EXPRESSION OF THE PLASMINOGEN ACTIVATOR SYSTEM IN THE VASCULAR WALL

ABSTRACT

The plasminogen activator system is thought to be a principal regulator of extracellular proteolysis. Although much work has been done on investigating its role in thrombosis, less is known about its role within the vascular wall, and in particular, in vessels undergoing neointimal hyperplasia.

The aim of this project was to confirm and extend previous work on this subject by studying the expression of t-PA, u-PA, PAI-1 and uPAR in the walls of human arteries and veins as well as in rabbit iliac arteries that were normal or undergoing neointima formation.

A variety of techniques were used including quantitative reverse transcriptase polymerase chain reaction (RTPCR) assays (developed for PAI-1 and uPA), *in-situ* hybridisation (to localize tPA, uPA and PAI-1 mRNA), immunohistochemistry (to localize all four proteins) and immunoassays to quantify (tPA, uPA and PAI-1) proteins and (uPA and PAI-1) activities.

In normal human internal mammary arteries and saphenous veins; immunohistochemistry showed all four proteins to be associated with endothelial cells with little present on smooth muscle cells. Surgical distension of saphenous veins resulted in increased immunostaining to all four proteins probably due to extravasation, as specific mRNA levels were lower in the distended compared to undistended vessels. Human atheroma demonstrated intense immunostaining to all four proteins in keeping with the increased antigens on immunoassay, increased (uPA and PAI-1) mRNA on quantitative RTPCR, and greater signal (for tPA, uPA and PAI-1) with *in-situ* hybridisation compared to normal artery. Although PAI-1 antigen was increased in atheroma, PAI-1 activity was decreased. In human saphenous vein grafts or saphenous vein in organ culture, tPA, uPA and uPAR immunostaining was increased compared to normal undistended saphenous vein in keeping with increased signal for tPA and uPA mRNA on *in-situ* hybridisation. PAI-1 immunostaining and mRNA signal was prominent on the endothelial cells of venous hyperplastic vessels but unlike arterial atheroma, they were largely absent on the neointimal smooth muscle cells. Experimental angioplasty of rabbit iliac arteries resulted in increased immunostaining for all four proteins and an increase in uPA activity.

These results are compatible with and extend previous reports implicating the plasminogen activator system in the control of cell migration and matrix remodeling during normal and pathological vessel growth and repair, but also emphasize the complexity of this process.

EXPRESSION OF THE PLASMINOGEN ACTIVATOR SYSTEM IN THE VASCULAR WALL

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

Mahomed Yazeed Salame BSc (Hons), MB BS, MRCP (UK).

University of Leicester

August 1999

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

This thesis is dedicated to my parents for

their unfailing support throughout my education.

PUBLICATIONS RESULTING FROM THIS THESIS

1) M.Y. Salame, N.J. Samani, I A Masood and D.P. deBono. The expression of the plasminogen activator system in the human vascular wall. (accepted for publication; ***Atherosclerosis***)

2) M.Y.Salame, N.J Samani, R.S. More, D.P. deBono. The plasminogen activators and inhibitors in atherosclerotic human arteries. ***J Hypertension*** *1996;14 Suppl 1: S85,324.*

3) M.Y. Salame, W Martin, R. More, H Mahadeva, N. Samani, D.P. deBono. Urokinase-type plasminogen activator and its receptor are increased in the vessel wall following experimental angioplasty. ***Eur Heart J*** *1996;17 Suppl: 98, P555.*

4) M.Y. Salame, N.J. Samani, R.S. More and D.P.deBono. The effect of surgical preparation of saphenous vein coronary artery conduits on vessel wall urokinase-type plasminogen activator and plasminogen activator inhibitor-1. ***Clinical Science*** *1996;90 Suppl: 23P,84.*

5) M.Y. Salame, R.S. More, W. Martin, H. Mahadeva, D.P. de Bono. Time sequence of changes in vessel wall urokinase-type plasminogen activator following experimental angioplasty. ***Heart*** *1996;75: 57.*

6) M.Y. Salame, I. Masood , R.S. More, N.J. Samani, D.P.deBono. Plasminogen

activator inhibitor-1 and urokinase in the organ culture model of intimal hyperplasia. ***J Hypertension*** *1996;14, Suppl1: S70,316.*

7) M.Y. Salame, I Masood, R.S. More, D.P. deBono. Vessel-wall von Willebrand factor in intimal hyperplasia. ***Fibrinolysis*** *1996;10: 141*

8) M.Y. Salame, R. More, W. Martin, H. Mahadeva, N.J. Samani, D.P. deBono. Changes in vessel wall urokinase-type plasminogen activator and its receptor following experimental angioplasty. ***Clin Science*** *1996;91 Suppl: 9P,M28*

9) M.Y. Salame, P Kimber, C O'Callaghan, S Frantz, DP deBono, B Williams. Cyclical mechanical strain induces plasminogen activator inhibitor-1 expression in human vascular smooth muscle cells. ***Heart*** *1996; 75: 79.*

10) M.Y. Salame, P. Kimber, C.O'Callaghan, D.P. deBono, B. Williams. Relationship between the magnitude of cyclical mechanical strain and plasminogen activator inhibitor-1 mRNA levels in human vascular smooth muscle cells. ***Eur Heart Journal*** *1996;17 Suppl: 196, P1064.*

11) M.Y. Salame, N.J. Samani, R.S. More, A. Thiara, D.P. de Bono. The effect of surgical distension of saphenous vein grafts on the plasminogen activator system. ***J Hypertension*** *1996;14: Suppl 1;S33.*

12) M.Y. Salame, W. Martin, R.S. More, H. Mahadeva, D.P. deBono. Time

sequence of changes in vessel wall tissue plasminogen activator, urokinase-type plasminogen activator and its receptor following experimental angioplasty. ***Fibrinolysis*** *1996;10: 73*

13) M.Y. Salame, G. Saldanha, I. Masood, D. Hemming, D.P. deBono. An immunohistological characterisation of the saphenous vein organ culture model of intimal hyperplasia. ***J Hypertension*** *1996;14 Suppl1: S103,423.*

14) M.Y. Salame, P. Kimber, C.J. O'Callaghan, S. Frantz, B. Williams.Cyclical stretch increases plasminogen activator inhibitor-1 expression in human vascular smooth muscle cells. ***J Hypertension*** *1996;14 Suppl 1: S33,111*

15) M.Y. Salame, I Masood, R.S. More, M Crowther, D.P. deBono. The localisation of von Willebrand factor in arterial and venous models of intimal hyperplasia. ***Eur Heart Journal*** *1996;17, Suppl: 84,P499.*

ORAL PRESENTATIONS

1) **The Northern Cardiology Group 1996 1st prize.** " The plasminogen activator system in intimal hyperplasia."

2) **British Cardiac Society 1996:** "Time sequence of changes in vessel wall urokinase-type plasminogen activator following experimental angioplasty."

3) **British Cardiac Society 1996** "Cyclical mechanical strain induces plasminogen activator inhibitor-1 expression in human vascular smooth muscle cells."

4) **13th International Congress on Fibrinolysis and Thrombolysis 1996, Barcelona.** "Time sequence of changes in vessel wall tissue plasminogen activator, urokinase-type plasminogen activator and its receptor following experimental angioplasty."

5) **XIXth Congress of the European Society of Cardiology 1997, Stockholm.** "Changes in urokinase-type plasminogen activator and plasminogen activator inhibitor-1 activities within the vessel wall during the development of intimal hyperplasia"

PAPERS IN PREPARATION

1. M.Y. Salame, N.J Samani, I. Masood and D.P. deBono. The expression of the plasminogen activator system in the saphenous vein organ culture model of neointimal hyperplasia; comparison to saphenous vein graft stenosis.

2. M.Y. Salame, N.J. Samani, IA Masood, D.P.deBono. An immunohistological characterisation of the saphenous vein organ culture model of intimal hyperplasia.

3. M.Y. Salame, R.S. More, NJ Samani, IA Masood, H. Mahadeva and D.P. de Bono. Time sequence of changes in the plasminogen activator system in the vessel wall

following experimental angioplasty.

4. M.Y. Salame, N.J. Samani I A Masood and D.P. deBono. Review article: The plasminogen activator system in the blood vessel wall.

5. M.Y. Salame, N.J. Samani I A Masood and D.P. deBono. The effects of surgical distension and preparation of saphenous veins on the expression of the plasminogen activator system.

ACKNOWLEDGEMENTS

I would especially like to thank Professor David deBono for having me as a Cardiology Research Registrar in his department and for his continued guidance, enthusiastic encouragement and friendship throughout this research project.

My sincere thanks to Professor Nilesh Samani for providing valuable critical appraisals throughout the project and for his kindness in allowing me the use of his research facilities.

I would also like to thank the British Heart Foundation for awarding me a British Heart Foundation Research Fellowship, without which this project would not have been possible.

I am extremely grateful to the Cardiothoracic surgeons at Glenfield Hospital and the Vascular surgeons at The Leicester Royal Infirmary for allowing me to collect fresh samples of excess blood vessels removed as part of routine operations.

I would like to thank Dr Ranjit More for donating frozen samples of rabbit common iliac arteries which underwent balloon injury and their non-balloon controls as well as samples of each of these vessels having been set in paraffin blocks.

My sincere thanks to Mr Simon Frantz, Mr Harin Mahadeva, Dr David Lodwick, Dr Amrik Thiara, Mr Michael Kaiser and Mr Martin Kelly for their friendship and for being a constant source of valuable advice on so much of the molecular biological methodology.

My thanks also to Mr Imran Masood for the demonstrations on setting up the saphenous veins in organ cultures.

My thanks to Mr Hash Patel for demonstrating the methodology for the mRNA *in-situ* hybridisation.

I would like to thank the Department of Histology at the Leicester Royal Infirmary for kindly allowing me the use of their facilities for cutting and staining histological sections.

I would also like to thank the Department of Surgery for kindly allowing me to constantly use their photographic equipment to make photographs of histology sections and for the use of their cell culture incubator for the study on the saphenous vein organ culture experiments.

Last but certainly not least, I would sincerely like to thank my wife Helena for being so supportive and understanding even though it often felt that evenings and weekends were non-existent.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 ABSTRACT

Three important aspects of ischaemic heart disease are the development of atherosclerotic plaques, thrombus formation and the development of restenosis in coronary arteries after percutaneous transluminal coronary angioplasty. Smooth muscle cell proliferation and migration as well as extracellular matrix turnover are important for the development of intimal hyperplasia in both atherogenesis and restenosis. Fibrin deposition occurs both in the progressive development of the atherosclerotic lesion as well as in the acute thrombotic occlusion of coronary arteries that leads to unstable angina or myocardial infarction.

The plasminogen activator system is thought to be a principal regulator of extracellular proteolysis. Plasmin, a potent serine protease, is the main effector of the plasminogen activator system. It has a broad range of activities including endogenous fibrinolysis, (the process by which fibrin is removed from the endothelial lining of blood vessels), extracellular matrix digestion (a prerequisite for smooth muscle cell migration) and the activation of a number of cytokines known to be involved in intimal hyperplasia development and atherogenesis. It seems likely that a balance must exist between the plasminogen activators (tissue plasminogen activator {tPA}, and urokinase-type plasminogen activator {uPA}) and the plasminogen activator inhibitors (PAIs).

Within the vessel wall, a local imbalance of the regulation of the plasminogen activator system resulting in increased extracellular proteolysis, consequent activation of cytokines and metalloproteinases, could result in smooth muscle cell proliferation, increased matrix turnover and thus facilitation of smooth muscle cell migration - all a

recipe for intimal hyperplasia development. On the other hand, a decrease in endogenous fibrinolysis on the endothelial surface at atheromatous sites could result in local decreased clearance of fibrin with consequent acute thrombotic closure of the coronary vessel.

There has been a great deal of work done on investigating the role of components of the plasminogen activator system in thrombosis. Less is known about the presence of components of the plasminogen activator system in the vascular wall, and in particular, in atherosclerotic or restenotic lesions. The aim of this project was therefore to confirm and extend previous work on this subject by studying the expression of t-PA, u-PA, PAI-1 and urokinase type plasminogen activator receptor (uPAR) in the walls of normal blood vessels, atheromatous lesions and in blood vessels undergoing the development of intimal hyperplasia. Normal arteries (human internal mammary arteries and rabbit common iliac arteries), normal veins (human saphenous veins), atheromatous arteries (human coronary endarterectomy and atherectomy and human carotid endarterectomy), arteries undergoing experimental restenosis (balloon-injured rabbit common iliac arteries), veins that have undergone restenosis (human saphenous vein graft stenosis) and veins that have undergone experimental intimal hyperplasia development (human saphenous veins in organ culture) were used for this project.

Quantitative reverse transcriptase polymerase chain reaction assays were developed and used to measure the level of expression of PAI-1 and uPA in normal human saphenous veins, internal mammary arteries and in human atheromatous lesions. *In situ* mRNA hybridisation was used in an attempt to localise the expression of tPA, uPA and PAI-1

mRNA. Immunohistochemical techniques were used to localise cells associated with tPA, uPA, PAI-1 and uPAR antigens. Cell identification was facilitated using monoclonal antibodies against α-actin, Factor VIIIRAg, CD31, CD45, CD68, desmin and vimentin. Immunoassays and immuno-activity assays were used to measure the level of tPA, uPA and PAI-1 antigen and activity respectively within the vessel wall. The temporo-spatial changes in levels of components of the PAS during the development of intimal hyperplasia were examined.

In normal arteries and veins, tPA, uPA, PAI-1 and uPAR proteins are associated with endothelial cells with very little present on the smooth muscle cells or extracellular spaces. Surgical distension of saphenous veins with blood in saline resulted in increased immunostaining to all four proteins on smooth muscle cells and within the extracellular spaces. This is probably due to extravasation from the lumen as quantitative RTPCR assays found PAI-1 and uPA mRNA levels to be lower in these vessels. Human atheromatous tissue and rabbit iliac arteries that had undergone balloon injury demonstrated an increase in all four proteins compared to normal controls. This was in keeping with the increases in antigens on immunoassay and the increases in mRNA seen with quantitative RTPCR and in-situ hybridisation in atheromatous vessels. Although PAI-1 antigen was increased in atheromatous vessels, immuno(activity)assay showed that PAI-1 activity was in fact decreased. In the neointima of saphenous veins (graft stenosis or vein culture), there was an increase in tPA, uPA and uPAR immunostaining on immunohistology. In-situ hybridisation of saphenous graft stenosis showed increased signal for tPA and uPA mRNA in the neointimal smooth muscle cells compared to normal saphenous veins. PAI-1

immunostaining and mRNA signal was prominent on the endothelial cells of these vessels but largely absent from the neointimal smooth muscle cells.

1.2 PATHOGENESIS OF CORONARY ARTERY DISEASE: OVERVIEW

Coronary atherogenesis results in the gradual formation of atherosclerotic plaques within the walls of coronary arteries, which leads to compromise of the vessel lumen and hence coronary blood flow resulting in myocardial ischaemia or infarction. Apparent divergent hypotheses regarding atherogenesis (Rokitanski 1852, Virchow 1856, Benditt 1977) had been drawn together in a unifying concept 'response to injury' (Ross and Glomsett 1976). This proposed that atherogenesis involves endothelial cell injury (physical or chemical), consequent release of factors into the subendothelium which results in the proliferation and migration of smooth muscle cells through the internal elastic lamina, and the synthesis by the smooth muscle cells of extracellular matrix molecules e.g. collagen, elastin and proteoglycans. Associated with this is the intracellular and extracellular lipid accumulation and thrombus formation. More recently more emphasis has been put on chronic inflammation and endothelial dysfunction (rather than denudation) as the principal driving forces behind atherogenesis (Ross 1999). Possible causes of endothelial dysfunction includes elevated or modified LDL; genetic alterations; elevated plasma homocysteine levels; free radicals from cigarette smoking, hypertension and diabetes mellitus; infectious organisms such as herpes viruses or chlamydia pneumoniae; or a combination of these or other factors. The process is thought to begin in childhood and the earliest changes observed are areas of diffuse thickening of the musculoelastic intima. As the disease progresses through adulthood three main lesions are described, namely, the fatty streak,

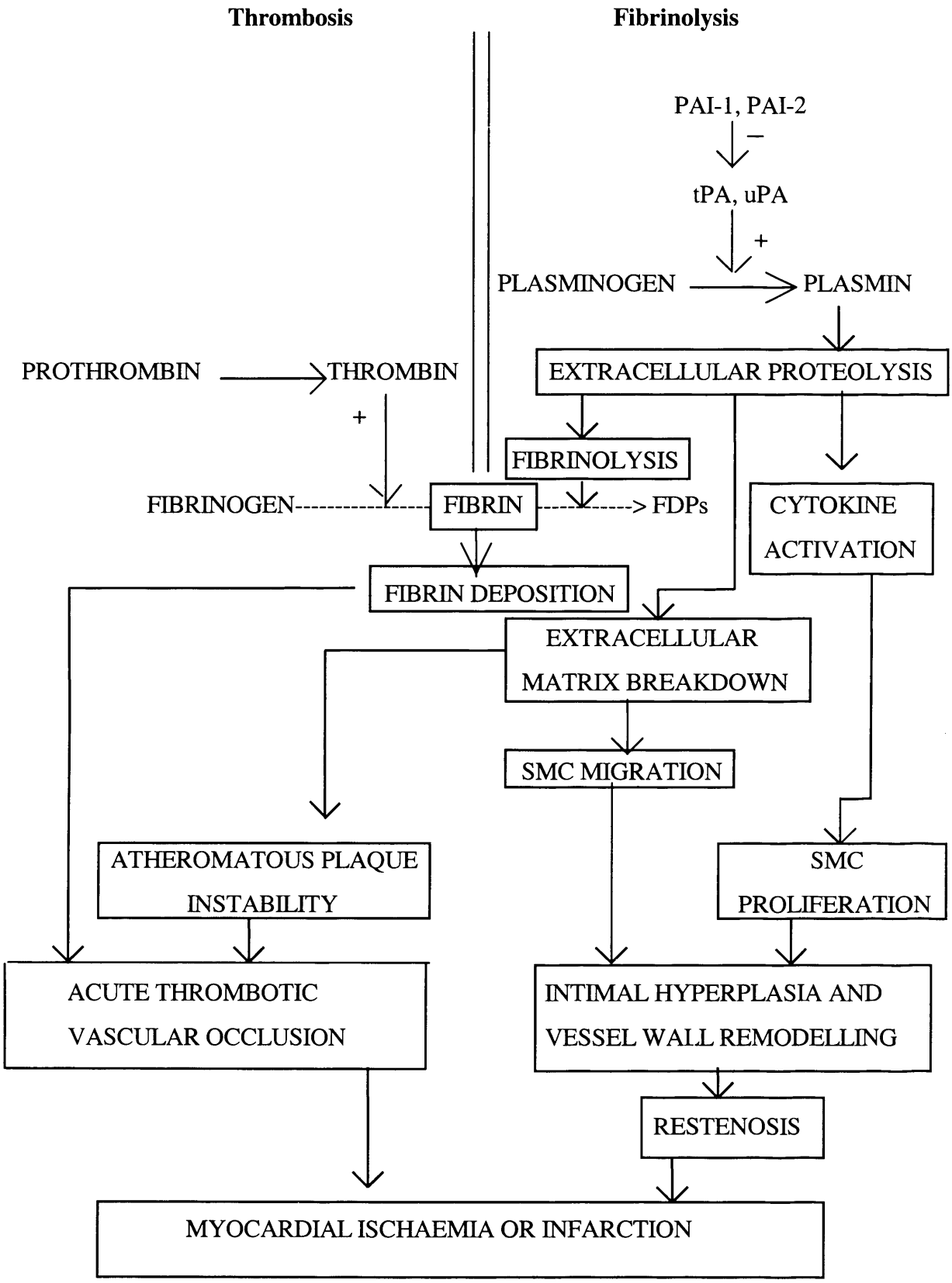
the fibrous plaque, and the complicated lesion. The essential histological feature of fatty streaks is the accumulation of small numbers of intimal smooth muscle cells surrounded by deposits of lipids. These fatty streaks are thought to be inflammatory in origin (Stary et al 1994). Lipid-laden macrophages and T lymphocytes are seen early in experimental models of atherosclerosis. It has been assumed that the fibrous plaques represent a progression from the fatty streaks. The fibrous plaque is composed of lipid-laden smooth muscle cells and macrophages with the deposition of collagen, elastin, and proteoglycans. Further development with calcification, cell necrosis, mural thrombosis, and haemorrhage results in the 'complicated lesion'. The fibrous plaque and to a greater extent the complicated lesion results in luminal narrowing and are associated with subsequent occlusion leading to unstable angina or myocardial infarction.

Restenosis results from the progressive re-narrowing of the vessel lumen that occurs typically weeks to months after percutaneous transluminal coronary angioplasty. Although the mechanisms responsible for this process are not completely understood (Mc Bride et al 1988, Forrester et al 1991), damage to the vascular endothelium triggers a complex series of events that results in transformation of a subset of smooth muscle cells from a resting 'contractile' state to a 'synthetic' state, which results in their proliferation and migration from media to the intima (Hassler 1970, Webster et al 1974). In the neointima, the newly arrived smooth muscle cells begin a second proliferative phase and finally enter a quiescent phase where they synthesise and secrete extracellular matrix. Concurrent with the above process, there is also vascular remodelling involving both the media and the adventitia in response to vessel injury

(Kakuta et al 1998).

Although the pathogenesis of atherosclerosis and restenosis are different, cellular proliferation and migration as well as extracellular matrix turnover are common important components of both. The plasminogen activator system may play an important role in coronary artery pathology by its effect on extracellular proteolysis thus affecting the proliferation and migration of cells as well as the turnover of the extracellular matrix. More specifically, the role of the plasminogen activator system in endogenous fibrinolysis may be important in coronary artery disease since fibrin deposition occurs both in the progressive development of the atherosclerotic lesion (Smith et al 1986, Schwartz et al 1988) as well as in the acute thrombotic occlusion of coronary arteries that leads to myocardial infarction (Davies et al 1984).

1.3 OVERVIEW OF THE PUTATIVE ROLE OF THE PLASMINOGEN ACTIVATOR SYSTEM IN THE VESSEL WALL



1.4 BACKGROUND

1.4.1 Proteolysis

A number of classes of proteolytic enzymes exist. These include the serine proteases (e.g. plasmin, trypsin and plasma kallikrein) and cysteine proteases (e.g. cathepsins B and L). Extracellular proteolysis of structural proteins may result in extracellular matrix breakdown, whilst its effect on regulatory proteins, could lead to activation or modulation. These proteolytic enzymes interact to activate and modulate each other, e.g. receptor-bound pro-urokinase can be activated by both cathepsin L (Goretzki et al 1992) and cathepsin B (Kobayashi et al 1993).

1.4.2 Plasmin

Plasmin, a broad-spectrum serine proteinase, is capable of readily degrading a host of extracellular proteins. The inactive proenzyme, plasminogen, is abundant in the vasculature and in most body fluids, and potentially serves as an almost limitless supply of proteolytic capacity with a phenomenal destructive potential. The transition of plasminogen to plasmin involves peptide bond cleavage and hence is irreversible.

The activity of plasmin and the activating enzymes is maximised by binding to the substrate (plasminogen and t-PA) or to the cell surface (t-PA, u-PA and plasminogen) (Vaheri et al 1990, Hajjar et al 1991). This arrangement serves to concentrate the reactants at the site of cleavage and under certain circumstances to protect against a variety of inhibitors including α2-antiplasmin, α2-macroglobulin, plasminogen activator inhibitors-1 and 2 (PAI-1 and PAI-2) (Menashi et al 1993, Collen et al 1976) The plasminogen activator system may be important, not only for its role in the

prevention or removal of fibrin from the vascular tree, but also for its role in the tissue repair (Astrup 1968), malignant transformation (Reich 1975), macrophage function (Reich 1975), ovulation and embryo implantation (Strickland & Beers 1976, Ohlsson et al 1991). Plasmin, is thought to be the effector of a host of proteolytic reactions e.g. activation of a number of cytokines, breakdown of the extracellular matrix (Schmitt et al 1992) and basement membrane glycoproteins either directly or through the activation of metalloproteinases e.g. collagenases, gelatinases and stromelysin (Santibanez et al 1993) and as such, the plasminogen activator system may be important in cellular growth, proliferation and migration, ubiquitous processes necessary in foetal development, ageing, wound healing, inflammation and neoplasia.

1.4.3 The plasminogen activators: tPA and uPA

Plasminogen-dependent proteolysis is initiated by secretion of one or both activating enzymes at the local tissue level. Human plasminogen activators also belong to the class of serine proteinases. They have been classified into two types: Tissue type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). These two proteins can be distinguished by differences in immunological cross reactivity and tissue localisation, with tPA, unlike uPA, having a strong affinity for fibrin (Rijken et al 1981, Wallen et al 1981, Thorson et al 1972).

1.4.3 a) Tissue plasminogen activator (tPA) within the blood vessel wall

Recombinant exogenous tPA is well known for its proven fibrinolytic role in the treatment of acute myocardial infarction and other acute thrombotic events (ASSET 1988, Bell et al 1976). However the walls of blood vessels are associated with an

endogenous fibrinolytic activity. This association has been recognised since the pioneering work of Todd et al 1959, and Astrup et al 1956. Almost two decades later Loskutoff and Eddington showed that the fibrinolytic activity of the endothelial cells from rabbit vena cava grown in culture depended upon the presence of plasminogen in the culture thus providing evidence that plasminogen activators are effectors of the fibrinolytic activity of these endothelial cells.

tPA, (MW 68,000), is the principal endogenous activator of plasminogen in the blood. It is derived predominantly by endothelial cells and present in the plasma at a concentration of approximately 70 pM. In vivo a variety of stimuli including catecholamines, thrombin and vasopressin induce rapid release of tPA from endothelium by a mechanism independent of protein synthesis (van Hinsberg 1988, Tranquille & Emeis 1989). It is a 527-residue serine protease of the plasminogen superfamily. It is synthesised as a single chain molecule and is cleaved to form a two-chain form that has minor differences in substrate affinity (Collen et al 1996). Cleavage by plasmin produces two-chain tPA consisting of a heavy chain (A chain) and a light chain (B chain). The B-chain contains the catalytically active site (Rijken & Groeneveld 1986), whilst the A-chain contains an epidermal growth factor domain, a finger domain and 2 kringle domains (Gaffney 1994). It is glycosylated at a number of sites. Cheng et al 1995, demonstrated that tPA binds to endothelial cells via a ligand on its B chain. This has the effect of both increasing its activity by approximately 100 fold and protecting it against inhibition by PAI-1. More recently Ellis et al 1997, provided evidence for a tPA receptor on vascular smooth muscle cells. Binding to fibrin is mediated by the finger and second kringle (Pannekoek et al 1988, de Munk et al 1989,

Higgins & Bennett 1990) and at the surface of polymerised fibrin it cleaves the zymogen plasminogen and hence initiate fibrinolysis.

A correlation between low endogenous plasma tPA activity and increased coronary risk has been shown by numerous epidemiological studies (Olofson et al 1989, Oseroff et al 1989, ECAT Angina study group 1993). However the majority of patients with coronary heart disease have normal fibrinolytic profiles (Collen 1988). This may be an indication that plasma measurements, whilst they give us a clue, may not be a sensitive enough marker to predict what is occurring locally within atherosclerotic lesions.

The role of tPA in the vessel wall is not fully known. It seems likely that its role on the endothelial lining is to prevent fibrin accumulation and thus help to prevent thrombotic occlusion. Within the deeper layers of the vessel wall, the role of tPA is less clear. Experimental studies in vitro have shown that tPA is a potent mitogen for human aortic smooth muscle cells (Herbert et al 1994) and may thus contribute to intimal smooth muscle cell proliferation in intimal hyperplasia. In keeping with its mitogenic role, De Petro et al 1994, showed tPA to be a growth factor of human fibroblasts. A role for tPA in angiogenesis has been suggested by the fact that it is increased in human endothelial cells during the formation of tubular capillary networks in vitro and that the capillary network formation is inhibited by anti tPA antibody (Ito et al 1995).

The immunolocalization of tPA antigen was assessed in vessels of various sizes from baboons and it was found that the tPA was mostly distributed in smaller sized vessels excluding the capillaries (Levin et al 1994). Experimental studies have shown that in

normal rat carotid arteries only low levels of t-PA was detected (Clowes et al 1990) and that its level increased after mechanical vascular injury suggesting that these molecules may be involved in post-angioplasty restenosis. tPA antigen and mRNA were found to be increased in atheromatous arteries compared to normal arteries (Lupu et al 1995). Whereas in normal arteries, the tPA antigen and mRNA were found on endothelial cells and medial smooth muscle cells, in atherosclerotic lesions, they were found to be increased in the intimal smooth muscle cells and macrophage-derived foam cells. tPA antigen was also present in the acellular extracellular spaces in atheromatous lesions co-localized with fibrin and in neomicrovessels within the plaque suggesting a role of tPA in plaque angiogenesis. In normal arteries, PA activity was largely present in the vasa vasorum and this was due to tPA rather than uPA (Lupu et al 1995). In the vessel wall of atherosclerotic abdominal aortic aneurysm, tPA and uPA mRNA were found in areas of inflammatory infiltrates in macrophage-like cells (Schneiderman et al 1995). The cellular distribution of tPA and uPA transcripts extended far beyond that of PAI-1 mRNA and suggests that the increased fibrinolytic capacity may promote proteolytic degradation of the aortic wall leading to physical weakening and active expansion of the aneurysm. Padro et al 1995, quantified tPA protein and activity in protein extracts from different layers of human aorta in relation to the presence and severity of atherosclerotic disease. They found that tPA protein was increased in necrotic areas of plaques, where tPA was mainly complexed to PAI-1. In the media, tPA antigen was higher in lesional segments and closely associated with smooth muscle cells. tPA activity however, was not detected in intimal nor medial extracts possibly due to the large amount of concomitant PAI-1 found.

1.4.3 b) Urokinase-type plasminogen activator (uPA) within the blood vessel wall

Sahli (1885) was the first investigator who discovered that urine contained enzymes capable of digesting fibrin and the name urokinase was coined by Sobel in 1952. uPA was originally isolated from human urine (White et al 1966). This protein was shown to be different from the major plasminogen activator found in human plasma (Kucinski et al 1968). In 1979 it became clear that the plasminogen activator activity in the circulating blood was due to tPA and not uPA (Astedt 1979, Kok 1979). The complete cDNA sequence consists of 2,304 base pairs and codes for the protein of 431 amino acids (Verde et al 1984). In the plasma it is at low concentration (approx. 150 pM). uPA is initially synthesised as a single-chain molecule (MW 54,000), scu-PA or pro-uPA (Wun 1982) which upon secretion binds to its glycosylphosphatidyl-inositol-anchored specific cell receptor uPAR. Pro-uPA undergoes extracellular activation by cleavage (Gunzler et al 1982, Skriver et al 1982) to a two chain form tcu-PA. This uPA cleavage can be mediated by plasmin (Longstaff et al, 1992), kallkrein (Ichinose et al 1986) and other proteases e.g. cathepsins and is accelerated by it binding to uPAR. Upon further limited proteolysis, 54 kD u-PA is converted to a low molecular weight form of uPA of 32 kD (Guenzler 1982). uPAR-bound uPA is susceptible to inhibition by PAI-1 and PAI-2.

uPA shows a similar domain structure to tPA. The N-terminal half contains the receptor binding site and 1 kringle (Appella et al 1987). The c-terminal portion of the protein consists of the serine protease domain. The kringle domain of uPA does not show a high affinity for fibrin and thus uPA in isolation is not clot specific in its activation of plasmin.

The role of uPA within the vessel wall is not fully known. uPA proteolytically cleaves fibronectin making it further open to attack by the enzymes plasmin and cathepsin. This predigestion of fibronectin may be an initial event in the local degradation of the extracellular matrix (Gold et al 1992). Analysis of atherosclerotic aorta in genetically altered mice indicated that deficiency of u-PA protected against media destruction and aneurysm formation, probably by means of reduced plasmin-dependent activation of pro-MMPs (Carmeliet et al 1997). uPA antigen and mRNA were found to be increased in atheromatous lesions compared to normal arteries (Lupu et al 1995). A particularly high uPA expression was noted on macrophages localised on the rims of the necrotic core. In advanced lesions, uPA activity was found to be increased. Strong uPA immunostaining was also present in neomicrovessels. Padro et al, 1995, found uPA to be increased in atherosclerotic aortic intima and co-localised with neointimal smooth muscle cells and tissue-infiltrating macrophages. However, uPA activity could not be detected in intimal or medial extracts; PAI-1 concentrations were found to be high in these areas of the vessel wall.

In an in vitro model of neovessel formation, namely culturing human umbilical vein endothelial cells on basement membrane preparation matrigel, which results in the formation of tubular endothelial structures, uPA is increased (Schnaper et al 1995). Inhibitors of plasminogen activators decreased the extent of the endothelial network. Over-expression of the uPA gene in transfected endothelial cells enhanced the invasive capacity of endothelial cells in a matrigel chemoinvasion assay (Gualandris et al 1997). Using a peptide comprising the amino-terminal domain of uPA that binds to the uPA receptor (uPAR) but lacking proteolytic activity, enhanced tube formation, showing

that uPAR occupancy may also be important for endothelial tubular formation and thus angiogenesis. The putative role of uPA in angiogenesis is strengthened by the observation that vascular endothelial growth factor (VEGF), a potent angiogenic factor and endothelial cell-specific mitogen, stimulates uPA activity and uPAR expression in vascular endothelial cells (Mandriota et al 1995). Using the technique of *in-situ* zymography uPA activity was found to be increased in sprouting capillaries, again suggesting a role in neoangiogenesis (deVries et al 1995). Clowes et al 1990 showed that in the normal rat carotid artery low levels of uPA are seen. uPA mRNA increases steadily after injury to reach a maximum at around 1 week whilst uPA activity in extracts of balloon-injured vessels reaches a maximum 16-24 hours after injury.

In keeping with the putative role of uPA in cellular migration, uPA activity was found to be significantly higher in malignant astrocytomas, especially in glioblastomas, than in normal brain tissue or low-grade gliomas (Yamamoto et al 1994). Antigen levels of u-PA and uPAR at the tumour-host interface correlated to tumour aggressiveness (Buo et al 1995). This may indicate that uPA in more aggressive tumours exceeds the inhibitory capacity represented by PAIs, resulting in enhanced migration of cells. However, others have found that a critical balance between uPA and PAI-1 is necessary for optimal cellular invasiveness in vitro (Liu et al 1995).

More recently, gene knockout mice studies have been used to investigate the relative importance of the plasminogen activators tPA and uPA in neointimal formation and re-endothelialization (Carmeliet et al (1997,[(1)]). They found that mechanical or electrical arterial injury resulted in less neointimal cell accumulation in uPA-/- and in tPA-/- /

uPA-/- mice compared to wild type or tPA-/- mice. Topographic analysis of vascular wound healing in electrically-injured wild-type and tPA-/- arteries revealed a similar degree of migration of smooth muscle cells from the non-injured borders into the necrotic centre. In contrast, uPA -/- and tPA-/- / uPA-/- arteries smooth muscle cells accumulated at the uninjured borders but failed to migrate into the necrotic centre. Proliferation of smooth muscle cells was not affected by uPA or tPA deficiency in this model.

1.4.4 The Plasminogen Activator Inhibitors

1.4.4 a) Plasminogen activator inhibitor-1 (PAI-1) within the blood vessel wall

PAI-1 (MW 50,000), a serine protease inhibitor with rapid and specific inhibition of both t-PA and u-PA, may be a prime regulator of plasminogen activation in vivo (Sprengers and Kluft 1987) in spite of its low concentration in the plasma (approx. 0.1 nM). PAI-1 cDNA has been cloned and sequenced (Ginsburg et al 1986) and the gene structure has been investigated (Loskutoff et al 1987).

In plasma and the extracellular matrix PAI-1 is associated with the adhesive glycoprotein vitronectin which may stabilise the active confirmation of PAI-1 (Lawrence et al 1994) and regulate the clearance of PAI-1-tPA complexes from the circulation by hepatic cells (Owensby et al 1991). It is synthesised by a number of cell types in culture including endothelial cells (Phillips et al 1984), hepatocytes (Healey et al 1994), platelets (Booth et al 1985) and vascular smooth muscle cells (Laug et al 1985). The cellular origin of plasma PAI-1 is not yet known although endothelial cells is strongly suspected. PAI-1 undergoes conformational change to form an inactive

'latent' form. PAI-1 reacts with both t-PA and u-PA with a second order rate constant of 10^6-10^7 mol/l/sec. The PA/PAI-1 complex has no activity (Kruithof et al 1983). These observations suggest that the expression of PAI-1 *in-vivo* must be precisely regulated. The inappropriate over-expression of this potent inhibitor, will suppress the normal fibrinolytic system, creating a local prothrombotic state with resultant pathological fibrin deposition whilst a deficiency of PAI-1 will result in bleeding tendencies (Shleef et al 1989, Dieval et al 1991).

A number of epidemiological studies have demonstrated high plasma PAI-1 levels in patients with coronary artery disease, particularly in those who presented with myocardial infarction, or unstable angina (Johnson et al 1984, Paramo et al 1985, Anzar et al 1988). Longitudinal clinical studies support the hypothesis that increased plasma PAI-1 levels may be important in the predisposition to myocardial infarction (Prins 1991). A direct relationship between deficient fibrinolysis, due to high plasma PAI-1 activity levels, and recurrent myocardial infarction was demonstrated in young men who had survived their first myocardial infarction 3 years previously (Hamsten et al 1987) and in a group of unselected patients (Munkvad et al 1990). However in an older group of patients followed over five years, PAI activity was not predicted for recurrent infarction (Jansson et al 1991).

Evidence of an etiologic role of PAI-1 in myocardial infarction was provided by a study showing the prevalence of the 4G allele (one of the alleles of the recently described 4G/5G polymorphism of the PAI-1 promoter) to be significantly higher in patients with myocardial infarction before the age of 45 than in population based

controls (Eriksson et al 1995). Both alleles bind a transcriptional activator, whereas the 5G allele also binds to a repressor protein to an overlapping binding site. In the absence of bound repressor (i.e. in 4G/4G alleles) the basal level of PAI-1 transcription is increased. However the data is conflicting as two large clinical trials failed to show an asociation between the 4G/5G polymorphism and arterial thrombosis (Ridker et al 1997, Ye et al 1995).

Elevation in plasma PAI-1 levels has also been noted in metabolic disorders associated with an increased risk of atherosclerosis. Significant correlation have been observed between PAI-1 levels and obesity (Vague et al 1986), non-insulin dependent diabetes mellitus (Auwerx et al 1988), hyperinsulinaemia (Juhan-Vague 1987), and hypertriglyceridaemia (Hamsten et al 1985). These observations imply that elevation in PAI-1 activity, in association with disorders of carbohydrate or lipid metabolism, may contribute to the development of atherosclerosis and coronary artery disease and thus myocardial infarction (Hamsten et al 1985). PAI-1 levels have been shown to be increased in hypertensive patients with compared to normotensive controls (Gleerup et al 1995, Keskin et al 1994)

Experimental studies have supported the importance of PAI-1 in thrombosis. Increased plasma PAI-1 levels decreases endogenous and exogenous fibrinolysis and increases the extension of thrombosis (Krishnamurti et al 1987, Vaughn et al 1992), whereas quenching of PAI-1 activity with a specific antibody induces an opposite effect (Levi et al 1992). Transgenic mice carrying human PAI-1 cDNA under the control of the mouse promoter are born with high levels of PAI-1 in their blood and develop

thrombotic problems in their extremities (Erikson et al 1990). In experimental animals subjected to thrombosis, PAI-1 mRNA was increased in endothelial cells, smooth muscle cells and in macrophages which were surrounding the lesion (Fujii et al 1992, Sawa et al 1992). Much more marked expression of PAI-1 mRNA was seen when the arterial injury was accompanied by diet induced hypercholesterolaemia (Sawa et al 1993). The coincident expression of PAI-1 and tissue factor (Wilcox et al 1989) as well as thrombin receptor (Nelkin et al 1992) within the plaque may have an important role in the regulation of extra cellular proteolysis during cellular growth and migration (Reilly et al 1991). Increased PAI-1 mRNA has been found in the vessel wall of human atherosclerotic abdominal aortic aneurysm wall (Schneiderman et al 1995). The analysis of adjacent tissue sections from the same patients by in situ hybridisation demonstrated an abundance of PAI-1 mRNA-positive cells within the intima of atherosclerotic arteries, principally around the base of the plaque. PAI-1 mRNA was also detected in cells scattered within the necrotic material and in the endothelial lining of adventitial vessels. PAI-1 frequently co-localised with uPA and tPA mRNA expressing cells. In contrast, in morphologically normal areas of aorta, PAI-1 mRNA was primarily found in endothelial cells lining the lumen. Lupu et al 1993, found increased PAI-1 expression in smooth muscle cells within the fibrous cap of atheromatous lesions compared to adjacent media or in normal arterial tissue. They also found PAI-1 mRNA in macrophages at the periphery of the necrotic core. In another study, PAI-1 mRNA and protein was localised to smooth muscle cells and macrophages in human coronary atherectomy specimens (Ludgren et al 1994). Increased vessel wall expression of PAI-1 mRNA was observed in balloon injured rabbit carotid arteries within 3 hours of injury whereas in uninjured normal vessels

PAI-1 mRNA was not detectable by northern blotting or in-situ hybridisation in endothelial and smooth muscle cells (Sawa et al 1994). In addition to raised PAI-1 mRNA, Shireman PK et al 1996, found atherosclerotic aortae to have increased PAI-1 protein as assessed by tissue explant supernatants. Similarly, Robbie et al 1996, also demonstrated increased PAI-1 protein in atheromatous lesions by tissue explant supernatants as well as immunohistochemistry.

The role of PAI-1 in neointimal formation was investigated in transluminal mechanical and perivascular electric injury in PAI-1 deficient (PAI-1 -/-) and wild-type mice (Carmeliet et al, 1997 [2]). These workers found that injury to PAI-1 -/- arteries resulted in increased neointimal formation with smooth muscle cells migrating more rapidly into the necrotic centre of the arterial wound compared to the wild-type arteries. There was no difference in re-endothelialization between the two types of mice. Adenoviral PAI-1 gene transfer into the PAI-1 deficient mice reduced the neointimal formation.

1.4.4 b) Plasminogen activator inhibitor-2 (PAI-2)

PAI-2 was originally isolated from human placenta and is abundant in the plasma in the third trimester of pregnancy (Kruithoff et al 1987). PAI-2 is synthesised by a number of cell types including monocytes-macrophages (Saksela et al 1985, Chapman et al 1985) and endothelial cells (Astred et al 1985). In contrast to PAI-1 which is a more effective inhibitor of t-PA, PAI-2 is a more effective inhibitor of u-PA than t-PA (Zoellner et al 1993). It is not known whether the production of PAI-2 from endothelial cells is a culture artefact or whether endothelial cells expresses PAI-2 in vivo. Except for monocytes/macrophages most cellular PAI-2 is in the non-glycosylated non-

secretory form (Wohlwend et al 1987). An intracellular role for PAI-2 has however not been clearly established but it is possible that cell associated PAI-2 play a role in inhibiting fibrinolysis following cell damage (Medcalf et al 1988). Bacterial endotoxins LPS are strong inducers of PAI-2 gene expression in human peripheral blood monocytes (Schwartz BS et al 1992). This suggest that PAI-2 regulation contributes to the modulation of extracellular proteolysis by mononuclear phagocytes in inflammatory processes resulting in fibrin deposition which accompanies the second phase of inflammatory reactions. The potential role of PAI-2 in inflammatory processes is strengthened by the observation that TNFα is a potent inducer of PAI-2 transcription in mononuclear phagocytes (Gyetko et al 1991) and fibroblasts (Kumar et al 1991). Since atherogenesis is now regarded as an inflammatory response to chronic vascular injury (Ross et al 1992) with deposition of fibrin, the hypothesis that PAI-2 may play a key role in this process needs to be investigated. PAI-2 may play a role in the dynamics of connective tissue remodelling since it could inactivate one of the serine proteases implicated in TNF-mediated cytolysis (Kumar et al 1991).

1.4.5 Receptors of the Plasminogen Activator System

1.4.5 a) Urokinase-type plasminogen activator receptor (uPAR)

A specific cellular receptor for urokinase (uPAR) was identified on blood monocytes and on the transformed U937 cell line (Vassalli et al 1985) and has been subsequently found on a number of cell lines in culture (Vassalli et al 1992; Blasi et al 1987). It is a glycosylphosphatidyl-inositol-linked glycoprotein, localised in micro-invaginations of the plasma membrane (Stahl et al 1995). The protein is composed of three homologous domains, the NH2- terminal of which is involved in the high affinity binding to the

epidermal growth factor-like (amino-terminal) domain of uPA.

Kinetic experiments indicate that the process of plasminogen activation occurs more rapidly when pro-uPA is bound to the cell-surface receptor and plasminogen (Conese et al 1995). Indeed uPAR have been found associated with plasminogen receptors (Plow et al 1986). The cell-surface uPAR may thus serve as a mean of localising the cell surface proteolytic activity in both time and space. Once bound, uPA retains its enzyme activity, is not internalised but is susceptible to inhibition by PAI-1 and PAI-2 (Reinartz et al 1994). uPAR is linked with the cell membrane via a glycosyl-phosphatidylinositol anchor (Plough et al 1991). uPAR therefore serves not only as an anchor for uPA but it also participates in a signal transduction pathway resulting in the phosphorylation of intracellular regulatory proteins (Busso et al 1994). In addition, uPAR is a major vitronectin-binding protein on endothelial cells (Kanse et al, 1996).

The distribution of uPAR on the cell surface was studied by immunofluoresence and immunoelectron microscopy, (Moyohanen et al 1993). Using two monoclonal antibodies, one able to bind to uPAR whether it was occupied or unoccupied, the other able to bind only to uPAR when unoccupied, it was shown that unoccupied uPAR was relatively mobile whilst uPAR occupied by uPA was localised to cell contact sites.

uPAR also exists as a ligand-free soluble form (Pedersen et al 1993). It lacks the lipid moiety of the glycolipid anchor present in the cellular-bound uPARs. The function of the soluble form of uPAR is not known. Binding of scuPA to its soluble uPAR results in the enhancement of the enzymatic activity and it may play a role in removing uPA

from the extracellular spaces (Higazi et al 1995).

A number of studies have suggested that uPAR is important for cell migration. Gyetko et al 1994 showed that uPAR is required for human monocyte chemotaxis in vitro; exposing adherent monocytes to a chemotactic gradient caused plasma membrane uPAR to localise strongly to the leading edge of cell migration. Treating these cells with anti-uPAR mAb or blocking uPAR expression with an antisense oligonucleotide ablated chemotaxis whilst blocking uPA expression with an antisense oligonucleotide had only a minimal effect. However, in an experimental and spontaneous metastasis model, an antibody to uPA inhibited invasion of cells through the matrigel or metastasis respectively (Kobayashi et al 1994). Similarly, T lymphocyte migration was also inhibited with anti-catalytic uPA mAb in a fibrin matrix in vitro (Kramer et al 1994). Binding of uPA to its receptor promoted migration of human melanoma cells in matrigel (Stahl et al 1994). A monoclonal antibody against uPAR (Mab 3936) inhibited migration through the matrigel up to 33% while a reduction of the uPA catalytic activity by PAI-2 resulted in a 46% inhibition of migration. Occupation of uPAR by enzymatically inactive uPA fragments reduced migration of cells through basement membranes in vitro (Kobayashi et al 1993). In another study uPA mediated bFGF-induced endothelial cell migration in vitro independent of its proteolytic activity but dependent on the occupancy of the uPA receptor (Odekon et al 1992).

uPAR was found to be increased in atheromatous lesions compared to normal arteries (Noda-Heiny et al 1995); uPAR immunostaining being absent in normal arteries whereas it was intensely positive in macrophages and neointimal smooth muscle cells.

However, uPAR mRNA expression was not significantly increased in migrating smooth muscle cells thus implying that altered post-transcriptional regulation may be contributing to the increased uPAR protein. The same authors found that antibodies to uPAR delayed the migration of cultured vascular smooth muscle cells.

More recently, gene knockout studies have been used to delineate the role of uPAR in neointimal formation after arterial electric injury in mice (Carmeliet et al 1998). Surprisingly, u-PAR deficient mice (uPAR -/-) had an equivalent degree of neointimal formation as (uPAR +/+) mice after arterial injury. It was observed on immunoelectron microscopy that uPA was bound to the cell surface of (uPAR +/+) cells, whereas it was present in the pericellular space around (uPAR -/-) cells. Pericellular plasmin proteolysis, evaluated by degradation of labelled fibrin and activation of zymogen matrix metalloproteinases was similar for both (uPAR -/-) and wildtype animals. These results therefore suggest that binding of uPA to u-PAR is not essential to provide sufficient pericellular uPA-mediated plasmin proteolysis to allow smooth muscle cell migration in neointimal formation.

1.4.5 b) Tissue plasminogen activator receptors

A 40 kD protein was found to specifically binds to tPA but not to uPA Hajjar et al 1991. It binds to both tPA and plasminogen in a manner that mimics the endothelial surface. tPA also specifically interacts with a human endothelial cell 45-kD plasminogen receptor, which, unlike the above, does not bind to antibodies to annexin II (Dudani et al 1994). tPA has been found to bind to endothelial cells with high affinity via a ligand on the B-chain (Cheng et al 1995). This binding resulted in an

almost 100 fold increase in the activation rate when compared to the enzyme in free solution. It is interesting to note that the ligand on the B-chain of tPA also binds to PAI-1 suggesting a possible competition between PAI-1 and the tPA receptor on endothelial cells.

