progress or in a final report detail the outcomes of your research.

NAME OF RESPONSIBLE MONITOR	*A/Professor Peter Leggat*
SCHOOL	*Public Health and Tropical Medicine*
APPROVED AT MEETING	***Date: 28 August 2002***
[forwarded by email without signature] Tina Langford Ethics Administrator	Date: 29 August 2002

Townsville Cairns Mackay

JAMES COOK UNIVERSITY
Townsville Qld 4811 Australia

Tina Langford, Ethics Administrator, Research Office, Ph: 07 4781 4342; Fax: 07 4781 5521

ETHICS REVIEW COMMITTEE
(Animal Ethics Sub-Committee)
APPROVAL FOR ANIMAL BASED RESEARCH OR TEACHING

PRINCIPAL INVESTIGATOR	**Dr Bradford Cullen**
CO-INVESTIGATOR	Frances Wood
SUPERVISOR	Associate Professor Jonathan Golledge
SCHOOL	Medicine
PROJECT TITLE	**PPAR-g ligands as a treatment for abdominal aneurysms**

APPROVAL DATE	8 Feb 2005	EXPIRY DATE	31 Dec 2008	CATEGORY	3

This project has been allocated Ethics Approval Number with the following conditions: **A 964**

1. All subsequent records and correspondence relating to this project must refer to this number.
2. That there is NO departure from the approved protocols unless prior approval has been sought from the Animal Ethics Sub-Committee.
3. The Principal Investigator is to advise the responsible Ethics Monitor appointed by the Ethics Review Committee:
   - periodically of the progress of the project;
   - when the project is completed, suspended or prematurely terminated for any reason.
4. In compliance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, and the *Queensland Animal Care and Protection Act 2001*, it is **MANDATORY** that you provide an annual report on the progress of your project. This report must also detail animal usage, and any unexpected event or serious adverse effect that may have occurred during the study.

NAME OF RESPONSIBLE MONITOR	Summers, Prof Phillip
EMAIL ADDRESS	phillip.summers@jcu.edu.au
ASSESSED AT MEETING	**Date:** 8 Feb 2005
APPROVED	**Date:** 8 Feb 2005
[forwarded by email without signature] Tina Langford Ethics Administrator **Research Office** Tina.Langford@jcu.edu.au	Date: 15 April 2005

C:\Ethics_Templates\ApprovalFormAnimal.doc

Attn: Corey Moran
07/4781 6986

THE UNIVERSITY OF WESTERN AUSTRALIA

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http://www.research.uwa.edu.au/human_ethics

Our Ref. RA/4/1/1224 4 May 2005

Associate Professor PE Norman
Surgery - M704
Fremantle Hospital

HUMAN RESEARCH ETHICS COMMITTEE

Project: Maintaining and developing the Health in Men Study

Please be advised that ethical approval of the above project has been granted by the Human Research Ethics Committee.

The Committee is bound by NHMRC Guidelines to monitor the progress of all approved projects until completion to ensure that they continue to conform to approved ethical standards.

The committee requires that all Chief Investigators report **immediately** anything that might affect or impact upon ethical approval of the project, including adverse events affecting subjects.

Approval should be sought in writing **in advance** for any amendments to the original application. You are also required as a condition of this approval to inform the Committee if for any reason the research project is discontinued before the expected date of completion.

An annual report form for completion will be sent to you twelve months from this date.

Please note that approval has been granted for a period of four years. Initial approval is for a period of one year, and, thereafter for future periods of one year at a time subject to the receipt of satisfactory annual reports. At the end of the four-year period you will be required to complete a new "*Application to Undertake Research Involving Human Subjects*" should you wish to continue with your research. However, in special circumstances, the Chair has the authority to extend the approval period in order to complete a project.

Please quote Project No RA/4/1/1224 all correspondence associated with this study.

Yours sincerely

KATE KIRK
Executive Officer
(Human Research Ethics Committee)

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