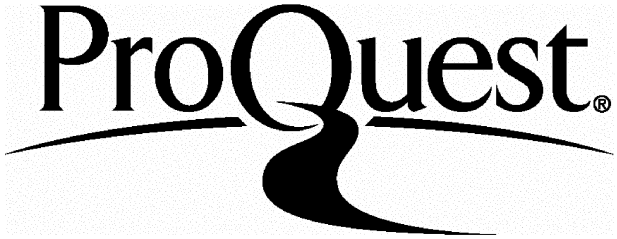
Matrix Metalloproteinase Inhibitors In Angioplasty Restenosis- Pharmacological And Gene Transfer Approaches.

Clare Margaret Dollery

1998

A thesis submitted to the University of London for the degree of Doctor of Philosophy (Ph.D.).

The candidate was registered at University College London.



ABSTRACT

The injury response associated with coronary angioplasty causes 25-50% of patients to develop restenosis at the site of their original lesion, due to the migration and proliferation of smooth muscle cells and failure of compensatory expansion of the arterial wall. Cell migration across matrix boundaries is a major component in the formation of neointimal hyperplasia following vascular injury, and is dependent on the alteration of the proteolytic balance within the arterial wall towards matrix breakdown. This study investigated the hypothesis that this change is mediated in part by the matrix metalloproteinases, (MMPs) and might be prevented by their inhibition, either by pharmacological means or by their natural inhibitors the tissue inhibitors of metalloproteinases (TIMPs).

Initial studies of a pharmacological inhibitor, CT1746, showed no reduction in neointimal hyperplasia after balloon injury in the rat carotid artery and highlighted problems with reliable delivery of this compound. Adenoviral and herpes gene transfer approaches were, therefore, developed and characterized in detail both *in vivo* and *in vitro* using a marker gene. A novel adenoviral vector, Av1.TIMP1, containing the TIMP1 cDNA was characterized, purified, and propagated. An increase in expression of biologically active and immunoreactive TIMP1 was seen *in vitro* in response to infection of smooth muscle cells (SMCs) with Av1.TIMP1. Infection of SMCs with Av1.TIMP1 reduced SMC invasion *in vitro* by 27% compared to control virally infected cells. The adenoviral vector was delivered to the rat carotid artery following balloon injury and four days later immunoreactive protein was identified and migration of smooth muscle cells reduced by 60%. The transmural expression of TIMP1 was associated with a 30% reduction in neointimal area two weeks after angioplasty. These studies support the hypothesis that arterial balloon injury involves MMP-dependent smooth muscle cell migration which can be attenuated *in vivo* by adenoviral gene transfer of TIMP1.

ACKNOWLEDGMENTS

It is impossible after three years to single out all the individuals who made contributions both scientific and otherwise to the completion of this work. I am grateful to them all.

First I would like to thank my supervisor Dr. Jean McEwan for offering me the chance to undertake this novel and exciting project. On the days when nothing worked her belief in the idea was sustaining. In addition to supervising my research throughout the three years, and despite a hectic schedule of commitments, Jean taught me the practical skills of the animal surgery. I particularly value the freedom that I was given in following up interesting findings.

Professor Steve Humphreys, my second supervisor, provided objectivity, practical advice, ideas, and most importantly encouragement. I am profoundly grateful to Steve for his advice and interest at a personal and professional level through out my time at UCL.

Dr. Adriano Henney was my initial second supervisor and with Jean introduced me to the fascinating field of matrix biology. The departure of his research group to Oxford was a practical blow to this project but I am grateful to him for his continuing positive attitude to my work and helpful advice.

This was the first project to establish adenoviral gene delivery at University College London. Professor David Latchman provided not only the necessary containment facilities but also supervision of the virus work. His group meetings and the advice of his postdoctoral fellows made this project possible. Dr. Alan McClelland, and his colleagues from Genetic Therapy Incorporated supplied the viral vectors and a number of protocols for virus propagation. Professor Derek Yellon showed interest in my project as well as providing the hospitable environment of The Hatter Institute.

In developing the adenovirus program and the many other new techniques of this project it has been necessary to seek facilities, reagents, and both

scientific and technical advice from many departments at UCL. In particular the help of Dr Jill Norman, Professor Geoff Laurent, and Professor Patrick Reilly and their colleagues stands out. It is a tribute to the institution that it functions as a true University where a Ph.D. student can glean enlightenment from so many sources. It is the personal contributions of the Heads of Department, Lecturers, Post Docs, fellow students, and Technicians, however, which is most remarkable. In helping me, they delayed their own grant applications, papers, and theses or were just late going to the pub. I am grateful.

The final acknowledgment must be to my family and friends. Chrys, Jenny, Gary, Sam, Laura, Jo and Hugh were both friends and colleagues. Kate and Greg, my closest friends, put up with three years of listening to the daily dilemmas, trying to gaze adoringly at my latest blot, and being stood up because I was feeding my cells. It is to their credit and my relief that we remain inseparable. I am grateful to my parents for everything.

All parts of this thesis were carried out by the candidate without technical assistance of any kind. The animal experiments described in chapter 3 were carried out jointly by Dr McEwan and myself according to home office regulations regarding supervision of animal experiments.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES.

CHAPTER 1. INTRODUCTION.