1.4.5 c) Receptors for tPA-PAI-1 and uPA-PAI-1 complexes

The PA/PAI-1 complex has no activity (Kruithof et al 1983). Both tPA-PAI-1 and uPA-PAI-1 complexes undergo receptor-mediated endocytosis and catabolism in hepatocytes (Grimsley et al 1995). uPA-PAI-1 complexes, but not free uPA, are readily internalised and degraded via the multiligand receptor α2-macroglobulin receptor/LDL receptor-associated protein (α2MR) (Conese et al 1995). Whereas uPA-PAI-1 complexes upon binding to uPAR resulted in its cleavage and internalisation via α2MR, uPA-PAI-2 complexes, upon binding to uPAR is cleaved into two fragments and are not internalised by the cell (Ragno et al 1995). u-PA-PAI-1 complexes also bind to VLDL receptors with high affinity and results in its internalisation leading to their degradation and removal. (Argraves et al 1995, Heegard et al 1995). tPA-PAI-1 complexes have been shown to be internalised in monocytes via a member of the α2MR and the complexes and are degraded by cathepsin D-like aspartyl proteases. Similarly, tPA is internalised and degraded by vascular smooth muscle cells in vitro (Grobmeyer et al 1993), a process mediated by the LDL receptor related protein/alpha 2-macroglobulin receptor (LRP) and inhibited by antibodies to PAI-1. This may indicate that tPA binds to smooth muscle cell-associated PAI-1 with subsequent tPA-PAI-1 internalisation and degradation. Smooth muscle cells may therefore play an important role in clearing tPA-PAI-1 complexes from within the vessel wall.

1.4.6 Cytokines and the Plasminogen Activator System

There seems to be an intricate network of interactions between the PAS and a number of cytokines; plasmin activates a number of cytokines e.g. TGFβ (Odekon et al 1994, Sato et al 1989) whilst a number of cytokines modulate the expression of components of the PAS. Gene knockout studies using transgenic mice has shown that uPA and tPA are required for the mitogenic and chemotactic effects of bFGF and PDGF-BB for vascular smooth muscle cells (Herbert et al, 1997). The development of atherosclerosis is under the control of several cytokines and growth factors known to influence the expression of the PAS in cultured cells.

The complex nature of the interaction between cytokines and components of the PAS is demonstrated by the following: IL-1 and TNF induces u-PA (Van Hinsberg et al 1990), induces PAI-1 and PAI-2 (Zoellner et al 1993) and reduces t-PA (Schleef et al 1988) in human vascular endothelial cells *in-vitro*. Basic-FGF, another cytokine thought to play an important role in the vessel wall, suppresses tPA release from cultured human umbilical vein endothelial cells, but enhances tPA from cultured human aortic endothelial cells (Yamamoto et al 1994), demonstrating that endothelial cells from different parts of the vascular tree may responds differently to the same cytokine *in-vivo*. In addition, basic FGF was also found to induce uPA in endothelial cells in vitro (Gualandris et al 1995), suppress the release of PAI-1 from cultured human umbilical vein endothelial cells (Kaji et al 1994), whilst it had no effect on uPA expression in smooth muscle cells in vitro (Kenargy et al 1995). TNFα treatment of human umbilical vein endothelial cells induces bFGF, contributes to the activation of TGF-β, which in turn opposes the effects of β-FGF by limiting synthesis and activity of the

plasminogen activators (Flaumenhaft et al 1992). PDGF induces tPA in human vascular smooth muscle cells in vitro resulting in increased cell migration and this is augmented by IL4 and inhibited by γ-interferon (Wang et al 1995). VEGF induces plasminogen activators, plasminogen activator inhibitor-1 (Pepper et al 1991) and uPAR (Mandriota et al 1995) in vascular endothelial cells. In mononuclear phagocytes TGF-β1 stimulates uPA expression and release of matrix-bound basic FGF (Falcone et al 1993) whilst IL1 and IL2 induces both uPA and PAI-2 with a resultant predominance of PAI-2 (Gyetko et al 1993).

Taken together it seems likely that the plasminogen activator system interacts intricately with the cytokine network in processes that play a determining role in vessel wall pathology including atherogenesis and restenosis.

1.4.7 Influence of physical factors in the vessel wall on the PAS

The vessel wall is exposed to haemodynamic forces due to both blood pressure and blood flow. These forces result in radial stretching of the wall and shear stresses on the endothelial surface. The effect of shear stress on components of the PAS was studied in endothelial cells (Kawai et al 1994). Increasing shear stress between 0-24 dyne/cm^2 resulted in an increase in tPA and uPA whilst PAI-1 was decreased. Shear stress attenuates the alteration in the balance in the fibrinolytic and coagulation system induced by cytokines (Kawai et al 1996). Similarly, Diamond et al 1990, found tPA messenger RNA levels increased in cultured human endothelial cells exposed to laminar shear stress. Repetitive stretch stimulated the secretion of tissue-type plasminogen activator by endothelial cells (Iba et al 1991). The cyclic strain-induced

PAI-1 secretion was found to be mediated by an increase of reactive oxygen species (Cheng et al 1996). These findings indicate that haemodynamic forces may play an important role in regulating the components of the plasminogen activator system in the vessel wall in vivo.

1.4.8 Other extracellular matrix proteinases and vessel wall pathology

Matrix metalloproteinases MMP-1, MMP-3 and MMP-9 (interstitial collagenase, stromelysin 1 and gelatinase B respectively) have been implicated in abdominal aortic aneurysm development (Newman et al 1994). Bendeck et al showed that MMP2 and 9 expression correlated with SMC migration into the intima of a balloon injured rat carotid artery. Inhibition of MMP with GM6001 resulted in a 97% reduction in SMC migration at 4 days, but by 14 days, there were no difference between the treated and control groups. The 'catch up' in neointimal growth occurred via increased smooth muscle cell replication. BB94 (Batimastat), a synthetic MMP inhibitor, however did decrease intimal hyperplasia by decreasing smooth muscle cell migration and proliferation (Zempo et al 1996). Metalloproteinases can be activated by plasmin (Lee et al 1996). Keski-Oja et al 1992, showed that the 72,000-Da gelatinase / type IV collagenase is activated by uPA. It is therefore possible that the PAS may play a role in metalloproteinases-mediated vessel wall pathology.

1.5 SCOPE OF THIS PROJECT

The aim of this project was to investigate the expression t-PA, uPA, PAI-1 and uPAR in the vascular wall. Expression was determined and quantified by quantitative reverse transcriptase polymerase chain reaction. The cells expressing specific mRNAs were

located by mRNA *in-situ* hybridisation. The respective proteins within the vessel wall were quantified using immunoassays and localised using immunohistochemical techniques. PAI-1 and uPA activities of the vessel wall were assessed using specific immunoactivity assays.

Samples of vascular tissues used for the project were as follows: Normal vessels (arteries - human internal mammary artery and veins - human saphenous veins) as well as vessels containing atheromatous lesions e.g. human coronary and carotid endarterectomies, and human saphenous vein graft stenosis. In addition, the expression of the Plasminogen Activator System were examined in two models of intimal hyperplasia, namely human saphenous veins in organ culture as well as the ballooned-injured rabbit common iliac arteries restenosis model.

1.6 STUDY HYPOTHESES

(1) There is differential expression of t-PA, uPA, PAI-1 and uPAR in the walls of normal veins and arteries compared to the vessels containing intimal hyperplasia.

(2) There is differential expression and content of tPA, uPA, PAI-1 and uPAR in the vessel wall between the two vascular conduits commonly used for coronary artery bypass grafting; distended saphenous vein and internal mammary artery.

CHAPTER 2

HISTOLOGICAL STUDY OF THE PLASMINOGEN ACTIVATOR SYSTEM IN THE HUMAN VASCULAR WALL

2.1 INTRODUCTION

In this chapter, the human blood vessel wall is examined using immunohistochemical and mRNA *in-situ* hybridisation techniques to determine the presence and localisation of both protein (tPA, u-PA, PAI-1 and uPAR) and mRNA (tPA, uPA, and PAI-1). In addition, immunohistochemical studies were performed using monoclonal antibodies against α-actin, CD31, CD45 and CD68 to localise smooth muscle cells, endothelial cells, leucocytes and macrophages respectively.

Structure of the vessel wall

Saphenous vein, internal mammary artery and coronary artery.

Normal saphenous veins contain an intima of variable thickness covered by endothelium and separated from the media by a rudimentary internal elastic lamina. The media consists of multiple layers of smooth muscle cells, separated by bundles of collagen, ground substance and occasional short elastic fibres. The inner layer of muscle fibres, usually arranged in a longitudinal fashion, are relatively inconspicuous, but a more prominent outer layer of circular muscle fibres is also present (Cormack 1987). The adventitia is of variable thickness and is composed of fibrous tissue (Marti et al 1971). Internal mammary artery and coronary artery have the same basic components as in the above description but there are some important differences. The intima of these vessels are comprised of the endothelium, very little subendothelial connective tissue and little or no smooth muscle cells present. The intima is separated from the media by a clearly defined internal elastic lamina. The media is prominent with a thick layer of smooth muscle cells separated from the adventitia by the external elastic lamina that is seen to be comprised of numerous elastic layers.

2.2 METHODS

2.2.1 General methods

2.2.1 a) Blood vessel collection

Human vascular tissue was obtained at the time of routine surgery. Saphenous veins (distended n=16) and (undistended n=11) (4 paired), internal mammary arteries (n=11) and coronary endarterectomies (n=11) were obtained from patients undergoing routine coronary artery bypass graft surgery. Carotid endarterectomies (n=9) and saphenous vein graft stenoses (n=3) were obtained from patients undergoing carotid and lower limb vascular surgery respectively.

2.2.1 b) Routine surgical distension of saphenous veins

Saphenous veins were gently dissected and side-branches as well as the proximal end ligated. After removal from the patient, the saphenous vein underwent routine careful but uncontrolled manual distension by the surgeon by cannulation using normal heparinised saline mixed with the patient's blood in a 20ml syringe at room temperature. Previous studies have shown that the pressure used for the routine surgical distension of saphenous veins consistently exceeds 600 mmHg (MJ Underwood 1994). Samples were analysed as unpaired all the immunoassays.

2.2.1 c) Preparation of blood vessels for the histological study

The vascular samples were immediately rinsed in cold TBS (filter sterilised, DEPC treated water), and divided into sections for the different investigations. Tissues allocated for mRNA *in situ* hybridisation and immunohistochemistry were divided into two pieces; one piece being fixed in 10% formaldehyde in saline overnight at 4°C for

paraffin embedding while the other piece was mounted in OCT on cork, immersed into isopentane pre-cooled in liquid nitrogen, placed in a bijoux and dropped into liquid nitrogen for further cooling before being stored at -80°C in freezer ready for cryostat sections.

2.2.1 d) Fixation and embedding of blood vessels

For histological studies, small samples of veins and arteries were chosen immediately adjacent to the piece of vessel used for the RNA extraction and protein studies. After fixation in formaldehyde saline, the tissues were dehydrated in graded alcohols then embedded in paraffin wax.

2.2.1 e) Cutting sections (paraffin & cryostat)

Paraffin sections (4μm) were cut on a Reichert Jung 3000 Microtome, floated on warm water, and picked up on slides previously coated with aminoalkyl-silane. The slides were then dried overnight at 42°C and stored until needed at room temperature. For cryostat sections, tissue mounted on cork in OCT (Optimum Cooling Temperature^{TM}) was cut into 4μm sections. Once cut, the sections were used after 3 hours of drying at room temperature in air for histological studies.

2.2.1 f) Coating slides with aminoalkylsilane

To ensure the tissue sections were not lost during the immunohistochemistry and *in-situ* hybridisation, the slides were coated in aminoalkyl-silane to increase the adherence of the sections. The slides were immersed in 1% Decon for 30 minutes, washed firstly in running tap water for 30 minutes, then in ultrapure water for 5 minutes then finally in

95% IMS twice for 5 minutes each. The slides were then dried in air in front of a fan for 10 minutes and dipped in a freshly prepared solution of 2% 3-amino-propyltriethoxysilane (Sigma) in dry acetone. The slides were then rinsed twice in dry acetone, rinsed 4 times in ultrapure water, dried overnight at 42°C and stored in a dust-free environment.

2.2.1 g) Haematoxylin and eosin staining

The paraffin-embedded sections on slides (in duplicates from each sample of tissue mentioned in 2.2.1(a)), were de-waxed in xylene and rehydrated in serial dilutions of ethanol (99%, 95%, 70%; each for 2 minutes), then immersed in haematoxylin for 40 seconds, washed in water then immersed in eosin for 25 seconds. The sections were then re-dehydrated in increasing concentrations of ethanol, immersed in xylene for 2 minutes, then protected by adhering the coverslips over the sections using DPX mountant (BDH).

2.2.2 Methods: Immunohistochemical analysis of the human blood vessel

Immunohistochemical studies were performed using mouse monoclonal antibodies to tPA, u-PA, PAI-1 and u-PAR. The conditions used are shown in Table 2.2.2 (a) and (b) overleaf. The blood vessels used in this part of the study were saphenous vein (undistended and surgically distended vein), internal mammary artery, coronary endarterectomy, carotid endarterectomy and saphenous vein graft stenosis. Three sections from each of the vessel samples mentioned in section 2.2.1 were used for each immunohistology study.

The mouse monoclonal anti-human tPA binds to both single and two-chain tPA and is able to bind to tPA bound to PAI-1. It is therefore able to bind to all forms of tPA in the vessel wall. The mouse monoclonal anti-human u-PA is directed against a B-chain epitope of human urokinase-type plasminogen activator, near the catalytic site. It reacts with free and receptor bound, single and two chain (high molecular weight) urokinase as well as the uPA B-chain (33kD) fragment. The mouse monoclonal anti-human PAI-1 binds to both free and bound PAI-1. The mouse monoclonal anti-human uPAR binds to both unoccupied and occupied uPAR on the surface of cells, to soluble uPAR and uPAR inside cells. The secondary antibody in the immunohistochemical procedures was biotin-labelled sheep anti-mouse antibody (Amersham) used at 1/200 dilution.

Table 2.2.2 a) Panel of monoclonal antibodies used to detect components of the plasminogen activator system

Epitope	Company	Identity No	Origin	Working Dilution of the Primary Antibody.	Incubation of the Primary Antibody	DAB Incubation
tPA	Biopool, Sweden	PAM 3	mouse	1:25	1 hour at 37°C	5 minutes
uPA	American Diagnostica	# 3689	mouse	1:25 in human vessels 1:100 in Rabbit vessels	1 hour at 37°C 1 hour at 37°C	5 minutes 2 minutes
PAI-1	Biopool, Sweden	MAI12	mouse	1:100	1 hour at room temperature	5 minutes
uPAR	American Diagnostica	#3936	mouse	1:25	1 hour at 37°C	5 minutes

Table 2.2.2 b) Panel of antibodies used to identify vessel wall cell phenotype

Epitope	Company	Identity No	Origin	Working Dilution of the Primary Antibody	Conditions for Primary Antibody Incubations
α-Actin	Dako Ltd, UK	M 0851	mouse	1:400	Overnight at 4°C
vWF	Dako Ltd, UK.	A 080	Rabbit	1:200	Overnight at 4°C Prior trypsin digestion.
CD31	Dako Ltd, UK.	M 0823	Mouse	1:50	Prior trypsin Overnight at 4°C
CD68	Dako Ltd, UK.	PG-M1	Mouse	1:100	Overnight at 4°C Prior trypsin digestion.
CD45 (LCA)	Dako Ltd, UK.	M 0701	Mouse	1:25	Overnight 4°C

Dako-CD68 detects an intra-cytoplasmic glycoprotein present in macrophages. According to the manufacturer, lymphoid, myocytic and cells of the granulocytic lineage are not immunoreactive with this antibody. Dako-CD45 (referred to as leucocyte common antigen) reacts with most leucocytes. The type of leucocyte can then be identified from its morphology. Dako-CD31 reacts with endothelium, granulocytes and monocytes. According to the manufacturer, Dako-Rabbit Anti-Human von Willebrand factor gives positive staining in endothelial cells and megakaryocytes; no staining of other cell types has been observed.

2.2.2 c) Protocols for the immunohistochemical staining.

The paraffin-embedded tissue sections on silane-coated slides were dewaxed in xylene, rehydrated in graded alcohols (99%, 99%, 95%, 70%) then distilled water. The slides were immersed in 3% hydrogen peroxide for 5 minutes to quench any endogenous peroxidase activity in the tissues, rinsed in distilled water and placed in 0.5 M TBS (pH 7.6) for 5 minutes. Normal sheep serum (Gibco BRL) was diluted 1:5 in 0.5 M TBS (pH 7.6) and 40µl of this was placed on the sections and incubated at (room temperature or 37°C as in Tables 2.2.2 (a) & (b) for 30 minutes. The serum was then tapped off the slides and any excess wiped off. Serial dilutions of primary antibody were made and 30µl of this were placed on the sections and incubated under the conditions shown in Tables 2.2.2 (a) & (b). The slides were then washed in TBS three times (5 minutes each wash) then 30µl of a 1/200 dilution of the secondary antibody was placed on the sections for 30 minutes at room temperature. The secondary antibody was sheep anti-mouse antibody (Dako) for all the immunohistochemical analyses except those for von Willebrand factor where sheep anti-rabbit antibody was

used. The slides were then washed three times in 0.5 M TBS as before and then 30 µl of reagent AB (Dako) (diluted according to manufacturer's instructions) was placed on the sections and incubated for 30 minutes. The slides were then washed three times in TBS and the sections were covered with a solution of DAB (3,3'-diaminoazobenzidine) in TBS and 0.3% hydrogen peroxide and then incubated at room temperature for varying time periods (5-15 minutes) to establish the optimum conditions. The slides were then placed in distilled water, counter-stained in Mayer's Haematoxylin for 30 seconds, dehydrated in graded alcohols, cleared in xylene and mounted in DPX mountant (BDH).

2.2.2 d) Determining the optimal conditions for the immunohistochemistry.

Pilot studies were needed to establish the best conditions for demonstrating the specific tissue antigens (PAI-1, uPA, uPAR and tPA), in particular, the optimum concentration for the primary antibody, the temperature of the primary antibody incubation and length of time for the DAB incubation.

2.2.2 e) Controls used for the immunohistochemistry

Both negative and positive controls were used in the immunohistochemical analyses. The negative controls used consisted of (i) omission of the primary antibody (i.e. leaving on the first layer of normal sheep serum instead of the primary antibody), (ii) replacing the primary antibody with mouse non-immune serum, (rabbit non-immune serum in the case of von Willebrand factor immunohistochemistry) and (iii) using negative control tissue (brain for CD45, CD69 and α-actin). Tissues known to express the respective antigens were used as positive control tissue e.g. bone marrow

for CD68, stomach for both anti-human tPA and anti-human uPA.

2.2.2 (f) Measuring the percentage of positive cells

Immunopositive cells were stained brown as the substrate used was diaminobenzidine. Cells (n=600) were counted with the help of a graticule on sections of tissue using a magnification of *x* 200. A random selection of slides was also counted by an independent person. Inter-observer and intra-observer variability were less than 11%.

2.2.3 Methods: mRNA *in-situ* hybridisation

2.2.3 a) cDNA probes used to detect specific mRNA species

The cDNA probes used to detect mRNA to tPA, uPA and PAI-1 in the *in-situ* study were obtained from the plasmids described in Section 3.2.7. The cDNA was released from the plasmid using the relevant restriction enzyme and run on an agarose gel. The band was cut out and purified using the Sephaglas Kit ®

2.2.3 b) Radiolabelling of cDNA probes

The cDNA probes were labelled with α^{32}P dCTP using the Radprime labelling kit (Gibco) as described in *Section 3.2.13 (c) iii.*

2.2.3 c) Protocol for the mRNA in-situ hybridisation.

Dewaxing and rehydrating slides

Paraffin -embedded sections on slides were de-waxed by immersing in xylene (for two periods of 10 minutes, changing the xylene after the first period) then rehydrated

serially in ethanols (99%, 99%, 95%, 70%) then finally in ultra pure DEPC water twice for 5 minutes ready for the proteinase K digestion.

Proteinase K digestion

The slides were immersed in pre-warmed (37°C) RNase-free P buffer (0.2 M Tris.HCl, 50mM EDTA, pH 8.0, in DEPC water) for 5 minutes then the sections covered with 150 µl of Proteinase K (Boehringer Mannheim) (5-20µg/ml) and incubated at 37° C for 1 hour. The slides were then immersed in DEPC-treated water for two 5 minute periods, changing the water after the first period and then in 0.2% w/v glycine in 1X PBS in DEPC water then in PBS for 5 minutes in each.

Re-fixing of tissue sections

The slides were then immersed in pre-cooled (4°C) DEPC-treated H_20 for two 5 minute periods and re-fixed in 0.4% paraformaldehyde in PBS for 20 minutes at 4°C.

Pre-hybridisation and hybridisation

Hybridisation and Pre-hybridisation solutions were made up as follows:

20 X SSPE	6.00	µl	
100% Formamide	12.50	µl	
100 X Denhardts	1.25	µl	
PEG Mix	4.50	mls	(PEG Mix = 1.25 mls 10% SDS, 2.25mls DEPC-treated water,1.5g 6% PEG 6000)

A 0.5 ml aliquot of salmon sperm (10 mg/ml) was incubated at 95°C for 10 minutes and added to 10 mls of the above to form the pre-hybridisation solution. This was gently mixed and 40 µl applied to each of the slides for a 3-hour pre-hybridisation incubation at 42 °C.

The hybridisation solution was made up as above but in addition the labelled probe was added. The cDNA probes were labelled with α^{32}P-dCTP using the Radprime Labelling Kit (Gibco) and purified with a G 50 Sephadex column, both as already described in the methodology used for 'Southern blotting and hybridisation' (3.2.14 c)). After the pre-hybridisation incubation was over, 40 µl of the hybridisation solution was gently added to the 40 µl of pre-hybridisation solution still present on each of the slides, a cover-slip placed over the solution and incubated overnight at 42 °C.

The wash

The coverslips were removed by quick immersion of the slides in 4X SSPE/ 0.1% SDS. The slides were washed twice in 3X SSPE/ 0.1% SDS for 15 minutes each at 42°C, then once in 1X SSPE/ 0.1% SDS for 15 minutes then dehydrated in graded alcohols at concentrations; 70%, 95%,100%, 100%, left to dry in air for 10 minutes

Autoradiography of the mRNA *in-situ* slides

The dried slides were exposed to film (Kodak) overnight. The autoradiograph was developed on an Agfa Currix machine. The slides were then exposed to photographic emulsion: Photographic K5 emulsion (Ilford UK) (10g) were weighed out in the dark and added to 10mls of ultrapure water containing 10 drops of glycerol, mixed and left to dissolve for 2 hours at 42°C in the dark. Sections were dipped in the emulsion at 37°C, left to dry in the dark for 2 hours, then placed in a light-tight box until appropriate exposure times (3-21 days).

Development

The slides were removed from the box under safe-light and immersed in developer (Kodak D19) for 4 minutes, 1% acetic acid for 1 minute, Hypam fixer for 4 minutes then washed in distilled water, all the immersions occurring at 15-17°C. The slides

were then counterstained in haematoxylin for 20 seconds, washed briefly in distilled water, dehydrated in graded IMS concentrations (75-99%) then immersed in xylene before placing the cover-slips on with DPX mountant (BDH).

2.2.3 d) Determining the optimal conditions for the mRNA in situ hybridisation

The following parameters were varied in order to find the optimal condition for the probes:-

Proteinase K concentration; (1-80μg/ml)

Probe concentration (1-25ng/ml)

Length of autoradiographic exposure - (1-3 weeks)

2.2.3 e) Negative control for the mRNA in-situ hybridisation

Control slides were treated with RNase (Sigma) in 2X SSC for 1 hour at 37 C, the test slides being incubated in 2 X SSC in the absence of RNase. It is acknowledged that a sense probe would have been a more ideal control, as it would have demonstrated the specificity of the hybridisation.

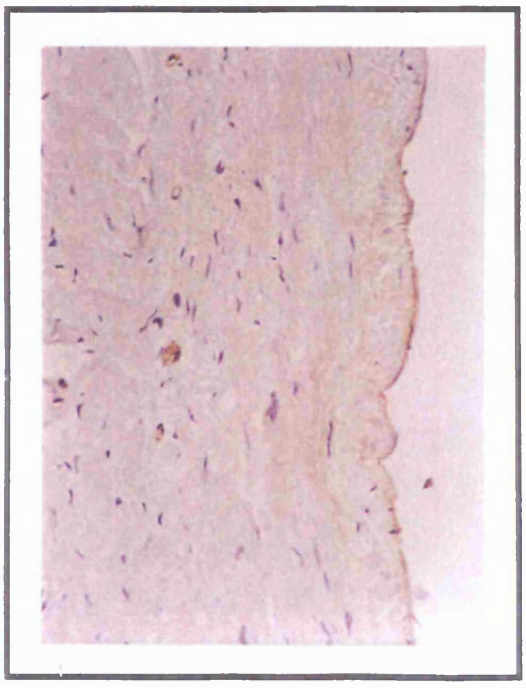
2.3 RESULTS

2.3.1 Saphenous vein coronary artery conduits

2.3.1(a) H&E staining (x100)

2.3.1(b) α-actin immunostaining (x100)

2.3.1(c) vWF immunostaining (x200)

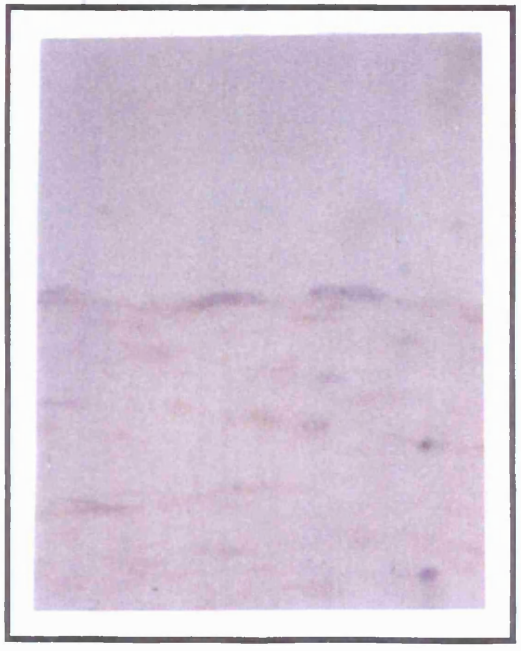


2.3.1(d) CD68 immunostaining (x100)

Figs 2.3.1 (a) - (d) The basic structure of the saphenous vein prior to its use as a bypass graft is shown; the intima (endothelium and subendothelium) is separated from the media by a flimsy internal elastic lamina (stained blue by Miller's reagent in Fig 2.3.1 (b). Spindle-shaped smooth muscle cells (α-actin immunoreactive) are present, not only in the media, but also in the intima and the adventitial vasa vasorum. In the saphenous vein coronary artery conduit sample shown, there is pre-existing intimal hyperplasia with numerous smooth muscle cells present in the subendothelial intima. All saphenous veins that were surgically distended retained their von Willebrand Factor (vWF) immunoreactivity. On higher magnification, vWF staining was seen to be associated with the endothelial cells as well as the extracellular spaces of the intima. It was noted that the thicker the intimal layer the deeper the vWF staining extended. Unlike all the saphenous vein graft stenosis; Fig 2.3.4 (d), macrophages and lymphocytes (as detected by CD68 and CD45) were absent within the wall of all microscopically normal saphenous veins.

2.3.1 (e) I tPA immunostaining

Undistended saphenous vein (x100) ***Distended saphenous vein (x100)***



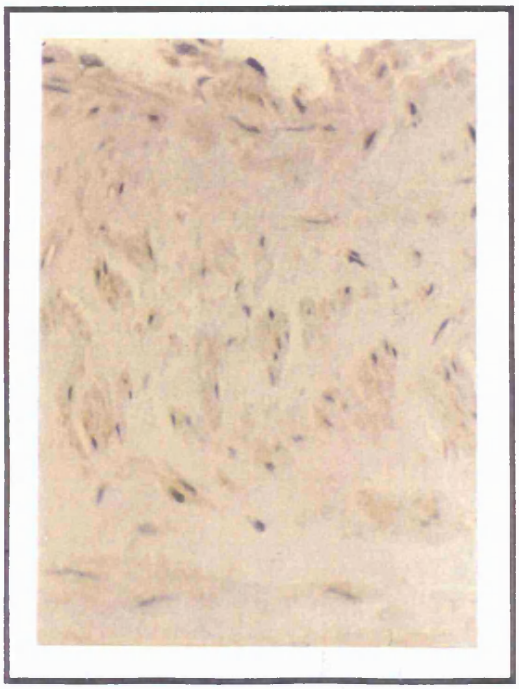
2.3.1 (e) II u-PA immunostaining

Undistended saphenous vein (x100)

Distended saphenous vein (x100)

2.3.1 (e) III PAI-1 immunostaining

Undistended saphenous vein (x100)



Distended saphenous vein (x100)

2.3.1 (e) IV uPAR immunostaining

Undistended saphenous vein (x200) ***Distended saphenous vein (x100)***

2.3.1 (e) I-IV

All saphenous veins were immunoreactive to tPA, uPA, PAI-1 and uPAR. tPA uPA and PAI-1 are seen to be associated with both smooth muscle cells (of the media, intima, and vasa vasorum) as well as the endothelium. Surgical distension of the saphenous vein coronary artery conduits resulted in a significant increase in immunopositivity of the medial smooth muscle cells for each of these three antigens. In undistended saphenous veins u-PAR antigen was associated with endothelial cells only, the smooth muscle cells being immuno-negative, however, distension of these veins resulted in the medial smooth muscle cells becoming uPAR immunopositive.

2.3.1 (f) Semiquantitation of the immunohistochemical findings for tPA, uPA, PAI-1 and uPAR in saphenous veins

The percentage of medial smooth muscle cells staining positive for tPA, u-PA and PAI-1 antigens before and after surgical preparation including distension are shown below:

Table 2.3.1 (f) The effect of surgical distending saphenous veins.

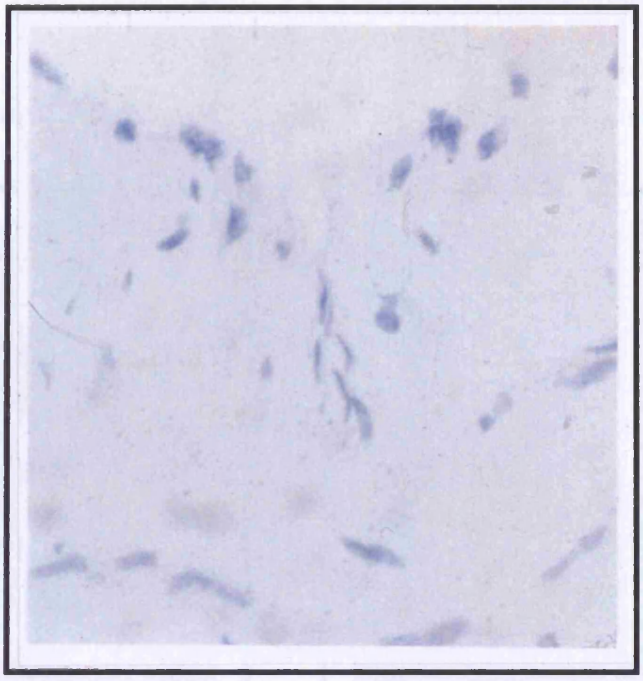
	Mean percentages of immunopositive SMCs		
Antigen	**Undistended vein (n=11)**	**Distended vein (n=16)**	**p value (unpaired)**
tPA	34%	63%	<0.0001
u-PA	7%	46%	<0.0001
PAI-1	12%	86%	<0.0001
uPAR	1%	7%	0.03

Distension of saphenous veins as part of their preparation for use as vascular conduits in coronary artery bypass grafting results in a significant increase in tPA, uPA, PAI-1 and uPAR antigens within the vessel wall at the time of grafting.

2.3.1 (g) Saphenous vein; tPA, uPA and PAI-1 mRNA in-situ hybridisation

Using the technique of *in-situ* mRNA hybridisation, PAI-1 mRNA was expressed by both endothelial and smooth muscle cells in all the saphenous veins. Figure 2.3.1 (g) shows the positive signal as small punctate black dots that are cellular in distribution. Using the same technique, signal for tPA and uPA were not observed in any of the saphenous veins.

2.3.1(g) PAI-1 mRNA in-situ hybridisation in saphenous vein (x200)

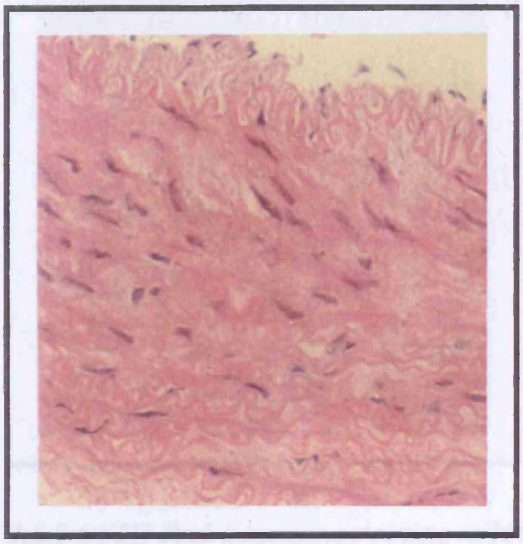


2.3.2 Results: Internal mammary arterial coronary artery conduits

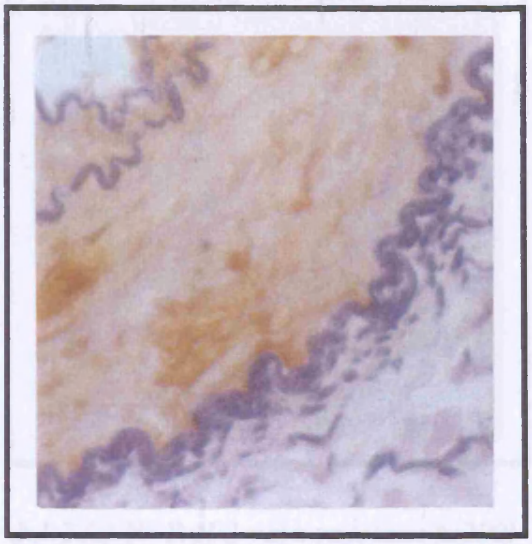
Fig 2.3.2 (a) shows, that unlike saphenous veins, the internal mammary arteries have a well-defined internal elastic lamina separating the intima from the media. The intima is largely devoid of smooth muscle cells. In places there is duplication of the internal elastic lamina. The spindle shape of the smooth muscle cells is less apparent in the media due to the abundance of elastic tissue with consequent recoil. Fig 2.3.2 (b) shows that the spindle shaped cells are immunopositive for α-actin confirming that they are smooth muscle cells. The vWF staining in Fig 2.3.2 (c) shows the endothelial cells lining both the vessel lumen as well as vasa vasorum in the adventitia. As in all the normal saphenous veins examined, all the normal internal mammary arteries also lacked macrophages and lymphocytes within the wall (unlike all the saphenous vein graft stenosis and arterial atheromatous tissues examined); see Figures 2.3.2 (d), 2.3.4 (d) and 2.3.3 (d).

2.3.2 Internal mammary artery

2.3.2 (a) H&E staining (x100)



2.3.2 (b) α-actin immunostaining (x100)

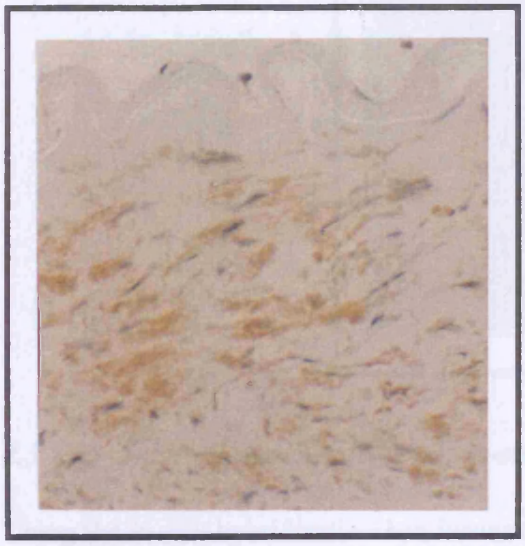


2.3.2 (c) vWF immunostaining (x100)

2.3.2 (d) CD68 immunostaining (x100)

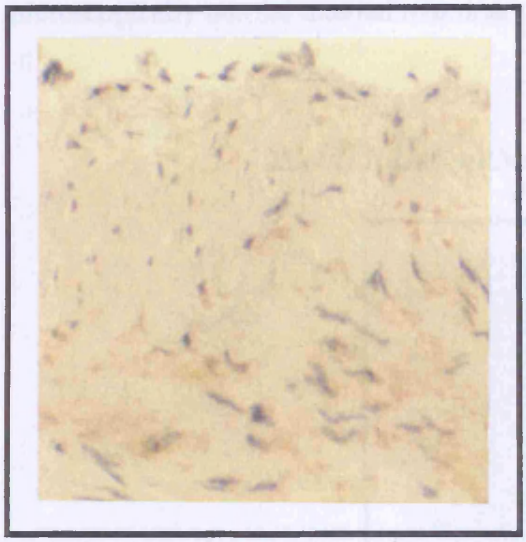
2.3.2 Internal mammary artery

2.3.2 (e) I tPA immunostaining (x100)

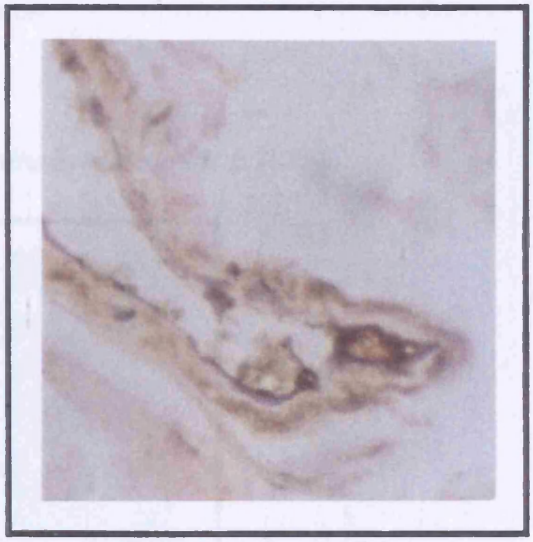


2.3.2 (e) II uPA immunostaining (x100)

2.3.2 (e) III PAI-1 immunostaining (x100)



2.3.2 (e) IV PAI-1 vasa vasorum (x200)



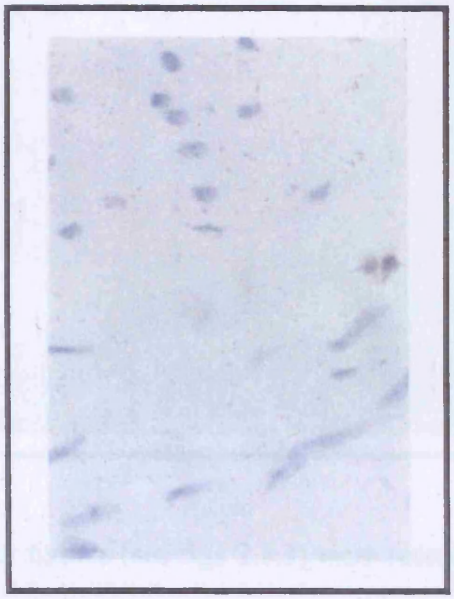
In internal mammary arteries, tPA, uPA and PAI-1 proteins were seen to be associated with both smooth muscle cells (of the media, intima, and vasa vasorum) and endothelium of the vessel lumen and the vasa in the adventitia, see figures 2.3.2 (e) I-V. However, uPAR protein, see Fig 2.3.2 (e) VI, is largely absent in the walls of internal mammary arteries, as is the case with undistended saphenous veins already shown in Fig 2.3.1 (e) IV.

2.3.2(e) VI uPAR immunostaining in IMA (x100)

2.3.2 (f) Internal mammary artery; in-situ mRNA hybridisation

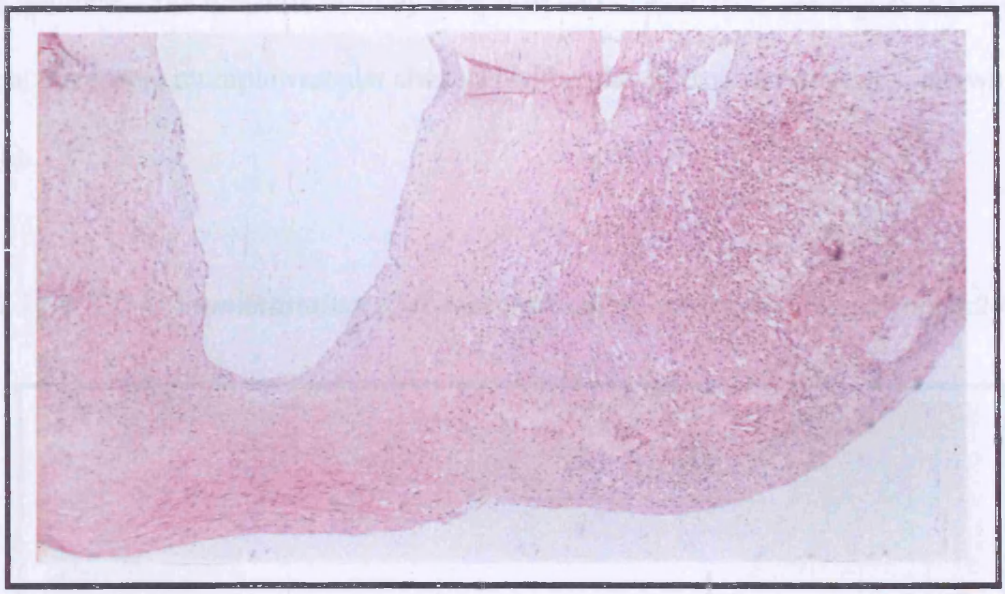
Using the *in-situ* hybridisation conditions described, there was signal for PAI-1 mRNA in the vessel wall (endothelium and media) and an absence of signal for uPA and tPA in microscopically normal internal mammary arteries.

2.3.2(f) PAI-1 mRNA in-situ hybridisation (x200)

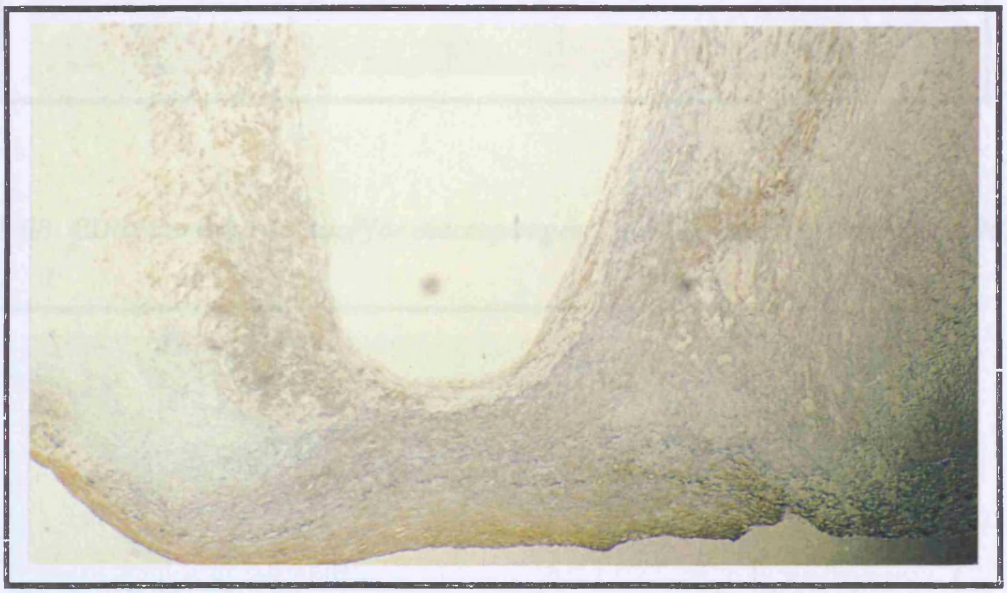


2.3.3 Atheromatous lesions

2.3.3 (a) H&E staining : Coronary endarterectomy (x100)



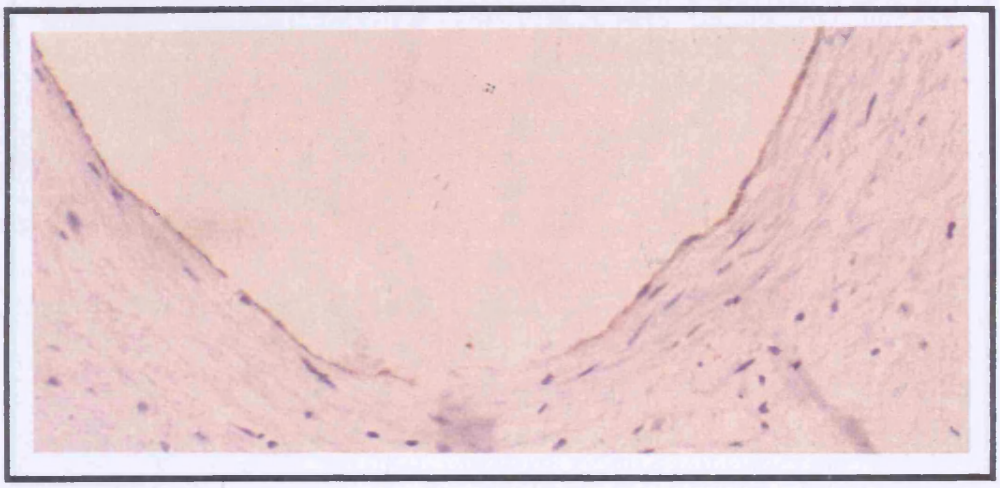
2.3.3 (b) α-actin immunostaining: Coronary endarterectomy (x100)



The coronary endarterectomy tissues (see figs 2.3.3) were seen to consist of an outer rim of α-actin immunoreactive smooth muscle cells which have been sheared off from the outer media during the endarterectomy procedure. There is a rim of smooth muscle cells around

the main lumen, see fig 2.3.3 (b). Within the atheromatous lesion there are sheaths of smooth muscle cells interspersed by inflammatory areas, typical cholesterol clefts and areas that are largely acellular. The lumen is lined by a layer of endothelial cells, see Figure 2.3.3 (c). In addition, there were multiple vascular channels within the atherosclerotic lesion, shown in fig 2.3.3 (a).

2.3.3 (c) CD 31 immunostaining for endothelium; coronary endarterectomy (x200)



2.3.3 (d) CD68 immunostaining for macrophages; coronary endarterectomy (x100)

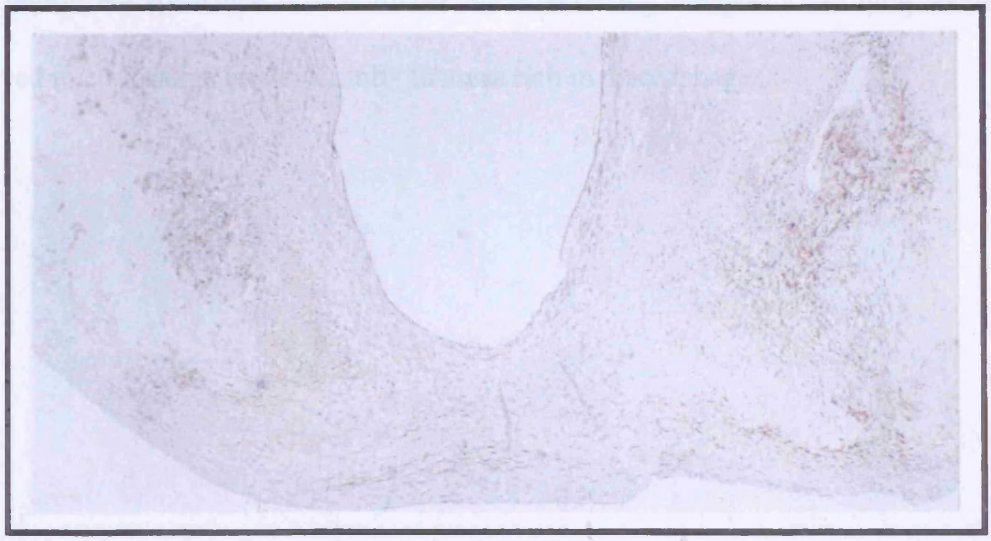


Figure 2.3.3 (d) shows CD68 immunopositive macrophages areas juxtaposition to areas infiltrated by α-actin immunopositive smooth muscle cells seen in figure 2.3.3 (b). The areas containing macrophages were predominantly seen periluminally and in clumps especially adjacent to vascular channels

2.3.3 (e) LCA immunostaining for lymphocytes in coronary atheroma (x100)

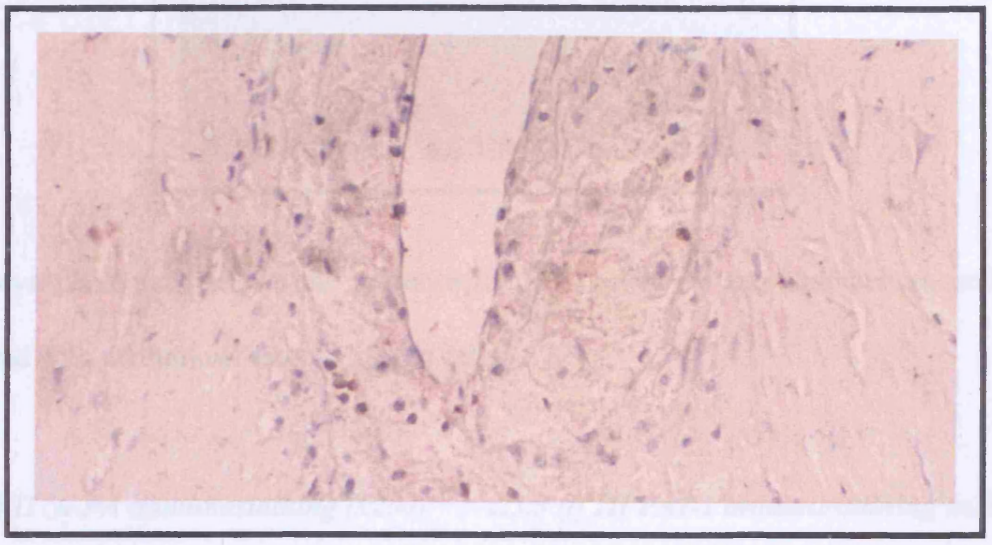
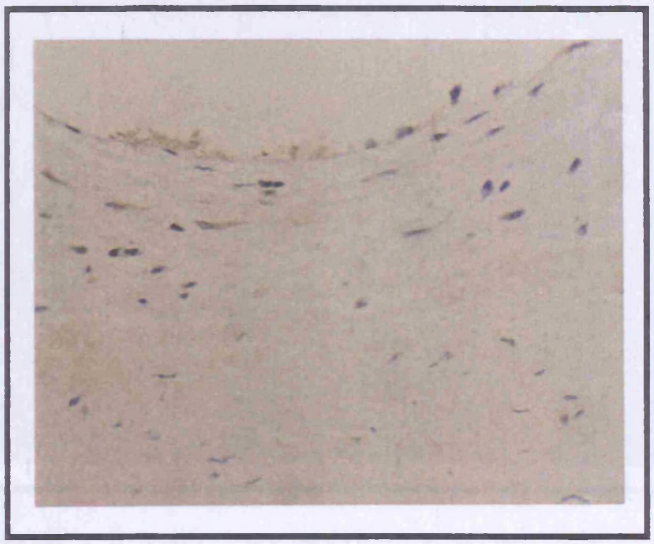


Figure 2.3.3 (e) demonstrates the presence of lymphocytes adjacent to a vascular channel deep within the substance of a coronary endarterectomy sample. The lymphocytes were observed to co-localize predominantly to areas rich in macrophages.

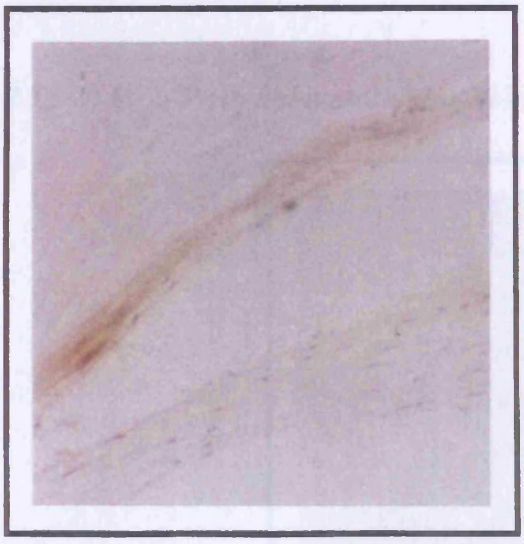
2.3.3 (f) tPA, uPA, PAI-1 and uPAR proteins in atheromatous lesions

2.3.3 (f) I tPA immunostaining (x200)

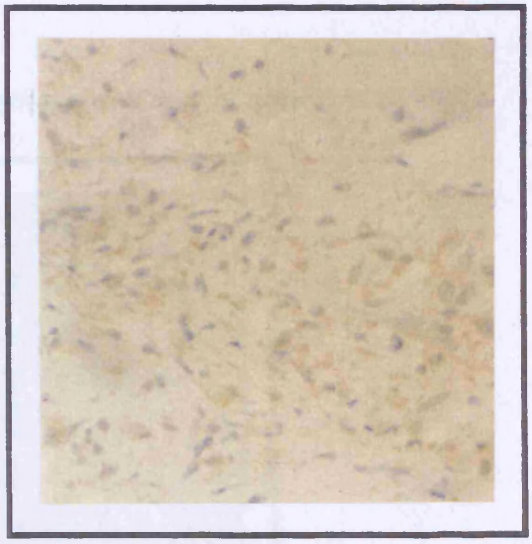


The above photograph shows the presence of tPA protein on the endothelium and also associated with periluminal smooth muscle cells.

2.3.3 (f) II u-PA immunostaining (x200)



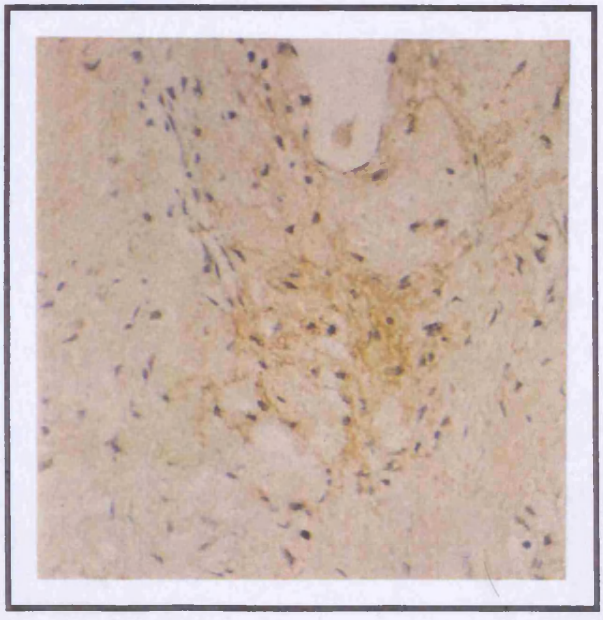
2.3.3 (f) III PAI-1 immunostaining (x100)



2.3.3 (f) IV u-PAR immunostaining coronary endarterectomy (x200)

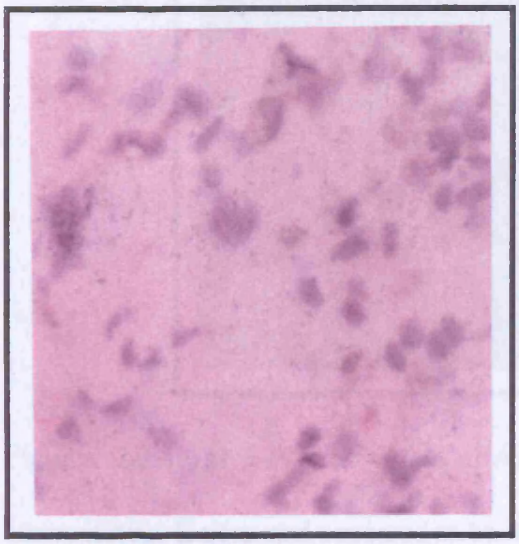
Figures 2.3.3 (f) I-IV show that tPA, uPA, PAI-1 and uPAR proteins were all present in the atherosclerotic lesions. tPA was predominantly associated with endothelial cells and periluminal smooth muscle cells. PAI-1, uPA and uPAR proteins were also associated with endothelial cells and periluminal smooth muscle cells, but in addition, were also found in areas rich in macrophages (see figure 2.3.3 (f)V)

2.3.3 (f) V u-PAR immunostaining in macrophage-rich area of atheroma (x100)

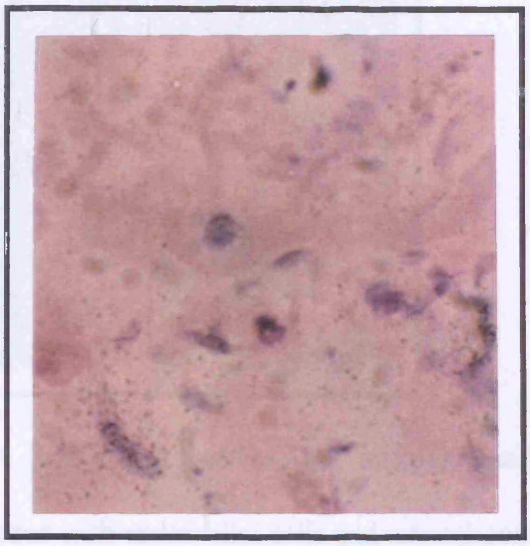


2.3.3 (g) tPA, uPA and PAI-1 mRNA in-situ hybridisation in atheroma

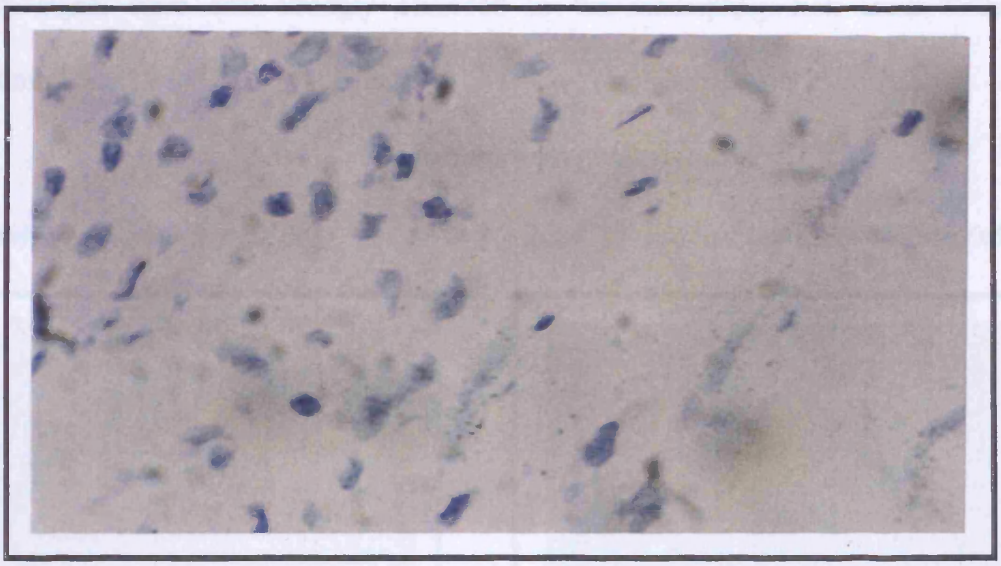
2.3.3 (g) I t-PA mRNA in-situ (x200)



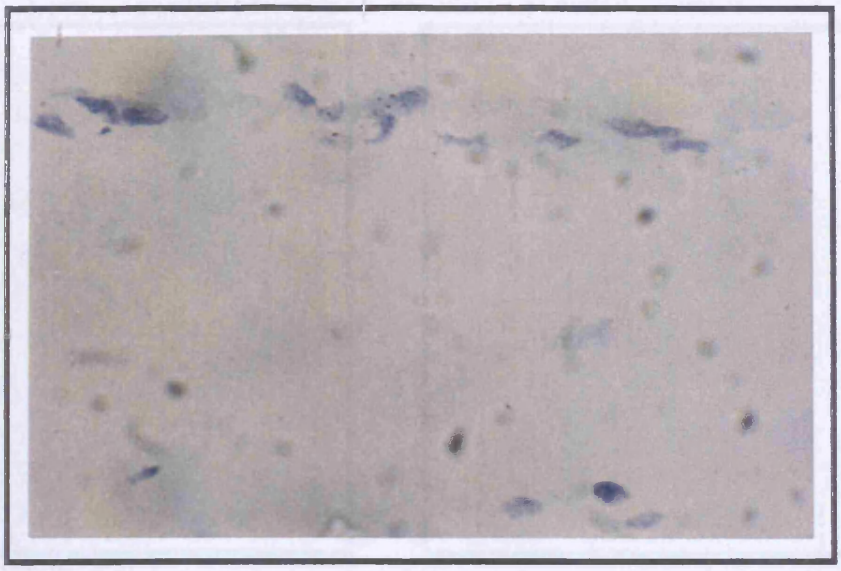
2.3.3 (g) II u-PA mRNA in-situ (x200)



2.3.3 (g) III PAI-1 mRNA in-situ on SMCs in atheroma (x200)



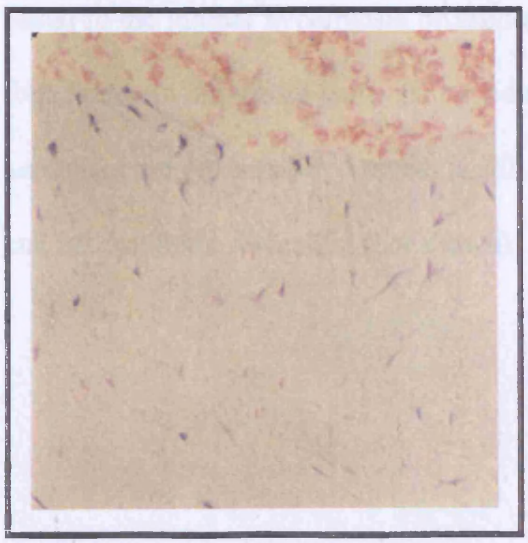
2.3.3 (g) IV PAI-1 mRNA in-situ on endothelial cells in atheroma (x200)



Figures 2.3.3 (g) I-IV show that tPA, uPA and PAI-1 mRNA are all expressed in atheromatous lesions and appeared to be associated with endothelial cells and smooth muscle cells of the neointima. The expression of these mRNA species was not homogenous in their distribution but rather periluminal, and in pockets within the neointima. PAI-1 and uPA mRNA signal were also strongly observed in the macrophage-rich areas within the neointima.

2.3.4 Saphenous vein graft stenosis.

2.3.4 (a) H&E staining (x100)



2.3.4 (b) α-actin immunostaining (x50)

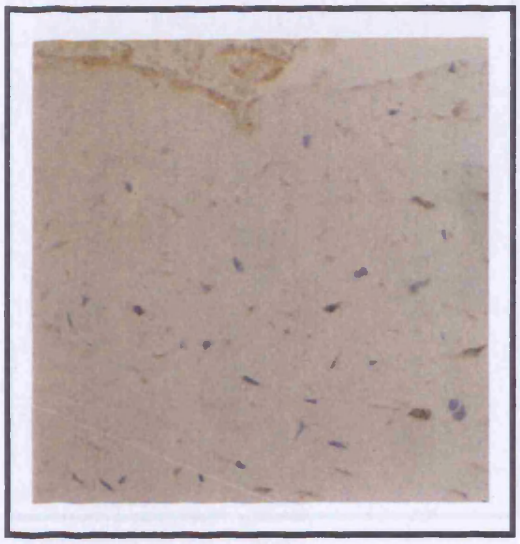
2.3.4 (c) vWF immunostaining (x100)

2.3.4 (d) CD68 immunostaining (x100)

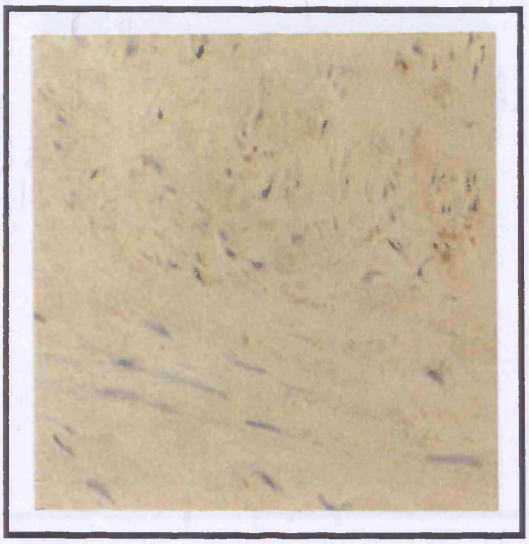
Figures 2.3.4 (a-d) show that the intimal hyperplasia of saphenous vein graft stenosis is principally composed of smooth muscle cells in a very thickened neointima. These smooth muscle cells are seen to have nuclei that are either stellate- or spindle-shaped, see fig 2.3.4 (a). The lumen is lined with endothelial cells as confirmed vWF immunostaining; fig 2.3.4 (c). The vWF immunostaining is seen to extend into the superficial layer of the intimal hyperplasia as it does in the intimal hyperplasia of the saphenous veins in organ culture, see fig 6.3.1 (d) II. Only a few macrophages were found in the intimal hyperplasia of saphenous vein graft stenotic lesions, and these are found deep in the vessel wall. The media, from which the smooth muscle cells of the neointima are presumably derived, is still recognisable deep in the vessel wall adjacent and internal to the adventitia (not shown).

2.3.4 (e) Saphenous vein graft stenosis; immunostaining

2.3.4 (e) I tPA immunostaining (x200)



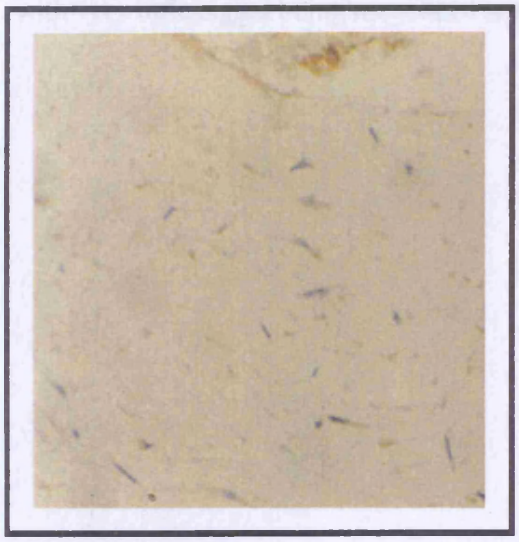
2.3.4 (e) II u-PA immunostaining (x200)



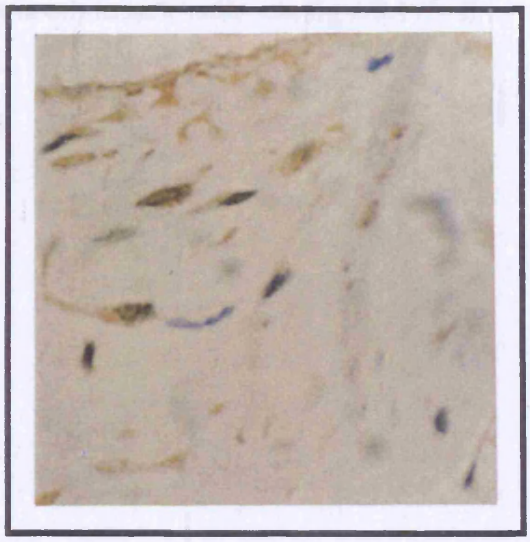
Both the smooth muscle cells within the neointima and the endothelial cells lining the lumen are immunopositive for tPA, uPA, PAI-1 and uPAR, see figs 2.3.4 (e) I-IV. Fig 2.3.4 (e) II shows the boundary between the media (the lower half of the photograph containing spindle shaped smooth muscle cells relatively unstained for uPA arranged with their axes parallel to the endothelium) and the neointima. The peri-medial part of neointima is seen to contain smooth muscle cells strongly immunopositive for tPA, uPA and uPAR with their long axes perpendicular to the endothelium and these cells are presumably migrating into the neointima proper (the upper half of the photograph). The media however, was consistently relatively immunonegative for all four proteins compared to the neointima.

2.3.4 (e) Saphenous vein graft stenosis; immunostaining

2.3.4 (e) III PAI-1 immunostaining (x100)

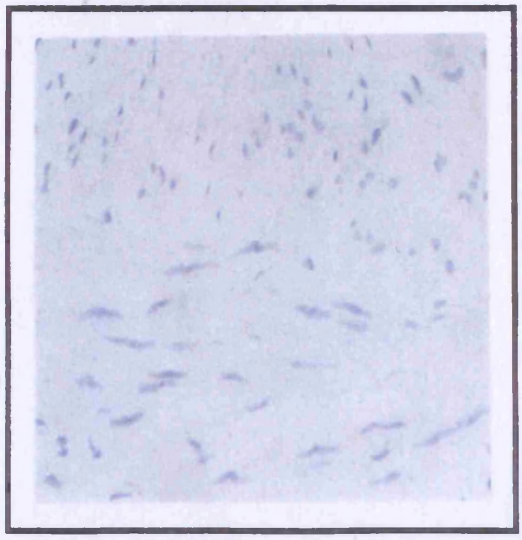


2.3.4 (e) IV uPAR immunostaining (x100)

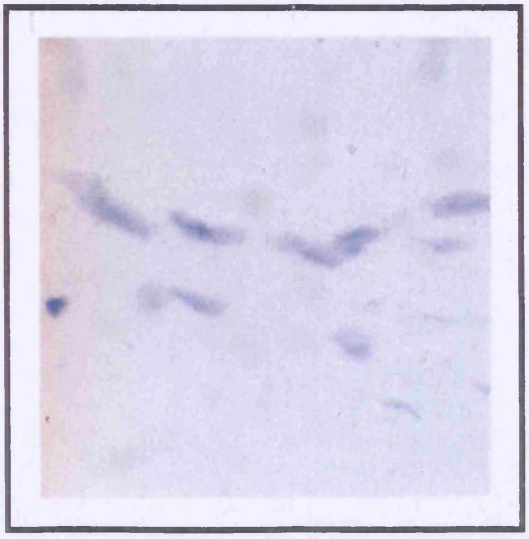


2.3.4 (f) Saphenous vein graft stenosis; *in-situ* hybridisation.

2.3.4 (f) I uPA mRNA in-situ (x100)



2.3.4 (f) II PAI-1 mRNA in-situ (x200)



Unlike undistended saphenous veins, grafted saphenous veins that had undergone graft

stenosis demonstrated strong signal for tPA and uPA mRNA in both smooth muscle cells and endothelial cells. PAI-1 mRNA signal was predominantly associated with endothelial cells with very little signal being associated with the smooth muscle cells, see fig 2.3.4 (f) II.

Table 2.4 (a) **Summary of results: immunohistology and mRNA *in-situ* hybridisation**

Human Blood Vessel	Immunohistochemistry % of immunopositive cells				mRNA in-situ hybridisation presence (+) or absence (-)		
	tPA	uPA	PAI-1	uPAR	tPA	uPA	PAI-1
Undistended SV							
Endothelial cells	100%	100%	100%	100%	-	-	+
Smooth muscle cells	34%	7%	12%	1%	-	-	+
Distended SV							
Endothelial cells	100%	100%	100%	100%	-	-	+
Smooth muscle cells	63%	46%	86%	7%	-	-	+
Internal mammary artery							
Endothelial cells	100%	100%	100%	100%	-	-	+
Smooth muscle cells	10%	10%	16%	3%	-	-	+
Atheroma (endarterectomy)							
Endothelial cells	100%	100%	100%	100%	+	+	+
Smooth muscle cells	*A	*A	*A	*A	*B	*B	*B
Saphenous vein graft stenosis							
Endothelial cells	100%	100%	100%	100%	+	+	++
Smooth muscle cells	*C	* C	* C	* C	*D	*D	*D

See Table on the following page.- *A--D -

Table 2.4 (b) Summary of results: immunohistology and mRNA *in-situ* hybridisation; atheroma and saphenous vein graft stenosis.

*A Immunohistology of atheroma	Unlike normal artery (internal mammary artery, n=11) which showed a small fraction of smooth muscle cells immunopositive to tPA, uPA, PAI-1 and uPAR proteins, atheromatous arteries (coronary; n=11, and carotid; n=9) demonstrated that the smooth muscle cells in peri-luminal intimal areas were predominantly immunopositive. Deeper still in the atheromatous intima immunopositivity was associated with smooth muscle cells scattered in sheets. Acellular areas within the atheroma were also positive for all 4 proteins. The medial smooth muscle cells (at the periphery of the endarterectomies) were mainly immunonegative. In addition it was noted that uPA and uPAR was also strongly positive in areas within the atheroma rich in macrophages.
*B mRNA in-situ hybridisation in atheroma	Unlike normal artery (internal mammary artery, n=11) which failed to show signal for tPA, uPA, and PAI-1 mRNA in the smooth muscle cells, atheromatous arteries (coronary; n=11, and carotid; n=9) demonstrated signal scattered throughout the atheromatous section (peri-luminal as well as deeper within the atheromatous intima) with cellular distributions. The signal for PAI-1 was particularly strong. The smooth muscle cells at the periphery of the endarterectomy sections i.e. within the media were largely negative.
*C Immunohistology of saphenous vein graft stenosis, n=3	In saphenous vein graft stenoses (n=3), both the endothelium and neointimal smooth muscle cells were immunopositive for tPA, uPA, and uPAR. PAI-1 showed an increased endothelial but decreased neointimal immunostaining compared to tPA, uPA and uPAR. The smooth muscle cells of the 'old' media were much less immunopositive and comparable to normal undistended saphenous veins.
*D mRNA in-situ hybridisation in saphenous vein graft stenosis	In saphenous vein graft stenoses (n=3), the endothelium and neointimal smooth muscle cells demonstrated the presence of tPA, uPA and PAI-1 mRNA. The endothelial cells consistently showed very strong signal for PAI-1 mRNA in comparison to normal saphenous veins

2.5 Discussion

This histological study (both immunohistological and mRNA *in-situ* hybridisation) has demonstrated that the presence and magnitude of components of the plasminogen activator system (tPA, uPA, PAI-1 and uPAR) in the vessel wall are dependent on - the type of vessel (artery or vein), whether the vessel has been surgically distended or not, and the presence of intimal hyperplasia either in the context of arterial atheroma or vein graft stenosis. It was observed that the endothelial cells in all the vessels mentioned were immunopositive for all four of the proteins. The differences between the vessel types therefore, laid mainly in the other cell types i.e. the smooth muscle cell phenotype, the presence or absence of macrophages and lymphocytes, as well as the staining of the extracellular matrix.

Discussion: Effect of surgical distension on the immunohistological findings in saphenous veins

Saphenous veins are distended during their preparation as vascular conduits in coronary artery bypass grafting. The distension is done by the surgeon after the vein has been removed from the leg by tying off one end of the vein and forcibly injecting heparinised saline (into which some of the patient's blood is added) into the other. It has been shown that gentle but uncontrolled manual distension of the saphenous veins generates surprisingly high intra-luminal pressures of greater than 600mmHg (Hasse et al 1981), and its detrimental effect on the vessel wall has been documented (Ramos et al 1976, Hofer et al 1981, Angelini et al 1987). It is likely therefore, that pressure-distension of the vessel results in the fissuring of the endothelial lining with the resultant introduction of luminal contents (i.e. heparinised saline mixed with the patient's plasma) into the

extracellular spaces of the vessel wall. Staining with H&E and immunostaining for both CD31 and vWF demonstrated that the vessel retained its endothelium and indeed the distended saphenous veins continued to show their immunopositivity to tPA, uPA, PAI-1 and uPAR on the endothelium after vessel distension. Interestingly, surgical distension resulted in an increase of immunopositivity to all four proteins within the media as is shown in Table 2.3.1. It is likely that the increase in these proteins in the vessel wall is a result of the extravasation of plasma containing these proteins into the extracellular spaces. tPA, uPA, and PAI-1 are known to exist in the plasma and a soluble form of uPAR has been described (Pedersen et al 1993). It is therefore possible that the presence of smooth muscle cell immunopositivity seen in the undistended veins could have also result from extravasation, as the vessel is known to undergo trauma during its mobilisation, dissection and handling. Although in theory the increase seen on distension could be explained by an increase in local production, the time between the surgical distension and tissue fixation was relatively short, i.e. approximately 15 minutes on average. In fact, when small pieces of these vessels (before distension and 15 minutes after distension) were used in quantitative RTPCR assays (see Chapter 3), the level of PAI-1 and uPA mRNA were found to decrease as a percentage of the total RNA extracted from the vessels as a result of distension (see Section 3.3.3 a). tPA and uPAR mRNA levels were not measured in these vessels and therefore the assertion that the increase in these two proteins in the vessel wall after distension may be due to extravasation is based on extrapolation. It has been shown that vessel injury *in-vivo* results in the increased synthesis of tPA, uPA (Clowes et al 1990) and PAI-1 mRNA (Sawa et al 1994). However the time interval between injury and sacrifice of the animal was longer than the above mentioned 15 minutes. In other words there may be

an initial loss of mRNA of these proteins, possibly by loss of some endothelial cells as a result of the injury, followed by an increase in synthesis as the areas of endothelial cell loss recovers and the smooth muscle cells increase their synthesis. It is clearly not possible to test this hypothesis in human saphenous veins *in-vivo.*

The mRNA *in-situ* hybridisation studies demonstrated that the signal for PAI-1 mRNA was present in both distended and undistended veins. This signal was associated with both endothelial cells and smooth muscle cells. It was not possible to quantify any differences in the strength of the signal between distended and native vessel. In spite of using the same technique under a range of *in-situ* hybridisation conditions, the presence of tPA and uPA mRNA was not detected. There are several possible explanations that could have accounted for this. It is possible that my *in-situ* hybridisation method was not sensitive enough since samples of the same vessels demonstrated the presence of tPA and uPA mRNA using RTPCR on RNA extracted from vessel homogenates. It could however be argued that whole vessel homogenates may contain a few blood cells e.g. neutrophils and monocytes which are known to synthesize components of the plasminogen activator system and this would have been enough the produce a PCR band due to the extreme sensitivity of PCR.

In summary therefore, surgical distension of saphenous veins increases the magnitude of all four proteins in the vessel wall as measured semiquantitatively by immunohistology. However, both uPA and PAI-1 mRNA were found to decrease soon after distension as measured by quantitative RTPCR (see Chapter 3). Surgical distension may therefore alter the extracellular proteolytic potential within the vessel

wall. This may have implications for the closure of the grafts due to both acute thrombosis as well as the vein graft stenosis due to intimal hyperplasia.

Discussion: Comparison of the immunohistological findings between undistended saphenous veins and internal mammary arteries.

Both saphenous veins and internal mammary arteries are used as vascular conduits for coronary artery bypass grafts and their attrition rate differs both in terms of subacute thrombotic closure as well as late closure. It would therefore be instructive to compare these two vascular conduits. Saphenous veins (undistended and distended) and internal mammary arteries both demonstrated that all four proteins (tPA, uPA, PAI-1 and uPAR) were associated with endothelial cells of the main lumen and the vasa vasorum. Similarly, tPA, uPA and PAI-1 but not uPAR was associated with the smooth muscle cells of the media of both undistended saphenous veins and internal mammary arteries. Immunohistologically, as far as these four proteins are concerned, it would appear that internal mammary arteries and undistended (but not distended) saphenous veins are similar. It is interesting to speculate on whether, distending saphenous veins, with its consequent alterations in the amount of these four proteins and hence resulting difference between the two vascular conduits, could help in explaining their differential attrition rates for both early and late vessel closure.

Discussion: mRNA *in-situ* hybridisation (tPA, uPA, PAI-1) and immunohistology (tPA, uPA, PAI-1 and uPAR); differences between atheromatous and non-atheromatous arteries

Internal mammary arteries used for coronary artery bypass grafting are usually free of

atheroma. On the other hand, coronary and carotid endarterectomies are performed on their respective vessels because there is a significant amount of atheroma present. Therefore these vessels were ideal for comparing atheromatous and non-atheromatous arteries with respect to the presence and localisation of tPA, uPA, PAI-1 and uPAR proteins in the vessel wall.

Using the technique of mRNA *situ* hybridisation, it was found that unlike normal internal mammary arteries, in which tPA and uPA mRNA were not demonstrated, there was detectable signal for these two mRNA species within the endarterectomy samples associated with endothelial cells and smooth muscle cells. Areas within the atheroma rich in macrophages as detected by CD68 immunopositive cells also demonstrated signal for tPA, uPA and PAI-1. Although PAI-1 mRNA was detected in both normal and atheromatous vessel walls, the atheromatous vessels consistently contained much more signal for PAI-1. In atheromatous specimens (both coronary and carotid) the synthesis of these molecules appeared to be smooth muscle cells, endothelial cells as well as macrophages. The PAI-1 mRNA signal in internal mammary arteries appeared to come from both the endothelium and smooth muscle cells. These mRNA findings are in keeping with previous work (Lupu et al 1995, Schneiderman et al 1995).

The immunohistochemical study on atheroma confirmed the associations of tPA, uPA, PAI-1 and uPAR antigens with endothelial cells, neointimal smooth muscle cells, and macrophages. The immunostaining for these antigens were much less (and in the case of uPAR, absent) in smooth muscle cells of normal internal mammary arteries. The relatively acellular parts of the atheromatous tissues demonstrated the presence of all

four proteins suggesting their secretion into the extracellular matrix. This concurs with previous published work on tPA, uPA and PAI-1 (Lupu et al 1993, Padro et al 1995). The finding for uPAR in atheroma is largely similar to that described in the only publication looking into the presence of uPAR in atheroma (Noda-Heiny et al 1995). However, the presence of u-PAR protein in acellular areas of atheroma has not been previously described. It is however in keeping with the finding of a ligand-free soluble form of uPAR (Pedersen et al 1993), which lacks the lipid moiety of the glycolipid anchor present in the cellular-bound uPARs. The function of the soluble form of uPAR is not known. Higazi et al 1995, has suggested that the binding of scuPA to its soluble uPAR results in the enhancement of the enzymatic activity and it may also play a role in removing uPA from the extracellular spaces. The mechanism by which uPAR works in the atheromatous lesion could be that the cell membrane-bound u-PAR localises u-PA and concentrates the extracellular proteolysis in the immediate vicinity of the smooth muscle cell membrane allowing the extracellular matrix to be dissolved in a controlled manner as the smooth muscle cell migrates into the space created. The soluble uPAR could be acting to remove uPA from the extracellular spaces.

Discussion: Immunohistology (tPA, uPA, PAI-1, uPAR) and mRNA in-situ hybridisation (tPA, uPA, PAI-1); differences between normal saphenous veins and saphenous veins graft stenosis

Human saphenous veins that had been used as arterial grafts to bypass occlusions in leg arteries and coronary arteries were examined. Saphenous veins when used as arterial bypass grafts are known to undergo intimal hyperplasia, atheromatous change and vessel remodelling over a period of months to years which results in graft stenosis or

occlusion. The samples of saphenous vein graft stenoses examined showed clear evidence of significant intimal hyperplasia and vascular remodelling with the overall vessel diameter being greatly increased. However none of these saphenous vein graft stenotic vessels demonstrated any classical atheromatous lesions e.g. cholesterol clefts, fibrous cap overlying a lipid pool, and indeed, although macrophages were present, they were very sparse unlike the atheromatous changes in endarterectomies described above. It was evident therefore that these saphenous vein grafts failed because of the development of a very thick neointimal layer of smooth muscle cells rather than the development of classical atheromatous lesions. Indeed the histological appearance was very similar to that of balloon injured normal arteries with the preponderance of both spindle-shaped and stellate-shape smooth muscle cell phenotypes in the neointima (See section 7.3.2). Even though these saphenous veins had undergone significant changes, it was still possible to distinguish the different layers, and in particular, it was possible to see where the initial media was, and thus from where the smooth muscle cells presumably originated to produce the neointima.

Whilst the histology of saphenous vein grafts that have undergone stenosis have been previously described (Dilley et al 1988, Davies et al 1994, Cox et al 1991) and is in keeping with the above mentioned findings, the expression of components of the plasminogen activator system in this type of vessels has not been previously published.

mRNA *in-situ* hybridisation demonstrated that unlike undistended saphenous veins, grafted saphenous veins that had undergone graft stenosis demonstrated signal for tPA and uPA mRNA in both smooth muscle cells and endothelial cells. PAI-1 mRNA

signal was predominantly and strongly associated with endothelial cells with very little signal being associated with the smooth muscle cells, see fig 2.3.4 (j). This may suggest that tPA and uPA play a role in the genesis of the neointima. This would be in keeping with previous data in the rat carotid injury model of intimal hyperplasia which suggests that tPA and uPA may be important for mitogenesis and migration of vascular smooth muscle cells (Clowes et al 1990).

The immunohistological findings were in keeping with the in-situ results. Smooth muscle cells of the neointima and endothelial cells of the main lumen and the vasa vasorum were immunopositive for all four proteins. The smooth muscle cells of the media were generally immunonegative. It was common to find the smooth muscle cells that were located just internal to the media, orientated with their long axes perpendicular to the endothelium and strongly immunopositive for tPA, uPA and uPAR. It is interesting to speculate that these smooth muscle cells may be migrating into the neointima and the strong immunopositivity may suggest that these components of the plasminogen activator system are important for regulating the migration.

Discussion: Specificities of the antibodies used

In the immunohistochemical studies performed, the monoclonal antibodies to tPA or to uPA were able to bind to tPA or uPA respectively whether or not the latter two were complexed to PAI-1. Similarly, the monoclonal antibody used against PAI-1, was able to bind to PAI-1 whether or not it was complexed to either tPA or uPA. Finally, the monoclonal antibody against uPAR was able to bind to uPAR whether or not it was bound to uPA. In other words the monoclonal antibodies used looked at total content

of tPA, uPA, PAI-1 and uPAR proteins within the vessel wall. The recent characterisation of two neoantigenic epitopes generated within the PAI-1 molecule after it forms a complex with either tPA or uPA, but not in native PAI-1 (Debrock et al 1995), should facilitate further study into whether the PAI-1 protein observed in the vessel wall is complexed to tPA or uPA.

CHAPTER 3

QUANTITATION OF mRNA TO COMPONENTS OF THE PLASMINOGEN ACTIVATOR SYSTEM IN HUMAN BLOOD VESSELS USING QUANTITATIVE RTPCR

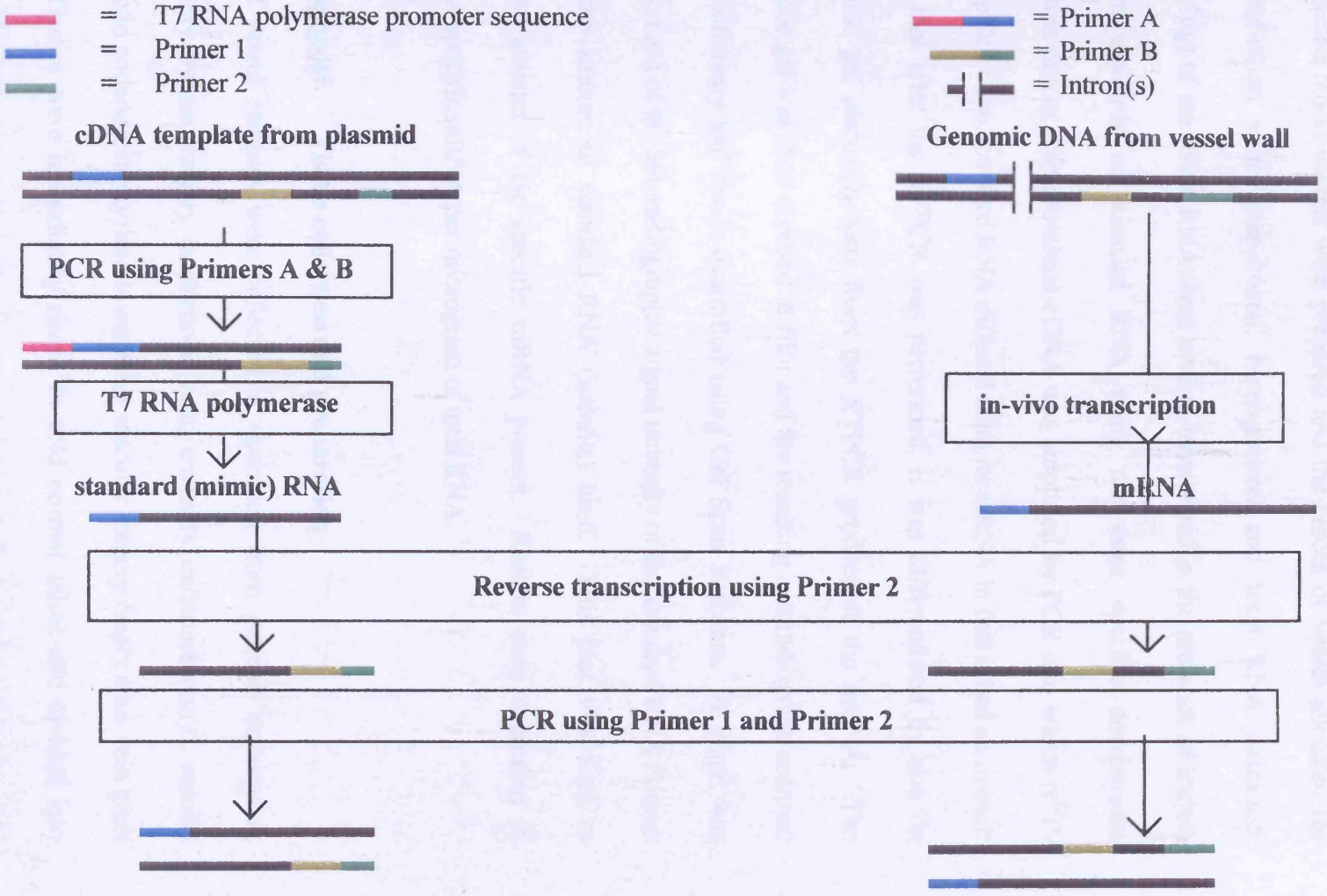
3.1 INTRODUCTION

Rationale for using quantitative RTPCR to measure expression

Due to the small amounts of vascular tissue in each blood vessel sample, the relatively small amount of extractable RNA, and the necessity to perform a number of other measurements on the same piece of vessel wall (including measuring tPA, uPA, and PAI-1 activities and antigen levels, immunohistochemistry as well as mRNA *in-situ* hybridisation), it was essential to use a highly sensitive method of measuring the levels of specific mRNAs. This precluded the use of Northern blotting. Quantitative reverse transcriptase polymerase chain reaction assays offered a highly sensitive and specific method for measuring expression of specific genes (Mullis & Faloona 1987). Accordingly, it was this method that was chosen to quantitate the mRNA to uPA and PAI-1and to detect tPA and PAI-2 mRNA expression.

The quantitative RTPCR assays had to be developed starting essentially with specifically designed oligonucleotide primers and donated plasmids containing specific sequences of cDNA. The quantitative RTPCR then had to be validated. Donated plasmids containing almost full-length specific cDNA fragments were amplified in competent bacteria in order to have enough material for the project. Some of this amplified cDNA was further amplified in a PCR using two composite primers. One composite primer contained the T7 RNA polymerase promoter sequence whilst the other contained an in-built deletion (see next page for strategy). The resultant DNA template was then used to make the RNA internal standards by the method of *in-vitro* transcription. The RNA internal standard was subsequently used in the QRTPCR assay.

3.1.1 Introduction : Overall design strategy of the quantitative RTPCR



3.2 METHODS

3.2.1 Overview

Freshly collected blood vessels were prepared and the pieces of vessel allocated for mRNA quantitation were snap-frozen, homogenised and total RNA extracted. Aliquots (0.5μg) of the tissue RNA were reverse-transcribed in the presence of known but different amounts of standard RNA using the same specific downstream oligonucleotide primer. The resultant cDNA was amplified by PCR into which α^{32}P-dCTP was spiked. The standard RNA differed from its mRNA in that it had an in-built deletion so that after the RTPCR was performed, it was differentiated by size on polyacrylamide gel electrophoresis from the RTPCR product of the mRNA. The polyacrylamide gel was then exposed to film and the resulting autoradiograph scanned by laser densitometry and bands quantified using Gel Scan Software. A graph was plotted of the ratio of the autoradiographic signal intensity of the standard RNA / tissue mRNA *vs* the amount of standard RNA (attmols) used. This plot was used to determine the amount of the specific mRNA present. Results were expressed in attmols of the specific mRNA per microgram of total RNA.

3.2.2 Methods: Tissue collection and preparation

Fresh blood vessel samples were collected at operation from patients undergoing coronary artery bypass surgery (saphenous vein, coronary endarterectomy), carotid surgery (carotid endarterectomy) and lower limb vascular surgery (saphenous vein graft stenosis). Tissues were immediately rinsed in cold normal saline and divided into segments for the different studies. Tissues allocated for mRNA analysis were placed in a clean bijoux, snap-frozen in liquid nitrogen, and stored at -80°C until needed.

3.2.3 Methods: Tissue homogenisation

Owing to the short length of the blood vessel samples available from the routine operations it was necessary to optimise its yield of RNA. Initially, different methods of tissue homogenisation were assessed. Manual homogenisation was done using a glass Teflon plunger for 5 mins. Homogenisation was also carried using an electrical homogeniser at 6000 rpm for 3 minutes.

3.2.4 Methods: RNA extraction from human blood vessels

Total RNA was extracted using the RNAzol B method (Chomczynski et al 1987) with slight modifications as outlined below. The tissues stored at -80 °C were pre-cooled in liquid nitrogen just prior to weighing thus allowing them to be weighed without thawing. Immediately after weighing the tissues were replaced in the liquid nitrogen. The tissues were then homogenised in the presence of RNAzol; 60 mg tissue per 1.0 ml RNAzol solution. The homogenate in suspension was transferred to an Eppendorf tube and 100 µl chloroform added for each ml of RNAzol used, shaken vigorously and left on ice for five minutes. The tubes were centrifuged at 13000rpm for 15 minutes and the clear aqueous supernatant transferred to fresh Eppendorf tubes. An equal volume of chloroform was added to each tube, vortexed for 30 seconds, placed on ice for 5 minutes then centrifuged at 13000rpm for 5 minutes. The clear supernatant was chloroform extracted once more as above. The resulting supernatant was transferred to a fresh Eppendorf tube and an equal volume of isopropanol added. The tubes were shaken gently and left on ice for 1 hour to precipitate the RNA, then centrifuged at 13000rpm for 15 minutes. The supernatant was discarded and the RNA pellet washed in 70% alcohol, centrifuged, and the supernatant discarded. The RNA pellets were left

to partially dry then dissolved in 400 µl of 1 mM EDTA (pH 7). The RNA was re-precipitated with an equal volume of isopropanol in the presence of 0.3 M sodium acetate at -20 C for 1 Hr, then centrifuged at 13000 rpm for 15 minutes. The pellet was washed in 70% alcohol as before, and finally dissolved in sterile DEPC-treated water. The RNA in solution was then ready for spectrophotometric analysis and reverse transcription.

3.2.5 Methods: Assessing extracted RNA; yield, purity and integrity

A measured amount of RNA solution was diluted in 1 ml of sterile water and the OD 260 (optical density at 260 A) was measured on a spectrophotometer (Phillips PU 8620). The concentration of the RNA in solution was calculated from the equation:

OD 260 of 1 = 40 µg RNA

The RNA purity was determined by the OD 260/280 ratio whilst its integrity was assessed by running 5µg of the RNA on a 0.8% agarose gel at 50 Volts for 1 hour in TAE Buffer. The RNA was observed with ethidium bromide staining under UV light.

3.2.6 Methods: Storage of RNA

Extracted RNA were stored as 2.5µg aliqouts at -80°C until needed and used once only when thawed.

3.2.7 Table: Structure of the plasmids used

cDNA	Plasmid	cDNA fragment	Restriction enzyme used	Bacteria
TPA	pSP65	607 base pairs	ECoR1 and Hind III	TG1
TPA	pBR322	2.0 kilobase pairs	Bgl II	NM522
UPA	pSP64	1426 base pairs	Hind III	TG1
PAI-1	pUC	2.5 kilobase pairs	Eco R1	TG1

The amplified cDNA sequences were used for the following tasks:

- To act as the positive control DNA template in the RTPCR assay.
- To provide a DNA template for optimising the PCR conditions.
- To act as a DNA template during the synthesis of the RNA internal standards.
- To act as a radiolabelled cDNA probe to:
    1) Hybridise with a Southern Blot of the PCR products produced in the RTPCR assay thus establishing the specificity of the assay.
    2) Hybridise with mRNA in the tissue sections as part of the histological *in-situ* hybridisation study.

The 2.5kb PAI-1 cDNA fragment inserted into a pUC plasmid (Ginsberg D et al 1986) was kindly donated by Dr D Ginsburg, Howard Hughes Medical Institute, USA.

3.2.8 Methods: Amplification of the plasmids

The four plasmids described above were amplified in E Coli TG1 to produce enough for this project.

3.2.8 (a) Preparation of competent cells

EColi (TG1) was streaked on selective plates overnight and a single colony was taken to inoculate 25 mls of LB medium. The latter was then incubated overnight with vigorous shaking. LB medium (500mls) was inoculated with 5 mls of cells from the overnight culture and the culture was vigorously shaken at 37°C until the A_{600} reached 0.45-0.55. The cells were chilled in ice water for 2 hours and collected by centrifugation at 2500g for 15 minutes at 4°C. The cells were resuspended in 15 mls of ice-cold freshly prepared, filter-sterilised trituration buffer (100 mM $CaCl_2$, 70 mM $MgCl_2$, 40mM Na Acetate, pH 5.5), diluted to 500mls with the same solution then incubated on ice for 45 minutes. The cells were then centrifuged at 1800g for 10 minutes and gently resuspended in 50 mls of ice-cold trituration buffer. Glycerol (80%) was added dropwise with gentle swirling to a final concentration of 15%(v/v). Aliquots in 1ml quantities were stored at -80°C until needed.

3.2.8 (b) Transformation of competent bacteria

A 200µl aliquot of competent TG1 cells was thawed on ice and 3 µl of DMSO added, mixed gently, and 20 ng of plasmid DNA added. The cells were incubated on ice for 30 minutes, then heat-shocked at 42°C for 90 seconds, followed by cooling on ice for 1 minute. LB medium (2mls) were added and shaken gently at 37°C for 1 hour to allow the cells to recover.

3.2.8 (c) Selection of transformants

To select for transformants using plasmids pSP64/65, the cells were plated on an LB plate containing 50µg/ml ampicillin and incubated at 37°C overnight. To screen

ampicillin-selected colonies for recombinants it was necessary to prepare plasmid DNA for restriction analysis.

3.2.8 (d) Amplification and isolation of the plasmids

10 µl of the stored TG1 was added to 10 mls of LB Broth in a Universal container, incubated overnight at 37°C with vigorous shaking then centrifuged at 2500rpm for 15 minutes. The supernatant was discarded to leave the pellet of cells which were resuspended in 100 µl of ice-cold Miniprep lysis buffer (Promega) (25mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose.) and incubated at room temperature. A freshly prepared solution (200 µl) containing 0.2M Na OH, 1% SDS was added and gently mixed, 150µl of ice-cold 5M potassium acetate (pH 4.8) added, mixed gently and incubated for 5 minutes on ice to precipitate the proteins then spin fastspeed for 5 minutes. The supernatant was transferred to a fresh tube, avoiding the white precipitate, RNAse A at a final concentration of 20 µg/ml was added, incubated at 37°C for 20 minutes then it was phenol/chloroform then chloroform:isoamyl alcohol (24:1) extracted. The DNA in solution was precipitated with 2.5 volumes of ethanol on dry ice, centrifuged at 12000g for 5 minutes, and the pellet washed in pre-chilled 70% ethanol. The pellet was dried under vacuum then dissolved in 30µl DEPC water.

3.2.9 Methods: Restriction digestion of the plasmids

The plasmids were digested by their respective enzymes as shown in Table 3.2.7.

3.2.9 (a) pSP65 containing tPA cDNA

The tPA cDNA sequence contained 607 basepairs. This sequence had been previously cut out of a fuller length cDNA sequence using Hpa II restriction enzyme that

recognises -:

C|CGG

GGC|C

The plasmid had been linearised using Acc1 which recognises -:

GT|CGAC

CAGC|TG

A ligase was used to insert the 607 bp tPA cDNA sequence into the linearised plasmid thus abolishing the restriction sites for Acc1 as well HpaII. Consequently, HindIII and EcoR1 were therefore used to release the tPA cDNA sequence from the plasmid. Conditions for releasing the tPA cDNA from the plasmid-:

STEP 1 The following were added to an Eppendorf tube:

Plasmid	2 µg
React 2 Buffer	2 µl
Water	14 µl
HindIII	1 µl

Incubated for 2 hours at 37°C

STEP 2 Add:

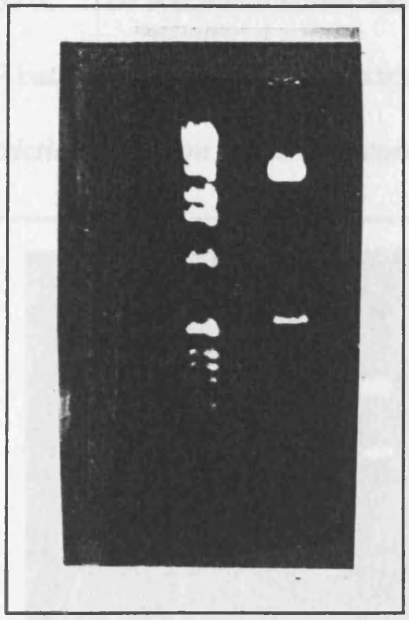
NaCl (1M)	0.5 µl
EcoR1	1.0 µl

Incubated for a further 2 hours at 37° C

STEP 3

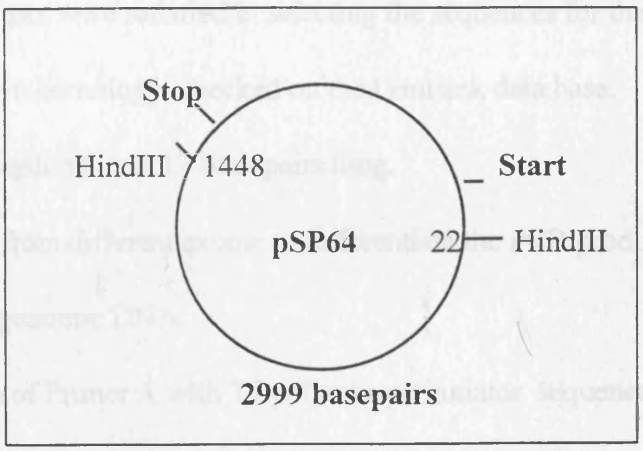
The reaction mixture was run on a 1.5 % agarose gel and the tPA fragment (607 base pairs) was Sephaglas extracted; see section 3.2.11 (c).

EcoR1/HindIII digest of pSP65 containing tPA cDNA



3.2.9 (b) pSP64 containing uPA cDNA

In the case of pSP64 containing uPA cDNA, a HindIII digest of the pSP64 plasmid resulted in the release of the 1427 basepaired uPA cDNA sequence.

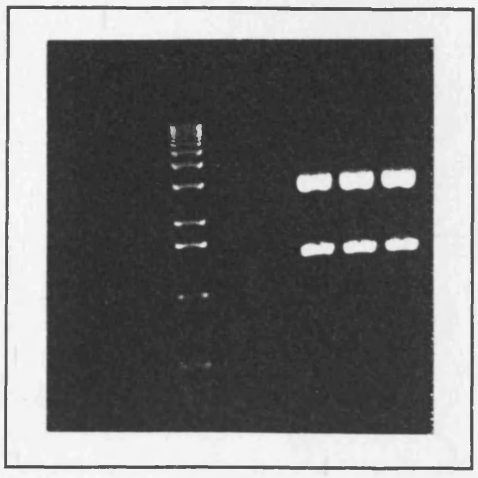


The following were added to an eppendorf tube for the Hind III digestion;

Plasmid	2 μg
React 2 Buffer	2 μl
Water	14 μl
HindIII	1 μl

Incubated for 2 hours at 37°C. The reaction mixture was run on a 1.5 % agarose gel and the tPA fragment (607 base pairs) was Sephaglas extracted; see section 3.2.11 (c).

HindIII restriction digestion of the uPA containing pSP 64



3.2.10 Methods: The oligonucleotide primers

3.2.10 a) Designing the oligonucleotide primers :

The following factors were satisfied in selecting the sequences for the primers used:

1. Lack of known homology: checked on the Genbank data base.
2. Minimum length: at least 17 base pairs long.
3. Primer pairs from different exons: to differentiate the PCR products of cDNA from that of genomic DNA.
4. Modification of Primer A with T7 polymerase initiator sequence; to enable the *in-*

4. Modification of Primer A with T7 polymerase initiator sequence; to enable the *in-vitro* transcription of the PCR product to synthesize the RNA standard.
5. Modification of Primer B with built-in deletion; to enable differentiation between tissue cDNA and standard DNA.
6. 50-60% G+C composition with balanced Tm for a given primer pair.
7. Avoidance of complimentarity at the 3' ends of primer pairs to decrease primer-dimer formation.

3.2.10 b) ***Primer sequences:***

3.2.10 b) (i) TISSUE PLASMINOGEN ACTIVATOR - (TPA)

(cDNA sequence; Pennica et al 1983)

tPA - Primer A (45 Base pairs)

| >>>>>>>>> RNA synthesis >>>>>

5'TAATACGACTCACTATAGGGACACCCCTGGCAGGCTGCCATCTTT 3'

T7 PROMOTER 1051-1072

tPA - Primer B (38 Base pairs)

5'GTGTCTCCAGCACACAGCATGTCATTGTCGTAAGTGTC 3'

1571-1550 1301-1285

tPA - Primer 1 (21 base pairs) Tm 68°C

5' CCCTGGCAGGCTGCCATCTTT 3'

1051-1071

tPA - Primer 2 (21 base pairs) Tm 66°C

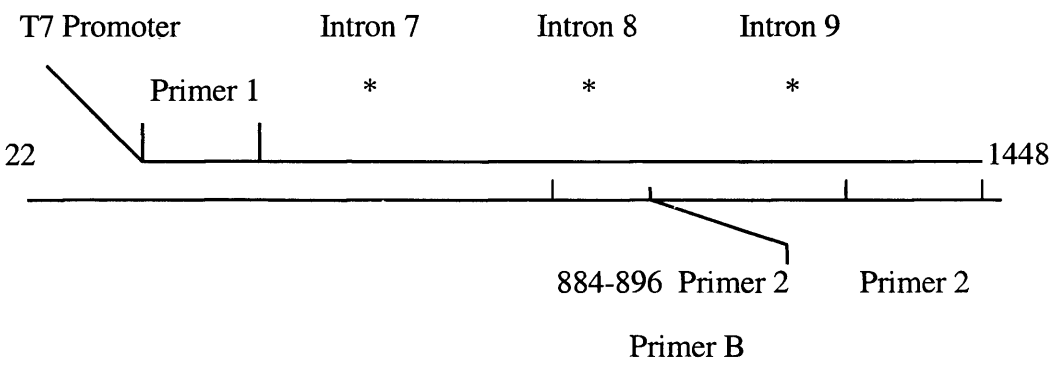
5' GTGTCTCCAGCACACAGCATG 3'

1571-1551

Primers 1 and 2 are on different exons of the tPA gene. These primers were used in the quantitative RT-PCR to amplify cDNA from both the tissue and the RNA Standard resulting in products of 522 and 274 base pairs respectively.

3.2.10 b) (ii) UROKINASE-TYPE PLASMINOGEN ACTIVATOR

(cDNA sequence; Holmes et al 1985.)



uPA PRIMER A

CTGTAATACGACTCACTATAGGGGGCACCGG

|------| T7 PROMOTER

649--651 672-679

uPA PRIMER B

TCCGGATAGAGATAGTCGGTGTGGTGAGCAAG

1065-1047 (Primer 2) 896-884

uPA PRIMER 1 (19 bp) Tm 60 C

GCTCTGTCACCTACGTGTG

684-702

uPA PRIMER 2 Tm 58 C

TCCGGATAGAGATAGTCGG

1065-1047

PCR PRODUCTS: NATIVE 382 bp, STANDARD 232 bp, DELETION 150 bp

3.2.10 b) (iii) PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1)

(cDNA sequence; Ginsburg et al 1986)

PAI-1 PRIMER A

5'AACCCAGCAGCTAATACGACTCACTATAGGGGTTC 3'

|----------|

|----------| T7 PROMOTER |------|

180-190 211-213

PAI-1 PRIMER B

5'CTGGCCGTTGAAGTAGAGGCTGGCTCTCTC 3'

|----------|

543-525 |----------|

(PAI-1 Primer 2) 419-408

PAI-1 PRIMER 1 (20bp) Tm 60 C

5'GGAACAAGGATGAGATCAGC 3'

|----------|

278-297

PAI-1 PRIMER 2 (19bp)

5'CTGGCCGTTGAAGTAGAGG 3'

|----------|

543-525

PCR PRODUCTS: NATIVE 266, STANDARD 161

3.2.10 b) (iv) PLASMINOGEN ACTIVATOR INHIBITOR-2 (PAI-2)

(cDNA sequence; Schleuning et al 1987)

PRIMER A Tm 72 C Fullmatch/Length = 25/34

GCTAGTAATACGACTCACTATAGGGTCAAGACTC

|----------|T7 PROMOTER|----------|

524-528 549-557

PRIMER B Tm 74 C Fullmatch/Length = 24/32

CTTCATCCACCATGGCTTGGTGGGTCCACTTG

|-------|PRIMER 2|----------|

938-935 916-907

PRIMER 1 (18bp) Tm 56 C

ATACCAGGATGGTCCTGG

|----------|

609-626

PRIMER 2 (18bp) Tm 56 C

ATCCACCATGGCTTGGTG

|----------|

1114-1097

PCR PRODUCTS:

NATIVE 506 Bases

MUTANT 326 Bases

3.2.10 c) Measuring the primer concentrations

Oligonucleotide primers were precipitated at -20°C in ethanol and 0.3 M sodium acetate and washed in 75% ethanol. The pellet was resuspended in DEPC-treated water and the concentration measured spectrophotometrically:- OD 260 of 1 = 20 μg oligonucleotide primer/ml . Aliquots of working dilution of 10 pmols/μl were made.

3.2.11 Methods: Synthesis of RNA internal standards: uPA and PAI-1

Overview

A series of PCRs were done using the plasmids (with their respective cDNA fragments) and the respective composite primers A and B (See Fig 3.1) to form the DNA standard templates which were then used to make the respective RNA standards. The PCR products were run on an ethidium-stained agarose gel, the desired band cut out under UV visualisation and the DNA and extracted using the Sephaglas kit™. The DNA template was *in-vitro* transcribed using T7 RNA polymerase to synthesize the RNA standard and then digested with DNase. The reaction solution was then phenol / chloroform- then chloroform-extracted and the RNA was precipitated in 2.5 volumes of ethanol on dry ice. The pellet was washed in chilled 70% ethanol and after spinning the pellet was dissolved in DEPC treated water. The concentration of the RNA standards were measured spectrophotometrically at OD260 and aliquots of the RNA standards were measured in batches of 10 picograms to use once only when thawed.

3.2.11 (a) Synthesis of the DNA standard templates; PCR using composite Primers A and B

A PCR was set up :

Plasmid with its cDNA	2.0	pg
10x PCR buffer(Bioline)	6.0	µl
$MgCl_2$(xmM)	1.8	µl
Taq polymerase(Bioline)	0.6	µl
Primer A	45.0	pmols
Primer B	45.0	pmols
dNTP (10mM mix)	4.5	µl
Sigma Water made up to	60.0	µl
TOTAL	60.0	µl

The respective Primers A and B were used in the above PCR with the relevant plasmid e.g. PAI-1 Primer A and PAI-1 Primer B was used in conjunction with the plasmid containing the PAI-1 cDNA fragment.

PCR PRODUCT USING PAI-1 PRIMERS A AND B.

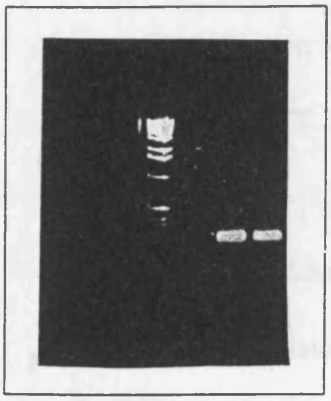
----------|----------|- - - - -|---------|----------|

180-190 T7 PROMOTER 211-213- - - - -(408-419)(525-543)

[----------262 base pairs----------]

PCR products of primers A and B

PAI-1 (262 bp) and uPA (266 bp)



tPA (293 bp)

3.2.11 (b) Agarose gel electrophoresis of the DNA template

The PCR products of the above PCR was run on a 2% agarose gel containing ethidium bromide at 70 Volts for 1 hour in 1 X TAE buffer. The band was visualised under UV light and excised.

3.2.11 (c) Extraction of the DNA standard template band from the agarose gel

The DNA gel-band excised from the ethidium stained agarose gel under UV light was chopped up and placed in an Eppendorf tube. Gel Solubiliser (250µl) (Sephaglas) was added and the tube was vortexed vigorously until the agarose was dissolved. 10µl Sephaglas BP was added, vortexed gently and incubated at room temperature for 5 minutes (vortexing every minute). The tube was then pulse-spun in a microfuge for 10 seconds and the supernatant discarded. The pellet was suspended in 80µl 'Wash Buffer' and pulse-spun for 10 seconds. The washing procedure was repeated twice. The pellet was then partially dried in air and 20µl 'Elution Buffer' was added, vortexed gently and spun at 12000 rpm in a microfuge. The supernatant containing the DNA in solution was carefully removed and stored at -20°C until needed.

3.2..11.(d) T7 RNA polymerase in-vitro transcription of the DNA standard template (tPA, uPA and PAI-1)

Transcription 5 X Buffer (Promega)	20	µl
100mM DTT	10	µl
RNA guard	100	units
NTP Mix (2.5mM each)	20	µl
DNA Template in water	5	µg
T 7 Polymerase Gibco BRL	30	units
Water to final volume of	100	µl

The above transcription reaction was incubated at 37°C for 1 Hour.

3.2.11.(e) Conditions for the DNase digestion

RNase-free DNase (1 µl; Sigma) was then added and incubated for a further 20 minutes at 37°C. This was necessary to digest any contaminating DNA.

3.2.11.(f) Phenol/chloroform extraction of the RNA standards

The cRNA was phenol/ chloroform extracted, precipitated with 2.5 volumes of ethanol, washed in 75% ethanol and finally dissolved in RNase-free water.

3.2.11(g) Measuring the concentration of the RNA standards:

An aliquot of the RNA solution was used for the spectrophotometric determination of the RNA concentration: -

OD 260 of 1 = 40µg RNA / ml.

Finally, the RNA solution was divided into 2.5μg aliquots at a concentration of 0.25 μg/μl, and stored at -80°C until needed.

3.2.12 Methods: Optimisation of the PCR conditions; using plasmids as the DNA templates

Before setting up polymerase chain reactions using Primers 1 and 2 on cDNA derived from blood vessels, it was necessary to work out the conditions so as to obtain a single clear band as the PCR product. The optimum PCR conditions for amplifying the specific DNA sequences (i.e. PAI-1, uPA and tPA) were determined by setting up PCRs using the relevant plasmid which has the cDNA sequence within it as the DNA template. The variables shown below were assessed in turn in a series of PCRs. The effect of the magnesium concentration was assessed in the range 1.0-5.0 mM final concentration and the minimum concentration that resulted in a clear band of PCR product was chosen as the optimum magnesium concentration. Primer concentrations were assessed in the range 0.25μM-2.0μM final concentration. Again the minimum primer concentration was chosen which resulted in a clear PCR product. The number of cycles was assessed in the range of 20-35 cycles to ensure that the PCR assay was in the exponential phase of the reaction. The annealing temperature was assessed from a few degrees below the calculated Tm of the primers to the temperature at which only the desired PCR product was obtained. The minimum amount of DNA template needed to obtain an easily detectable amount of PCR product was chosen.

The following parameters were found to be important-:

- Magnesium concentration.

- Primer concentration
- Number of cycles
- Annealing temperatures
- Denaturing the cDNA
- Amount of DNA template at start of PCR.

The optimal conditions for the PCR were as follows:

PAI-1 : 26 cycles

60°C annealing temperature

Primer concentration = 1.5 mM

Mg^{2+} concentration = 15.0 pmol

Amount of cDNA at the start of the PCR = 2 µl of the RT mixture.

u-PA : 31 cycles.

60°C annealing temperature

Primer concentration = 1.5 mM

Mg^{2+} concentration = 15.0 pmol

Amount of cDNA at the start of the PCR = 2 µl of the RT mixture

3.2.13 Methods: RT-PCR pilot study of mRNA expression in vessel wall

Using the primers 1 and 2 (shown in 2.4.6) a pilot RTPCR study was done to ascertain whether the specific mRNA expression (tPA, uPA, and PAI-1) could be demonstrated from the RNA extracted from blood vessels.

3.2.13.(a) Reverse transcription using the specific downstream primers: (Primer 2)

Vessel wall total RNA	0.5	µg
5 X Buffer (Gibco)	2.0	µl
0.1M DTT	0.2	µl
RNase Inhibitor	0.1	µl
MMLV enzyme	0.1	µl
dNTP (10 mM mix)	1.0	µl
Downstream Primer	10.0	pmols
DEPC Water made up to	10.0	µl

The tissue total RNA was heated to 92°C for 5 minutes under mineral oil (to denature the RNA) then the tube was quick-chilled on ice for 3 minutes. The rest of the reaction mixture was then added. After incubating at 37°C for 1 hour the MMLV (reverse transcriptase) enzyme was destroyed by incubating at 94°C for 5 minutes.

3.2.13 (b) Polymerase chain reaction of vessel wall cDNA

Vessel wall cDNA	2.0	µl	
NTP mix (2.5 mM each)	1.5	µl	
10 x Buffer (NH_4)	2.0	µl	
MgCl2 (50 mM)	0.6	µl	(1.5 mM final concentration)
Taq Polymerase	0.2	µl	
Upstream primer (10 pmol/ul)	15.0	pmol	(5 pmols for tPA)
Downstream primer (10 pmol/ul)	13.0	pmol	(5 pmols for tPA)
Water	10.8	µl	
TOTAL	20.0	µl	

Controls: 1) Water instead of cDNA

2) Water instead of Upstream primer

3) Water instead of the enzyme

3.2.13 c) Identification of the PCR product

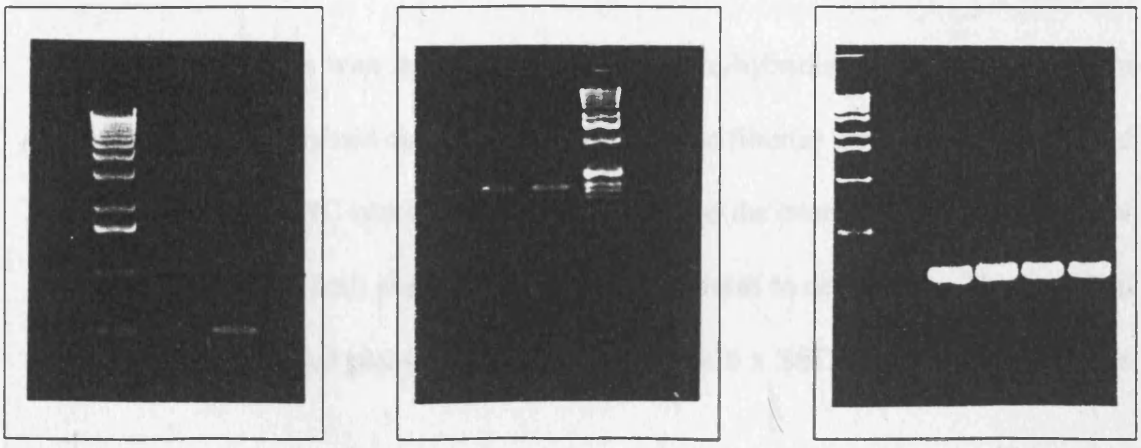
The PCR product was run on an agarose gel that was Southern blotted and hybridised with specific radiolabelled DNA probes. Finally, the PCR products were sequenced by the cycle sequencing method.

3.2.13 c) i Agarose gel electrophoresis of the vessel wall PCR product

The PCR products were run on a 2 % Agarose gel in TBE buffer at 50 volts. Ethidium bromide 5 ul was added to the boiled agarose solution (50 mls). 1KB ladder (Gibco BRL) was used as the DNA marker. In this way the size of the expected PCR product was confirmed.

Fig 3.2.13 c) RT-PCR products from vessel wall

tPA (522 bp) *uPA (382 bp)* *PAI-1 (266 bp)*



3.2.13 (c) (ii) Southern blotting

The agarose gel was denatured in 300 mls of Denaturing Solution (see Appendix) for two 15 minute periods on a shaking table, the solution being changed after the first period, rinsed in de-ionised water, placed in 300 mls of Neutralising Solution (see Appendix) for two periods of 15 mins, again the solution being changed after the first period, Southern blotted overnight onto a Hybond N filter using 20 x SSC as the Transferring Solution (see Appendix). The filter was then gently soaked in 6 x SSC to remove any debris, dried in air on 3MM paper for 1 hour, wrapped in Saran wrap and exposed to light from a UV illuminator for 1 minute to fix the DNA to the filter.

3.2.13 (c) (iii) ***Hybridisation of the Southern blots of the PCR products with radio-labelled cDNA probes***

Pre-hybridisation:

Pre-hybridising solution was freshly made up:

20 x SSC	9.0	mls
DEPC treated water	12.5	mls
PEG Mix	5.4	mls
Denhardts solution	1.5	mls

The Hybond N filters with their fixed DNA were pre-hybridised at 65 C for 3 Hrs in Hybaid bottles in a Hybaid oven. During this time the filter(s) were placed in a Hybaid bottle, 50 mls of 6xSSC added and the bottle placed in the oven at 65°C. An aliquot of salmon sperm DNA (1ml) was heated to 95 C for 10 mins to denature it. This was then added to the pre-heated prehybridising solution. The 6 x SSC was decanted from the

Hybaid bottle and was replaced by the prehybridising solution. The filters were prehybridised for 3 Hrs.

Labelling the cDNA probes:

The probe was labelled using the Radprime labelling kit (Gibco BRL) : To the 21 µl of denatured probe, the following were added in order:-

Radprime Buffer (+ Random hexanucleotides)	20	µl
AGT mix	3	µl
dCTP-P32	5	µl
Klenow fragment	1	µl

Incubated for 1 Hr at 37 C.

Purifying the radioactively labelled probe

The radioactively labelled probe was separated from the unincorporated dCTP (and the other dNTPs) by passing it through a G50 Sephadex column and collecting the first peak of radioactive eluate.

The hybridisation

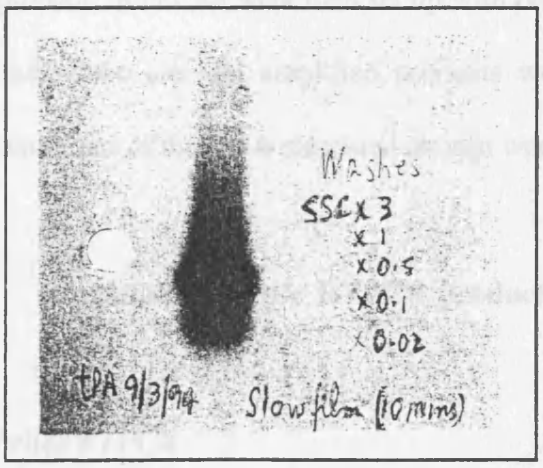
The hybridisation solution was made up as for the prehybridisation solution but in this case the pre-heated salmon sperm DNA was added to the labelled probe which was quickly added to the Hybaid bottle; the prehybridising solution being discarded. The hybridisation was done at 65°C overnight.

Stringency of washing the Hybond-N filters

The filter(s) were washed at 65 C using the following protocol;

3X SCC	15 mins
1X SCC	15 mins
0.1X SCC	15 mins
0.05 X SCC	5 mins

Confirmation of the vessel wall RT-PCR product by Southern blotting and hybridisation with specific cDNA probe



3.2.14 Methods: RTPCR of RNA standards

3.2.14 (a) Testing the RNA standards in a RTPCR

An RTPCR on the RNA standard synthesised by in vitro transcription was set up to establish that the expected PCR product was obtained.

3.2.14 (b) Establishing the range of concentrations needed of the RNA standards for the QRTPCRs

RNA standards were diluted to form a series of RNA concentrations ranging from 15 ng / µl to 0.1 fg/µl (i.e. 15x 10^6 dilution). Each of the RNA samples was reverse-transcribed to form cDNA which was then amplified by PCR. The conditions chosen for both the RT reaction and the PCR were identical to the conditions stipulated above for the respective tissue RNA reactions. The PCR products were loaded on an ethidium-stained agarose gel and electrophoresed and the intensity of the bands observed under UV light. A range of dilutions of the standard RNA was chosen so that the intensities of its PCR product spanned the range of intensities being obtained from the vessel wall PCR product. RTPCRs were then set up with both the tissue RNA and the standard in the same tube and the amplified products were again observed to confirm that the concentrations of the RNA standards chosen were appropriate.

3.2.15 Methods: Quantitation of the RTPCR products of both tissue and standard RNA.

3.2.15 a) Radio-labelled RTPCR

RT-PCRs were set up as described in 3.2.13 b) and 3.2.14 b), except that on these occasions α32 dCTP was spiked in the PCR. The radiolabelled PCR products were loaded on an 8 % polyacrylamide gel (see 3.2.15 a) and electrophoresed at 100 volts for 1.5 hours. The gel was placed on a piece of 3MM paper, covered by Saran wrap, and placed in a film cartridge in a dark room. The cartridge was placed in a -70°C freezer for 2 Hours then developed in an Agfa Currix machine. The Autoradiograph was then scanned on a LKB Laser Densitometer.

3.2.15 b) Polyacrylamide gel electrophoresis

8% Polyacrylamide gel :	10 X TBE	2	mls
	Water	14	mls
	40 % Polyacrylamide	4	mls

De-gassed for 15 minutes by stirring under vacuum and the following were added:

10 % APS	140	µl
TEMED	7	µl

Stirred gently and poured between the plates. The gels were run at 50 Volts for 2 $^{1}/_{2}$ hours on a mini gel kit (BioRad).

3.2.15 c) Radio-labelling 1kb DNA ladder.

In order to check that the amplified DNA were of the expected size, the radio-labelled PCR reaction products were run on a 8 % polyacrylamide gel with radiolabeled marker. The following were added to a 0.5 ml microcentrifuge tube on ice:

10X T4 DNA polymerase reaction buffer	1.0	µl
1 Kb DNA Ladder	2.5	µg
T4 DNA polymerase	10.0	units
Autoclaved water made up to	10.0	µl

The mixture was incubated for 2 minutes at 37 C then cooled on ice. Added to the above reaction mixture was a 30 ul mixture comprising of the following components:

Autoclaved water	23	µl
10X T4 DNA polymerase reaction buffer	3	µl

dATP (10mM)	1	µl
dGTP (10mM)	1	µl
dTTP (10mM)	1	µl
[α-^{32}P] dCTP (3000 Ci/mmol; 10 mCi/ml)	1	µl

The mixture was incubated for 2 minutes at 37 C, then 1 µl of 10 mM dCTP was added and incubated for a further 2 minutes. The reaction was stopped by adding 2.5 µl of 0.5 M EDTA. The tube was centrifuged for 10 seconds.

3.2.15 d) Autoradiography of the RTPCR products

The gels were placed on 3MM paper and covered in Saran wrap, exposed to Kodak film in a dark room. The film was then developed in an Agfa Currix machine.

3.2.15 e) Scanning of autoradiographs

The PCR DNA bands on the autoradiograph were scanned by laser densitometry on an LKB Laser Densitometer.

3.2.15 f) Analysis of scans

The signals detected were analysed by Gel Scan XL 2400 LKB-Produkter AB to give a numerical value proportional to the signal intensity of the band.

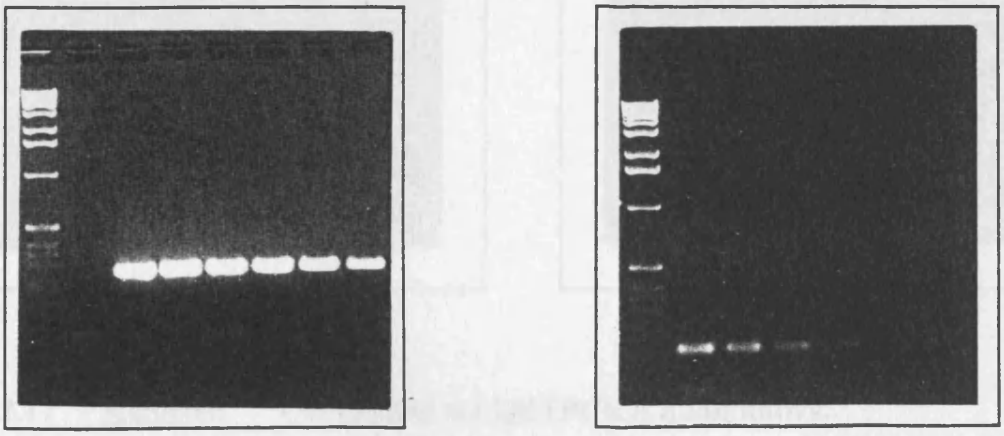
3.2.16 Methods: Determination of the exponential phase and the efficiency of amplification in the RTPCR assays

The efficiency of the RTPCR assays were determined by performing a radio-labelled

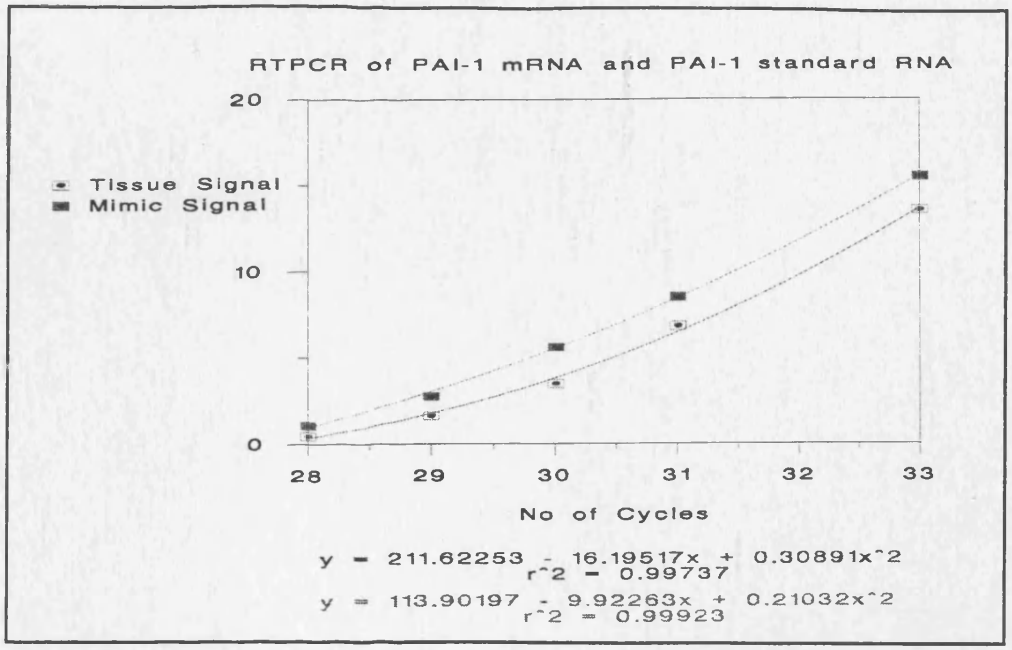
RTPCR of both the tissue RNA and the standard RNA in the same tube (at roughly equal starting concentrations of tissue mRNA and standard RNA, see next paragraph). The RTPCR were set up as in 3.2.14 and 10 μl aliquots of the reaction mixture were collected after different number of cycles. These aliquots were loaded into separated lanes on a 8% polyacrylamide gel. After electrophoresis, the gel was exposed to film, the autoradiograph developed and scanned. A plot of signal intensity of PCR product (corrected for length of PCR product) *vs* number of cycles of PCR was carried out for both bands (the tissue RNA and the standard RNA). Initial experiments were required to establish that the starting concentrations of the specific tissue mRNA and the standard RNA were roughly equal. This was done using RTPCR pilot runs at different starting concentrations of each in turn and observing the strength of the signal on ethidium stained agarose gels as shown below.

Fig 3.2.16 RTPCR of vessel wall PAI-1 mRNA and PAI-1 standard (mimic) RNA at differing starting concentrations

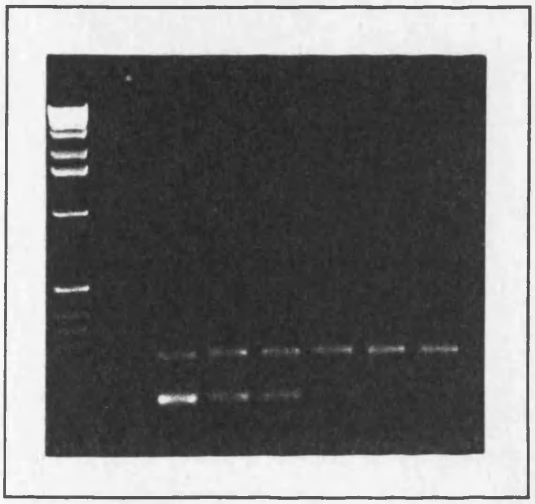
PAI-1 tissue cDNA (266 base pairs) ***PAI-1 standard cDNA (161base pairs)***



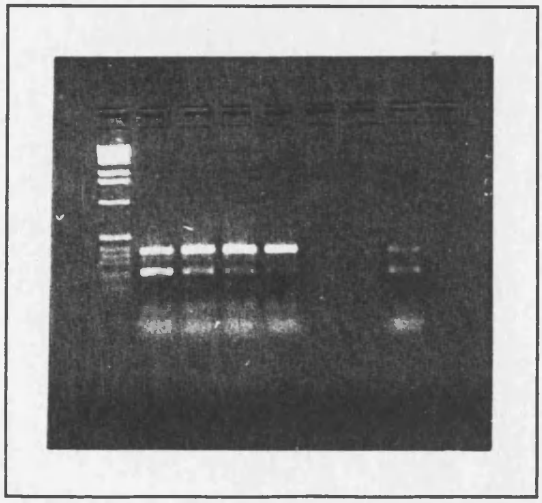
Plot of the number of PCR cycles versus strength of autoradiographic signals from both tissue PAI-1 cDNA and standard cDNA



Quantitative RTPCR assay (PAI-1)



Quantitative RTPCR assay (uPA)



3.2.17 Methods: Confirming the QRTPCR is quantitative.

Measured amounts of tissue RNA (and multiples thereof) in the presence of a range of concentrations of standard RNA were put through a series of QRTPCR. In this way,

for example, a doubling of the tissue RNA in the QRTPCR was reflected in a doubling of the length-adjusted signal intensity

3.2.18 Methods: Assessing the reproducibility of the quantitative RTPCR (PAI-1 and uPA)

The 'Hot' QRTPCR assay was performed three times on each of three different RNA samples. The assays were done using master mixes for the reverse transcription reaction and then for the PCR. The inter-assay variability was thus determined.

3.2.19 Methods: Statistical analysis

Results were analysed using means and SEM and groups were compared using using the non-parametric Mann Whitney U test. Differences were considered statistical significant when $p \leq 0.05$.

3.3 RESULTS

3.3.1 RNA extraction from human vessel wall

3.3.1 a) Homogenisation and yield of total RNA from the vessel wall

Due to the large number of assays needed to be done on each piece of vessel, it was necessary to optimise the yield of RNA. Electrical homogenisation of vascular tissue improved the RNA yield by approximately 25 % when compared to manual homogenisation using a Potter homogeniser. Since the total content of RNA in the tissue was unknown it is not possible to comment on the absolute efficiency of extraction.

Table 3.3.1 (a) Yield of total RNA from the vessel wall

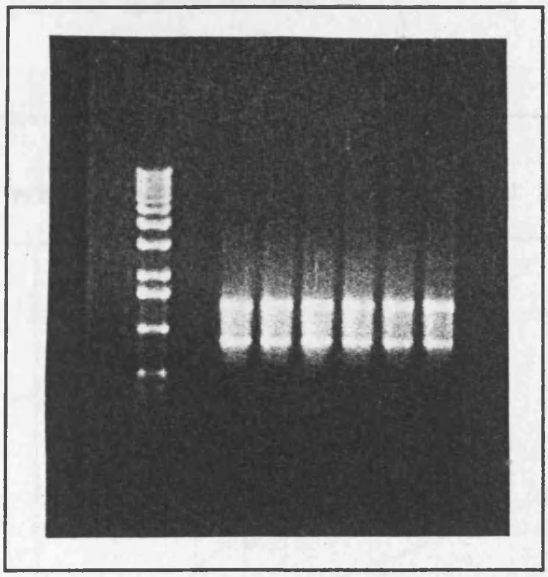
Type of Vessel	Yield of Total RNA (μG / 100mg Tissue)
Undistended saphenous vein	17.0 (1.5) *
Distended saphenous vein	9.8 (2.0) **
Internal mammary artery	19.6 (2.4) +
Atheromatous endarterectomy	7.9 (1.1) ++
* vs **	p = 0.02
* vs +	p = 0.4
** vs +	p = 0.03
+ vs ++	p = 0.002

3.3.1 b) Purity and integrity of extracted total RNA from vessel wall

Using the RNAzol method for extracting RNA from vascular tissue consistently resulted in OD 260/280 ratios between 1.30 -1.45, whereas, modifying the method by performing a further chloroform-extraction, resulted in an increase in the OD 260/280 ratios to 1.6-1.9. RNA integrity was assessed by running the extracted RNA on an ethidium stained 0.8% agarose gel and examining it under UV light as shown in the photograph below. The photograph shows the vessel wall total RNA as a smear with the two characteristic ribosomal RNA bands.

Fig 3.3.1 (b)

The integrity of extracted total RNA from vessel wall

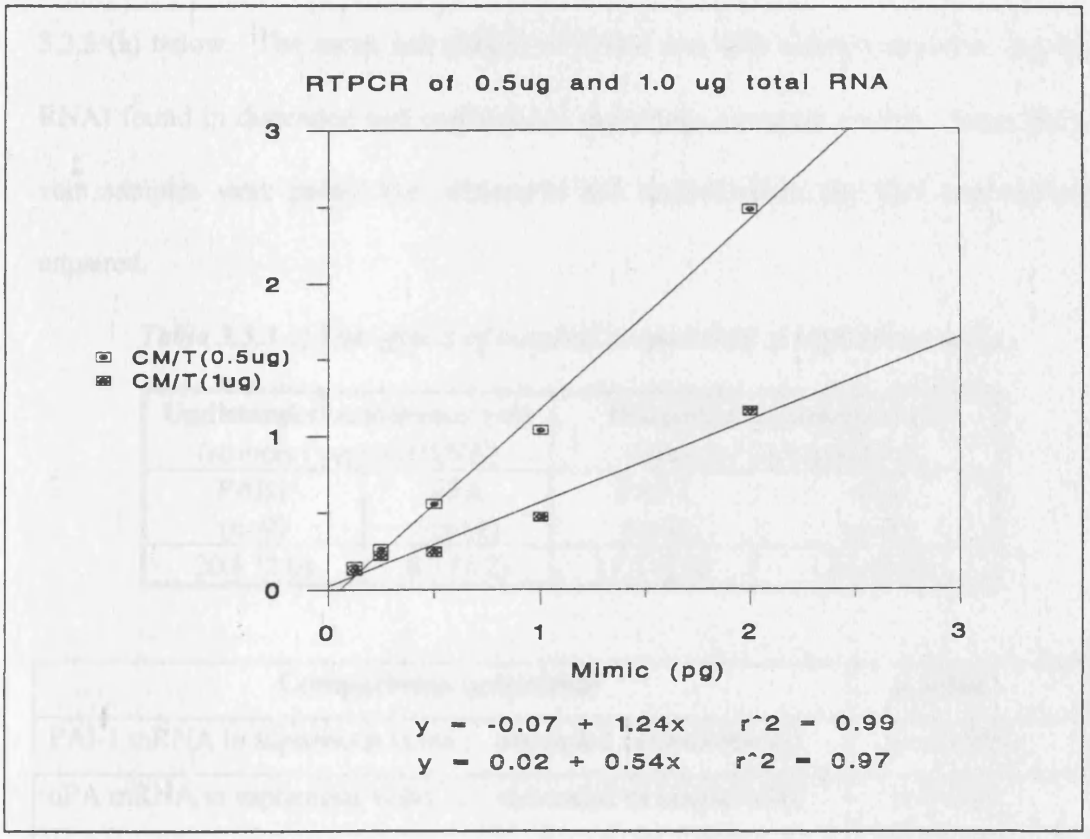


3.3.2 Results: Validation of the QRTPCR assay

The RT-PCR assays were set up using serial dilutions of standard RNA with either 0.5μg or 1μg of total tissue RNA in the same tube so that the RNA underwent co-reverse transcription and co-amplification. The plot below demonstrates the validity of the assay in that it found that the amount of specific mRNA present in the 1.0 μg of total tissue RNA was twice that found in the 0.5 μg..

Fig 3.3.2

RT-PCR assays using serial dilutions of PAI-1 standard RNA with either 0.5μg or 1μg of total tissue RNA.



The amount of standard RNA required to achieve equivalence between the signal intensity of the tissue RNA and standard RNA is determined from the graph above; i.e. when CM/T =1

3.3.3 Results: PAI-1 and uPA mRNA in the vessel wall measured by RTPCR

3.3.3 a) Saphenous veins; examining the effects of surgical preparation on PAI-1 and uPA mRNA.

Distending saphenous veins as part of the routine surgical preparation of the vessels for use as vascular conduits in coronary artery bypass grafting results in a significant reduction of both PAI-1 mRNA (p=0.004) and uPA mRNA (p=0.04) as shown in Table 3.3.3 (a) below. The mean and (SEM) of PAI-1 and uPA mRNA (attmols / μg total RNA) found in distended and undistended saphenous veins are shown. Since only 4 vein samples were paired (i.e. distended and undistended), the data was analysed unpaired.

Table 3.3.3 a) The effects of surgical preparation of saphenous veins

Undistended saphenous vein (attmols / μg total RNA)		**Distended saphenous vein** (attmols / μg total RNA)	
PAI-1 (n=9)	uPA (n=11)	PAI-1 (n=8)	uPA (n=7)
20.8 (2.0)	6.3 (1.2)	11.2 (2.0)	3.1 (0.4)

Comparisons (unpaired)	**p value**
PAI-1 mRNA in saphenous veins ; distended *vs* undistended	p = 0.004
uPA mRNA in saphenous veins ; distended *vs* undistended	p = 0.04
Undistended saphenous veins ; PAI-1 *vs* uPA mRNA	p = <0.0001
Distended saphenous veins ; PAI-1 *vs* uPA mRNA	p = 0.002

However the ratio of uPA/PAI-1 mRNA measured before and after surgical preparation; was similar 0.30 *vs* 0.27 respectively. It was also noted that PAI-1 mRNA was present in significantly greater amounts than uPA mRNA in saphenous vein as a proportion of the total RNA extracted from the vessel wall. This relationship holds true for both distended and undistended veins.

3.3.3 b) Comparison of vascular conduits used for CABG; content of PAI-1 mRNA and uPA mRNA at the time of grafting.

Distended saphenous veins and internal mammary arteries are used as vascular conduits in coronary artery bypass grafting. The content of PAI-1 and uPA mRNA in these vessel walls at the time of grafting was measured using the technique of quantitative RTPCR. The results are shown in Table 3.3.3 b) below and expressed in (attmols / μg total RNA)

Table 3.3.3 b) PAI-1 mRNA and uPA mRNA in vascular conduits used for CABG

Internal mammary artery (attmols / μg total RNA)		**Distended saphenous vein** (attmols / μg total RNA)	
PAI-1 (n=5)	uPA (n=8)	PAI-1 (n=8)	uPA (n=7)
21.4 (3.5)	0.8 (0.1)	11.2 (2.0)	3.1 (0.4)
uPA/PAI-1 = 0.037		uPA/PAI-1 = 0.27	

Comparisons	**p value**
PAI-1 mRNA (internal mammary artery *vs* distended saphenous vein)	p = 0.02
uPA mRNA (internal mammary artery *vs* distended saphenous vein)	p = 0.0002

Significant differences between surgically prepared saphenous veins and internal mammary arteries in the amount of PAI-1 and uPA mRNA were present in the vessel wall at the time of coronary artery bypass grafting. When internal mammary arteries were compared to undistended normal saphenous veins, there was no significant difference in the level of PAI-1 mRNA ($p = 0.8$), whereas there was a significant difference in the amount of uPA ($p = 0.0009$).

3.3.3 c) Comparison between normal and atheromatous arteries in their content of PAI-1 mRNA and uPA mRNA

There was a significant difference in the quantities of in the levels of both PAI-1 and uPA mRNA as measured by quantitative RTPCR between the internal mammary artery and coronary endarterectomy

Internal Mammary Artery (attmols / μg total RNA)		**Atheromatous Artery** (attmols / μg total RNA)	
PAI-1 (n=5)	uPA (n=8)	PAI-1 (n=5)	uPA (n=3)
21.4 (3.5)	0.8 (0.1)	65.1 (19.5)	8.5 (0.2)
uPA/PAI-1 = 0.037		uPA/PAI-1 = 0.131	

Comparison	**P Value**
PAI-1; Internal mammary artery vs Atheromatous artery	$p = 0.02$
uPA ; Internal mammary artery vs Atheromatous artery	$p < 0.0001$

Both PAI-1 and uPA expression was found to be significantly greater in atheromatous arteries compared to normal internal mammary artery. PAI-1 expression was higher than uPA in both normal internal mammary arteries and coronary endarterectomy samples.

3.4 DISCUSSION

Given that a number of assays had to be performed on relatively small samples of vessel wall, the choice of assay needed to quantify the amount of specific mRNAs was critical to the project. Quantitative RTPCR provided an essential and very sensitive method.

3.4 (a) Discussion: Theoretical considerations for the RT-PCR assay

By definition, the PCR process is a chain reaction. The products from one cycle of amplification serve as substrate for the next. Therefore the amount of product increases exponentially and not linearly as in most enzymatic processes. Under ideal or theoretical conditions, the product doubles during each cycle of the PCR reaction according to Equation 1.

Equation 1: $N = N_o 2^n$

where:

N = The number of amplified molecules.

N_o = The number of molecules present before amplification.

n = The number of amplification cycles.

Equation 1 indicates a linear relationship between the number of target molecules and the initial number of target molecules. Experimentally, the efficiency of amplification (E) is less than perfect, and the PCR process is thus described by Equation 2.

Equation 2 : $N = N_0 (1+E)^n$

Therefore, for the tissue cDNA, the following equation apply:

$$n_t = n_{0t} (1+E_t)^n$$

Where N_t = No of tissue cDNA molecules present after amplification.

N_{0t} = No of tissue cDNA molecules present at the start of the PCR.

Similarly, for the standard (mimic) cDNA the following equation apply:

$$n_S = n_{0S} (1+E_S)^n$$

If Es = Et, then for a given number of cycles, n, the following equation is true:

$$n_S / n_t = n_{0S} / n_{0t}$$

A plot of N_S/N_t vs N_{oS} should theoretically have a gradient of $1/ n_{0t}$

As would be predicted, the ratio of standard template per unknown (tissue) template plotted against standard template is a hyperbolic relationship that approaches an asymptote when one species is present in vast excess. For this reason the most accurate results are obtained when the competitive template and the unknown template are amplified at nearly equivalent concentrations. Thus, an initial titration in log increments to determine the approximate concentration of the unknown RNA was done, then, a finer titration was performed to obtain more accurate results.

However, the main constraints in obtaining quantitative data are inherent in the amplification process. Because amplification is (at least initially) an exponential process, small differences in any of the variables that control the reaction rate will dramatically affect the yield of the PCR product. The number of variables that can influence the rate of a PCR include the concentrations of DNA polymerase, dNTPs, Mg^{2+}, DNA templates, and primers; annealing, extension, and denaturing temperatures; cycle length and cycle number; ramping times; rate of primer-dimer formation; and presence of contaminating DNA. Moreover, prior to the amplification process, the mRNA needed to be reverse-transcribed into cDNA and the efficiency of this reaction has been shown to be highly variable. Quantitative RTPCR provided a technique that obviates these problems and allows the precise quantitation of specific mRNA species. The strategy involves co-reverse-transcription of the mRNA and the internal standard cRNA using the same 3'primer followed by co-amplification of the competitive template that uses the same primers as those of the target cDNA. The competitive template can be distinguished from the target cDNA after amplification by their difference in size.

The advantages of this technique are that 1) quantitation is independent of the many variables that affect both the reverse-transcription as well as the amplification, 2) it is much more sensitive than Northern Blotting or ribonuclease protection assays for quantitating specific mRNAs and 3) it is highly specific.

However, two important conditions had to satisfied before the RTPCR assay could be assumed to be valid: First, it was necessary to show that the efficiency of reverse transcription and subsequent amplification for the standard RNA is identical to that of the tissue mRNA. Secondly, it was necessary to show that the RTPCR assay was quantitative; in other words, altering the amount of mRNA present at the start of the RTPCR (e.g. doubling it) should result in a proportionate change in the amount of the product obtained. Both of these conditions were satisfied for the PAI-1 and uPA RTPCR assays.

3.4 (b) Discussion: RNA yield from human vessel wall

It was noted that the yield of RNA μg/100mg of tissue differed significantly between distended 9.8 (2.0) and undistended saphenous veins 17.0 (1.5); p=0.02. A possible explanation for this could be that the surgical distension of the saphenous veins would have necessarily resulted in aqueous media infiltrating into the wall of the vessel and thus adding to its wet weight. As a result the proportion of cell weight to total weight would be less for the distended vessels and consequently the yield of RNA would be less. It is noteworthy that there was no statistical difference in the yield between undistended saphenous veins 17.0 (1.5) and internal mammary arteries 19.6 (2.4); p=0.4. The comparatively low yield of RNA from endarterectomy specimens 7.9 (1.1)

is probably a reflection of the fact that atheromatous lesions contain significant amounts of acellular material e.g. lipid deposits and extracellular matrix, which contributes to weight but not RNA content. Consequently there was significant difference in RNA yield between atheromatous endarterectomies and normal arteries (internal mammary arteries) 7.9 (1.1) vs 19.6 (2.4); p=0.002

These findings demonstrated the limitations of comparing the quantities of specific mRNA (PAI-1 or uPA) between different vessel type by expressing yield of specific mRNA as a proportion of tissue weight. In this study the yield of specific mRNA was expressed as a proportion of total RNA present.

3.4 (c) Discussion: The effects of surgical preparation of saphenous veins on their PAI-1 and uPA mRNA content.

Distending saphenous veins, as part of the routine surgical preparation of these vessels for use as vascular conduits in coronary artery bypass grafting, results in a significant reduction of both PAI-1 mRNA [from 20.8 (2.0) to 11.2 (1.2); p=0.004] and uPA mRNA [from 6.3(1.2) to 3.1(0.4); p=0.04]. The cause of this is unknown but there are a number of possible explanations. Distension could have removed cellular elements of the blood that would have otherwise been adherent on the endothelial surface of the vessel; blood monocytes and macrophages are known to contain mRNA to both PAI-1 and uPA (Hildenbrand et al 1998).

The same argument could be presented for endothelial cells, some of which may be lost during the distension process due to the inevitable tearing of the intima (Angelini et al 1987, Sayers et al 1992). It was also noted that PAI-1 mRNA was present in

significantly greater amounts than uPA mRNA in saphenous vein as a proportion of the total RNA extracted from the vessel wall. This relationship holds true whether distended ($p=0.002$) or undistended veins ($p < 0.0001$) are examined. However the ratio of uPA/PAI-1 mRNA measured before and soon after surgical preparation was similar 0.30 *vs* 0.27 respectively. Although at the time of grafting the fibrinolytic potential appears unaltered at the mRNA level, it is not possible to say what happens once the conduit has been grafted.

3.4 (d) Discussion: Comparison of vascular conduits used for CABG; content of PAI-1 mRNA and uPA mRNA at the time of grafting.

The content of PAI-1 and uPA mRNA in the vessel wall at the time of grafting could, in theory, determine the amount of these proteins present within the wall after the graft has been inserted and this may have an effect on graft survival. There were significant differences between the two vascular conduits in the content of uPA and PAI-1 mRNA levels at the time of grafting. In internal mammary arteries, there was a significantly lower level of uPA expression ($p=0.0002$) and a significantly higher level of PAI-1 expression ($p=0.02$) compared to distended saphenous veins. Thus the uPA/PAI-1 ratio in distended saphenous veins is much higher than in internal mammary arteries. Intuitively, this should result in a lower rate of acute or subacute thrombotic closure of distended saphenous veins compared to internal mammary artery conduits. However, the rate of acute thrombotic closure in internal mammary artery conduits is less than for saphenous vein conduits. This may be a reflection of the fact that whilst vascular uPA and PAI-1 may be important for endogenous fibrinolysis, a host of other important factors may be more relevant to acute thrombotic closure, e.g. blood flow, exposure of

the prothrombotic subendothelium in distended saphenous veins, prostacycline production, etc. The higher uPA/PAI-1 ratio in distended saphenous veins, with the consequent increased vessel wall extracellular proteolytic activity and thus increased putative cell migratory potential, may however contribute to the much increased tendency of the saphenous veins to undergo vein graft stenosis. When internal mammary arteries were compared to undistended normal saphenous veins, there was no significant difference in the level of PAI-1 mRNA ($p = 0.8$), whereas there was a significant difference in the amount of uPA mRNA ($p = 0.0009$).

3.4 (e) Discussion: Comparison between normal and atheromatous arteries in their content of PAI-1 mRNA and uPA mRNA

Both PAI-1 and uPA expression was found to be significantly greater in atheromatous arteries compared to normal internal mammary artery as measured by quantitative RTPCR. PAI-1 expression was higher than uPA in both normal internal mammary arteries and coronary endarterectomy. This suggests that expression components of the plasminogen activator system are upregulated in atheromatous lesion. The uPA/PAI-1 ratio is also significantly increased in coronary atheroma as compared to internal mammary artery. Again, this increased ratio may contribute to the increased fibrinolytic activity known to exist in atheromatous lesion (Underwood et al 1993). Although the increased uPA/PAI-1 mRNA ratio within the atheromatous vessel wall should intuitively also result in less thrombus formation, it is known that fibrin deposition and thrombus development at the atheromatous site is a recurrent phenomenon (Smith et al, 1986). This may be explained by a number of factors including rheology e.g. poor blood flow as well as the altered production of a number

of other molecules produced by the endothelium. Also, the measured increased uPA/PAI-1 ratio does not in itself give us any information as to where within the vessel wall this ratio is increased since the ratio was measured on RNA extracts from homogenised vessels. In other words, it may be possible that whilst the uPA/PAI-1 ratio is increased in the vessel wall in total, the actual uPA/ PAI-1 activity ratio on the endothelial surface may be the reverse. From the PAI-1 and uPA *in-situ* hybridisation study of atheromatous lesion, see section 2.3.3 (g), it was noted that the expression of the molecules was not homogeneous. It is to be expected therefore that the atheromatous lesion will have areas within it that has increased extracellular proteolytic potential and thus increased cell migratory potential whilst other areas may predispose to fibrin deposition and thrombosis.

CHAPTER 4

QUANTITATING SPECIFIC PROTEINS OF THE PLASMINOGEN ACTIVATION SYSTEM IN THE HUMAN BLOOD VESSEL WALL

4.1 INTRODUCTION

In the preceding chapters, evidence was presented on the expression and localisation of components of the plasminogen activation system in the human blood vessel wall. However, measurement of the specific proteins (tPA, uPA, PAI-1 and uPAR) by counting the percentages of immunopositive cells was only semi-quantitative. In addition, the latter method of quantitating the proteins although specific and sensitive, may be misleading as far as the total load of these proteins present in the vessel wall. This is because these proteins, in addition to being associated with cells (both intracellular and on the cell membrane), are also present within the extracellular matrix. For example, in atheromatous lesions there are relatively large acellular areas that are immunopositive to these proteins.

In this chapter, work on quantitating tPA, uPA and PAI-1 antigens from samples of homogenised human blood vessels (distended saphenous veins, internal mammary arteries and endarterectomies from atheromatous lesions) using enzyme linked immunosorbent assays (ELISA) is presented. The quantities of these proteins are presented as a proportion of the vessel weight as well as a proportion of the total protein content of the vessel wall.

4.2. METHODS:

4.2.1 General Methods

4.2.1 (a) Tissue collection and preparation

Fresh blood vessels for this part of the study were collected as described in section 2.2.1. The blood vessel samples were immediately and thoroughly rinsed in cold TBS

(filter sterilised, DEPC-treated water) to remove any blood or debris, and divided into sections for the different investigations. Tissues allocated for protein (antigen) assays were placed in a bijoux, snapped-frozen in liquid nitrogen and stored at -80°C until needed.

4.2.1 (b) Tissue homogenisation.

The tissues were weighed and homogenised in 1 ml of acid acetate extraction buffer (75 mM acetic acid, 225 mM NaCl, 75 mM KCl, 10 mM EDTA, 100 mM arginine, and 0.25% [vol/vol] Triton X-100, pH 4.2) for 3 mins (3 periods of 1 minute with intervals cooling in ice). The homogenate was transferred to eppendorfs, spun at full speed for 15 mins at 4°C, the supernatant filtered using 0.45μm filters and stored at -80°C until needed.

4.2.2 Methods: Total protein estimation

The total protein concentration of the vessel wall homogenate was determined by the Lowry method (Sigma Diagnostics) with protein precipitation using TCA; filtered homogenate (150μl) was added to 850μl of ultrapure water, 100μl of Na deoxycholate (1.5 mg/ml) added, left for 10 minutes, then 100μl of 72% trichloroacetic acid (TCA) was added to precipitate the protein. The Eppendorf tubes were spun at 12000 g for 5 minutes, the supernatant decanted and blotted away. Lowry reagent (1 ml) was added to the pellet to dissolve it and this was transferred to a bijoux. A further 1 ml of water was added to the eppendorf tubes and this was quantitatively added to the respective bijoux and left to stand for 20 minutes. Fiolin & Ciocalteau's reagent (0.5 ml) was

added to the bijoux, mixed well, left for 30 minutes before transferring to disposable cuvettes for spectrophotometric determination at 750 nm. The known protein concentration of the vessel wall filtered homogenate was used subsequently to express specific protein antigen and activities per μg of total protein.

4.2.3 Methods: Measurement of tPA, uPA and PAI-1 (antigen) levels.

4.2.3 (a) Measurement of tPA antigen.

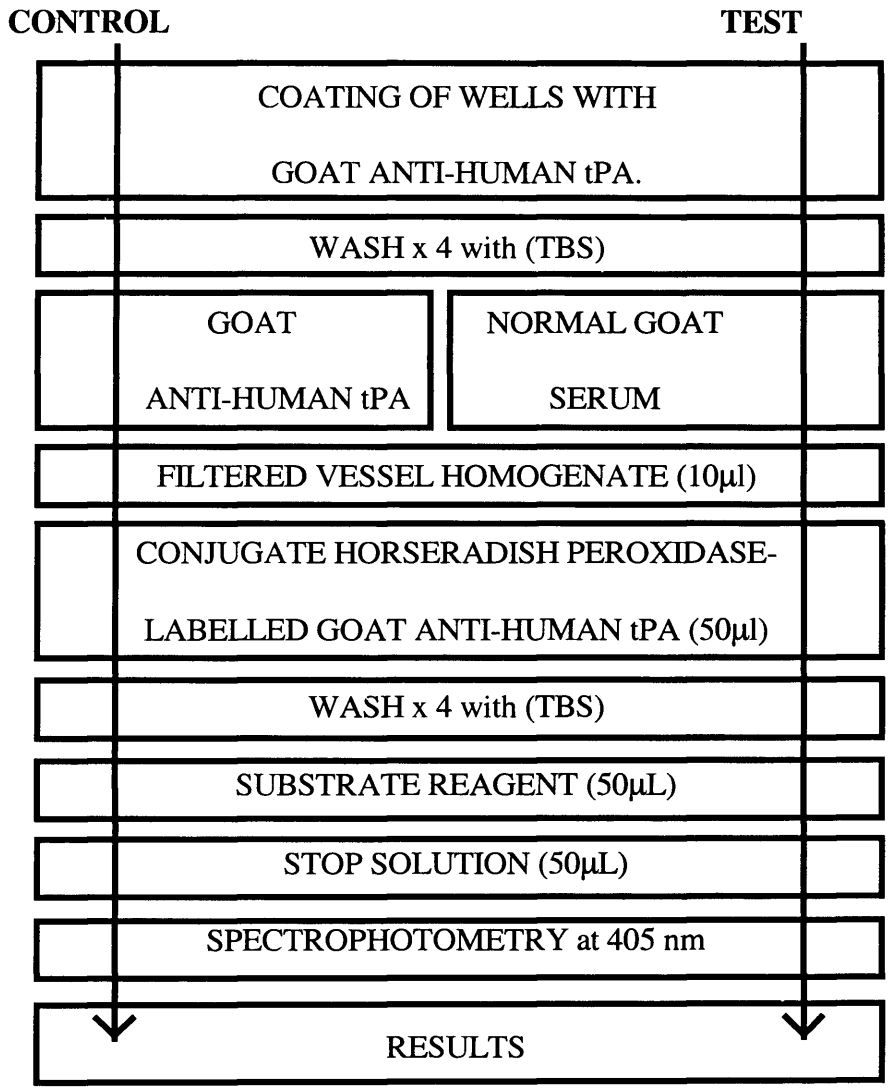
Tissue plasminogen activator antigen was measured by an enzyme linked immunosorbent assay (ELISA) using Imulyse tPA® (Biopool) according to the manufacturer's instructions. The immunoassay utilises the double antibody principle (Bergsdorf et al 1983).

1) Goat anti-human tPA (which was diluted in 0.1M $NaHCO_3$ to a final concentration of 10μg/ml) was incubated overnight (16-18 hrs) at 25°C in the wells of the assay plate thus coating the wells with the antibody.

2 The contents of the wells were discarded and the wells washed 4 times using PET Buffer (PBS-EDTA-Tween, Biopool) for periods of 3 minutes for each wash finally emptying the wells.

3 Normal goat serum (150μl of a 12.5μg/ml solution) was added to the test wells whilst 150μl of a 12.5μg/ml solution of goat anti-human tPA was added to the control wells. (In the test wells, tPA in the blood vessel wall sample binded to the coat antibodies whilst in the control wells, the tPA was prevented from binding the

coat antibodies by the goat anti-human tPA antibodies in solution.



4 Samples (10µl) of the filtered tissue homogenate of blood vessel wall was added to their respective wells (each samples being put into two adjacent wells, the wells in the even-numbered columns acting as the controls; see below). The plate was incubated for 3 hours at 25°C with gentle agitation.

5 Conjugate (50µl) was added to the wells and incubated for 2 hours at 25°C with gentle agitation.

6. The contents of the wells were discarded and the wells washed (x4) with PET

buffer.

7 Substrate (orthophenylenediamine di-HCl) was reconstituted according to the manufacturer's instructions and 200µl added to the wells and incubated for 20 minutes in the dark.

8 The reaction was stopped by 4.5 M H_2SO_4.

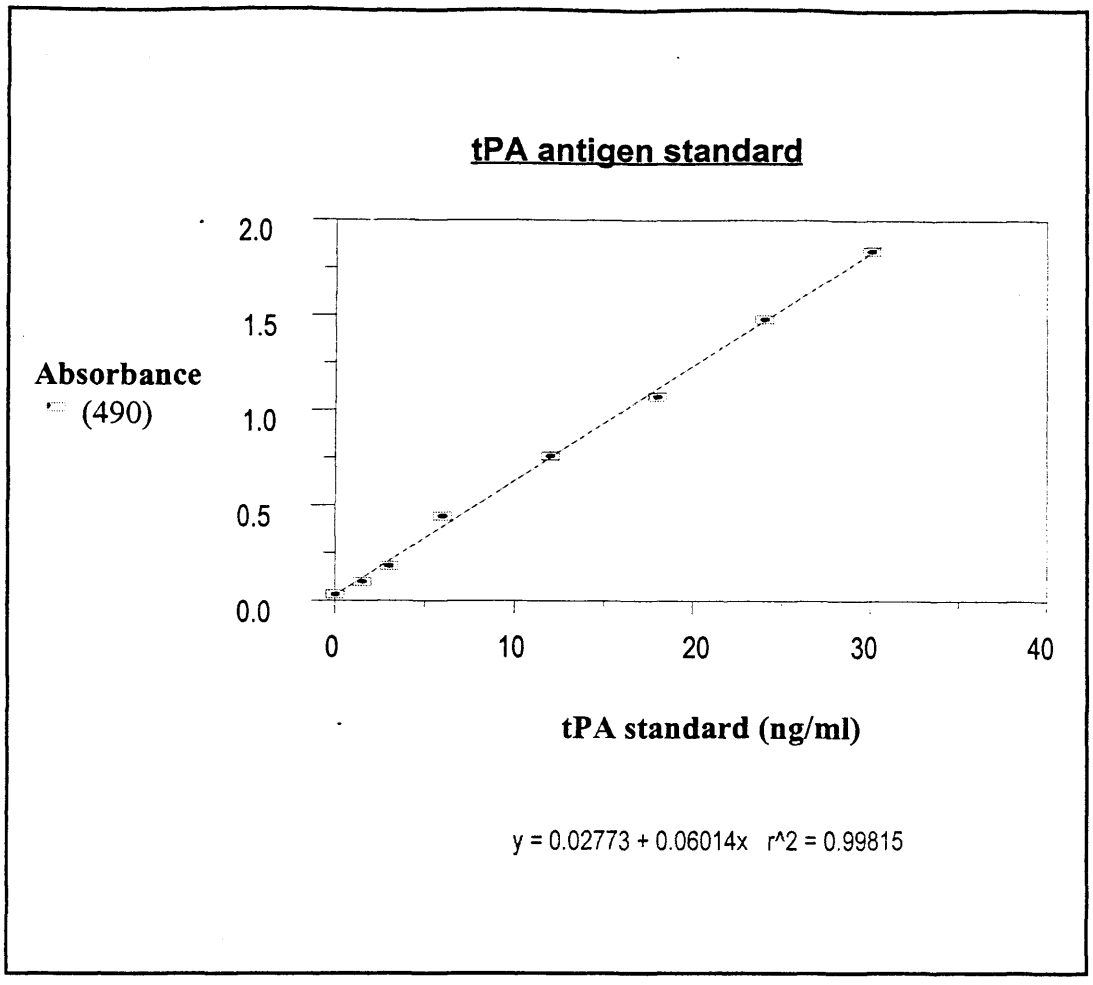
9 Absorbance of the wells were measured on a automated spectrophotometer at 492nm.

The tPA calibration standards were prepared as shown below and to the wells of columns 1 and 2 of the assay plate, 10 µl of the appropriate standard was added.

tPA standard concentration (ng/ml)	Volume (µl) of tPA standard (30ng/ml)	PET Buffer (µl)
30.0	100µl	0
24.0	80	20
18.0	60	40
12.0	40	60
6.0	20	80
3.0	10	90
1.5	5	95
0	0	100

The difference in absorbance between the test and controls for each sample (standards or filtered blood vessel homogenate) were calculated; this represent the tPA specific part of the response = Δ Abs. The standard curve was obtained by plotting Δ Abs against the amount of the tPA in the standard dilutions.

Fig 4.2.3 (a) Standard curve of tPA antigen



An initial titration experiment was needed to determine the quantity of filtered blood vessel homogenate needed for the assay in order for the spectrophotometer's reading to lie in the range of the standard tPA concentrations. It was found that for tPA, 10µl of the filtered homogenate was adequate. The quantity of tPA antigen in the vessel wall was expressed in ng/µg of total protein.

4.2.3 (b) Measurement of uPA antigen.

Urokinase-type plasminogen activator antigen was measured by ELISA using TintElize u-PA® (Biopool) according to the manufacturer's instructions. The immunoassay

utilises the double antibody principle. The methodology was similar to the tPA ELISA detailed above and so it is not reiterated. Again, as was the case with tPA, an initial titration experiment was needed to determine the quantity of filtered homogenate needed for the assay in order for the spectrophotometer's reading to lie within the range of readings of the uPA antigen standard. It was found that for uPA 30 µl of the filtered tissue homogenate were required. The quantity of uPA antigen in the vessel wall was expressed in ng/µg of total protein.

4.2 3 (c) Measurement of PAI-1 antigen.

PAI-1 antigen was measured by ELISA using TintElize PAI-1 (Biopool) according to the manufacturer's instructions. The methodology (Declerck et al 1993) was similar to the tPA ELISA detailed above and so it is not reiterated. Again an initial titration experiment was needed to determine the quantity of filtered homogenate needed for the assay in order for the spectrophotometer's reading to lie within the range of readings of the PAI-1 antigen standard. It was found that for PAI-1, 10 µl of the filtered homogenate were required. The quantity of PAI-1 antigen in the vessel wall was expressed in ng/µg of total protein.

4.3 RESULTS

4.3.1 Results: Conduits used for coronary artery bypass grafting; comparison between distended saphenous veins and internal mammary arteries in their content of tPA, uPA and PAI-1 antigens.

When expressed as a fraction of the total protein content, there were significant differences between distended saphenous veins and internal mammary arteries in their content of tPA antigen (p=0.009) whilst there were no significant difference noted for uPA (p = 0.77) or PAI-1 (p = 0.16), see Table 4.3.1 (a). However, when the quantities of these proteins were expressed as a proportion of the weight of tissue, significant differences between the two types of surgical conduits were found in their content of tPA (p = 0.03) and PAI-1 (p < 0.0001), see Table 4.3.1 (b).

Table 4.3.1(a) Vessel wall content (Mean, SEM) of tPA, uPA and PAI-1 (μg/g total protein)

Protein (antigen)	Saphenous Veins	Internal mammary arteries	p value
tPA	3.9 (0.9) n=11	1.2 (0.4) n=8	0.009
uPA	0.07 (0.02) n=16	0.06 (0.01) n=7	0.77
PAI-1	0.4 (0.1) n=9	0.2 (0.04) n=9	0.16

Table 4.3.1 (b) Vessel wall content (Mean, SEM) of tPA, uPA and PAI-1 (ng/g tissue)

Protein (antigen)	Saphenous Veins	Internal mammary arteries	p value
tPA	311 (38) n=11	141 (37) n=7	0.03
uPA	4.5 (0.8) n=16	3.9 (0.6) n=7	0.9
PAI-1	72 (11.9) n=9	16.3 (2.5) n=9	<0.0001

4.3.2 Results: Comparison between internal mammary artery and coronary endarterectomy in their content of tPA, uPA and PAI-1 antigens.

A significantly greater amount of tPA (p = 0.001), uPA (p = 0.0003) and PAI-1 (p = 0.004) were found in atheromatous endarterectomy samples compared to normal

internal mammary arteries, see 4.3.2 (b). However, when the amounts of these antigens are expressed as a fraction of the total protein content, only PAI-1 (p = 0.004) was found to be significant, see table 4.3.2 (a).

Table 4.3.2 (a) Vessel wall content** (Mean, SEM) **of tPA, uPA and PAI-1 (μg/g total protein)

Protein (Antigen)	Endarterectomy	Internal mammary arteries	p value
tPA (ng / mg total prot)	2.3 (0.4) n=6	1.2 (0.4) n=8	0.11
uPA (ng / mg total prot)	0.08 (0.02) n=11	0.06 (0.03) n=7	0.54
PAI-1 (ng / mg total prot)	2.6 (0.3) n=6	0.2 (0.04) n=9	0.0004

Table 4.3.2 (b) Vessel wall content** (Mean, SEM) **of tPA, uPA and PAI-1 (ng/g tissue)

Protein (Antigen)	Endarterectomy	Internal mammary arteries	p value
tPA (ng / g tissue)	652 (99) n=6	141 (37) n=7	0.001
uPA (ng /g tissue)	16.4 (2.4) n=11	3.9 (0.6) n=7	0.0003
PAI-1 (ng / g tissue)	272 (84) n=6	16.3 (2.4) n=9	0.0004

4.4 DISCUSSION

4.4.1 The immunoassays

The 'Imulyse tPA' assay (Biopool), detects single-chain and two-chain tPA in complex with PAI-1, PAI-2 or α-2 antiplasmin. 'TintElize uPA' assay (Biopool), detects scu-PA, HMW uPA (complexed to PAI-1 or PAI-2 or not) and LMW uPA. 'TintElize PAI-1®' (Biopool) detects active or latent forms of PAI-1, as well as complexes of PAI-1 with tPA or uPA. In other words these immunoassays measured the total content of these three proteins in the blood vessel homogenate.

4.4.2 Discussion: Comparison between conduits used for coronary artery bypass grafting (distended saphenous veins and internal mammary arteries) in their content of tPA, uPA and PAI-1 antigens.

There were significant differences between distended saphenous veins and internal mammary arteries in their content of tPA antigen when expressed as a fraction of the total protein content, whilst there were no significant difference noted for uPA or PAI-1, see Table 4.3.1 (a). However, when the results were expressed as a fraction of weight of tissue, significant differences were between these two types of surgical conduits in their content of both tPA and PAI-1, see Table 4.3.1 (b).

It is difficult to be certain as to why there was a difference in the results when expressed with different denominators, i.e. tissue weight and total protein. This lack of concordance is particularly evident for PAI-1, where there was a highly significant difference in PAI-1 proteins between internal mammary arteries and distended saphenous veins. It possible that surgically distending the veins resulted in a large amount of other plasma proteins (e.g. albumin) to enter the vessel wall in addition to PAI-1. Nevertheless, it is apparent that distended saphenous veins have a higher content of tPA and PAI-1 proteins within the vessel wall compared to internal mammary arteries at the time of grafting when being used as vascular conduits. These results are in broad agreement with the semi-quantitative immuno-histochemical findings on tPA and PAI-1 presented in Section 4.2.

However, whilst there was an increased content of uPA protein in the distended saphenous veins compared to internal mammary arteries when measured semi-

quantitatively using immunohistochemistry, this was not noticed with the immuno(antigen) assays. This may appear to be a paradox, but it should be borne in mind that these two methods of measuring the specific protein levels have some fundamental differences. Firstly, different antibodies were used which may have had different affinities and thus different sensitivities. Secondly, the semi-quantitative immunohistochemical method relied on the percentage of smooth muscle cells in the media that were immunopositive. It did not measure the degree of relative positivity of the cells, in other words, the amount of protein associated with a particular cell could have been a little or a lot, but, the cell was counted as being immunopositive if it appeared to take up the DAB stain. Thirdly, the semi-quantitative immunohistochemical method looked only at cell positivity of the tunica media of the vessel. However, from the immunohistochemistry it was very clear that the adventitia was strongly immunopositive for all of these proteins and this antigen load would not have been reflected in the semi-quantitative data. A further point worthy of mention is that the immunohistochemical method essentially examines for the presence of a specific epitope present in the tissue, and, whilst the epitope may be present, the protein may be partially degraded and therefore not be measured in the specific immuno(antigen) assay.

4.4.3 Discussion: Comparison between internal mammary artery and coronary endarterectomy in their content of tPA, uPA and PAI-1 antigens.

A significantly greater amount of tPA ($p = 0.001$), uPA ($p = 0.0003$) and PAI-1 ($p = 0.004$) were found in atheromatous endarterectomy samples compared to normal internal mammary arteries, see 4.3.2 (b). However, when the amounts of these

antigens are expressed as a fraction of the total protein content, only PAI-1 ($p = 0.004$) was found to be significant, see table 4.3.2 (a).

The significantly higher levels of tPA, uPA and PAI-1 proteins in the atheromatous lesion as compared to normal artery (internal mammary artery) found by this method is in concordance with the findings by Padro et al 1995. It is also in keeping with the increased immunopositivity found in these immunohistological sections shown in Chapter 2 as well as with the findings of higher levels of PAI-1 and uPA mRNA found in atheromatous lesions, see Chapter 3.

The method of quantitating specific proteins in the vessel wall by homogenisation of the vessel has its limitations. Although an attempt was made to rinse the vessels to try and remove as much of blood as possible, there was always blood sometimes with thrombi present on the endothelium and in the vasa vasorum. Thrombi and advetititia are seen to contain these proteins on immunohistology. In atheromatous endarterectomy lesions, there were usually multiple areas containing adherent thrombi and these were not forcibly washed off during the preparation of these samples for homogenisation. It is therefore possible that in these vessels, the quantitation of these proteins were higher than in vessels without adherent clots. In some instances, particularly for internal mammary arteries the vessels were dissected with a lot of extravascular tissue around them and in these samples, an attempt was made to further dissect them away from the extravascular tissues. Another limitation of this gross method of quantitating specific proteins is that it does not tell us where within the vessel wall were these proteins predominantly present, nor does it tell us

anything about the activities of the proteins. Finally, it has to be recognised that differences observed between the atheromatous lesion examined (i.e. coronary endarterectomy) and normal IMA, may have been partially due to the fact that these vessels were from different vascular beds. Coronary arteries may be intrinsically different from internal mammary arteries; for example, they have a greater preponderance to develop atheroma unlike the latter.

CHAPTER 5

QUANTITATING THE ACTIVITIES OF uPA AND PAI-1 IN THE HUMAN BLOOD VESSEL WALL

5.1 INTRODUCTION

The preceeding chapters provide evidence of vessel wall expression of tPA, uPA and PAI-1 mRNAs, the localisation of these specific mRNAs by *in-situ* hybridisation, the quantitation of tPA, uPA and PAI-1 proteins by immunoassay, and the immunohistological localisation of tPA, uPA, PAI-1 as well as uPAR. However the presence of both plasminogen activators and plasminogen activator inhibitor makes it difficult to predict the activities of these proteins in the walls of the various vessels.

In this chapter, work on quantitating uPA and PAI-1 activities from samples of homogenised human blood vessels (distended saphenous veins, internal mammary arteries and endarterectomy from atheromatous lesions) using enzyme linked immunosorbent activity assays (ELISA) is presented. The ELISA is an antibody capture assay, in which the captured protein is then measured functionally. The activities of these proteins are presented as a proportion both of the vessel weight as well as the total protein content of the vessel wall.

5.2. METHODS:

5.2.1 General Methods

5.2.1 (a) Tissue collection and preparation

Fresh blood vessels for this part of the study was collected as described in section 2.2.1. The blood vessel samples were immediately but thoroughly rinsed in cold TBS (filter terilised, DEPC-treated water) to remove any blood or debris, and divided into sections for the different investigations. Tissues allocated for protein (antigen) assays were placed in a bijoux, snapped-frozen in liquid nitrogen and stored at -80°C until needed.

5.2.1 (b) Tissue homogenisation.

The tissues were weighed and homogenised in 1 ml of acid acetate extraction buffer (75 mM acetic acid, 225 mM NaCl, 75 mM KCl, 10 mM EDTA, 100 mM arginine, and 0.25% [vol/vol] Triton X-100, pH 4.2) for 3 mins (3 periods of 1min with intervals cooling in ice). The homogenate was transferred to eppendorfs, spun at full speed for 15 mins at 4°C, the supernatant filtered using 0.45μm filters and stored at -80°C until needed.

5.2.1 (c) Total protein estimation

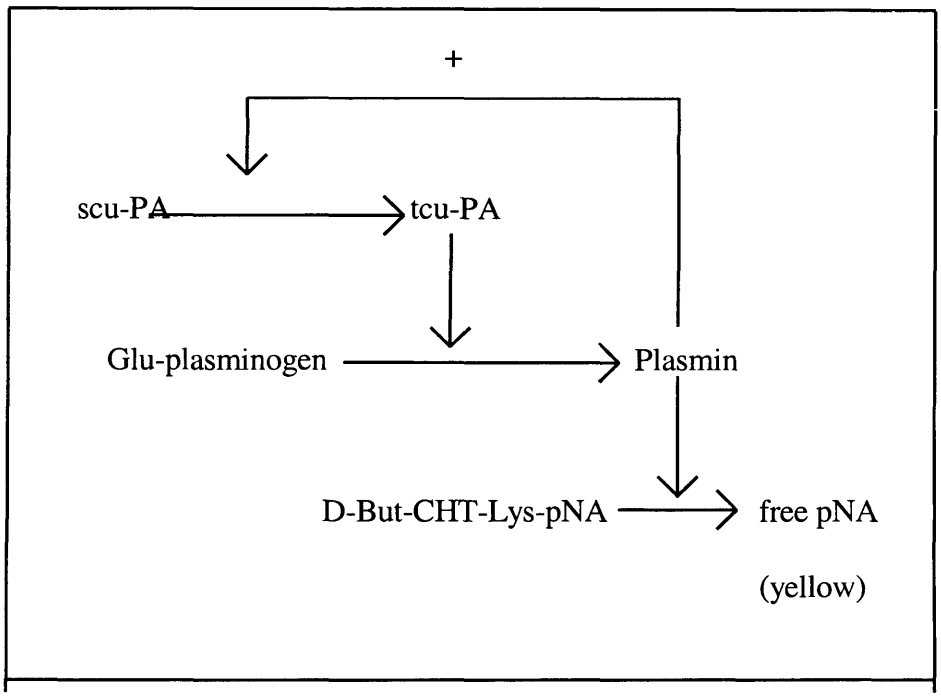
The total protein concentration of the vessel wall homogenate was determined by the Lowry method (Sigma Diagnostics) with protein precipitation as was previously described in *Section 4.2.3 (c).*

5.2.2 Methods: Measurement of the fibrinolytic activity

5.2.2 (a) Quantitating uPA activity in the vessel wall

uPA activity was measured using an immunosorbent assay (Jonsson-Berg et al 1989), Chromolize uPA® (Biopool). Single chain uPA is allowed to quantitatively adsorb to the micro-test plate wall. After washing away non-adsorbed material with buffer, the adsorbed scuPA is activated by the addition of a plasmin solution. The plasmin converts scuPA to the active two-chain molecule but does not dislodge it from the micro-test plate wall. The plasmin which was added to both the test and control wells were subsequently thoroughly washed away before the uPA was determined by adding

a reagent containing plasminogen, poly-D-lysine and a plasmin sensitive chromogenic substrate; the uPA activates the plasminogen to plasmin which in turn cleaves the chromogenic substrate to result in a yellow colour which is quantified spectrophotometrically by measuring the absorbance at 405nm. The amount of yellow colour developed is proportional to the amount of scuPA present in the sample or standard. Apart from scu-PA, the assay also responds to the 53kD form of uPA but not the 35kD form of uPA.



5.2.2 (b) Quantitating PAI-1 activity in the vessel wall

PAI-1 activity was measured using Spectrolyse ®(pL) PAI-1 (Biopool) according to the manufacturer's instructions. This is based on a two-stage, indirect enzymatic assay, Chmielelewska et al 1983. In stage one, a fixed amount of tPA (50µl 40 IU/ml) was added to the 50 µl of filtered vessel homogenate (or to the PAI-1 standards) and allowed to react for 15 minutes at 25°C with the PAI-1 present. The standards were

prepared as follows: PAI-1 standard "40" was made up with 50μl tPA/PAI-1 depleted plasma plus 50μl assay buffer. PAI-1 standard "0" was made up with 50μl tPA/PAI-1 depleted plasma plus 50μl of 40 IU/ml tPA. The two standards were prepared in triplicates. The sample was then acidified with 100μl acetate buffer (1.0 mol/L sodium acetate buffer, pH 3.0) and mixed for 20 minutes at 37°C to destroy α2-antiplasmin and other plasmin inhibitors which could interfere with the assay, and the sample was subsequently diluted with 2.0 ml water. In stage two, the residual tPA activity was measured by putting 20μl of the sample into the micro test plate well and 200μl ice cold PAR/pL mix (a mixture of Glu-plasminogen, poly-D-lysine and chromogenic substrate at neutral pH) was added. The test plate was sealed and incubated at 37°C for 90 minutes then 50 μl of 'Stop' solution was added. The residual tPA activity in the samples catalysed the conversion of plasminogen to plasmin, which in turn hydrolysed the chromogenic substrate, the amount of colour developed being proportional to the amount of tPA activity in the sample. Poly-D-lysine was present as a stimulator of the tPA catalysed conversion plasminogen to plasmin. The PAI-1 content of the samples was then identified as the difference between the amount of tPA added and the amount of tPA found. One unit of PAI-1 activity is defined as the amount of PAI-1 inhibits one international unit of human single chain tPA.

5.3 RESULTS

5.3.1 uPA and PAI-1 activities in conduits used for coronary artery bypass grafting: comparison between distended saphenous veins and internal mammary arteries

When expressed as a proportion of tissue weight, PAI-1 activity was significantly

lower in surgically distended saphenous veins compared to internal mammary arteries at the time of their use as bypass grafts in coronary artery surgery, see Table 5.3.1 (a). When expressed as a proportion of total protein extracted form the vessel wall, PAI-1 activity was also lower in surgically distended saphenous veins compared to internal mammary arteries but this did not achieve statistical significance, see *Table 5.3.1 (b)*; showing Mean and (SEM).

Table 5.3.1 (a) Activities of uPA and PAI-1 in vessel wall expressed per weight of tissue

Protein activity	Distended Saphenous Vein	Internal Mammary Artery	p value
uPA Act (ng/mg tissue)	0.035 (0.003) n=16	0.047 (0.007) n=11	0.25
PAI-1Act(U/mgtissue)	1.5 (0.2) n=15	6.5 (0.9) n=10	<0.0001

Table 5.3.1 (b) Activities of uPA and PAI-1 in vessel wall expressed per amount of total protein in tissue

Protein activity	Distended Saphenous Vein	Internal Mammary Artery	p value
uPA Act(ng/mg t prot)	0.58 (0.14) n=16	0.35 (0.09) n=9	0.20
PAI-1Act(U/mg t prot)	23.5 (5.5) n=15	28.6 (5.8) n=9	0.39

5.3.2 uPA and PAI-1 activities in normal and atheromatous arteries; comparison between internal mammary artery and atheromatous endarterectomy

When expressed as a proportion of tissue weight or as a proportion of total protein extracted form the vessel wall, PAI-1 activity was significantly lower in atheromatous endarterectomy compared to normal internal mammary arteries, see *Table 5.3.2 (a)* and *(b)*. There was no statistical difference in uPA activity between atheromatous endarterectomy and normal internal mammary artery.

Table 5.3.2 (a) Activities of uPA and PAI-1 in vessel wall expressed per weight of tissue

Protein activity	Internal Mammary Artery	Endarterectomy	p value
uPA Act (ng/mg tissue)	0.047 (0.007) n=11	0.036 (0.011) n=7	0.29
PAI-1Act(U/mgtissue)	6.5 (0.9) n=10	1.1 (0.2) n=7	0.0002

Table 5.3.2 (b) Activities of uPA and PAI-1 in vessel wall expressed per amount of total protein in tissue

Protein activity	Internal Mammary Artery	Endarterectomy	p value
uPA Act(ng/mg t prot)	0.35 (0.09) n=9	0.43 (0.11) n=7	0.05
PAI-1Act(U/mg t prot)	28.6 (5.8) n=9	12.7 (2.1) n=7	0.54

5.4 DISCUSSION

5.4.1 The immuno-activity assays

The Chromolyse uPA activity assay (Biopool), detects scu-PA, the 53 kD form of uPA, but not the 35kD form of uPA. Spectrolyse ®(pL) PAI-1 (Biopool) detects PAI-1 activity. tPA activity was not measured due to limitation of time. Unlike the immuno-antigen assays, which measured the presence of a specific protein epitope, the immunoactivity assays used measured the active content of the specific proteins in the blood vessel homogenate.

5.4.1 Discussion: uPA and PAI-1 activities in conduits used for coronary artery bypass grafting: comparison between distended saphenous veins and internal mammary arteries

When expressed as a proportion of tissue weight, PAI-1 activity was significantly lower in surgically distended saphenous veins compared to internal mammary arteries at the time of their use as bypass grafts in coronary artery surgery, ($p< 0.0001$) see Table 5.3.1 (a). When expressed as a proportion of total protein extracted form the

vessel wall, PAI-1 activity was also lower in surgically distended saphenous veins compared to internal mammary arteries but this did not achieve statistical significance, ($p = 0.2$) see *Table 5.3.1 (b).* The lower PAI-1 activities in the distended saphenous veins is somewhat surprising in view of the findings of both the immunohistochemical and immuno(antigen) assay studies presented in Sections 2.3.1 (f) and 4.3.1 respectively. A possible explanation could however be that the higher level of tPA antigen seen immunohistochemically in these distended veins resulted in the lowering of the PAI-1 activity. A study comparing tPA activities between distended saphenous veins and internal mammary artery would help in addressing this issue. Although uPA immunostaining was found to be significantly increased in distended saphenous veins compared to internal mammary arteries and there was a non significant increase when immuno(antigen) assay was used (Section 4.3.1), there was no significant difference in uPA activities found. Again, it is difficult to give an explanation on these findings given the difference in PAI-1 activities and the absence of data on tPA activity.

5.4.2 Discussion: uPA and PAI-1 activities in normal and atheromatous arteries; comparison between internal mammary artery and atheromatous endarterectomy

Whilst there was no statistical difference in uPA activity between atheromatous endarterectomy and normal internal mammary artery (either expressed as a proportion of tissue weight or as a proportion of total protein extracted) from the vessel wall, PAI-1 activity was significantly lower in atheromatous endarterectomy samples compared to normal internal mammary arteries, see *Table 5.3.2 (a)* and *(b).*

This was somewhat surprising given the previous findings of greater PAI-1 antigen levels present in atheroma on the immuno(antigen) assay as well as the immunohistochemistry. The more modest increased levels of tPA in atheroma could help to explain this apparent anomaly. The lower PAI-1 activity in atheromatous endarterectomy could provide an explanation for the increased fibrinolytic activity of atheromatous lesions as measured by the fibrin plate method (Underwood et al 1995).

The methodology of assessing the specific protein activities within the vessel wall using vessel homogenates clearly has intrinsic limitations. It measures the activity of the vessel wall as a whole and does not give any information on where in the vessel wall the protein activity resides e.g. endothelium, neointima, media and/or adventitia. Information on localisation of the specific protein activities can be obtained from *in-situ* zymography.

CHAPTER 6

THE PLASMINOGEN ACTIVATION SYSTEM IN THE SAPHENOUS VEIN ORGAN CULTURE MODEL OF INTIMAL HYPERPLASIA

6.1 INTRODUCTION

The natural history of aorto-coronary saphenous vein grafts has been well documented. During the first post-operative month, approximately 14% of grafts may occlude as a consequence of thrombosis (Sanz et al 1990). At the end of the first post-operative year, between 13-25% of grafts become occluded (Grondin et al 1984, FitzGibbon et al 1991). Although this may be related to thrombosis, an increasing proportion of graft-failures is secondary to intimal hyperplasia (Batayias et al 1977). During the next five post-operative years, further intimal hyperplasia occurs and the attrition rate during this time period is approximately 2% per year. (Bourassa et al 1991). Saphenous vein graft surviving beyond five years develop the changes associated with atherosclerosis such as necrosis, haemorrhage, calcification and thrombosis (Mautner et al 1992, Bulkey & Hutchins 1977) but with important differences; saphenous vein atherosclerosis is typically diffuse, concentric, usually do not have fibrous caps and usually contain a heavy inflammatory infiltrate (Lytle et al 1992, Ratliff and Myles 1992)

6.1.1 Blood vessel organ culture

Organ culture of saphenous veins results in the proliferation and migration of smooth muscle cells as well as the secretion of extracellular matrix by the latter cells. These are processes known to be central to the development of vein graft stenosis, atherogenesis and restenosis post angioplasty. Hence organ culture of saphenous veins may be a useful model for studying the changes within the vessel wall that occurs during the development of intimal hyperplasia.

The first report of vessel organ culture was in 1959 by Trowell et al, who showed that

lengths of mesenteric arteries could be maintained in culture with no evidence of cell death. This is perhaps not surprising since nutrients only have a small distance to diffuse and vessels are already adapted to this mode of nutrient transfer, making them ideal for organ culture. Barett et al 1979 described the intimally directed proliferation of smooth muscle cells in an organ culture of human aorta. The requirement for the endothelium to be present in order for intimal hyperplasia to develop in a porcine organ culture model was demonstrated by Koo et al 1989. Subsequently, it was shown that intimal thickening and smooth muscle proliferation was reduced in de-endothelialised human internal mammary arteries compared to their controls with intact endothelium (Holt et al 1992).

The use of organ culture of human saphenous veins as a model for saphenous vein graft intimal hyperplasia was originally described by Soyombo et al 1990. They demonstrated the migration of proliferating smooth muscle cells was intimally directed resulting in a neointima as it is in vein grafts and in atherosclerotic arteries. However there were low levels of staining for elastin and absence of organised elastic fibres. In this respect the model does not entirely mimic arteriovenous bypass grafting. Another observation made by this group was the presence of endothelial microlumeni within the neointima, a feature sometime seen in advanced atherosclerotic lesions (Gown et al 1986) and described in rabbit veins transplanted into the carotid arterial circulation (Zwolak et al 1987).

For this project, the use of the vein culture model for studying changes in the plasminogen activator system during the development of intimal hyperplasia was an

attractive one for a number of reasons. It was relatively easy both to obtain human saphenous veins and to set up the veins in culture. The length of time it takes for the neointima to develop is relatively short and, unlike isolated smooth muscle cell experiments, the structural elements of endothelium, smooth muscle cells and extracellular matrix are relatively preserved. In addition, this model is based on human tissue. There are a number of studies showing important differences between intimal hyperplasia development in humans and in animal models (Ferrel et al 1992 and Park et al 1993). Finally, experiments focusing on arterial intimal hyperplasia may not be wholly relevant to vein graft stenosis (Yang et al 1991, Predel et al 1992, Davies et al 1993)

In this chapter, work is presented on the organ culturing human saphenous veins, on further immunohistological characterisation of the model, on documentation of expression of tPA, uPA and PAI-1 mRNA by both RTPCR of vessel wall homogenates as well as mRNA *in-situ* hybridisation, on immunohistochemical findings using antibodies against tPA, uPA, PAI-1 and uPAR and finally on immuno(antigen)- as well as immuno(activity)-assays on vessel wall homogenates.

6.2 METHODS

6.2.1 Saphenous vein organ culture

Fresh surplus samples of distended saphenous veins (n=16) were obtained at operation from patients undergoing coronary artery bypass grafting. The samples were transported to the laboratory at room temperature in calcium-free Krebs solution (NaCl 6.9g, KCl 0.34g, $MgSO_4.7H_2O$ 0.25g, KH_2PO_4 0.14g, $NaHCO_3$ 2.1g, glucose 1.08g; in

1 litre of H_2O) and set up in culture within 4 hours. Exercising great care to keep handling to a minimum, the excess adventitial tissue was dissected off using fine forceps and micro-scissors whilst the vein remained immersed in the calcium-free Krebs solution. The damaged ends of the veins were removed using a clean scalpel blade and discarded. The remaining piece of vein was divided into 5mm segments and opened out endothelial surface uppermost. Veins were set up in culture by transferring them to vein culture dishes that were made up using Pyrex 60 x 20mm dishes into which sylgard 184 resin (Dow Corning Ltd, Belgium) was set at a depth of 5 mm. The vein segment was extended to approximately its *in-situ* length (with the endothelial surface uppermost) by pinning them onto a coarse polyester cloth (P500, Henry Simon, Cheshire, UK.) using 4 minuten A1 pins (Watkins & Doncaster, Kent, UK). The veins were then immersed in 6 mls of vein culture medium. The vein culture medium was composed of the following i) RMPI 1640 tissue culture medium (Northumbria Biologicals Ltd, Cramington, UK), ii) foetal calf serum 30% v/v. iii) Penicillin 50 U/ml, Streptomycin 50μg/ml (Sigma, Poole, Dorset) and iv) L-glutamine 2 mM.(Northumbria Biologicals, Cramington). The organ culture was performed in a humidified chamber (Queue Systems, West Virginia, USA) at 37°C equilibrated with 5% (v/v) CO2 in air, the medium being replaced with fresh medium every 48-72 hours.

Fig 6.2.1 (a) Saphenous vein being cleared of excess adventitial tissue

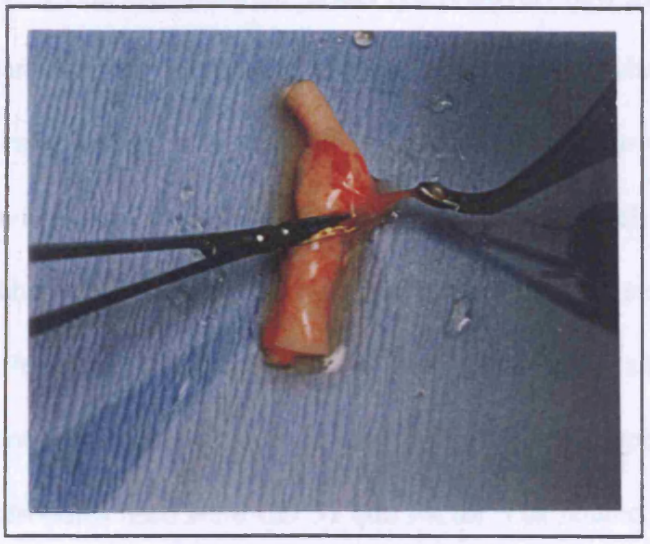
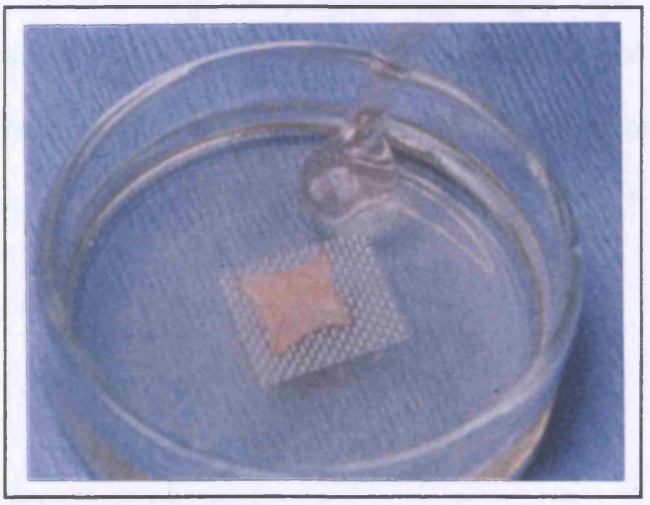


Fig 6.2.1 (b) Human saphenous vein prepared for organ culture



6.2.2 Methods: Immunohistological characterisation of the human saphenous vein organ culture *in-vitro* model; comparison to *ex-vivo* saphenous vein graft stenosis

Before the vein culture model is used to investigate the changes in the plasminogen activation system during the development of intimal hyperplasia, it was necessary to

demonstrate that the model closely resembles saphenous vein intimal hyperplasia *in vivo*. The saphenous vein organ culture model is a relatively new model and it has not yet been fully characterised. An attempt was therefore made to characterise it further immunohistologically and the findings were compared with the *in-vivo* venous intimal hyperplasia of *ex-vivo* femoro-saphenous vein grafts (n=3), which had failed due to graft stenosis and had to be re-grafted. The immunohistochemical study was performed to identify the different cellular phenotypes present in the vein wall and the temporo-spatial changes that occurred during the development of intimal hyperplasia. The panel of monoclonal antibodies used were CD 31 and Factor VIII related antigen to identify endothelial cells; α-actin, desmin, vimentin for smooth muscle cell phenotypes, vimentin for fibroblasts, CD 68 for macrophages, CD45 for lymphocytes and Ki67 for proliferating cells. The vein was cultured for specified time periods (0-21 days) and the temporo-spatial relationship of intimal hyperplasia development and the changes in cell phenotype were observed. The conditions used for the immunohistochemical study are summarised below.

Table 6.2.2 Panel of antibodies used to identify cell phenotypes in the human saphenous vein organ culture

Epitope	Cell	Company	Identity no	Origin	Working	Conditions for primary incubation
α-Actin	SMC	Dako	M 0851	Mouse	1:400	Overnight 4°C
vWF	EC	Dako	A 080	Rabbit	1:200	Prior trypsin digestion, overnight at 4°C
CD31	EC	Dako	M 0823	Mouse	1:50	Prior trypsin digestion, overnight at 4°C
Vimentin	SMC, EC	Dako	Vim 3B4	Mouse	1:100	1 hour at room temperature
Desmin	SMC	Dako	DE-R-11	Mouse	1:250	Overnight at 4°C
CD68	Macrophage	Dako	PG-M1	Mouse	1:100	Overnight at 4°C
Ki67	Proliferating	Dako	Ki67	Mouse	1:200	Overnight at 4°C
CD45	Lymphocyte	Dako	M 0701	Mouse	1:25	Overnight at 4°C

6.2.3 Methods: Immunohistological study of tPA, uPA, PAI-1, and uPAR in the human saphenous vein organ culture model of intimal hyperplasia

Immunohistochemical studies were performed on organ cultures from saphenous veins (n=16), their uncultured controls (n=16) and saphenous vein graft stesoses (n=3) using monoclonal antibodies to tPA, uPA, uPAR, and PAI-1. The conditions used in the immunohistochemical protocol were the same as summarised in Table 2.2.2 (a). Surgically distended saphenous veins were organ-cultured for 0-21 days to assess temporo-spatial changes of the PAS in the vessel wall during the development of intimal hyperplasia. In this histological study cultured veins were qualitatively compared to their uncultured control as well as the saphenous vein graft stenoses (n=3).

6.2.4 Methods: Quantitating tPA, uPA and PAI-1 proteins in the vessel wall using immuno(antigen) assays

Following the organ culturing process, samples of saphenous veins were rinsed in normal saline to wash away any serum, snap-frozen in liquid nitrogen and stored at -80°C until needed. The samples were weighed then homogenised in acetate buffer as described previously in Section 4.2.1 (b). The total protein was determined as previously described in Section 4.2.1 (c) and the tPA, uPA and PAI-1 antigens were quantified as previously described in Chapter 4.

6.2.5 Methods: Quantitating uPA and PAI-1 activities in the vessel wall using immuno(activity) assays

Following the organ culturing process, samples of saphenous veins were rinsed in normal saline to wash away any serum, snap-frozen in liquid nitrogen and stored at -

80°C until needed. The samples were weighed then homogenised in acetate buffer as described previously in Section 4.2.1 (b). The total protein was determined as previously described in Section 4.2.1 (c) and the uPA and PAI-1 activities were quantified as previously described in Chapter 5.

6.2.6 Methods: mRNA expression of tPA, uPA and PAI-1 in the human saphenous vein organ culture model

Two approaches were used to detect the presence of mRNA to tPA, uPA and PAI-1 in the vein culture, namely, RTPCR of total RNA extracted from the cultured vein, and mRNA *in- situ* hybridisation.

6.2.6 (a) RTPCR of RNA extracted from saphenous vein organ culture

The sample of saphenous vein allocated for RTPCR was snap-frozen in liquid nitrogen and stored at -80°C until needed. The protocols used for the total RNA extraction and RTPCR of vein cultures were exactly as described previously for blood vessels to detect tPA, uPA and PAI-1, see Sections 3.2.4 and 3.2.13. The RTPCR products were separated by agarose gel electrophoresis and size determined using KB ladder (Gibco BRL) as described in Section 3.2.113 (c) I.

6.2.6 (b) mRNA in situ hybridisation study of saphenous vein organ culture

After the culturing process a sample of the vessel was placed in 10% formaldehyde in saline and fixed at 4°C overnight. The protocol used for the *in-situ* hybridisation was as described previously in Section 2.2.3.

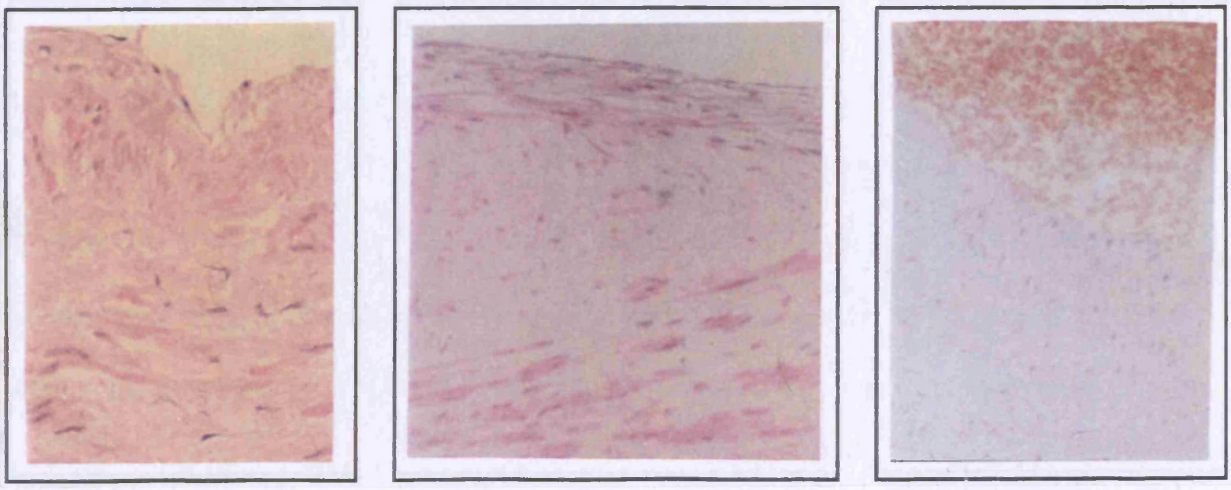
6.3 RESULTS

6.3.1 Immunohistological characterisation of the saphenous vein organ culture; comparison with normal uncultured (control) veins and saphenous vein graft stenosis.

All the saphenous veins organ-culture for 2 weeks (n=16) developed a thick neointimal layer of spindle-shaped cells which overlaid the media as shown in the H&E stained section in Fig 6.3.1(a) II below. The media of the vessel walls were still recognisable but were depleted of smooth muscle cells suggesting that migration of these cells were intimally directed and occurred during the formation of the neointima. The saphenous vein graft stenoses (n=3) (an example shown in Fig 6.3.1 (a) III) all contained a neointima which compromised the lumen. The neointimal cells were seen on higher magnification to be either spindle-shaped or stellate-shaped. As in all the control veins, all the vein cultures and the vein graft stenoses were lined by endothelium. In contrast to normal saphenous veins, the lumenal diameter of saphenous veins which had undergone arterialised vein graft stenosis was considerably smaller whilst the cross sectional area of the vessel was considerably larger indicating that the vessels had undergone an adaptive remodelling process during the development of graft stenosis.

6.3.1 (a) H& E staining

(I) Saphenous vein control *(II)Saphenous vein organ culture* *(III) Vein graft stenosis*



Figures 6.3.1 (b) II demonstrates the universal finding (seen in all 16 samples) that the spindle-shaped cells of the neointima of the saphenous vein organ culture consists of α-actin positive smooth muscle cells. The internal elastic lamina stained blue by Miller's reagent is seen to be flimsy and discontinuous. The thick medial layer of smooth muscle cells seen in the uncultured control saphenous vein (see Fig 6.3.1 (b) I) is replaced by a media much depleted of α-actin positive cells in the vein culture. The very thick layer of neointima in the vein graft stenosis (Fig 6.3.1 (b) III) consists of α-actin positive cells. On higher magnification these cells are seen to be both spindle-shaped and stellate-shaped whilst the media is seen to be diminished in thickness compared to the usual thickness one would expect to see in normal saphenous veins.

6.3.1 (b) α-actin/Miller's stain

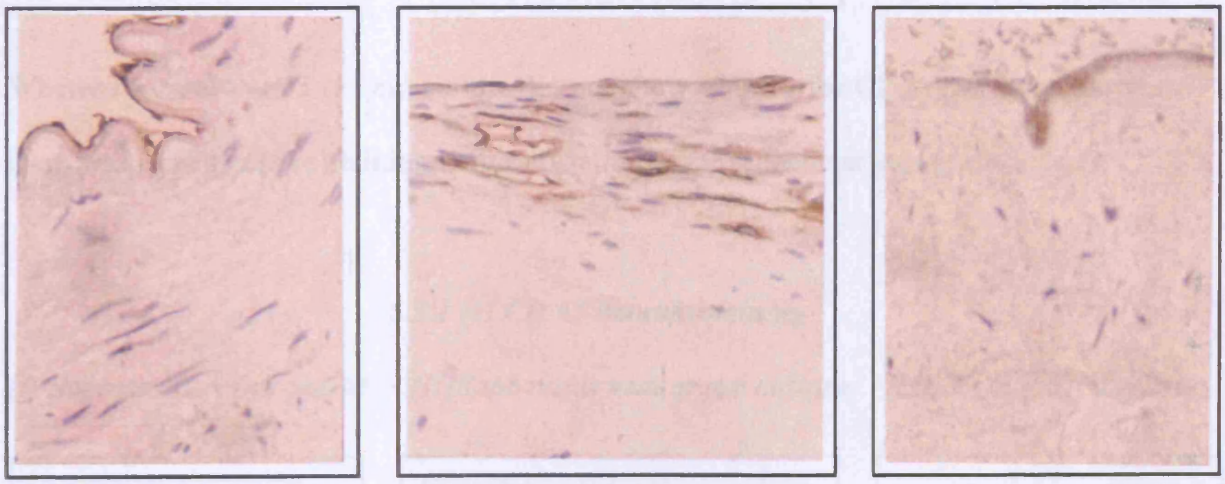
(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***

Immunostaining for CD31 as shown in Fig 6.3.1 (c), demonstrated the presence of endothelial cells overlying the neointima of all the saphenous veins in organ culture. This neointima was also found to have numerous endothelial cells some of which were isolated whilst others appeared to be arranged as tubular structures. CD31 immunostaining of

uncultured (control) saphenous veins demonstrated the presence of endothelium as expected, but unlike the organ culture, there was the absence of positive immunostaining in the rest of the intima, and, in particular there was the absence of these tubular endothelial structures. In the saphenous vein graft stenosis, in addition to the positive immunostaining on its endothelium, CD31 immunostaining was also positive deep within the neointima presumably indicating neovascularisation within this type of vessel.

6.3.1 (c) CD 31 immunostaining

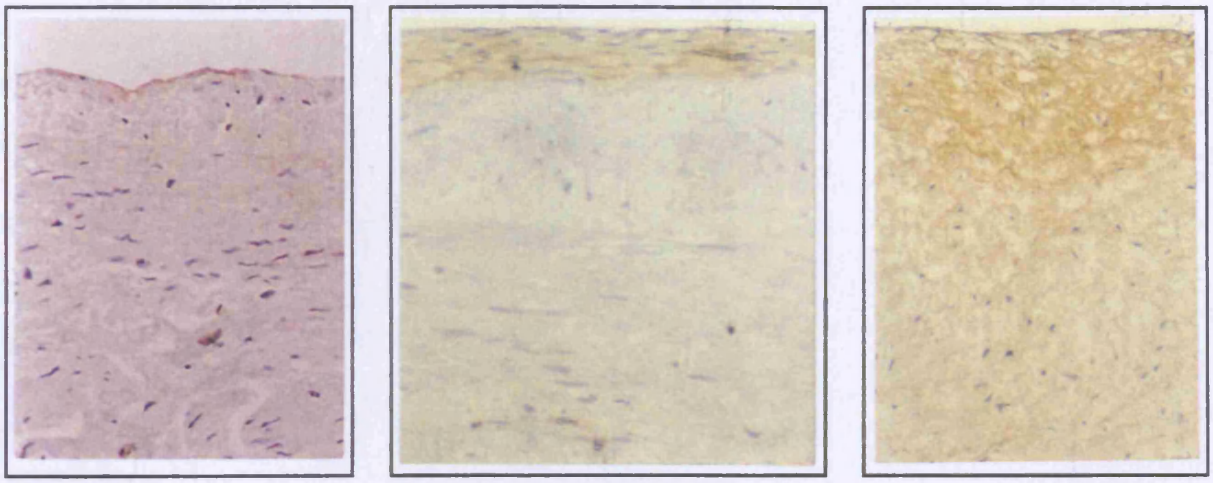
(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



Immunostaining for Factor VIII, Fig 6.3.1(d), was associated with endothelial cells as was seen for CD31 immunostaining, but, unlike the latter, Factor VIII staining was also abundant in the extracellular spaces of the neointima of both the saphenous vein culture as well as the saphenous vein graft stenosis.

6.3.1 (d) Factor VIII immunostaining

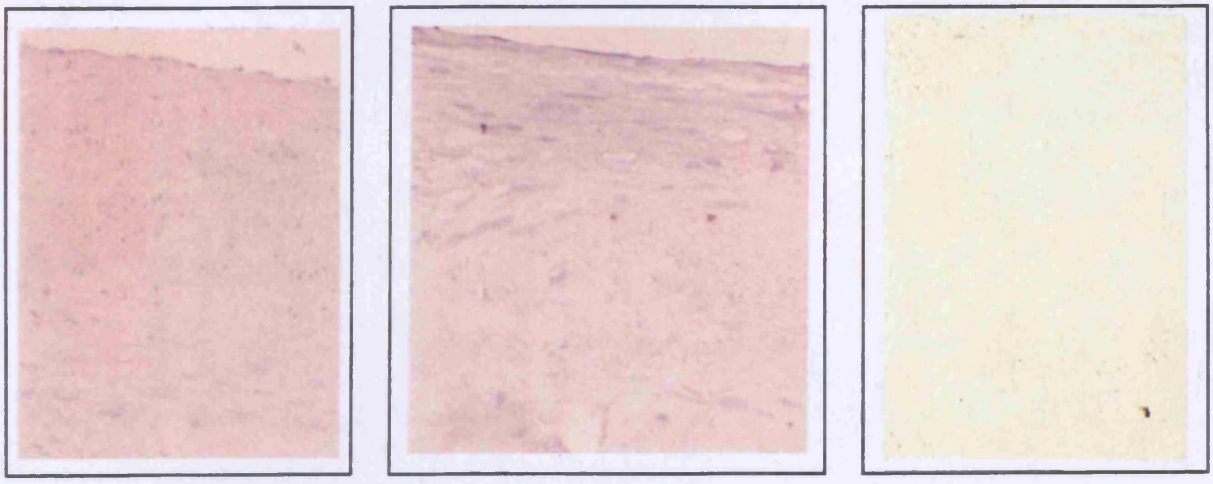
(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



Whereas control vein and vein culture did not show staining for CD45 (for lymphocytes), there was some positive staining in the adventitia of the saphenous vein graft stenosis.

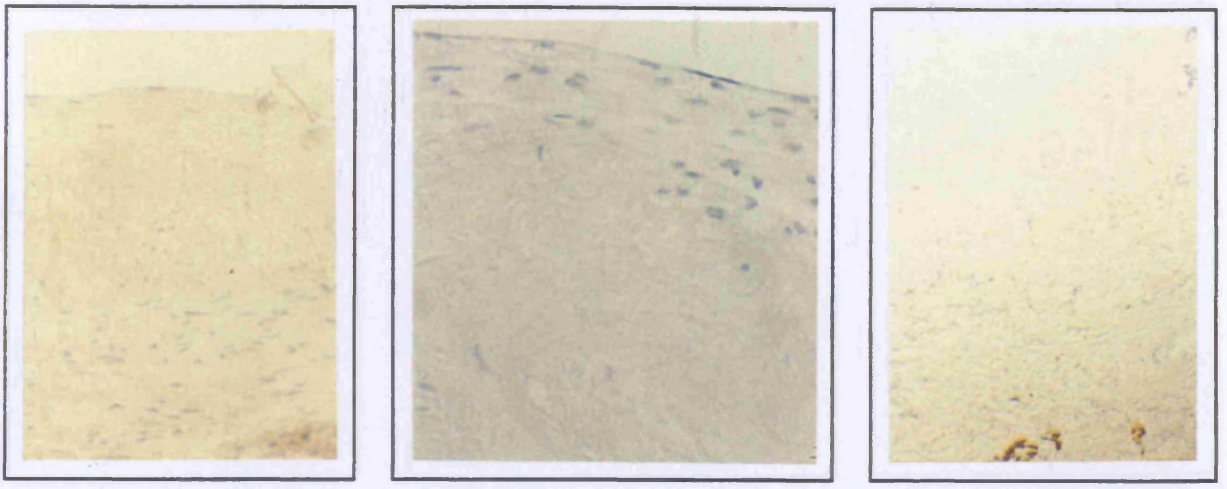
6.3.1 (e) CD 45 immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



6.3.1 (f) CD 68 immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



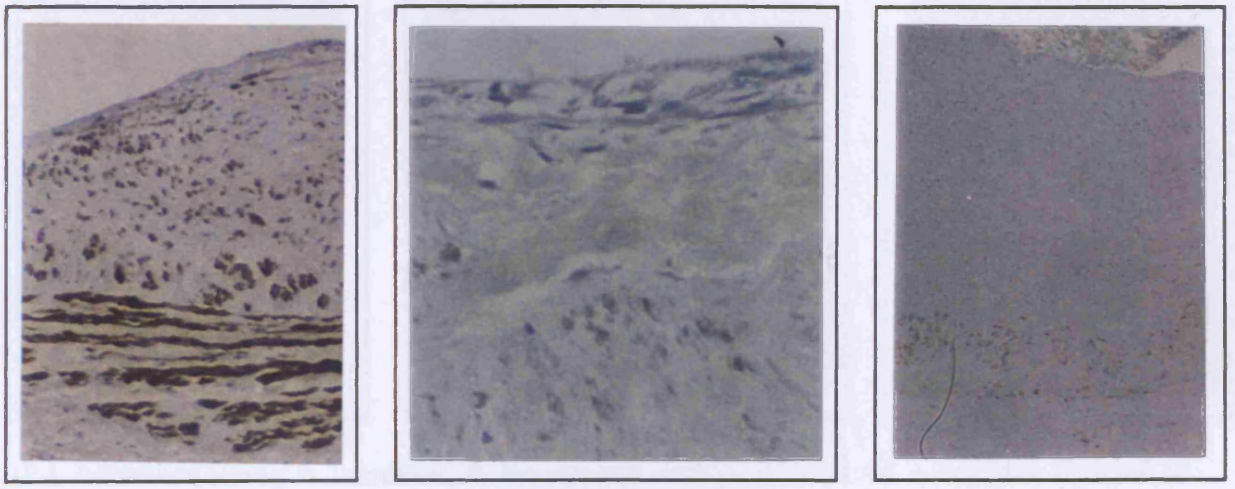
Similarly, whereas control vein and vein culture universally did not show staining for CD68 (for macrophages), there was some positive staining in the adventitia of one of the three saphenous vein graft stenosis samples.

There were significant phenotypic changes in smooth muscle cells noted during the development of intimal hyperplasia in the saphenous vein organ culture. These changes were exemplified by immunostaining for two intermediate filaments, desmin and vimentin. Desmin immunostaining was very strong in the media of all the normal control saphenous veins as shown in Fig 6.3.1 (g) I. However desmin immunostaining was largely absent in the intimal hyperplasia both of saphenous veins in organ culture (present in only 2 of 16 vessels cultured for 2 weeks) and saphenous vein graft stenosis (in a few isolated cells in all three samples), see Figs 6.3.1 (g) II and 6.3.1 (g) III respectively. Interestingly, desmin immunostaining could be found, albeit at a greatly reduced amount, in the media of all three vein graft stenoses. In all 16 uncultured control saphenous veins, vimentin

immunostaining was largely absent from both saphenous vein culture and saphenous vein graft stenosis.

6.3.1 (g) Desmin immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



6.3.1 (h) Vimentin immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***

6.3.2 Results: Immunohistological study of tPA, uPA, PAI-1 and uPAR in saphenous veins in organ culture.

6.3.2 (a) tPA imunostaining

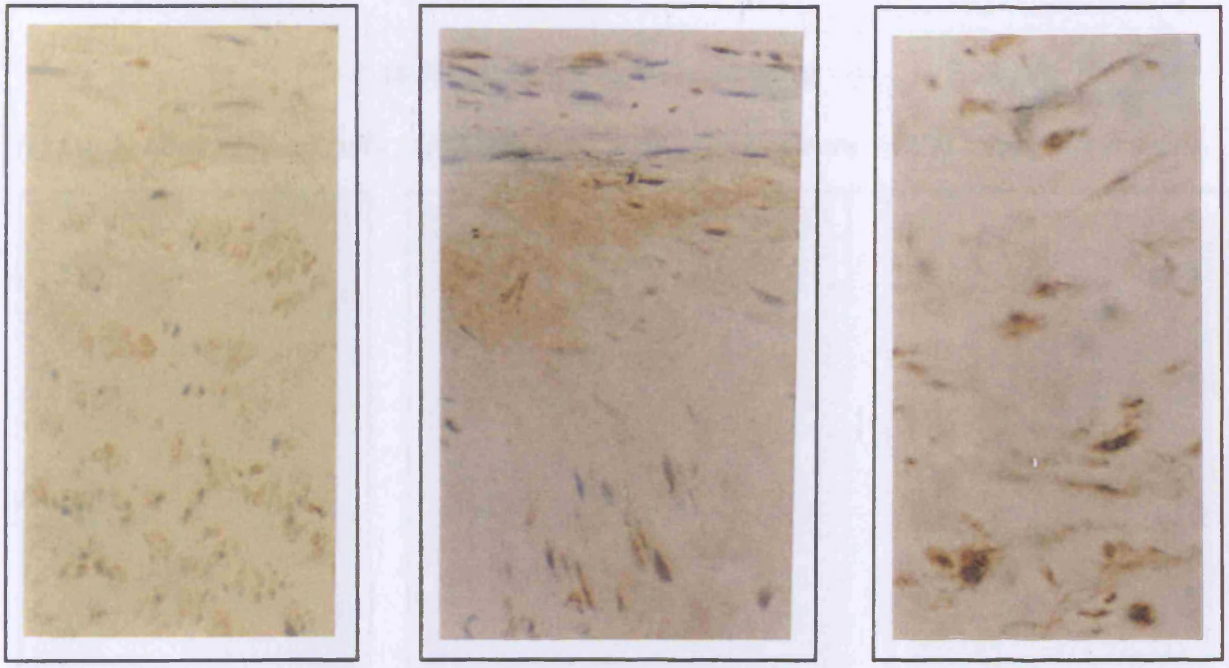
(I) Saphenous vein control *(II)Saphenous vein organ culture* *(III) Vein graft stenosis*

tPA immunostaining is increased in the smooth muscle cells of the neointima both of all saphenous veins cultured for 2 weeks as well as all 3 saphenous vein graft stenoses compared to the smooth muscle cells of normal undistended saphenous veins. The degree of tPA staining of the media of saphenous vein graft stenosis is similar to that of normal undistended saphenous veins. tPA staining is also present on the endothelium of all of these three types of vessels.

uPA immunostaining is increased in the smooth muscle cells of the neointima of all saphenous veins cultured for 2 weeks as well as all 3 saphenous vein graft stenoses compared to the smooth muscle cells of undistended saphenous veins. The extent of uPA staining of the media of the saphenous vein graft stenosis, is similar to that of normal undistended saphenous veins. uPA staining is also present on the endothelium of all of these three types of vessels.

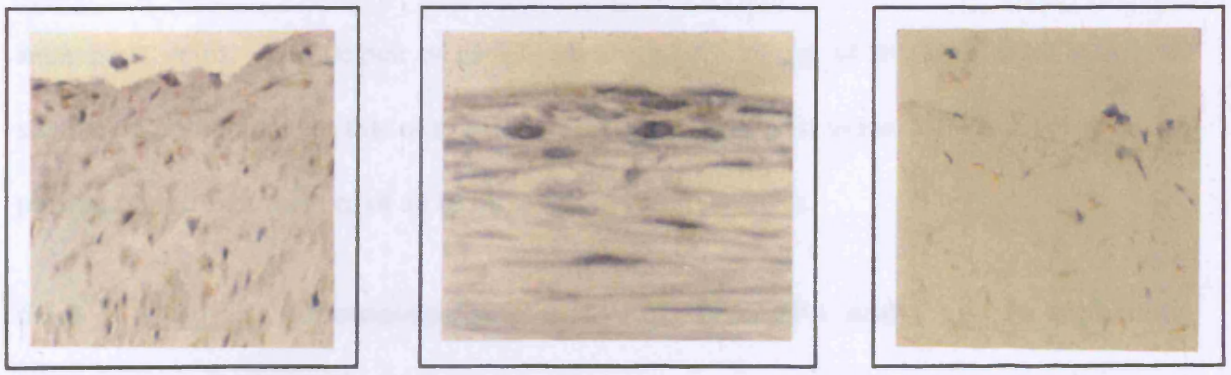
6.3.2 (b) uPA immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



6.3.2 (c) PAI-1 immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***

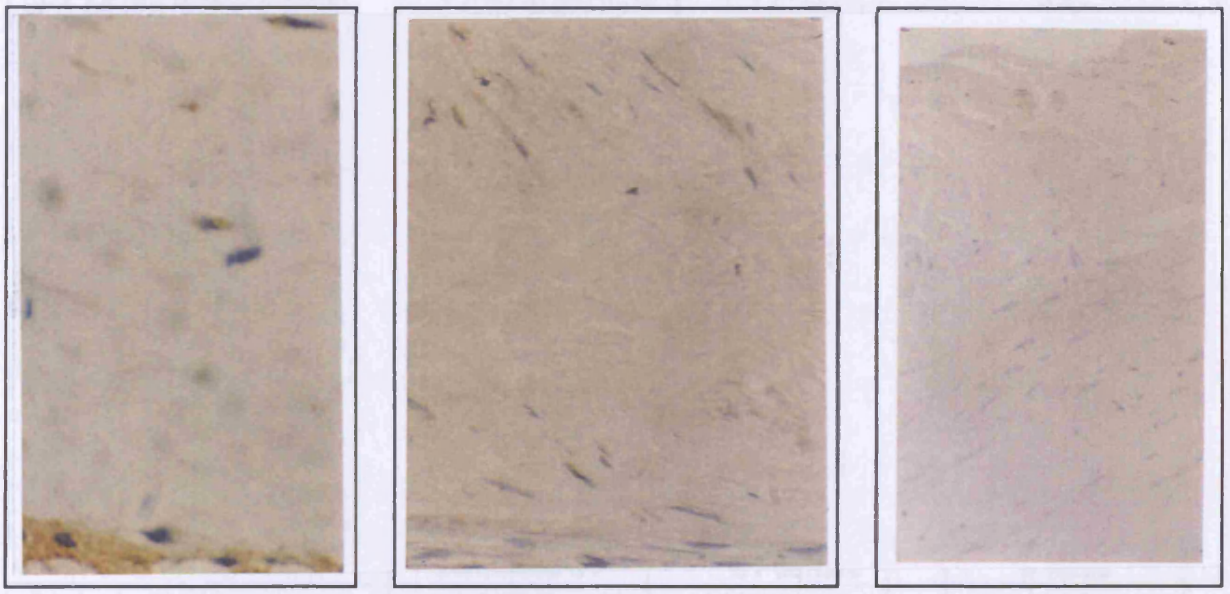


PAI-1 staining is present on the endothelium of saphenous veins, saphenous vein graft stenosis and saphenous veins in culture with the degree of immunostaining greatly increased in the case of the latter. However, PAI-1 staining was reduced in the smooth muscle cells of

the neointima of both the saphenous vein graft graft stenosis and saphenous vein organ culture compared to normal native saphenous veins.

6.3.2 (d) uPAR immunostaining

(I) Saphenous vein control ***(II)Saphenous vein organ culture*** ***(III) Vein graft stenosis***



As was found with tPA and uPA, uPAR immunostaining is increased in smooth muscle cells of the neointima both of saphenous veins cultured for 14 days (n=16) as well as the saphenous vein graft stenoses (n=3) compared to the smooth muscle cells of undistended saphenous veins. The degree of uPAR staining of the media of the saphenous vein graft stenosis is comparable to that of normal undistended saphenous veins. uPAR staining is also present on the endothelium of all of these three types of vessels.

6.3.3 Results: Immuno(antigen) assays of tPA, uPA and PAI-1 in saphenous veins in organ culture.

When expressed as a fraction of total protein content, there was significantly less tPA antigen and significantly more PAI-1 antigen in organ-cultured saphenous veins compared to distended saphenous veins, $p=0.019$ and $p<0.0001$ respectively (unpaired), see Table 6.3.3

(a), showing the Mean and (SEM). uPA antigen was not significantly different between the distended saphenous veins and organ-cultured saphenous veins (unpaired).

Table 6.3.3 (a) Content of tPA, uPA and PAI-1 antigens in distended saphenous veins and saphenous veins in organ culture expressed per quantity of total protein.

	Distended SV	SV culture	p value
tPA Ag (μg/g total protein)	3.9(0.9) n=11	1.3 (0.4) n=5	0.019
uPA Ag (μg /g total protein)	0.07 (0.02) n=16	0.1 (0.06) n=9	0.64
PAI-1 Ag (μg/g total protein)	0.4 (0.1) n=9	12.0 (5.4) n=9	<0.0001

When expressed as a fraction of tissue weight, there was significantly greater PAI-1 antigen in organ-cultured saphenous veins compared to distended saphenous veins, $p<0.0001$, (unpaired) see Table 6.3.3 (b). uPA and tPA antigens were not significantly different between the distended saphenous veins and organ-cultured saphenous veins (unpaired).

Table 6.3.3 (b) Content of tPA, uPA and PAI-1 antigens in distended saphenous veins and saphenous veins in organ culture expressed per quantity of tissue weight

	Distended SV	SV culture	p value
tPA Ag (ng/g tissue)	310.7(37.9) n=11	291.8(90.0) n=5	0.66
uPA Ag (ng/g tissue)	4.5 (0.8) n=16	6.2 (1.2) n=9	0.23
PAI-1 Ag (ng/g tissue)	72 (11.9) n=9	609 (73) n=9	<0.0001

6.3.4 Results: Immuno(activity) assays of uPA and PAI-1 in distended saphenous veins and saphenous veins in organ culture.

uPA activity was found to be significantly greater in saphenous veins undergoing organ culture compared to uncultured surgically distended saphenous veins (unpaired comparison), see Table 6.3.4 (a) below. When expressed as a proportion of total protein, uPA activity was also greater in the vein culture but this did not achieve statistical significance. An explanation of this apparent discrepancy is given in the discussion section of this chapter. PAI-1 activities were not found to be significantly different between vien culture and native distended saphenous veins.

(a), showing the Mean and (SEM). uPA antigen was not significantly different between the distended saphenous veins and organ-cultured saphenous veins (unpaired).

Table 6.3.3 (a) Content of tPA, uPA and PAI-1 antigens in distended saphenous veins and saphenous veins in organ culture expressed per quantity of total protein.

	Distended SV	SV culture	p value
tPA Ag (μg/g total protein)	3.9(0.9) n=11	1.3 (0.4) n=5	0.019
uPA Ag (μg /g total protein)	0.07 (0.02) n=16	0.1 (0.06) n=9	0.64
PAI-1 Ag (μg/g total protein)	0.4 (0.1) n=9	12.0 (5.4) n=9	<0.0001

When expressed as a fraction of tissue weight, there was significantly greater PAI-1 antigen in organ-cultured saphenous veins compared to distended saphenous veins, $p<0.0001$, (unpaired) see Table 6.3.3 (b). uPA and tPA antigens were not significantly different between the distended saphenous veins and organ-cultured saphenous veins (unpaired).

Table 6.3.3 (b) Content of tPA, uPA and PAI-1 antigens in distended saphenous veins and saphenous veins in organ culture expressed per quantity of tissue weight

	Distended SV	SV culture	p value
tPA Ag (ng/g tissue)	310.7(37.9) n=11	291.8(90.0) n=5	0.66
uPA Ag (ng/g tissue)	4.5 (0.8) n=16	6.2 (1.2) n=9	0.23
PAI-1 Ag (ng/g tissue)	72 (11.9) n=9	609 (73) n=9	<0.0001

6.3.4 Results: Immuno(activity) assays of uPA and PAI-1 in distended saphenous veins and saphenous veins in organ culture.

uPA activity was found to be significantly greater in saphenous veins undergoing organ culture compared to uncultured surgically distended saphenous veins (unpaired comparison), see Table 6.3.4 (a) below. When expressed as a proportion of total protein, uPA activity was also greater in the vein culture but this did not achieve statistical significance. An explanation of this apparent discrepancy is given in the discussion section of this chapter. PAI-1 activities were not found to be significantly different between vien culture and native distended saphenous veins.

Table 6.3.4 (a) uPA and PAI-1 activities [Mean (SEM)] in distended saphenous veins and saphenous veins in organ culture expressed per weight of tissue.

	Distended SV	SV culture	p value
uPA Activity (ng/g tissue)	34.9 (3.1) n=16	58.9 (6.3) n=9	0.005
PAI-1Activity (U x10^3/g tissue)	1.5 (0.2) n=15	2.3 (0.4) n=10	0.16

Table 6.3.4 (b) uPA and PAI-1 activities in distended saphenous veins and saphenous veins in organ culture expressed per 10mg of total protein.

	Distended SV	SV culture	p value
uPA Activity (ng/g total protein)	0.6 (0.1) n=16	1.2 (0.5) n=9	0.25
PAI-1 Activity (U x 10^3/g total protein)	23.5 (5.5) n=15	23.5 (7.1) n=9	0.91

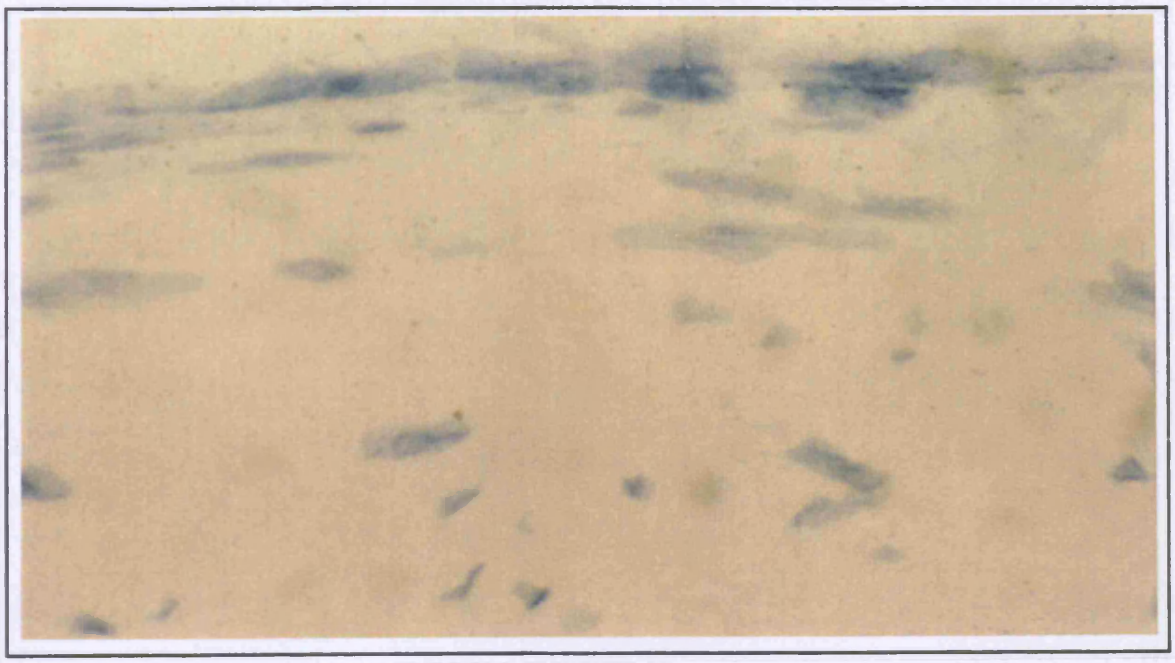
6.3.5 (a) Results: Detection of tPA, uPA and PAI-1 mRNA expression in saphenous veins in organ culture using RTPCR of vessel wall homogenate

The RNA extracted from organ cultured saphenous veins were used in RTPCR using the specific primers as previously described in Section 3.2.13. The appropriate bands were seen on ethidium stained agarose gels under ultra-violet light confirming expression of these mRNA species in the vessels that underwent vein culture.

6.3.5 (b) Results: Detection of mRNA expression in saphenous veins in organ culture using *in-situ* hybridisation.

In-situ hybridisations for tPA, uPA and PAI-1 were performed on 14 day saphenous vein organ cultures. At this time point only PAI-1 mRNA signal was detected and this was strong on the endothelium but largely absent in the underlying neointima.

Fig 6.3.5 PAI-1 mRNA on insitu hybridisation in saphenous vein organ culture



6.4 DISCUSSION

6.4.1 Suitability of the saphenous vein organ culture model in studying the changes in the plasminogen activation system in intimal hyperplasia.

Since the saphenous vein organ culture model was to be used to investigate the temporospatial changes in expression of components of the plasminogen activation system, and, since this model had not been fully characterised, an attempt was made to characterise the cell phenotypes using a panel of monoclonal antibodies in immunohistological studies. Like others, (Barrett et al 1979,and Soyombo et al 1990), we also observed that the migrating smooth muscle cells were intimally directed. The use of desmin, vimentin and α-actin immunohistology demonstrated a close similarity between the model and *ex-vivo* saphenous vein graft stenosis. Both desmin and vimentin immunopositivity of the smooth muscle cells within the neointima were greatly reduced in whilst α-actin staining was unchanged. Interestingly, desmin immunostaining could be found, albeit at a greatly

reduced amount, in the 'old' media of the vein graft. This is in keeping with desmin being associated with contractile smooth muscle cell phenotype (Johansson et al 1999). The concordance between the vein organ culture model and *ex-vivo* saphenous vein graft stenosis was further demonstrated with vWF immuostaining; the vWF immunostaining was not only present on the endothelium but extended deep into the extracellular tissue of the neointima of both vein culture and vein graft stenosis, but not in native undistended saphenous veins. In keeping with Soyombo et al 1990, both vWF and CD31 immunostaining demonstrated the presence of multiple tubular endothelial structures within and underneath the neointima of saphenous veins undergoing organ culture. This may suggest that the saphenous vein organ culture, in addition to its use as a model for studying intimal hyperplasia development, also has the potential for use as a model to study angiogenesis and neovascularisation. However, immunostaining against CD45 (leucocyte common antigen), and CD68 was present in vein graft stenosis indicating the presence of macrophages; these antigens were not seen in normal saphenous veins and consequently not present in vein organ culture. In addition, the cells within the neointima of the organ culture model were seen to be closely packed without much extracellular matrix. In summary therefore, whilst the vein organ culture model shares a number of similarities with *in-vivo* venous intimal hyperplasia, there are differences which may be important.

6.4.2 Discussion: The effects of organ culturing saphenous veins on the expression of the PAS; similarities to in-vivo saphenous vein graft stenosis.

Increased immunostaining to tPA, uPA and uPAR was associated with the smooth muscle cells of the neointima of all the saphenous veins in culture (n=16) as well as all the saphenous vein graft stenosis (n=3) compared to the smooth muscle cells of normal

undistended saphenous veins (n=16). The increase was observed immunohistologically as early as 3 days of vein organ culture and persisted at 3 weeks; the longest time point the saphenous veins were cultured. Unlike the three mentioned proteins, PAI-1 immunostaining was much less apparent within the neointima of all the organ-cultured veins and saphenous vein graft stenoses. In the vein organ culture, smooth muscle cells (with their long axes perpendicular to the cells of both the media and the neointima) that appeared to be migrating to form the neointima, were associated with increased uPA and uPAR immunostaining, see Figs 6.3.2 (b)(II) and 6.3.2 (d)(II). The degree of immunostaining of smooth muscle cells of tPA, uPA, PAI-1 and uPAR in the media of the saphenous vein graft stenosis is comparable to that of the media of normal undistended saphenous veins.

All four proteins were present on the endothelium of all three types of vessels mentioned above. In the case of PAI-1, there was a strong increase in PAI-1 immunostaining of the endothelium overlying the newly formed neointima of the vein organ culture. This strong immunohistological signal of PAI-1 on the endothelium of the vein culture is likely to be due to increased local synthesis since the *in-situ* hybridisation study demonstrated very stong PAI-1 mRNA signal in endothelial cells at 14 days of culture. Similarly, in the samples of saphenous vein graft stenoses studied, PAI-1 mRNA signal was also observed on the endothelium. However, unlike the saphenous vein graft stenosis which demonstrated signal for tPA and uPA mRNA, vein organ culture at 2 weeks of culture did not show signal for these two mRNA species. This difference between saphenous veins in organ culture and saphenous vein graft stenosis may be temporal phenomenon.

Quantitation of the specific proteins by immunoantigen assay of the vessel wall homogenate demonstrated that there was significantly more PAI-1 antigen and no change in uPA antigen

when organ-cultured saphenous veins were compared to distended saphenous veins. tPA antigen was found to be at significantly higher levels in distended saphenous veins compared to vein organ culture when expressed as a fraction of total protein but not when expressed as a fraction of tissue weight. The reason for the latter discrepancy is not clear and merits further study.

Although there appears to be differences in the results between the immunohistological and immunoantigen assays on vessel wall homogenates, it has to be remembered that the former study is at best semi-quantitative; for example it is possible that PAI-1 antigen which was found to be very high in the vein organ culture homogenate came predominantly from the endothelium as the neointimal smooth muscle cells were not observed to be strongly immunopositive iimmunohistologically. This would be compatible with the relatively strong PAI-1 mRNA signal obtained from the endothelium of the vein organ culture

It is interesting to speculate as to why the expression of these proteins should be altered during the process of intimal hyperplasia development. As suggested by Reidy et al 1996, a possible explanation could be that these proteins are needed for the regulation of proliferation and migration of the smooth muscle cells to result in neointimal formation. The concurrent alterations in the presence of agonists (tPA and uPA), facilitator (uPAR) and antagonist (PAI-1) of the plasminogen activation system may indicate the need for modulating the degree of extracellular proteolysis during smooth muscle cell proliferation and migration. The strong signal for PAI-1 mRNA and protein associated with the endothelium of saphenous vein organ culture may explain the propensity of saphenous vein grafts to thrombotic closure.

CHAPTER 7

THE PLASMINOGEN ACTIVATOR SYSTEM IN EXPERIMENTAL ANGIOPLASTY.

7.1 INTRODUCTION

Depending on the coronary lesion type, restenosis accounts for the recurrence of angina pectoris in approximately 15-50% of patients following coronary angioplasty and thus remains a significant problem. One of the classic models used in investigating this phenomenon is the animal experimental angioplasty model in which an artery is mechanically injured by inflating an appropriately sized angioplasty balloon and the resultant changes in the vessel wall observed over time.

For this section of the project, frozen (-80°C) samples of rabbit common iliac arteries that had undergone experimental angioplasty and their contralateral non-balloon controls, together with paraffin embedded samples of the same vessels were used.

The frozen tissues were homogenised and used to quantify uPA and PAI-1 activities using immuno(activity) assays. The paraffin embedded tissues were used immunohistologically to localise tPA, uPA, PAI-1 and uPAR proteins and the amounts of these proteins were measured semi-quantitatively by measuring the percentage of immunopositive cells.

7.2 METHODS

7.2.1 Experimental angioplasty in the rabbits

The experimental angioplasty procedures on rabbit common iliac arteries were performed. Briefly, a 3 mm (2 cm length) angioplasty balloon (Medtronic) was inflated in the common iliac artery of New Zealand white male rabbits (fed on normal diet and weighing 3-3.5 kg), to a pressure of 8 atmospheres for three periods

of 60 seconds. The rabbits (n = 24; 3 for each time period) were sacrificed at 30 min, 2 hrs, 1 day, 3 days, 7 days, 14 days, 1 month and 3 months. The contralateral common iliac arteries were used as control.

7.2.2 Methods: Fixation of rabbit common iliac arteries

The common iliac arteries were perfusion-fixed *in-situ.* The animals were sacrificed with an overdose of phenobarbital and the upper abdominal aorta, cannulated distally and flushed with heparinised saline 1000 units in 500 mls normal saline before pressure perfusion fixation *in-situ* at 80 mmHg with 0.1 % gluteraldehyde / 4% fomaldehyde in phosphate buffer (pH 7.4) for 30 minutes. After removal, the vessels underwent further emersion fixation for 24 hrs before processing.

7.2.3 Methods: Assessment of arterial injury due to balloon angioplasty in rabbit common iliac artery

The extent of balloon injury of the rabbit common iliac artery was assessed in multiple sections of each artery with light microscopy using histochemical (see Section 2.2.1 for methodology) and immunohistochemical staining (see Section 2.2.2 for methodology on vWF and α-actin). Scanning and transmission electron microscopy of the balloon-injured arteries (1 vessel/timepoint and >10 fields/artery) were used to assess ultrastructural details including endothelialization and gaps in the internal elastic lamina.

7.2.4 Methods: Assessment of smooth muscle cell proliferation following experimental angioplasty in rabbit common iliac artery

Cell proliferating at different time points after balloon injury was assessed using a

monoclonal antibody against proliferation cell nuclear antigen (PCNA). The percentage of immunopositive (proliferating) smooth muscle cells was determined for both the media and the neointima in ballooned-injured arteries and controls.

7.2.5 Methods: Immunohistological changes in the vessel wall changes in tPA, uPA, PAI-1 and uPAR proteins following balloon angioplasty of rabbit common iliac arteries

The immunohistochemical study was performed using mouse monoclonal antibody to human tPA, u-PA, PAI-1 and uPAR. The protocol for the immunohistological procedure were exactly as described earlier in Section 2.2.2 and Table 2.2.2 (a) with two exceptions; the dilution of the anti-uPA antibody for the primary incubation was 1/100 and the length of time for the DAB incubation was 2 minutes. A minimum of 600 cells was counted in each of the 3 vessels per time point and the percentage of positive cells in the different layers of the vessel wall was counted.

7.2.6 Methods: Changes in uPA and PAI-1 activities within the vessel wall after balloon injury to the rabbit common iliac arteries.

The vessels for assessing protein activity and antigen levels did not undergo perfusion fixation. Following flushing with heparised saline, the vessels immediately removed and then snapped frozen in liquid nitrogen and stored at -70°C. The changes in activities of uPA and PAI-1 in the vessel wall after balloon injury were determined on vessel homogenates using the methodology previously described in Sections 5.2.2 (a) and 5.2.2 (b) using the commercial kits Chromolyse uPA (Biopool) and Spectrolyse PAI-1 (Biopool). Three vessels for each time point (1, 3, 7, 14, 30 days) and 5 contralateral common iliac arteries were used as unpaired controls.

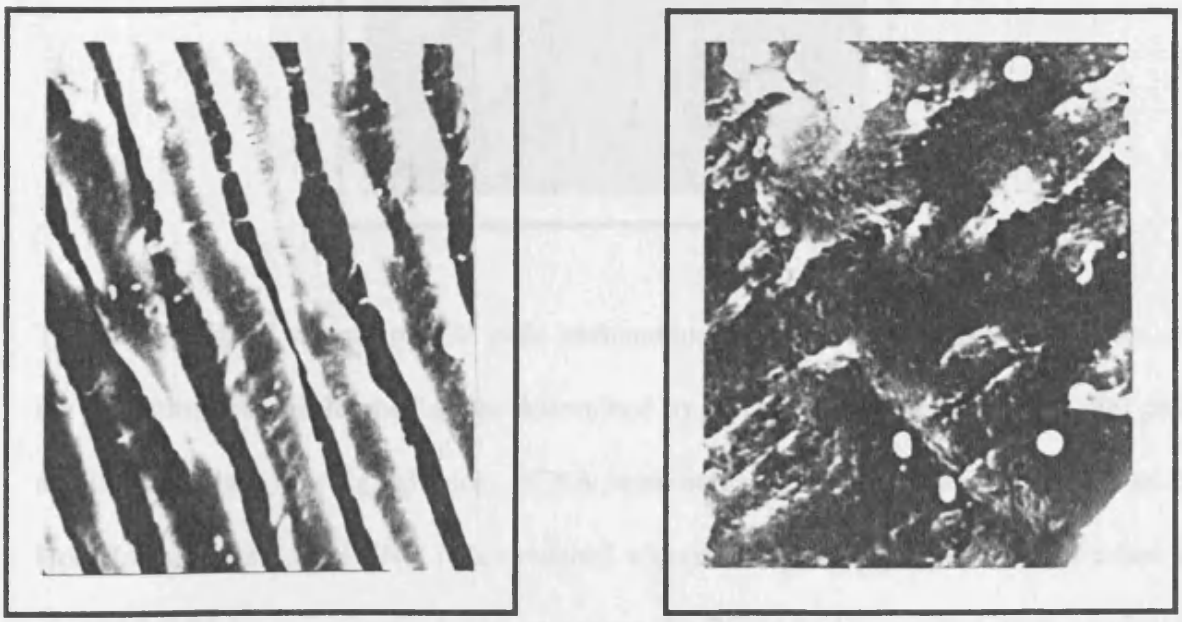
7.3 RESULTS

7.3.1 Arterial changes after balloon injury in rabbit common iliac arteries

7.3.1 (a) The endothelium

Balloon injury to the rabbit common iliac arteries resulted in the development of a neointima. There was considerable loss of endothelial cells evident in the balloon-injured area with no obvious damage to the internal elastic lamina. This immediate loss of endothelium can be seen in the balloon-injured arteries of animals sacrificed 1/2 hour after injury. Platelets were seen to be adherent to the exposed subendothelial intima.

Fig 7.3.1 (a) Scanning electron microscopy of endolumenal surface of the common iliac artery; endothelium (left) and the result of balloon injury (right).

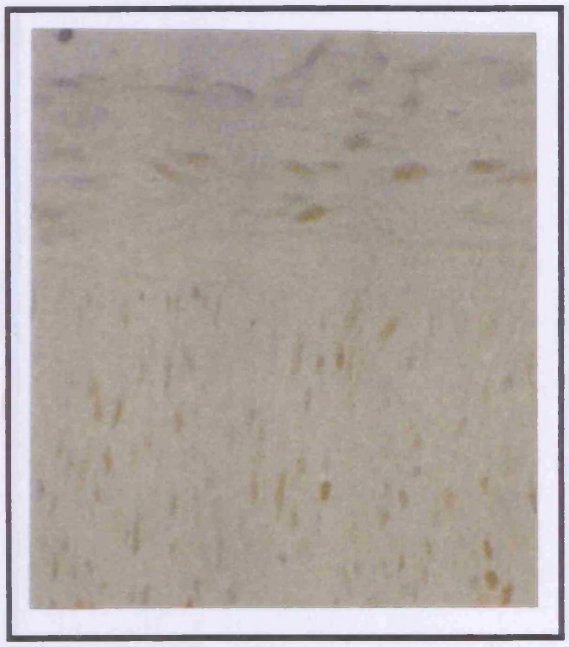


Re-endothelialisation was completed at one month post-injury in all vessels as assessed by scanning electron microscopy and vWF immunostaining.

7.3.1 (b) Results: The kinetics of smooth muscle cell proliferation in the balloon-injured rabbit common iliac artery

Immunoreactivity to PCNA was used to detect proliferating smooth muscle cells at the different time points as a result of the balloon injury.

Fig 7.3.1 (b) [I] Immunostaining for proliferating cell nuclear antigen (PCNA) seven days after balloon injury of rabbit common iliac artery.



The percentage of smooth muscle cells immunopositive for PCNA (the PCNA index) in the neointima and in the media was determined by cell counting a minimum of 600 cells and is shown below in fig 7.3.1 (c). PCNA immunoreactivity was observed as early as 24 Hrs. In the intima the PCNA index reached a maximum at 7 days and this decreased to control (<1%) by one month. In the neointima the PCNA index reached its maximum at two weeks, then decreased to baseline by one month. The percentage of PCNA positive cells in control arteries was consistently less than 1%. Fractures in the internal elastic lamina developed by 3 days when pseudopodia of smooth muscle cells were observed. The

smooth muscle cells were observed to migrate through the defects in the internal elastic lamina, see fig 7.3.1 (c). Complete re-endothelialisation was observed at 1 month.

Fig 7.3.1 (b) [II] The proliferation index using PCNA

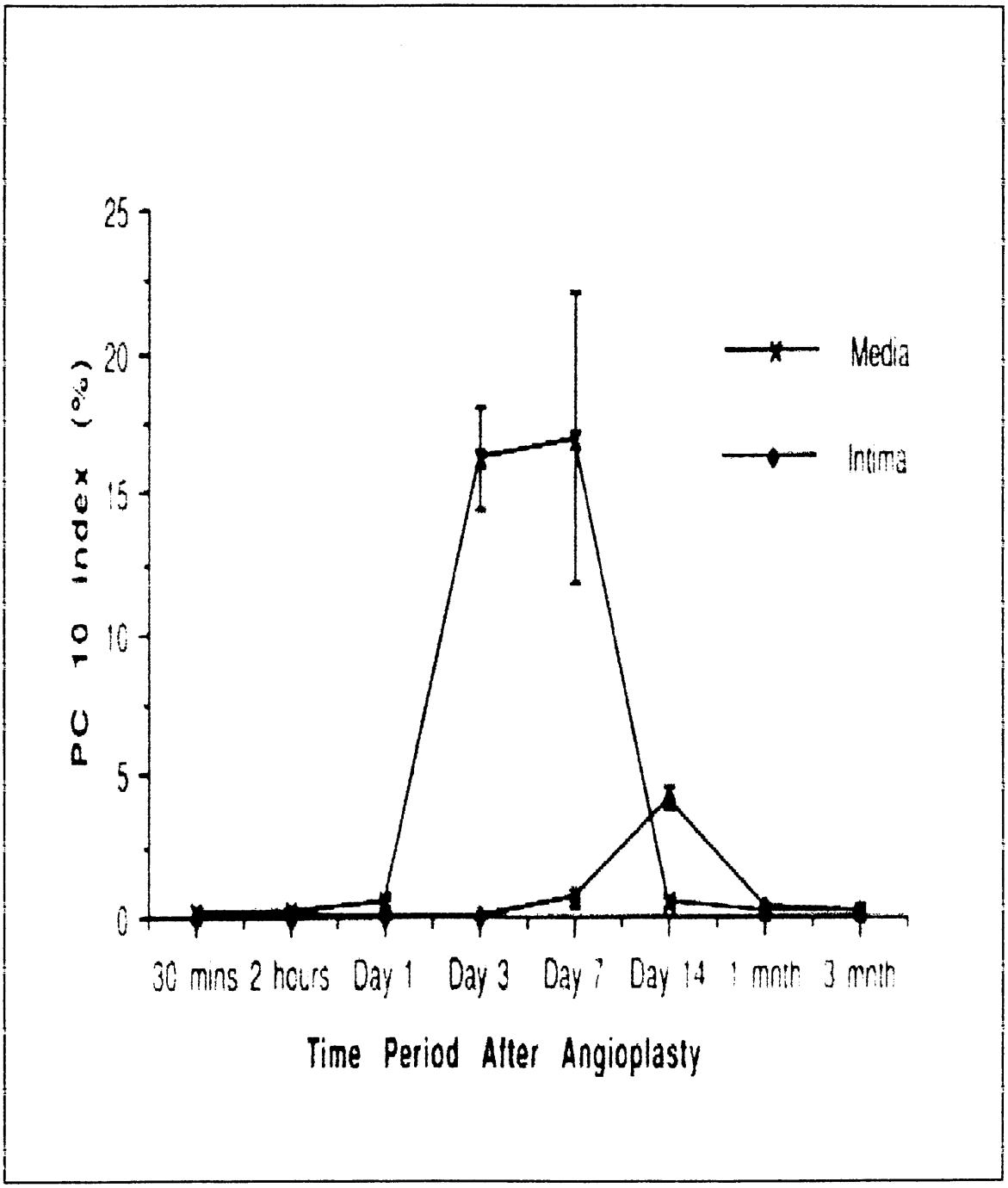
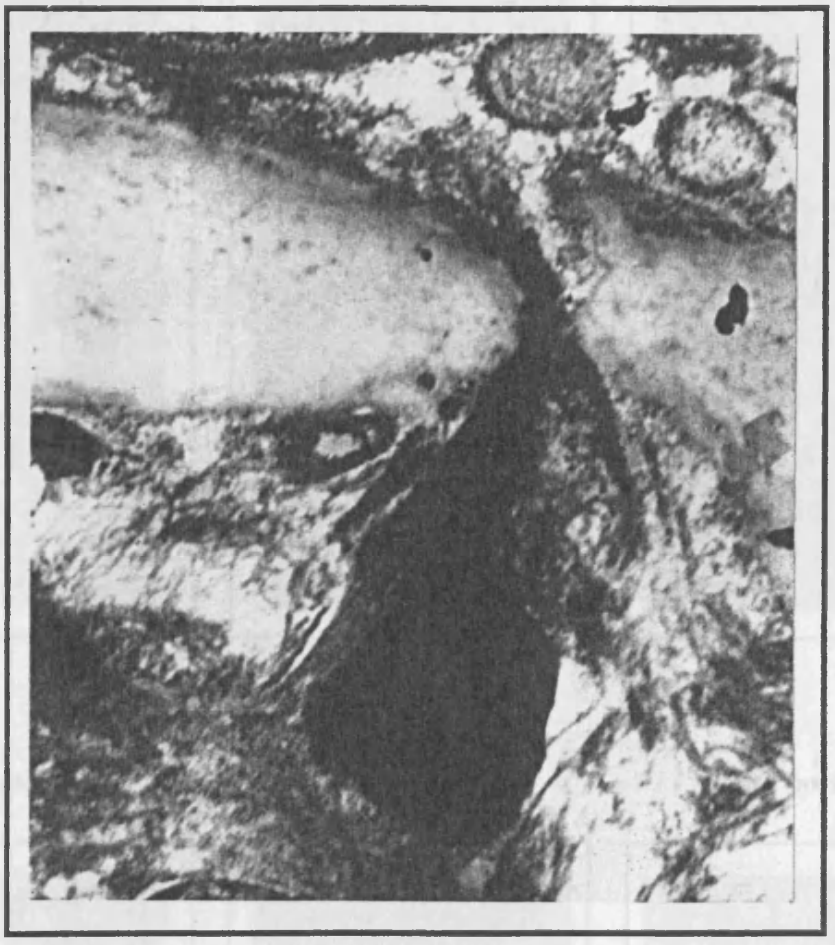


Fig 7.3.1 (b) [III] Transmission electron microscopy showing a smooth muscle cell migrating through a defect in the internal elastic lamina.



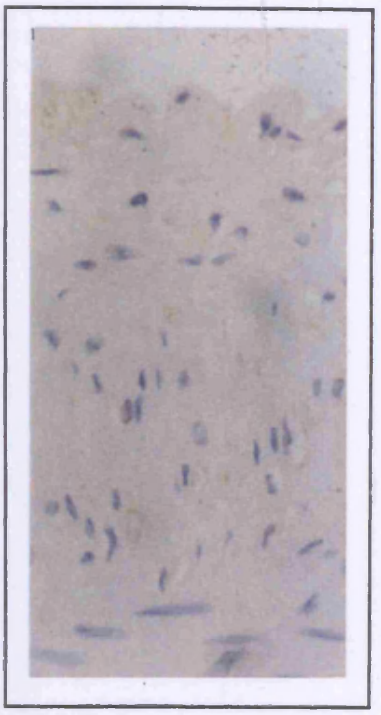
7.3.2 Result: Temporo-spatial changes in tPA, uPA, PAI-1 and uPAR proteins on immunohistochemistry in the rabbit common iliac artery following balloon injury.

The presence of these proteins in control (non-injured) rabbit common iliac arteries was essentially restricted mainly to the endothelium, with very little signal present in the media. However, after balloon injury, the amounts of these proteins all increased within the vessel wall, but with different temporospatial profiles.

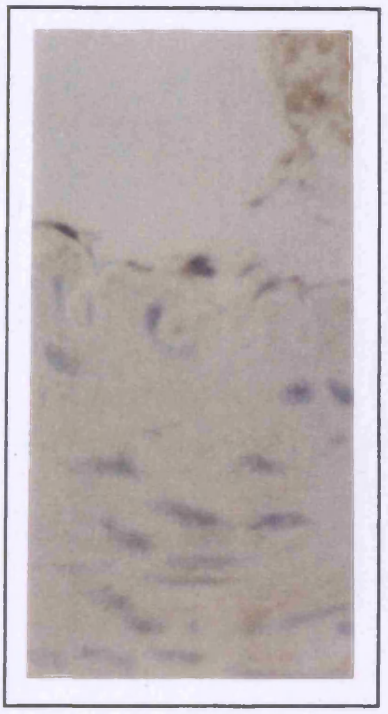
7.3.2 (a) I-VI Immunohistological changes in tPA protein in rabbit common iliac artery

I Non-balloon control

II 2 hrs post injury

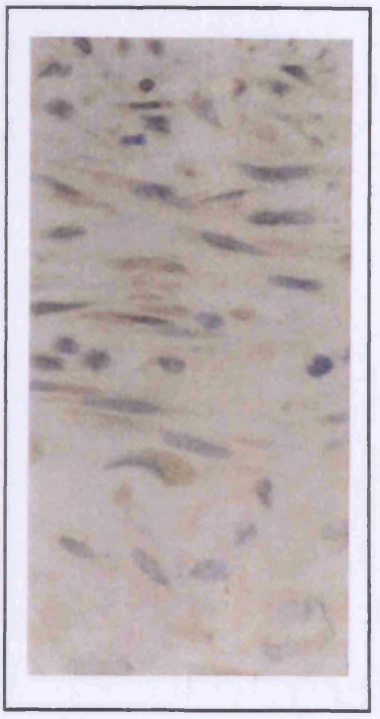


III 24 hr post injury

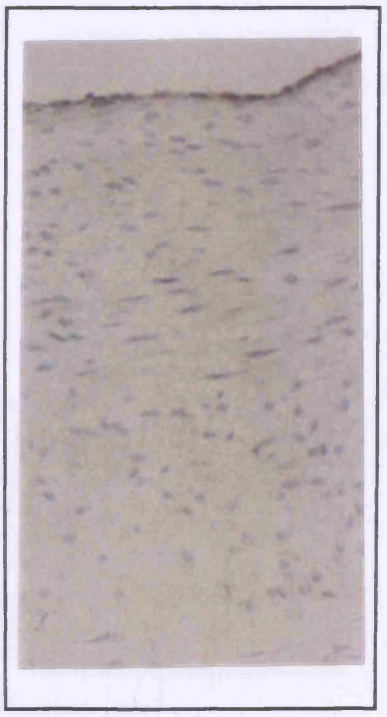


IV 1 week post injury

V 2 weeks post injury



VI 1 month post injury



7.3.2 (b) I-VI Immunohistological changes in uPA protein in rabbit common iliac artery

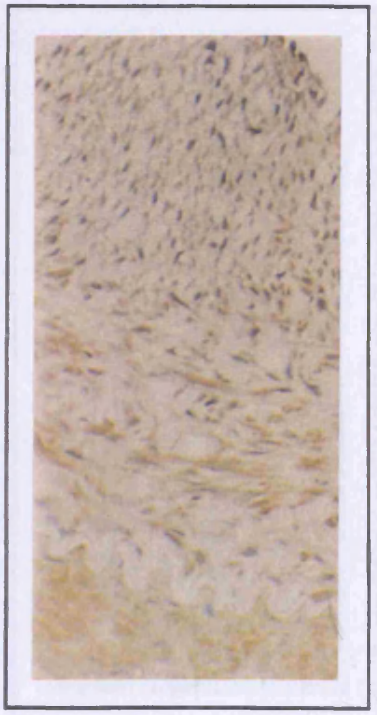
I Non-balloon control

II 1/2 hr post injury

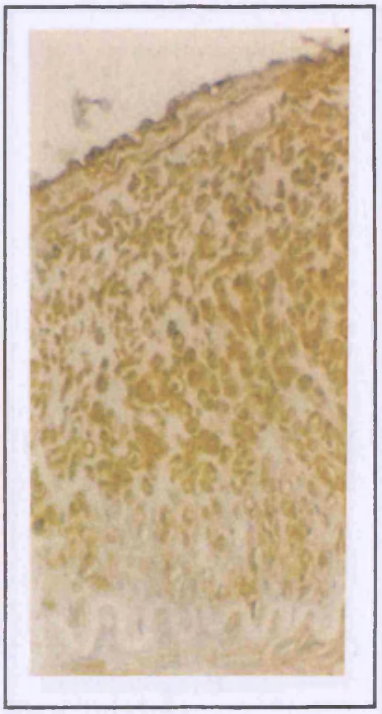
III 24 hr post injury

IV 1 week post injury

V 2 weeks post injury



VI 3 months post injury



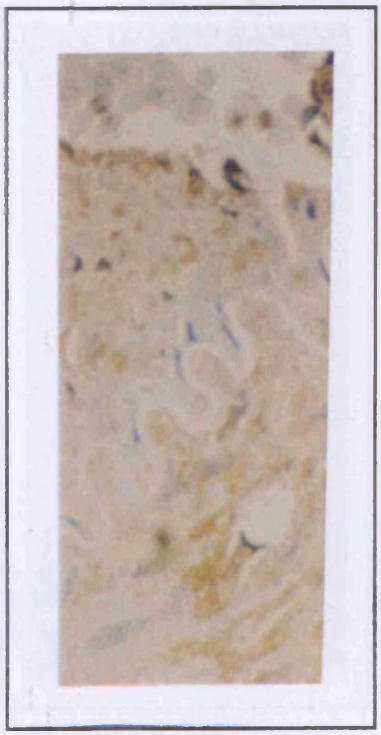
7.3.2 (c) I-VI Immunohistological changes in PAI-1 protein in rabbit common iliac artery

I Non-balloon control

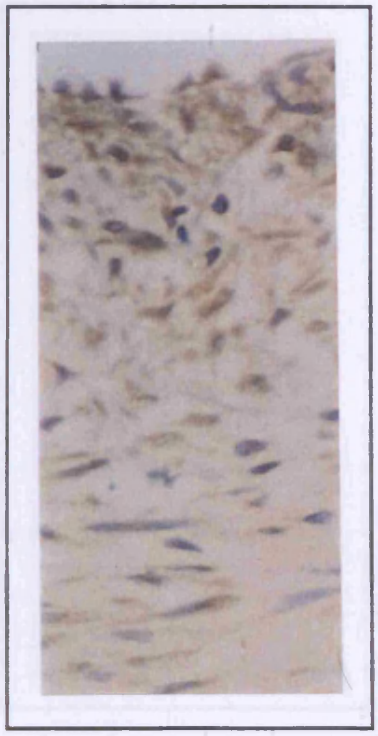
II 1/2 hr post injury

III 24 hr post injury

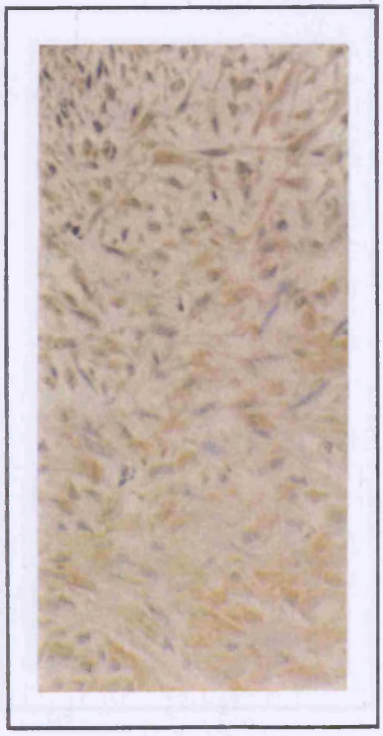
IV 1 week post injury



V 2 weeks post injury

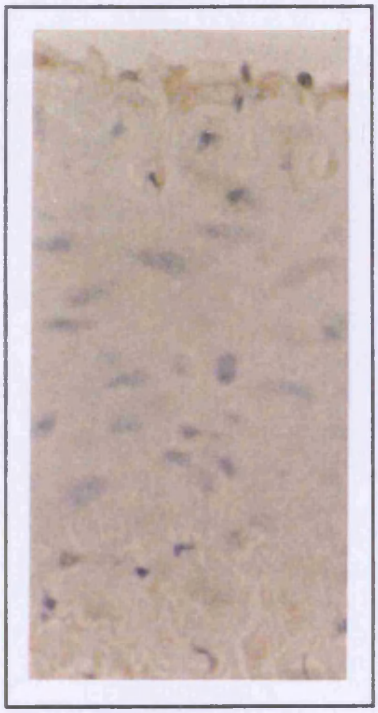


VI 3 months post injury



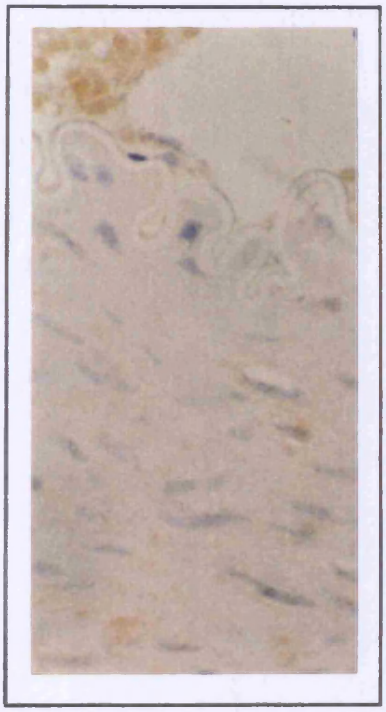
7.3.2 (d) I-VI Immunohistological changes in uPAR protein in rabbit common iliac artery

I Non-balloon control

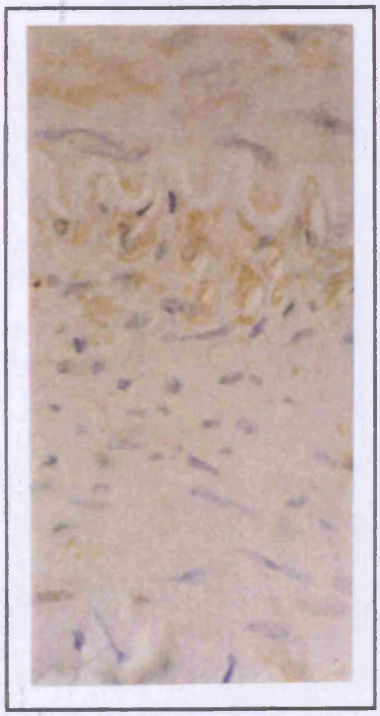


II 1/2 hr post injury

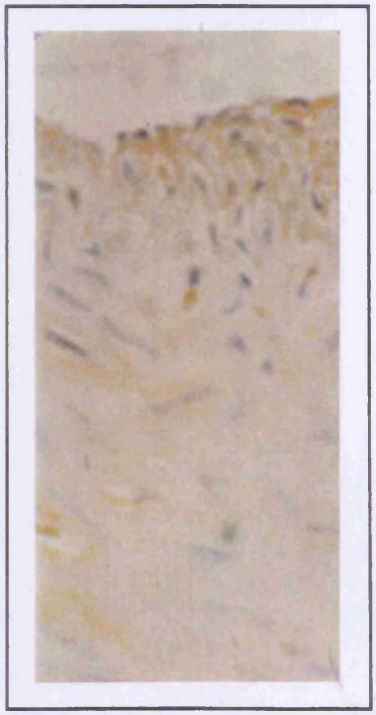
III 24 hr post injury



IV 1 week post injury



V 2 weeks post injury



VI 3 months post injury

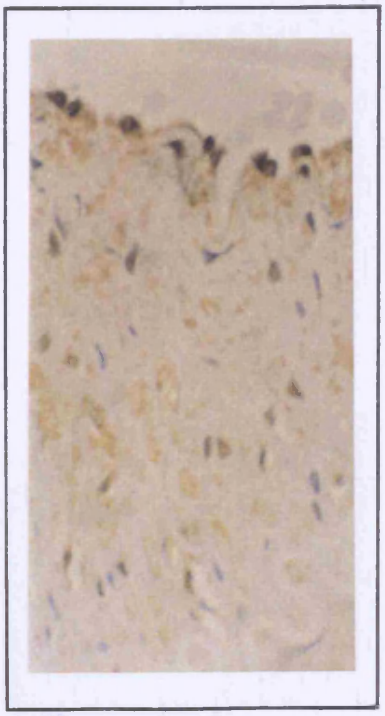
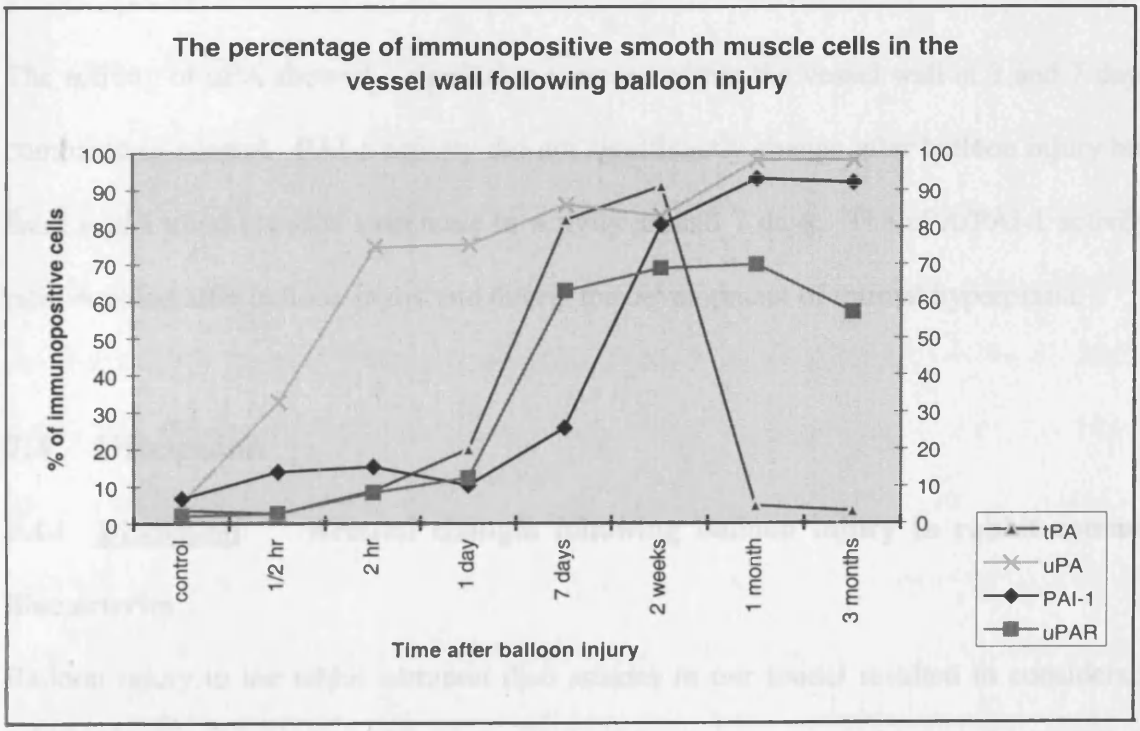


Table 7.3.2 The mean percentage and (SEM) of immunopositive smooth muscle cells to tPA, uPA, PAI-1 and uPAR

	Control	1/2hr	2 hrs	1day	7 days	14 days	30 days	90 days
t-PA	4 (2)	3 (2)	9 (2)	20 (4)	82 (5)	91 (2)	5 (2)	3 (1)
u-PA	6 (2)	33 (4)	75 (4)	75 (6)	86 (4)	83 (8)	>90	>90
u-PAR	2 (1)	3 (1)	8 (4)	12 (6)	63 (5)	69 (3)	70 (7)	57 (15)
PAI-1	7 (1)	14 (3)	15 (2)	10 (1)	26 (3)	81 (7)	>90	>90

The proteins (t-PA, u-PA, PAI-1 and u-PAR) all significantly increased in the vessel wall following balloon injury. Tissue plasminogen activator protein initially increased and then returned to pre-injury levels by 1 month whilst u-PA, PAI-1 and uPAR proteins remained significantly raised even at 3 months.



7.3.3 Results: Changes in uPA and PAI-1 activities within the vessel wall after balloon injury to the rabbit common iliac arteries.

Since both urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) proteins are increased in the vessel wall during the development of intimal hyperplasia, the changes in the activities of uPA and PAI-1 in the vessel wall homogenate after balloon injury was investigated.

Table 7.3.3 uPA and PAI-1 activities in the wall of the rabbit common iliac artery after balloon injury

Activity	Control	1day	3days	7days	14 days	30 days
u-PA(ng/g tissue) injury vs control	35 (3.3)	28.4 (6.6) p=0.4	53 (3.6) p=0.02	65 (5.8) p=0.01	47 (3.2) p=0.06	40 (3.4) p=0.4
PAI-1(U/mg tissue) injury vs control	5.6 (1.2)	6.9 (2.2) p=0.6	4.1 (1.0) p=0.4	3.1 (1.4) p=0.2	5.0 (1.8) p=0.8	6.2(2.5) p=0.8
uPA/PAI-1 ratio	10.0	4.1	12.9	21.0	9.4	6.5

The activity of uPA showed a significant increase within the vessel wall at 3 and 7 days compared to control. PAI-1 activity did not significantly change after balloon injury but there was a trend towards a decrease in activity around 7 days. The uPA/PAI-1 activity ratio doubled after balloon injury and during the development of intimal hyperplasia.

7.4 Discussion

7.4.1 Discussion: Arterial changes following balloon injury in rabbit common iliac arteries

Balloon injury to the rabbit common iliac arteries in our model resulted in considerable loss of endothelial cells evident in the balloon injured area with no obvious initial damage to the internal elastic lamina. In this respect the degree of medial damage was less than

that described by others in which histologically a tear in the internal elastic lamina and the media can be seen. Nevertheless all our balloon injured vessels developed neointima.

The immediate loss of endothelium seen in the balloon-injured arteries of animals sacrificed 1/2 hour after injury resulted in the exposure of subendothelium and platelets were seen to be adherent to the exposed subendothelial intima. This is thought to result in the activation of platelets with release of a number of molecules that may be important for the restenotic process.

The immunoreactivity to PCNA was used to detect proliferating smooth muscle cells at the different time points as a result of the balloon injury. This showed that in the intima the PCNA index reached a maximum at 7 days and this decreased to baseline by one month. In the neointima the PCNA index reached its maximum at two weeks, then decreased to baseline by one month. This would be compatible with the idea of smooth muscle cells proliferating then migrating into the neointima where they proliferate further. The internal elastic lamina was observed to be intact on sections of electron microscopy earlier than 3 days post injury. However, gaps in the internal elastic lamina developed by 3 days when pseudopodia of smooth muscle cells were observed. This may imply that these defects in the internal elastic lamina may have resulted, not from direct trauma of the balloon injury, but from the dissolution of the matrix by various proteases, see Fig 7.3.1 (b) [III]. The smooth muscle cells were observed to occupy these defects and appeared to be migrating to form the neointima. By one month there was complete re-endothelialisation of all injured vessels and some vessels appeared fully re-endothelialised at two weeks.

7.4.2 Discussion: Temporo-spatial changes in tPA, uPA, PAI-1 and uPAR proteins on immunohistochemistry in the rabbit common iliac artery following balloon injury.

The presence of tPA, uPA, PAI-1 and uPAR proteins in control (non-injured) rabbit common iliac arteries were essentially restricted mainly to the endothelium, with very little signal present in the media. However, after balloon injury, the amounts of these proteins all increased within the vessel wall, but with different temporospatial profiles. Tissue plasminogen activator protein initially increased and then returned to pre-injury levels by 1 month whilst u-PA, PAI-1 and uPAR proteins remained significantly raised even at 3 months.

It is possible that some of the PAI-1, tPA and uPA proteins seen in the vessel soon after injury originated from the plasma and made its way by diffusing through the injured endothelial layer. *In-situ* mRNA hybridisation studies on these vessels would have answered the question as to whether these proteins were synthesized locally. It is however likely that in the injured rabbit vessels some of the proteins were indeed synthesized locally, as others have shown that tPA and uPA (Clowes et al 1990) as well as PAI-1 (Sawa et al 1992) syntheses are increased locally following vessel injury in rat carotid artery. These studies found the increase in mRNA expression to be relatively short lived. However as Table 7.3.2 shows the respective proteins were found the be increased in the vessel wall for weeks (tPA) or months (uPA, PAI-1 and uPAR). The fact that tPA levels within the substance of the vessel wall fell back to pre-injury levels by one month seems to imply that the increased uPA, PAI-1 and uPAR protein found in the vessel wall beyond one month must either have been sustained by

continued increased local synthesis or by a slower rate of tissue degradation compared to tPA.

7.4.3 Discussion: Changes in uPA and PAI-1 activities within the vessel wall after balloon injury to the rabbit common iliac arteries.

Having previously shown that both urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) proteins are increased in the vessel wall during the development of intimal hyperplasia, the changes in the activities of uPA and PAI-1 in the vessel wall after balloon injury resulting in intimal hyperplasia formation was investigated.

The activity of uPA showed a significant increase within the vessel wall at 3 and 7 days compared to control. PAI-1 activity did not significantly change after balloon injury but there was a trend towards a decrease in activity around 7 days. The uPA/PAI-1 activity ratio doubled after balloon injury and during the development of intimal hyperplasia. This change in the proteolytic balance may facilitate extracellular proteolysis, activation of cytokines with consequent smooth muscle cell migration and resulting intimal hyperplasia. Reversing the changes in proteolytic balance in the vessel wall after PTCA may be a potential target for the prevention of restenosis.

CHAPTER 8

SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

Considerable research effort over the last decade has been directed towards understanding the molecular mechanisms underlying atherosclerosis, restenosis following angioplasty and vein graft stenosis. Smooth muscle cell proliferation and migration are fundamental to the pathology of intimal hyperplasia. Migration requires the digestion of extracellular matrix surrounding the smooth muscle cells. Multiple metallo-enzymes secreted by smooth muscle cells are involved in this process, and there is evidence that components of the PAS may be important in their activation (Lee et al 1996). In addition tPA has been shown to be mitogenic for smooth muscle cells (Herbert et al 1995) and may also contribute to the proliferative aspect of the pathology.

Many studies have considered animal models to dissect the molecular aspects of intimal hyperplasia, however these studies have their limitations (Ferrel et al 1992), and as such use of human vessels was largely employed in this study. To understand the role of the plasminogen activation system in the vessel wall and its contribution to the genesis of intimal hyperplasia, a variety of normal and abnormal vessels were studied with respect to expression and activity of the various components of the PAS.

Chapters 2 to 5 concentrated on the human blood vessels (normal arteries, normal veins and atheromatous arteries and vein graft stenosis), examining the expression of components of the PAS (tPA, uPA, PAI-1 and uPAR) and employing the techniques of quantitative RTPCR on extracted RNA, immunohistochemistry and mRNA *in-situ* hybridisation on fixed paraffin-embedded tissues, immuno(antigen) assay and immuno-(activity) assay on human blood vessel homogenates. Chapter 6

concentrated on the human saphenous vein organ culture model of intimal hyperplasia. Chaper 7 examined the changes in the PAS in the rabbit angioplasty model.

Saphenous vein is a commonly employed conduit for coronary artery bypass grafts. Prior to use the vein is distended, to counteract spasm occurring during dissection and to identify any leaks. Since this results in vessel injury, distension may predispose the vein to undergo intimal hyperplasia and subsequent graft stenoses. As such, expression of components of the PAS was compared between distended and undistended vein. Immunohistological data showed that tPA, uPA, PAI-1 and uPAR were all increased in the smooth muscle cells of the distended veins as compared to undistended controls. This was in contrast to quantitative RTPCR data which demonstrated a reduction in both uPA and PAI-1 mRNA in distended veins, the ratio of the two however was comparable to that in the control veins. The apparent discrepancy between the immunochemical and RTPCR data has been explained to some extent earlier, but it is important to highlight the fact that the fall in mRNA levels may indeed have been a transient phenomenon due to vessel injury and that once implanted into the circulation the expression may well have increased. Also given that the ratio uPA/PAI-1 mRNA remains unchanged it seems that distension does not alter functional regulation of the PAS at the mRNA level especially in the early phase. It is difficult to know what happens once the graft has been implanted, unless grafts that have failed acutely (<30 days) are studied using the above techniques.

Internal mammary artery is also a commonly used conduit for coronary artery bypass grafting, and has been shown to have superior long term patency in comparison to saphenous vein. Saphenous veins and internal mammary arteries were compared for differential expression of components of the PAS which might partly explain differences in patency. Immunohistochemical data (Chapter 2) showed that the levels of uPA, tPA, PAI-1 and uPAR were all increased in distended veins compared to IMA. This increase, in the face of a fall in mRNA levels was ascribed to extravasation of plasma proteins during distension. Indeed ELISA depicted a similar picture i.e. considerably elevated levels of tPA, uPA and PAI-1 antigens in distended veins compared to IMA. Distending the vessels with saline rather than with blood plus saline could have tested the extravasation hypothesis, however distension was done as part of the routine surgery and one had to rely on surplus samples for use in this study. Interestingly, the activities of both uPA and PAI-1 (Chapter 5) were actually increased in the IMA, a possible explanation is that the extravasated proteins were functionally inactive. In the face of these conflicting protein data, the RTPCR study on veins and internal mammary arteries demonstrated very interesting results. In both distended and undistended vein samples the ratio of uPA/PAI-1 mRNA was ten fold greater than that for IMA. If this is an indicator of basal vessel physiology, then smooth muscle cell migration may occur more rapidly in vein since the conditions are in favour of proteolysis.

In atheromatous lesions, there was intense immunostaining for tPA, uPA, PAI-1 and uPAR localised to intimal smooth muscle cells and endothelial cells. In addition, macrophages within the plaque showed strong staining for uPA and uPAR and waker staining for tPA and PAI-1. However in normal arteries (IMA) staining for all the

four proteins was reduced in comparison to the atherosclerotic vessel. The RTPCR data complemented this finding showing elevated levels of both PAI-1 and uPA mRNA. Interestingly, the ratio uPA/PAI-1 mRNA was elevated implying enhanced proteolytic potential, in keeping with ideas about smooth muscle cell migration discussed earlier. ELISA studies confirmed the immunocytochemical data, showing higher levels of tPA, uPA and PAI-1 antigen in the atheromatous vessel wall, in comparison to the control artery. The ratio of the activities of uPA/PAI-1 was significantly higher in the atheromatous vessel further confirming the presence of a highly proteolytic environment within the atherosclerotic plaque, this was in keeping with the findings of Padro et al 1995 and Underwood et al 1993. This part of the study confirmed the hypothesis that the expression (of tPA, uPA, PAI-1 and uPAR) is increased in the atherosclerotic plaque. It is interesting to speculate whether by modulating components of the plasminogen activation system, the proteolytic balance could be altered favourably to prevent extracellular matrix degradation and therefore plaque instability.

Saphenous vein graft stenosis is a common clinical problem, and results in the recurrence of ischaemic symptoms. Animal studies of veno-arterial grafting have limitations, and also rarely translate into clinical efficacy in human. Therefore organ culture of human saphenous vein was used to study the PAS in this form of intimal hyperplasia. Firstly, the model was further validated by phenotypic comparison using monoclonal antibodies to α-actin, vimentin, desmin, CD31 and vWF. This demonstrated a good correlation between the in vitro model and actual stenotic segments of vein excised from patients with failed grafts.

Consistent with intimal hyperplasia in atherosclerotic arteries the levels of uPA, tPA, PAI-1 and uPAR were all increased in saphenous vein graft stenosis. However in the neointima of cultured saphenous veins tPA, uPA and uPAR antigens were increased whilst PAI-1 was decreased. PAI-1 antigen and mRNA was however prominent in the endothelial cells of the cultured veins. This difference between saphenous veins in culture and established saphenous vein graft stenosis may be a temporal phenomemon. In addition, the ratio uPA/PAI-1 activity was increased in cultured vein demonstrating increased proteolytic potential during the development of IH. Organ culture may be a useful model to study the development of intimal hyperplasia and given that the proteolytic activity is enhanced, it would be interesting to study the effect of suppressing uPA on the development of intimal hyperplasia.

Rabbit arterial balloon injury model: The temporo-spatial changes in the plasminogen activation system were examined using a rabbit arterial balloon injury model. Semi-quantatative immunohisto-chemistry showed that tPA, uPA, uPAR and PAI-1 antigens were all significantly increased following injury. tPA levels fell back to pre-injury levels by the end of 1 month, however levels of the other components remained elevated even at 3 months. The ratio uPA, tPA/PAI-1 remained elevated until around two months, this is in keeping with immuno-activity data which show enhanced uPA activity compared to PAI-1. This is favourable for the process of migration, which is undoubtedly an important part of the pathological process. Again it is interesting to speculate whether inhibiting uPA activity or enhancing PAI-1 activity could reduce the magnitudeof intimal hyperplasia, and result in a clinically effective approach to dealing with restenosis.

This thesis has highlighted the importance of the PAS in the process of intimal hyperplasia in three different models and will conclude by addressing future directions that research in this area may take.

FUTURE PERSPECTIVES

Inhibition of the plasminogen activators within the subendothelial intima and the media may inhibit vascular smooth muscle cell migration. As such, this could be a potential target for the treatment of vascular diseases in which smooth muscle cell migration is a prominent feature e.g. intimal hyperplasia of atherogenesis, post angioplasty restenosis and vein graft stenosis. Furthermore alteration of the process endothelial cell migration may provide a new approaches to prevent angiogenesis for example in atherosclerotic lesions to limit its growth, or to increase angiogenesis in clinical situations of chronic ischaemia e.g. myocardial ischaemia. In addition the plasminogen activator system may be manipulated on the endothelial surface so as to prevent thrombotic risk in situations of unstable angna or recurrent transient ischaemic attacks.

To date no specific inhibitors of the plasminoen activators have been tried clinically to inhibit intimal hyperplasia development. The race is on to find and evaluate low molecular weight inhibitors of the plasminogen activators that are safe to use and with favorable pharmacokinetic profiles. Much of the impetus for the development of these agents is coming from investigators interested in preventing malignant cell migration.

Suramin, a polysulphonated napthylurea compound, increases the production of the

plasminogen activator inhibitors and decreases the production of the plasminogen activators in cells and thus may be able to decrease cell migration. Indeed, this molecule has been shown to do just this with a correlated decrease in metastasis of cancer cells (Marutsuka et al 1995). Whilst this molecule is known to be toxic, it nevertheless demonstrates the possibility for therapeutic inhibiting cell migration with small molecules.

Blockage of uPAR by small molecules may also be a target to inhibit cellular migration. Lu et al 1996, showed that ATF-HSA inhibits in-vitro the motility of endothelial cells in a dose-dependent way by decreasing cell deformability. 4-substituted benzo(b)thiophene-2-carboxamidines are being developed as potent and selective inhibitors of urokinase (Bridges et al 1993).

Another approach would be to inhibit the plasminogen activator system at the gene or mRNA level. Antisense oligonucleotide inhibition of urokinase reduces spread of human ovarian cancer in mice (Wilhelm et al 1995) indicating that inhibition of uPA may result in decreased migration of cells. PAI-1 production by both endothelial cells and smooth muscle cells have been inhibited using antisense technology. (Sawa et al 1994). Knock-out mice for tPA, uPA, PAI-1 and plasmin have now been developed (Lijnen et al 1995, Bugge et al 1995). These mice provide a means to test the proposed function of the plasminogen activator system in intimal hyperplasia development after experimental angioplasty.

Retroviral vector-mediated transfer and expression of human tPA gene in human

endothelial cells has been reported (Ektherae et al 1995) and this represents a necessary finding in the development of this gene therapy in the prevention of thrombotic complications of vascular disease. Retroviral vectors have been used to increase both tPA and uPA expression in primate endothelial cells (Dichek et al 1995). More recently adenovirus-mediated transfer of tPA gene to human endotheial cells resulted in a 3 fold increase of tPA secretion (Sugawara et al 1997). Transgenic mice overexpressing uPA have been developed (Meiri et al 1994) and these can be used to study the role of increased uPA in vessel wall under pathological conditions e.g. neointimal formation.

The exploitation of the plasminogen activator system on the endothelial surface for acute therapeutic goals has already had a huge impact in cardiology and is presently being assessed for the treatment of acute ischaemic strokes. However, it is possible that using a strategy of long-term alteration of the plasminogen activator system within the substance of the vessel wall other beneficial effects such as prevention of restenosis and atherogenesis may be observed. This area of reasearch is therefore worthy of further pursuit.

REFERENCES

Angelini GD, Passani SL, Breckenridge IM, Newby AC. Nature and pressure dependence of damage induced by distension of human saphenous vein coronary artery bypass grafts. *Cardiovasc Res 1987;21: 902-907.*

Aznar J, Estelles A, Tomo G, Sapena P, Tormo V, Blanch S, Espana F. PAI activity and other fibrinolytic variables in patients with coronary artery disease. *Br Heart J 1988;59: 535-541.*

Appella E, Robinson EA, Ullrich SJ, Stoppelli MP, Corti, A, Cassani G, and Blasi F. The receptor-binding sequence of urokinase. A biological function for the growth-factor module of proteases. *J Biol Chem 1987;262: 4437-4440.*

Argraves KM, Battey, FD, MacCalman CD, McCrae KR, Gafvels M, Kozarsky KF, Chappell DA, Strauss JF 3rd, and Strickland DK. The very low density lipoprotein receptor mediates the cellular catabolism of lipoprotein lipase and urokinase-plasminogen activator inhibitor type 1 complexes. *J Biol Chem 1995; 270: 26550-26557.*

ASSET: Trial of tissue plasminogen activator for mortality reduction in acute myocardial infarction. Anglo-Scandanavian Study of early thrombolysis. *Lancet 1988;2: 525-530*

Astedt B. No cross reaction between circulating plasminogen activator and urokinase. *Thrombosis Research 1979;14: 535-539*

Astred B, Lecander I, Brodin T, Lundblad A, Low K. Purification of a specific plasminogen activator inhibitor by monoclonal antibody and its complex formation with plasminogen activator. *Thromb Haemost 1985;53: 122*

Astrup T. The biological significance of fibrinolysis. *Lancet 1956;25: 569*

Astrup T. Blood coagulation and fibrinolysis in tissue culture and tissue repair. *In: Houck JC, Forscher BK (eds) Chemical Biology of Inflammation. Pergamon, New York, 1968: p242-257.*

Auwerx J, Bouillon R, Collen D, Geboers J. Tissue-type plasminogen activator inhibitor activity in diabetes mellitus. *Arteriosclerosis 1988;8: 68-72.*

Barrett LA, Mergner WJ, Trump BF. Long term of human aortas. Development of atherosclerotic-like plaques in serum-supplemented medium. *In Vitro 1979;15: 957-966.*

Batayias GE, Barboriak JJ, Korns ME, Pintar K. The spectrum of pathological changes in aortocoronary saphenous vein grafts. *Circulation 1997:56 : 18-22.*

Behrendt N, E.Ronne, M Ploug, Petri T, Lober D, Nielson LS. The human receptor for urokinase plasminogen activator. *J Biol Chem 1990;256: 6453-6460.*

Bell WR. Streptokinase and Urokinase in the treatment of pulmonary thromboli: from a National Cooperative Study. *Thromb Haemostas 1976;35: 57-69.*

Bendeck MP, Irvin, C, and Reidy, MA. Inhibition of matrix metalloproteinase activity inhibits smooth muscle cell migration but not neointimal thickening after arterial injury. *Circulation Research 1996;78: 38-43.*

Benditt EP. Implications of the monoclonal character of human atherosclerotic plaques. *American Journal of Pathology 1977;*86: 693-702.

Bergsdorf N, Nilsson T, and Wallen P. An enzyme linked immunosorbent assay for determination of tissue plasminogen activator applied to patients with thromboembolic disease. *Thrombosis & Haemostasis 1983;*50: 740-744.

Blasi F, Vassalli JD, and Dano K. Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. *Journal of Cell Biology 1987;104: 801-804.*

Booth NA, Anderson JA, Bennett B. Platelet release protein which inhibits plasminogen activators *J Clinical Pathol 1985;38: 825-830.*

Bourassa MG, Campeau L, Lesperance J. Changes in grafts and in coronary arteries after coronary artery bypass surgery. *Cardiovasc Clin. 1991;21: 83-100.*

Bridges AJ, Lee A, Schwartz CE, Towle MJ and Littlefield BA. The synthesis of three 4-substituted benzo[b]thiophene-2-carboxamidines as potent and selective inhibitors of urokinase. *Bioorg Med Chem 1993;1: 403-410.*

Bugge TH Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K, and Degen JL The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J Biol Chem 1995;270: 16886-16894.*

Bulkley BH, Hutchins GM. Accelerated atherosclerosis. A morphological study of 97 saphenous vein coronary bypass grafts. *Circulation 1977;55: 163-169.*

Buo L, Meling GI, Karlsrud TS, Johansen HT, and Aasen AO. Antigen levels of urokinase plasminogen activator and its receptor at the tumor-host interface of colorectal adenocarcinomas are related to tumor aggressiveness. *Hum Pathol 1995;*26: 1133-1138.

Busso N, Masur SK, Lazega D, Waxman S, and Ossowski L. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *J Cell Biol 1994;126: 259-270.*

Carmeliet P, Moons L, Herbert JM, Crawley J, Lupu F, Lijnen R, and Collen D. Urokinase but not tissue plasminogen activator mediates arterial neointima formation in mice. *Circulation Research 1997;81: 829-839.*[(1)].

Carmeliet P, Moons L, Lijnen R, Janssens S, Lupu F, Collen D, and Gerard RD. Inhibitory role of plasminogen activator inhibitor-1 in arterial wound healing and neointima formation: a gene targeting and gene transfer study in mice. *Circulation 1997;96: 3180-3191* [2]

Carmeliet P, Moons L, Dewerchin M, Rosenberg S, Herbert JM, Lupu F, Collen D. Receptor-independent role of urokinase-type plasminogen activator in pericellular plasmin and matrix metalloproteinase proteolysis during vascular wound healing in mice. *Journal of Cell Biology 1998;140: 33-245.*

Chapman HA, Stone OL. Characterization of a macrophage- derived plasminogen-activator inhibitor. Similarities with placental urokinase inhibitor. *Biochem J 1985;230: 109*

Cheng JJ, Chao, YJ, Wung BS, and Wang DL. Cyclic strain-induced plasminogen activator inhibitor-1 (PAI-1) release from endothelial cells involves reactive oxygen species. *Biochemical & Biophysical Research Communications 1996;225: 100-105.*

Cheng XF, Brohlin M, Pohl G, Back O, and Wallen P. Binding of tissue plasminogen activator to endothelial cells. the effect on functional properties. localization of a ligand in the b-chain of tPA. *Thromb Res 1995;77(2):149-164.*

Chmielewska J, Ranby M, Wiman B. Evidence of a rapid inhibitor to tissue plasminogen activator in plasma. *Throm Res 1983;31: 427-436.*

Chomczynski P and Sacchi N. Single-step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem 1987;162: 156-159.*

Clowes AW, Clowes MM, Au YPT, Reidy MA, Belin D. Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circulation Res 1990;67: 61-67.*

Collen D & Juhan-Vague I. Fibrinolysis and atherosclerosis. *Semin Thromb Haemostasis 1988;14: 180-183.*

Collen D. Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. *Eur J Biochem 1976;69: 209-216.*

Collen D. Fibrin selective thrombolytic therapy for myocardial infarction. *Circulation 1996; 93: 857-865.*

Conese M and Blasi F. Urokinase/urokinase receptor system: internalization/ degradation of urokinase-serpin complexes: mechanism and regulation. *Biol Chem Hoppe Seyler 1995;376: 143-155.*

Cormack DH. In: JB Lippincott (9th): Ham's Histology. Phildelphia, Pennsylvania, *1987: p444-445.*

Cox JL, Chaisson DA, Gotlieb AI. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences between veins and arteries. *Prog Cardiovasc Dis. 1991;34: 45-68.*

Davies MG, Klayachkin ML, Kim JH, Hagen PO. Endothelin and vein bypass grafts in experimental atherosclerosis. J Cardiovasc Pharmacol 1993;22: S348-S351.

Davies MG, Hagen PO. Structural and functional consequences of bypass grafting with autologous vein. *Cryobiology. 1994;31: 63-70.*

Davies MJ, Thomas A. Thrombosis and acute coronary lesions in sudden cardiac ischaemic death. *New Eng J Med 1984;310: 1137-1140.*

Debrock S and Declerck, P.J. Characterization of common neoantigenic epitopes generated in plasminogen activator inhibitor-1 after cleavage of the reactive center loop or after complex formation with various serine proteinases. *FEBS Lett 1995;376: 243-246.*

DeClerck PJ, Moreau H. Jespersen J. Gram J. Kluft C. Multicenter evaluation of commercially available methods for the immunological determination of plasminogen activator inhibitor-1 (PAI-1). *Thrombosis & Haemostasis. 1993;70: 858-863.*

DeMunk GAW, Caspers MPM, Chang GTG, Pouwels PH, Enger-Valk BE, Verheijen JH. Binding of tissue plasminogen activator to lysine, lysine anologues and fibrin fragments. *Biochemistry 1989;28: 7318-7325.*

De Petro G, Copeta A, and Barlati S. Urokinase-type and tissue-type plasminogen activators as growth factors of human fibroblasts. *Exp Cell Res 1994;213: 286-294.*

de Vries TJ, Kitson JL, Silvers WK, and Mintz B. Expression of plasminogen activators and plasminogen activator inhibitors in cutaneous melanomas of transgenic melanoma-susceptible mice. *Cancer Res 1995;*55: 4681-4687.

Diamond SL, Sharefkin JB, Dieffenbach C, Frasier-Scott K, McIntire LV, and Eskin SG. Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. *Journal of Cellular Physiology 1990;1432: 364-371.*

Dichek DA, Lee SW, and Nguyen NH. Characterization of recombinant plasminogen activator production by primate endothelial cells transduced with retroviral vectors. *Blood 1994;*84: 504-516.

Dieval J, Nguyen G, Gross S, Delobel J, Kruithof EKO. A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1. *Blood 1991;77: 528-532.*

Dilley RJ, McGeachie JK, Prendergast FJ. Review of the histological changes in vein to artery grafts, with paeticular reference to intimal hyperplasia. *Arch Surg. 1988;123: 691-696.*

Dudani AK, Pluskota A, and Ganz PR. Interaction of tissue plasminogen activator with a human endothelial cell 45-kilodalton plasminogen receptor. *Biochem Cell Biol 1994;72: 126-131.*

ECAT Angina pectoris study group. ECAT angina pectoris study: baseline associations of haemostatic factors with extent of arteriosclerosis and other coronary risk factors in 3000 patients with angina pectoris undergoing coronary arteriography. *Eur Heart J 1993;14: 8-17.*

Ellis V, Whawell SA. Vascular smooth muscle cells potentiate plasmin generation by both urokinase and tissue plasminogen activator-dependent mechanisms: evidence for a specific tissue-type plasminogen activator receptor on these cells. *Blood 1997;90: 2312-2322.*

Ekhterae D and Stanley JC. Retroviral vector-mediated transfer and expression of human tissue plasminogen activator gene in human endothelial and vascular smooth muscle cells. *J Vasc Surg 1995;*216: 953-962.

Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, and Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci USA 1995; 92: 1851-1855.*

Erikson LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR. Development of venous occlusion in mice transgenic for the plasminogen activator inhibitor 1 gene. *Nature 1990;346: 74-76.*

Falcone DJ, McCaffrey T.A, Haimovitz-Friedman A, and Garcia M. Transforming growth factor-beta 1 stimulates macrophage urokinase expression and release of matrix-bound basic fibroblast growth factor. *J Cell Physiol 1993;1553: 595-605.*

Falk E. Unstable angina with fatal outcome: dynamic coronary thrombosis leading to infarction and/or sudden death: autopsy evidence of recurrent mural thrombosis with peripheral embolism culminating in total vascular occlusion. *Circulation 1985; 71: 699-708.*

Ferrell M, Fuster V, Gold HK, Chesebro JH. A dilema for the 1990's, choosing the appropriate experimental model for the prevention of restenosis. *Circulation 1992;85: 1630-1631.*

FitzGibbon GM, Leach AJ, Kafka HP, Keon WJ. Coronary bypass graft: long-term angiographic study. *J Am Coll Cardiol. 1991;17: 1075-1080.*

Flaumenhaft R, Abe M, Mignatti P, and Rifkin DB. Basic fibroblast growth factor-induced activation of latent transforming growth factor beta in endothelial cells: regulation of plasminogen activator activity. *J Cell Biol 1992;*118: 901-909.

Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventative therapies. *J Am Coll Cardiol. 1991;79: 758-769.*

Fujii S, Sawa H, Saaitz JE, Lucore CL, Sobel BE. Induction of endothelial cell expression of the plasminogen activator inhibitor type 1 gene by thrombosis in vivo. *Circulation 1992;86: 2000-2010.*

Gaffney PJ and Longstaff C *In: Haemostasus and Thrombosis (eds) Bloom AL, Forbes CD, Thomas DP, Tuddingham EGD. Churchill Livingstone 1994: Vol 1, p550.*

Ginsburg D, Zeheb R, Yang AW. cDNA cloning of human plasminogen activator inhibitor from endothelial cells. *J Clin Invest 1986;78: 1673-1680.*

Gleerup G, Vind J, and Winther K. Platelet function and fibrinolytic activity during rest and exercise in borderline hypertensive patients. *European J Clin Invest 1995;*25: 266-270.

Gold LI, Rostagno A, Frangione B, and Passalaris T. Localization of the cleavage sites on fibronectin following digestion by urokinase. *J Cell Biochem 1992;50: 441-452.*

Goretzki L, Schmitt M, Mann K, Calvete J, Chucholowski N, Kramer M, Gunzler WA, Janicke F, and Graeff H. Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-upa) by the cysteine protease cathepsin l. *FEBS Lett 1992;297: 112-118.*

Gown AM, Tsukada T, Ross R: Human atherosclerosis. II Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am J Pathol 1986;125: 191-207.*

Grimsley PG, Normyle JF, Brandt RA, Joulianos G, Chesterman CN, Hogg PJ, and Owensby DA. Urokinase binding and catabolism by hep g2 cells is plasminogen activator inhibitor-1 dependent, analogous to interactions of tissue-type plasminogen activator with these cells. *Thromb Res 1995;79: 353-361.*

Grobmyer SR, Kuo A, Orishimo M, Okada SS, Cines DB, and Barnathan ES. Determinants of binding and internalization of tissue-type plasminogen activator by human vascular smooth muscle and endothelial cells. *J Biol Chem 1993;268: 13291-13300.*

Grondin CM, Campeau L, Lesperance J. Comparison of late changes in internal mammary artery and saphenous vein grafts in two consecutive series of patients 10 years after operation. *Circulation 1984:70,Suppl I: 208-212.*

Guenzler W, Steffens G, Oetting F. Structural relationship between human high and low molecular weight mass urokinase. *Hoppe-Seyler's Z. Physiol. Chem 1982; 3363: 133-141.*

Gualandris A, Lopez Conejo T, Giunciuglio D, Albini A, Sabini E, Rusnati M, Dell'Era P, and Presta M. Urokinase-type plasminogen activator overexpression enhances the invasive capacity of endothelial cells. *Microvascr Res 1997;53: 254-260.*

Gualandris A and Presta, M. Transcriptional and posttranscriptional regulation of urokinase-type plasminogen activator expression in endothelial cells by basic fibroblast growth factor. *J Cell Physiol 1995;162: 400-409.*

Gyetko MR, Schollenberger SB, Sitrin RG. Urokinase expression in mononuclear phagocytes: Cytokine-specific modulation by interferon-gamma and tumour necrosis factor- alpha. *J Leucocyte Biol 1991;51: 256-263.*

Gyetko MR, Todd, RF 3rd, Wilkinson CC, and Sitrin RG. The urokinase receptor is required for human monocyte chemotaxis in vitro. *J Clin Invest 1994;*93: 1380-1387.

Gyetko MR, Wilkinson CC, and Sitrin RG. Monocyte urokinase expression: modulation by interleukins. *J Leukoc Biol 1993;*53: 598-601.

Hamsten A, Wiman B, Defaire U, Blombaeck M. Increased plasma levels of a rapid inhibitor of t-PA in young survivors of myocardial infarction. *N Eng J Med 1985;313: 1557-63.*

Hajjar KA. The endothelial cell tissue plasminogen activator receptor: Specific interaction with plasminogen. *J Biol Chem 1991;266: 21962-21970.*

Hamsten A, De Faire Y, Walldius G. PAI in plasma. Risk factor for recurrent myocardial infarction. *Lancet 1987;2: 3-9.*

Hasse J, Graedel E, Hofer H. Morphological studies in saphenous vein grafts for aorto-coronary bypass surgery. *J Thorac Surg. 1981:29: 38-40.*

Hassler O. The origin of cells constituting arterial intima thickening. An experimental autoradiographic study with the use of H^3-thymidine. *Lab Invest 1970; 22: 286-93.*

Healy AM and Gelehrter TD. Induction of plasminogen activator inhibitor-1 in hepg2 human hepatoma cells by mediators of the acute phase response. *J Biol Chem 1994;269: 19095-19100.*

Heegaard CW, Simonsen AC, Oka K, Kjoller L, Christensen A, Madsen B, Ellgaard L, Chan L, and Andreasen PA. Very low density lipoprotein receptor binds and mediates endocytosis of urokinase-type plasminogen activator-type-1 plasminogen activator inhibitor complex. *J Biol Chem 1995;270: 20855-20861.*

Herbert JM, Lamarche I, Prabonnaud V, Dol F, and Gauthier T. Tissue-type plasminogen activator is a potent mitogen for human aortic smooth muscle cells. *J Biol Chem 1994;269: 3076-3080.*

Higazi A, Cohen RL, Henkin J, Kniss D, Schwartz BS, and Cines D.B. Enhancement of the enzymatic activity of single-chain urokinase plasminogen activator by soluble urokinase receptor. *J Biol Chem 1995;*270: 17375-17380.

Higgins DL, Benditt WF. Tissue plasminogen activator: the biochemistry and pharmacology of variants produced by mutagenesis. *Annual Review of Pharmacology and Toxicology 1990;30: 91-121.*

Hildenbrand R, Jansen C, Wolf G, Bohme B, Berger S, von Minckwitz G, Horlin A, Kaufmann M and Stutte HJ. Transforming growth factor-beta stimulates urokinase expression in tumor-associated macrophages of the breast. *Lab Invest 1998;*78: 59-71.

Hofer H, Mihatsch MJ, Guggenheim R. Morphological studies in saphenous vein grafts for aorto-coronary bypass surgery..*J Thorac Cardiovasc Surg. 1981;29: 32-40.*

Holmes WE, Pennica D, Blaber M, Rey MW, Guenzler WA, Steffens GJ, Heyneker HL. Cloning and expresion of pro-urokinase in escherichia coli. *Bio/technology 1985;3: 923-929.*

Holt CM, Francis SE, Rogers S, Gadsdon PA, Taylor T, Cleland C, Soyombo A, Newby AC, Angelini GD. Intimal proliferation in an organ culture of human internal mammary artery. *Cardiovasc Res 1992;26: 1189-1194.*

Iba T, Shin T, Sonoda T, Rosales O, and Sumpio BE. Stimulation of endothelial secretion of tissue-type plasminogen activator by repetitive stretch. *J Surg Res 1991;50: 457-460,.*

Ichinose A, Fujikawa K. Suyama T. The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. *J Biol Chem. 1986;261: 3486-3489.*

Ito K Ryuto M, Ushiro S, Ono M, Sugenoya A, Kuraoka A, Shibata Y, and Kuwano, M. Expression of tissue-type plasminogen activator and its inhibitor couples with development of capillary network by human microvascular endothelial cells on matrigel. *J Cell Physiol 1995;162: 213-224.*

Jansson JH, NilssonTK, Johnson O. Von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. *Br Med J 1991;66: 351-5.*

Johansson B, Eriksson A, Ramaekers F, Thornell LE. Smoothelin and intermediate filament proteins in human aortocoronary saphenous vein by-pass grafts. *Histochem Journal. 1999;31: 723-7.*

Johnson O, Mellbring G, Nilsson T. Defective fibrinolysis in survivors of myocardial infarction. *Int J Cardiol 1984;6: 380-2.*

Jonsson-Berg AK, Ranby M. An immunosorbent activity assay for single-chain urinary plasminogen activator (scuPA or pro-uPA) in plasma. Thromb Haemostas 1989;62: 233 (abstr 726).

Juhan-Vague I, Vague P, Alessi MC, Badier C,Valadier J, Aillaud MF, Atlan C. Relationship between plasma insulin, triglyceride, body mass index, and plasminogen activator inhibitor-1. *Diabetes Metab 1987;13: 331-6.*

Kaji T, Yamamoto C, Sakamoto M, Kozuka H, and Koizumi F. Suppression of plasminogen activator inhibitor type 1 release from cultured human umbilical vein endothelial cells by basic fibroblast growth factor. *Life Sci 1994;54: 1563-1569.*

Kakuta T, Usui M, Coats WD Jr, Currier JW, Numano F, and Faxon DP. Arterial remodeling at the reference site after angioplasty in the atherosclerotic rabbit model. *Arteriosclerosis, Thrombosis & Vascular Biology 1998;*18: 47-51.

Kanse SM, Kost C, Wilhelm OG, Andreasen PA, and Preissner KT. The urokinase receptor is a major vitronectin-binding protein on endothelial cells. *Experimental Cell Research 1996;224: 344-353.*

Kawai Y, Matsumoto Y, Watanabe K, Yamamoto H, Satoh K, Murata M, Handa M, and Ikeda Y. Hemodynamic forces modulate the effects of cytokines on fibrinolytic activity of endothelial cells. *Blood 1996;87: 2314-2321.*

Kawai Y, Matsumoto, Y., Ikeda, Y., Yamamoto, H., Satoh, K., and Watanabe, K. [Effect of shear stress on hemostatic regulation in endothelium]. *Rinsho Byori - Japanese J Clin Path 1994;42: 1128-1136.*

Kenagy RD and Clowes AW. Regulation of baboon arterial smooth muscle cell plasminogen activators by heparin and growth factors. *Thromb Res 1995;77: 55-61.*

Keskin A, Tombuloglu M, and Buyukkececi F. Fibrinolytic activity and platelet release reaction in essential hypertension. *Jpn Heart Journal 1994;*35: 757-763.

Keski-Oja J, Lohi, J., Tuuttila, A., Tryggvason, K., and Vartio, T. Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. *Exp Cell Res 1992;202: 471-476.*

Kobayashi H, Moniwa N, Sugimura M, Shinohara H, Ohi H, and Terao T. Effects of membrane-associated cathepsin b on the activation of receptor-bound prourokinase and subsequent invasion of reconstituted basement membranes. *Biochim Biophys Acta 1993;1178: 55-62.*

Kobayashi H, Gotoh J, Shinohara H, Moniwa N, and Terao T. Inhibition of the metastasis of lewis lung carcinoma by antibody against urokinase-type plasminogen activator in the experimental and spontaneous metastasis model. *Thromb Haemost 1994;71: 474-480.*

Kok P. Separation of plasminogen activators from human plasma and a comparison with activators from human uterine tissue and urine. Thrombosis and Haemostasis *1979:734: 744.*

Koo EWY, Gotlieb AI. Endothelial stimulation of intimal cell proliferation in a porcine aortic organ culture. *Am J Pathol. 1989:134: 497-503.*

Kramer MD, Spring, H., Todd, R.F., and Vettel, U. Urokinase-type plasminogen activator enhances invasion of human t cells (jurkat) into a fibrin matrix. *J Leukocyte Biol 1994;56: 110-116.*

Krishnamurti C, Barr CF, Hassett MA, Young GD, Alving BM. Plasminogen activator inhibitor: a regulator of ancrod induced fibrin deposition in rabbits. *Blood 1987;69: 798-803.*

Kruithof EKO, Tran-Thang C, Gudinchet A, et al. Fibrinolysis in pregnancy: a study of plasminogen activator inhibitors. *Blood 1987;69: 460-466.*

Kruithof EKO. Inhibition of tissue plasminogen activator by human plasma, in Davidson JF, Bachmann F, Bouvier CA, (eds): *Progress in Fibrinolysis, vol VI. Edinburgh, Churchill Livingstone, 1983: p 365.*

Kumar S, Baglioni C. Protection of tumour necrosis factor- mediated cytolysis by over expression of type 2 plasminogen activator inhibitor. *J Biol Chem 1991;266: 20960-20969.*

Laug WE. Vascular smooth muscle cells inhibit the plasminogen activators secreted by endothelial cells. *Thrombis & Haemostasis 1985;*53: 165-169.

Lawrence DA, Berkenpas MB, Palaniappan S, and Ginsburg D. Localization of vitronectin binding domain in plasminogen activator inhibitor-1. *J Biol Chem 1994;269: 15223-15228.*

Lee E, Vaughan DE, Parikh SH, Grodzinsky AJ, Libby P, Lark MW, Lee RT. Regulation of matrix metalloproteinases and plasminogen activator inhibitor-1 synthesis by plasminogen in cultured human vascular smooth muscle cells. *Circulation Research 1996;78: 44-49.*

Levi M, Biemond BJ, Van Zonneveld AJ, Ten Cate JW, Pannekoek H. Inhibition of plasminogen activator inhibitor activity results in promotion of endogenous thrombolysis and inhibition of thrombus extension in models of experimental thrombosis. *Circulation 1992;85: 302-312.*

Levin EG, and del Zoppo GJ. Localization of tissue plasminogen activator in the endothelium of a limited number of vessels. *Am J Pathol 1994;144: 855-861.*

Lijnen HR, Moons L, Beelen V, Carmelie P, and Collen D. Biological effects of combined inactivation of plasminogen activator and plasminogen activator inhibitor-1 gene function in mice. *Thromb Haemost 1995;74: 1126-1131.*

Liu G, Shuman MA., and Cohen RL. Co-expression of urokinase, urokinase receptor and pai-1 is necessary for optimum invasiveness of cultured lung cancer cells. *Int J Cancer 1995;60: 501-506.*

Longstaff C, Clough AM, Gaffney PJ. Kinetics of plasmin activation of single chain urinary-type plasminogen activator (scuPA) and demonstration of a high affinity interaction between scu-PA and plasminogen. *J Biol Chem 1992:82: 1391-1397.*

Loscalo J, Brunwald E. Tissue plasminogen activator. *N Eng J Med 1988;319: 925-931.*

Loskutoff DJ, Edgington TS. Synthesis of fibrinolytic activator and inhibitor by endothelial cells. *Proc Nat Acad Sci USA 1977;74: 3903-7.*

Loskutoff DJ, Linders M, Keijer J, Veerman H, van Heerikhuizen H, Pannekoek H. Structure of the human plasminogen activator inhibitor 1 gene: nonrandom distribution of introns. Biochemistry 1987;26: 3763-3768.

Luag WE. Vascular smooth muscle cells inhibit plasminogen activators secreted by endothelial cells. *Thromb Hemost 1985;20: 165*

Lundgren CH, Sawa H, Sobel BE, and Fujii S. Modulation of expression of monocyte/macrophage plasminogen activator activity and its implications for attenuation of vasculopathy. *Circulation 1994;*90: 1927-1934.

Lupu F, Bergonzelli GE, Heim DA, Cousin E, Genton CY, Bachmann F, and Kruithof EK. Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. *Arterioscler Thromb 1993;13: 1090-1100.*

Lupu F, Heim DA, Bachmann F, Hurni M, Kakkar VV, and Kruithof EKO. Plasminogen activator expression in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol 1995;15: 1444-1455.*

Lytle BW, Loop FD, Tayler PC. Vein graft disease: The clinical impact of stenosis in saphenous vein bypass grafts to coronary arteries. *J Thorac Cardiovasc Surg 1992;103: 831-840.*

Mandriota SJ, Seghezzi, G, Vassalli JD, Ferrara N, Wasi S, Mazzieri R, Mignatti P, and Pepper MS. Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. *J Biol Chem 1995;*270: 9709-9716.

Marti MC, Bouchardy B, Cox JN. Aorto-coronary bypass with autogenous sasphenous vein grafts: Histopathological aspects. *Virchow Arch 1971;(352): 255-260*

Marutsuka K, Hasui Y, Asada Y, Naito S, Osada Y, and Sumiyoshi A. Effects of suramin on metastatic ability, proliferation, and production of urokinase-type plasminogen activator and plasminogen activator inhibitor type 2 in human renal cell carcinoma cell line sn12c-pm6. *Clin Exp Metastasis 1995;*13: 116-122.

Mautner SL, Mautner GC, Huntsberger SA, Roberts WC. Comparison of composition of atherosclerotic plaques in saphenous veins used as aortocoronary bypass conduits with plaques in native coronary arteries in the same men. *Am J Cardiol. 1992;70: 1380-1387.*

Mc Bride W, Lange RA, Hillis LD. Restenosis after successful coronary angioplasty: pathophysiology and prevention. *N Eng J Med 1988;318: 1734-1737.*

Medcalf RL, Kruithof EKO, Schleuning WD. Plasminogen activator inhibitor 1 and 2 are tumour necrosis factor/cachectin responsive genes. *J Exp Med 1988;168:751-759*

Meiri N, Masos T, Rosenblum K, Miskin R, and Dudai Y. Overexpression of urokinase-type plasminogen activator in transgenic mice is correlated with impaired learning. *Proc Natl Acad Sci USA 1994;91: 3196-3200.*

Menashi S, Lu, H., Soria, C., and Legrand, Y. Endothelial cell proteases: physiological role and regulation. *Baillieres Clin Haematol 1993;6: 559-576,.*

Mullis K, Faloona F. In: *Methods in enzymology, Vol 155 (ed R Wu), p335. Academic Press, New York and London.*

Munkvad S, Gram J, Jespersen J. A depression of active tissue plasminogen activator in plasma characterizes patients with unstable angina pectoris who develop myocardial infarction. *Eur Heart J 1990;11: 525-8.*

Nelkin NA, Soifer SJ, O'Keefe J, Vu TKH, Charo IF, Coughlin SR. Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest 1992;90: 1614-1621.*

Newman KM, Jean-Claude J, Li H, Scholes JV, Ogata Y, Nagase H, and Tilson MD. Cellular localization of matrix metalloproteinases in the abdominal aortic aneurysm wall. *J Vasc Surg 1994;20: 814-820.*

Noda-Heiny H, Daugherty A, and Sobel BE. Augmented urokinase receptor expression in atheroma. *Arterioscler Thromb Vasc Biol 1995;15: 37-43.*

Ny T, Bjersing L, Hsueh AJW, Loskutoff DJ. Cultured granulosa cells produce two plasminogen activators and an inactivator, each regulated differently by gonadotropins. *Endocrinology 1985;116: 1666.*

Odekon LE, Sato Y and Rifkin DB. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. *J Cell Physiol 1992;*150: 258-263.

Odekon LE, Blasi F, and Rifkin DB. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta. *J Cell Physiol 1994;158: 398-407.*

Ohlsson M, Peng XR, Liu YX, Jia XC, Hsueh AJW,Ny T. Hormone regulation of tissue plasminogen activator expression and plasminogen activator-mediated proteolysis. *Seminars in Thrombosis and Haemostasis 1991:17: 286-290.*

Olofson BO, Dahlen G, Nilsson TK. Evidence for increased levels of plasminogen activator inhibitor and tissue plasminogen activator in plasma of patients with angiographically verified coronary disease. *Eur Heart J 1989;10: 77-82.*

Oseroff A, Krishnamurti T, Hassett A, Tang D, Alving B. Plasminogen activator and plasminogen activator inhibitor activities in men with coronary heart disease. *J Lab Clin Med 1989;113: 88-93.*

Padro T, Emeis J.J, Steins M, Schmid KW, and Kienast J. Quantification of plasminogen activators and their inhibitors in the aortic vessel wall in relation to the presence and severity of atherosclerotic disease. *Arteriosclerosis, Thrombosis & Vascular Biology 1995;15: 893-902.*

Pannekeok H, de Vries C, van Zonneveld AJ. Mutants of human tissue plasminogen activator: structural aspects and functional properties. *Fibrinolysis 1988;2: 123-132.*

Paramo JA, Colucchi M, Collen D, Van De Werf F. PAI in the blood of patients with coronary artery disease. *Br Med J 1985;291: 575-576.*

Park TC, Harker TC, Edwards JM, Moneta GL, Taylor LM, Porter JM. Human saphenous vein grafts explanted from the arterial circulation demonstrate altered smooth-muscle and endothelial responses. *J Vasc Surg 1993;18: 61-69.*

Pedersen N, Schmitt M, Ronne E, Nicoletti MI, Hoyer-Hansen G, Conese M, Giavazzi R, Dano K, Kuhn W, Janicke F. A ligand-free, soluble urokinase receptor is present in the ascitic fluid from patients with ovarian cancer. *J Clin Invest 1993;92: 2160-2167.*

Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. *Nature. 1983;301: 214-221.*

Pepper MS, Ferrara N, Orci L, and Montesano R. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun 1991;181: 902-906.*

Philips M, Juul AG, Thorsen S. Human endothelial cells produce a PAI and a tissue-type plasminogen activator-inhibitor complex. *Biochim Biophys Acta 1984;802: 99*

Plow EF, Freaney DE, Plescia J, Miles LA The plasminogen system and the cell surface: evidence for plasminogen and urokinase receptors on the same cell type. *J Cell Biol. 1986;169: 296-300.*

Ploug M, Ronne E, Behrendt N, Jensen AL, Blasi F, Dano K. Cellular receptor for urokinase plasminogen activator. Carboxyl terminal processing and membrane anchoring by glycosyl-phosphadylinositol. *J Biol Chem 1991:266; 1926-1933.*

Predel GH, Yang Z, von Segesser, Turina M, Buhler FR, Luscher TF. Implications of pulsatile stretch on growth of saphenous veins and mammary artery smooth muscle cells. *Lancet 1992:340; 878-879.*

Prins MH, Hirsh J. A critical review of the relationship between impaired fibrinolysis and myocardial infarction. *Am Heart J 1991;122: 545-551.*

Ragno P, Montuori N, and Rossi G. Urokinase-type plasminogen activator/type-2 plasminogen-activator inhibitor complexes are not internalized upon binding to the urokinase-type-plasminogen-activator receptor in thp-1 cells. interaction of urokinase-type plasminogen activator/type-2 plasminogen-activator inhibitor complexes with the cell surface. *Eur J Biochem 1995;*233: 514-519.

Ramos JR, Berger K, Mansfield PB, Sauvage LR. Histological fate and endothelial changes of distended and nondistended vein grafts. *Ann Surg 1976;183: 205-228.*

Ranby M Studies on the kinetics of plasminogen activator. *Biochim Biophys Acta 1982;704: 461-469.*

Ratliff NB, Myles JL. Rapidly progressive atherosclerosis in aortocoronary saphenous vein grafts. *Arch Pathol Lab Med 1989;113: 772-776.*

Reich E. Plasminogen activation of neoplastic cell and macrophages. *In: Reich E, Rifkin DB, Shaw E (eds) Proteases and biological control. Cold Spring Harbor Symposium, New York, 1975: p491-500.*

Reidy MA. Irvin C. Lindner V. Migration of arterial wall cells. Expression of plasminogen activators and inhibitors in injured rat arteries. *Circulation Research. 1996;78: 405-14.*

Reilly CF, Macfall RC. Platelet derived growth factor and transforming growth factor beta regulate plasminogen activator inhibitor synthesis in vascular smooth muscle cells. *J Biol Chem 1991;266: 9419-9427.*

Reinartz J, Link J, Todd RF., and Kramer MD. The receptor for urokinase-type plasminogen activator of a human keratinocyte line (hacat). *Exp Cell Res 1994;*214: 486-498.

Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Miletich JP. Arterial and venous thrombosis is not associated with the 4G/5G polymorphism in the promoter of the plasminogen activator inhibitor gene in a large cohort of US men. *Circulation 1997;95: 59-62.*

Rijken DC , Hoylaerts M. and Collen D. Fibrinolytic properties of one and two chain extrinsic Tissue-type plasminogen activator. *J Biol Chem. 1982;257: 2920-2925.*

Rijken DC and Groeneveld E. Isolation and functional characterisation of the heavy and light chains of human tissue plasminogen activator. *J Biol Chem 1986;261: 3098-3102.*

Rijken DC and Collen, D.. Purification and characterisation of the plasminogen activator secreted by human melanoma cells in culture. *J. Biol. Chem* 1981;*256: 7073-7041.*

Robbie LA, Booth NA, Brown AJ, Benditt B. Inhibitors of fibrinolysis are elevated in atherosclerotic plaque. Arteriosclerosis, Thrombosis & Vascular Biology. 1996;16: 539-545.

Ross R. The process of Atherogenesis - cellular and molecular interaction: from experimental animal models to humans. *Diabetologia 1992;35: S34-S40.*

Ross R, Glomset JA. The pathogenesis of atherosclerosis. *N Eng J Med. 1976;295: 369-377.*

Ross R. Atherosclerosis--an inflammatory disease. *New England Journal of Medicine. 1999;340: 115-126.*

Sahli W. Ueber das Vorkommen von Pepsin und Trypsin im normalen menschlichen Harn. *Pflugers Archiv fur die gesamte Physiologie 1885:36: 209-229.*

Saksela O, Hovi T, Vaheri A. Urokinase-type plasminogen activator and its inhibitor secreted by cultured human monocyte-macrophages. *J Cell Physiol 1985;122: 125.*

Santibanez JF and Martinez, J. Membrane-associated procollagenase of leukemic cells is activated by urokinase-type plasminogen activator. *Leuk Res* 1993;17: 1057-1062.

Sanz G , Pajaron A, Alegria E. Prevention of early aortocoronary bypass occlusion by low-dose aspirin and dipyridamole. *Circulation 1990;82: 765-773.*

Sato Y, Rifkin DB. Inhibition of endothelial movement by pericytes and smooth muscle cells: Activation of a latent transforming growyh factor Beta 1 like molecule by plasmin during co-culture. *J Cell Biol 1989;109: 309-315*

Sawa H, Fujii S, Sobel BE. Augmented arterial wall expression of type 1 plasminogen activator inhibitor induced by thrombosis. *Arterioscler Thromb 1992;12: 1507-1515.*

Sawa H, Sobel, B.E., and Fujii, S. Potentiation by hypercholesterolemia of the induction of aortic intramural synthesis of plasminogen activator inhibitor type 1 by endothelial injury. *Circ Res 1993;*73: 671-680.

Sawa H, Lundgren C, Sobel BE, and Fujii S. Increased intramural expression of plasminogen activator inhibitor type 1 after balloon injury: a potential progenitor of restenosis. *J Am Coll Cardiol 1994;24: 1742-1748.*

Sawa H, Sobel, BE, and Fujii S. Inhibition of type-1 plasminogen activator inhibitor production by antisense oligonucleotides in human vascular endothelial and smooth muscle cells. *J Biol Chem 1994;269: 14149-14152.*

Sayers RD, Watt PAC, Muller S. Endothelial cell injury secondary to surgical preparation of reversed and non-reversed (in-situ saphenous vein bypass grafts. *Eur J Vasc. 1992;6: 354-361.*

Schmitt M, Janicke F, Moniwa N, Chucholowski N, Pache L, and Graeff H. Tumor-associated urokinase-type plasminogen activator: biological and clinical significance. [review]. *Biol Chem Hoppe Seyler 1992;373: 611-622.*

Schleef RR, Bevilaqua MP, Sawdey M, Gimbourne MA, Loskutoff DJ. Cytokine activation of endothelium. *J Biol Chem 1988;263: 5797-5803.*

Schleef R, Higgins DL, Pillemer E, Levitt LJ. Bleeding diathesis due to decreased functional activity of type1 plasminogen activator inhibitor. *J Clin Invest 1989:83; 1747-1752.*

Schleuning WD, Medcalf RL, Hession C, Rothenbuhler R, Shaw A, Kruithof EK. Plasminogen activator inhibitor 2: regulation of gene transcription during phorbol ester-mediated differentiation of U-937 human histiocytic lymphoma cells. *Molecular & Cellular Biology. 1987;7: 4564-4567.*

Schnaper HW, Barnathan ES, Mazar A, Maheshwari S, Ellis S, Cortez SL, Baricos WH, and Kleinman HK. Plasminogen activators augment endothelial cell organization in vitro by two distinct pathways. *J Cell Physiol 1995;165: 107-118.*

Schneiderman J, Sawdey MS, Keeton MR, Bordin GM, Bernstein EF, Dilley RB, Loskutoff DJ. Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. *Proc Nat Acad Sci USA 1992;89: 6998-7002.*

Schneiderman J, Bordin GM, Engelberg I, Adar R, Seiffert D, Thinnes T, Bernstein EF, Dilley RB, and Loskutoff DJ. Expression of fibrinolytic genes in atherosclerotic abdominal aortic aneurysm wall. a possible mechanism for aneurysm expansion. *J Clin Invest 1995;96: 639-645.*

Schwartz BS, Bradshaw JD. Regulation of plasminogen activator inhibitor mRNA levels in liposaccheride-stimulated human monocytes. Correlation with the production of the protein. *J Biol Chem 1992;267: 7089-7094.*

Schwartz CJ, Valente AJ, Kelly JL, Sprague EA, Edwards EH. Thrombosis and development of atherosclerosis: Rokitansky revisited. *Semin Thromb Hemostasis 1988;14: 189-195.*

Shireman PK, McCarthy WJ, Pearce WH, Patterson BK, Shively VP, Cipollone M, Tamarina N, Verrusio EN, and Kwaan HC. Elevated levels of plasminogen-activator inhibitor type 1 in atherosclerotic aorta. *J Vasc Surg 1996;23: 810-818.*

Skriver L, Nielsen LS, Stephens R, and Dano K. Plasminogen activator released as inactive proenzyme from murine cells transformed by sarcoma virus. *European J Biochem 1982;124: 409-414.*

Smith EB. Fibrinogen, fibrin and fibrin degradation products in relation to atherosclerosis. *Clinics in Haematolog. 1986;*15: 355-370

Sobel GW, Mohler SR, Jones NW, Dowdy ABC, Guest MM. Urokinase: an activator of plasma profibrinolysin extracted from human urine. *Am J Physiol 1952;171: 768*

Soyombo AA, Angelini GD, Bryan AJ, Jasani B, Newby AC. Intimal proliferation in a organ culture of human saphenous vein. *Am J Pathol. 1990;137: 1401-1410.*

Sprengers ED, Kluft C: Pasminogen activator inhibitors. *Blood 1987; 69: 381-387.*

Stahl A and Mueller BM. The urokinase-type plasminogen activator receptor, a gpi-linked protein, is localized in caveolae. *J Cell Biol 1995;129: 335-344.*

Stahl A and Mueller, B.M. Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells in vitro. *Cancer Res 1994;54: 3066-3071.*

Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation 1994;89: 2462-2478.*

Strickland S, Beers WH. Studies on the role of plasminogen activator in ovulation. *J Biol Chem. 1976:251: 5694-5702.*

Sugawara Y, Sakata Y, Minowada S, Hamada H, Yoshida Y, Sato O, Deguchi J, Kimura H, Namba T, Makuuchi M, and Miyata T. Adenovirus-mediated transfer of tissue-type plasminogen activator gene to human endothelial cells. *Surgery 1997; 122: 91-100.*

Thorsen S, Glas-Greenwalt P and T Astrup. Differences in the binding of Fibrin of urokinase and tissue plasminogen activator. *Thrombos. Diathes. Haemorr. 1972; 28: 65-74.*

Todd AS. Histological localization of plasminogen activator. *J Pathol Bacteriol 1959;78: 281-7*

Tranquille N, Emeis JJ. Protein synthesis inhibition by cycloheximide does not affect the acute release of tissue-type plasminogen activator. *Thrombosis and Haemostasis 1989;61: 442-447.*

Trowell OA. The culture of mature organs in synthetic medium. *Exp Cell Res. 1959;16: 118-147.*

Underwood MJ, deBono DP. Increased fibrinolytic activity in the intima of atheromatous coronary arteries: protection at a price. *Cardiovasc Res 1993;27: 882-885.*

Underwood MJ. The fibrinolytic activity of the vessel wall and its modification. *MD Thesis, University of Leicester, 1994.*

Vague P, Juhan-Vague I, Aillaud MF. Correlation between blood fibrinolytic activity, PAI level, plasma insulin level and relative body weight in normal and obese subjects. *Metabolism 1986;35: 250-253.*

Vaheri A, Stephens RW, Salonen EM, Pollanen J, and Tapiovaara H. Plasminogen activation at the cell surface-matrix interface. *Cell Differentiation & Development 1990;32: 255-262.*

Van Hinsberg VWM, Van Den Berg EA, Fiers W, Dooijewaards G. Tumour necrosis factor induces the production of urokinase type plasminogen activator by human endothelial cells. *Blood 1990;75: 1991-1998.*

Van Hinsberg VWM. Regulation of the synthesis and secretion of plasminogen activators by endothelial cells. *Haemostasis 1988:18: 307-327.*

Vassalli JD, Baccino D and Belin D. A cellular binding site for the 55,000 form of human plasminogen activator, urokinase. *J Cell Biol 1985;100: 86-92.*

Vassalli JD, Wohlwend A, and Belin D. Urokinase-catalyzed plasminogen activation at the monocyte/macrophage cell surface: a localized and regulated proteolytic system. *Curr Top Microbiol Immunol 1992;*181: 65-86.

Vaughan DE, Declerck PJ, Van Houte E, De Mol M, Collen D. Reactivated recombinant plasminogen activator inhibitor 1 (rPAI-1) effectly prevents thrombolysis in vivo. *Thromb Haemost 1992;68: 60-63.*

Verde P, Stoppelli MP, Galeffi P, Di Noceri P, Blasi F. Identification and primary sequence of an unspliced human urokinase poly(A)+ RNA. *Proc Nat Acad Sci, USA 1984;81: 4727-4731.*

Virchow R. Gesammette abdandlungen zur. *Wissenschaftliscen Medicin Frankfurt, M. Meidinger Sohn 1856.*

Wallen P, Ranby M, Bergsdorf N, and Kok P. (1981). Purification and characterisation of tissue plasminogen activator: on the occurrence of two different forms and their enzymic properties,p.16-23. In: *Progress in fibrinolysis, Vol 5. Davidson, J.F., Nillson, I.M. and Astedt, B. (eds.). Churchill Livingstone, Edinburgh..*

Wang W, Chen HJ, Giedd KN, Schwartz A, Cannon PJ, and Rabbani LE. T-cell lymphokines, interleukin-4 and gamma interferon, modulate the induction of vascular smooth muscle cell tissue plasminogen activator and migration by serum and platelet-derived growth factor. *Circ Res 1995;77: 1095-1106.*

Webster WS, Bishop SP, Geer SC. Experimental aortic intimal thickening. Morphology and source of intimal cells. *Am J Pathol 1974;76: 245-264.*

White WF, Barlow GH, and Mozen MM. The isolation and characterisation of plasminogen activators (urokinase) from human urine. *Biochemistry 1996;5: 2160-2169.*

Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci USA 1989; 86: 2839-2843.*

Wilhelm O, Schmitt M, Hohl S, Senekowitsch R, and Graeff H. Antisense inhibition of urokinase reduces spread of human ovarian cancer in mice. *Clin Exp Metastasis 1995;13: 296-302.*

Wohlwend A, Belin D, Vassalli JD. Plasminogen activator-specific inhibitors produced by human monocytes/macrophages. *J Exp Med 1987;165: 320-339.*

Wun TC, Ossowski L, and Reich E. A Proenzyme form of human urokinase. *J. Biol. Chem. 1982;257: 7262-7268.*

Yamamoto C, Kaji T, Furuya M, Sakamoto M, Kozuka H, and Koizumi F. Basic fibroblast growth factor suppresses tissue plasminogen activator release from cultured human umbilical vein endothelial cells but enhances that from cultured human aortic endothelial cells. *Thromb Res 1994;73: 255-263.*

Yang Z, Shultz P, von Segesser L, Bauer E, Turina M, Luscher TF. Different interactions of platelets and arterial venous bybass vessels. *Lancet 1991;337: 939-943.*

Ye S, Green FR, Scarabin PY, Nicaud V, Bara L, Dawson SJ, Humphries SE, Evans A, Luc G, Cambou JP, et al. The 4G/5G genetic polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene is associated with differences in plasma PAI-1 activity but not with risk of myocardial infarction in the ECTIM study. Etude CasTemoins de I'nfarctus du Mycocarde. *Thrombs & Haemos. 1995;74: 837-841.*

Zempo N, Koyama N, Kenagy RD, Lea HJ, and Clowes AW. Regulation of vascular smooth muscle cell migration and proliferation in vitro and in injured rat arteries by a synthetic matrix metalloproteinase inhibitor. *Arteriosclerosis, Thrombosis & Vascular Biology 1996;16: 28-33.*

Zoellner H, Wojta J, Gallicchio M, McGrath K, Hamilton JA. Cytokine regulation of the synthesis of plasminogen activator inhibitor-2 by human vascular endothelial cells. *Thromb Haemost 1993;69: 135-140.*

Zwolek RM, Adams MC, Clowes AW. Kinetics of vein graft hyperplasia: Association with tangential stress. *J Vasc Surg 1987;5: 126-136.*

Appendix Reagents

Ammonium persulphate (APS)

Ammonium persulphate 1 g

Add water to 10 mls.

Denaturing Solution

1.5 M NaCl

0.5 M NaOH

Dye for electrophoresis of DNA

Glycerol	6.7	mls
DEPC Water	10.9	mls
50X E Buffer	2.4	mls
Bromophenol Blue	0.25%	

Denhardts X 50

Ficoll	5 g
Polyvinyl pyrilodine	5 g
Bovine serum albumin	5 g

Neutralizing Solution

Tris 1.0 M (pH 7.4)

NaCl 1.5 M

PEG Mix (Polyethylene glycol)

PEG 1.5 g

Water 2.25 ml

SDS 1.25 g

Phosphate buffered saline (PBS)

NaCl	8.0 g)	
KCl	0.2 g)	made up to 1 litre with water; pH 7.4
Na_2HPO_4	1.44 g)	
KH^2PO_4	0.24 g)	

10% SDS

Electrophoresis-grade SDS	100 g
Water	900 mls

Heat to 68°C to help dissolution. Adjust to pH 7.2 with a few drops of HCl then make up to 1 litre with water.

20X SSC

Na Cl	175.3 g)	
NaH_2PO_4	27.6 g)	made up to 800 mls with water and adjusted to pH 7.4 with
EDTA	7.4 g)	approximately 6.5 mls 10N NaOH, then finally made up to 1 litre with water.

ABBREVIATIONS

APS	Ammonium persulphate (see appendix)
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethelyene diamine tetra-acetic acid
HUVECS	Human umbilical vein endothelial cells
IMA	Internal mammary artery
IMS	Industrial Methylated Spirit.
kb	Kilobase
kD	Kilodaltons.
α^{32}P dCTP	α^{32} Phosphorus-labelled deoxycytosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
MMLV	Murine reverse transcriptase enzyme
OD	Optical density
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PCR	Polymerase chain reaction
rNTP	Ribonucleotide triphosphate
RT-PCR	Reverse transcriptase polymerasation chain reaction
RNA	Ribonucleic acid
SDS	Soodium dodecyl sulphate
SV	Saphenous vein
HSVC	Human saphenous vein culture
SVG	Saphenous vein graft
SSC	Sodium chloride/sodium citrate solution (see appendix)
SSPE	Sodium chloride/sodiumphosphate/EDTA solution (see appendix)
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
tPA	Tissue plasminogen activator
uPA	Urokinase-type plasminogen activator
VSMC	Vascular smooth muscle cells.