1.1 BACKGROUND

The properties and regulation of the matrix metalloproteinase (MMP) family of matrix breakdown enzymes and their inhibitors were initially studied in tumour metastasis and arthritis (Basset *et al.* 1990; Brinckerhoff, 1991; Greenwald *et al.* 1992; Greenwald, 1994; Mignatti and Rifkin, 1993; Melchiori *et al.* 1992; Urbanski *et al.* 1992). Their powerful role in these diseases led others to study their expression in the vasculature in normal and atherosclerotic arteries and in balloon injured arteries (Strauss *et al.* 1996; Nikkari *et al.* 1995; Bendeck *et al.* 1994; Zempo *et al.* 1994; Nikkari *et al.* 1994; Southgate *et al.* 1996; Galis *et al.* 1995b; Galis *et al.* 1994b; Henney *et al.* 1991; Webb *et al.* 1997; Vine and Powell, 1991). Parallels can be drawn between wound healing and the response to vascular injury and between the liberation of metastatic malignant cells into the blood stream and the migration of smooth muscle cells from the intima to the media in an injured artery (Dollery *et al.* 1995). Evidence for MMP upregulation after vascular injury led to the hypothesis under investigation in this study that inhibition of MMPs may attenuate the stenotic response to vascular injury.

The vessel wall is an integrated functional component of the circulatory system which is continually remodeling in response to haemodynamic conditions and disease states. The endothelium releases locally active hormones, such as nitric oxide and endothelin, which have immediate vasoactive properties and longer term trophic effects on the medial smooth muscle cells (Moncada, 1997). The medial smooth muscle cells are also exerting a longer term effect on the physical properties of the vessel wall by controlling the synthesis of its major structural components such as the collagens (Type I, III, IV and VI , elastin, proteoglycans, and glycoproteins (Murphy and Reynolds, 1993). The smooth muscle cells (SMCs) synthesize these structural components and control matrix turnover via the matrix

metalloproteinase (MMP) enzymes, the plasmin/plasminogen system and their inhibitors. The matrix composition determines the physical elastic properties of the vessel wall, while its breakdown can release growth factors stored in the matrix, changing the cellular components of the wall. The matrix, therefore, rather than being merely a system of scaffolding for the surrounding cells, is central to the control of vascular remodeling.

1.2 The Matrix Metalloproteinases

MMPs are a family of Zn^{2+}- dependent enzymes which have the capacity to degrade virtually all of the constituents of the extracellular matrix (Galis *et al.* 1994a). They are important in the resorption of extracellular matrices in both normal physiological processes and pathological states. There are four potential areas of importance for the MMPs in vascular biology: atherogenesis, the development of the unstable plaque, migration and proliferation of SMC after angioplasty, and vascular remodeling.

Sixteen metalloproteinases have been identified, cloned, and sequenced. They can be divided into four groups: Firstly the Collagenases that degrade structural type I-III collagens only; secondly the type IV Collagenases/Gelatinases which act on the basement membrane and partially degraded collagen; and thirdly the Stromelysins which have a broad substrate specificity (proteoglycans, laminin, fibronectin, gelatin, and basement membrane collagens) (see Table 1: The Matrix Metalloproteinases). The newest group in the MMP family are an integral part of the plasma membrane, rather than secreted proteins, and have been named the membrane type MMPs (MT-MMPs 1-4) (Sato *et al.* 1994; Takino *et al.* 1995; Puente *et al.* 1996; Will and Hinzmann, 1995).

Taken together the MMPs, once activated, can degrade all extracellular matrix components. It is important, therefore, that the activity of these enzymes is kept under tight control, and this operates at three levels: transcription, activation of the latent proenzymes and inhibition of proteolytic activity (see Figure 1) (Matrisian, 1990).

SUBGROUP	NAME	MMP NUMBER	MOLECULAR WEIGHT KD (latent form)	SUBSTRATE	REGULATOR IN HUMAN VSMC	NATIVE INHIBITOR
COLLAGENSASES	Interstitial Collagenase	1	55	Collagen type III[1], I, II, VII, X, gelatin and proteoglycans	IL-1, TNFα, PDGF, Phorbol	TIMP 1 and 2
	Neutrophil Collagenase	8	75	Collagen type I, III, II, proteoglycans		
	Collagenase 3	13				
GELATINASES	Gelatinase A	2	72	Collagen type IV, V, VII, X, gelatin	Constitutive	TIMP 2 and 1
	Gelatinase B	9	92	Collagen type IV, V, VII, X,	IL1 and TNFα	TIMP 1 and 2
STROMELYSINS	Stromelysin 1	3	57	Collagen type III, IV, V, IX, Laminin, fibronectin, elastin, gelatin, proteoglycans, Progelatinase B, Procollagenase		TIMP 1 and 2
	Stromelysin 2	1	57	As for stromelysin 1.		TIMP 1 and 2
	Stromelysin 3	11	51	Gelatin, fibronectin, proteoglycans		TIMP 1 and 2
	PUMP-1	7	28	Gelatin, fibronectin, laminin, collagen type IV, procollagenase, proteoglycan core protein		TIMP 1 and 2
	Metalloelastases	12	57	Elastin		TIMP 1 and 2
MEMBRANE TYPE MMPs	MT1-MMP	14	66	Collagen type IV, gelatin, casein, fibronectin, progelatinase A		TIMP 1
	MT2-MMP	15	64	progelatinase A		TIMP 1 and 2
	MT3-MMP	16	50			
	MT4-MMP	17	70			
	MMP-18	18				
	MMP-19	19	60	?similar to stromelysin	Not Known	TIMP-2

Table 1 The Matrix Metalloproteinases

[1] Underlined words denote a potent interaction between the MMP enzyme and the substrate or inhibitor indicated.

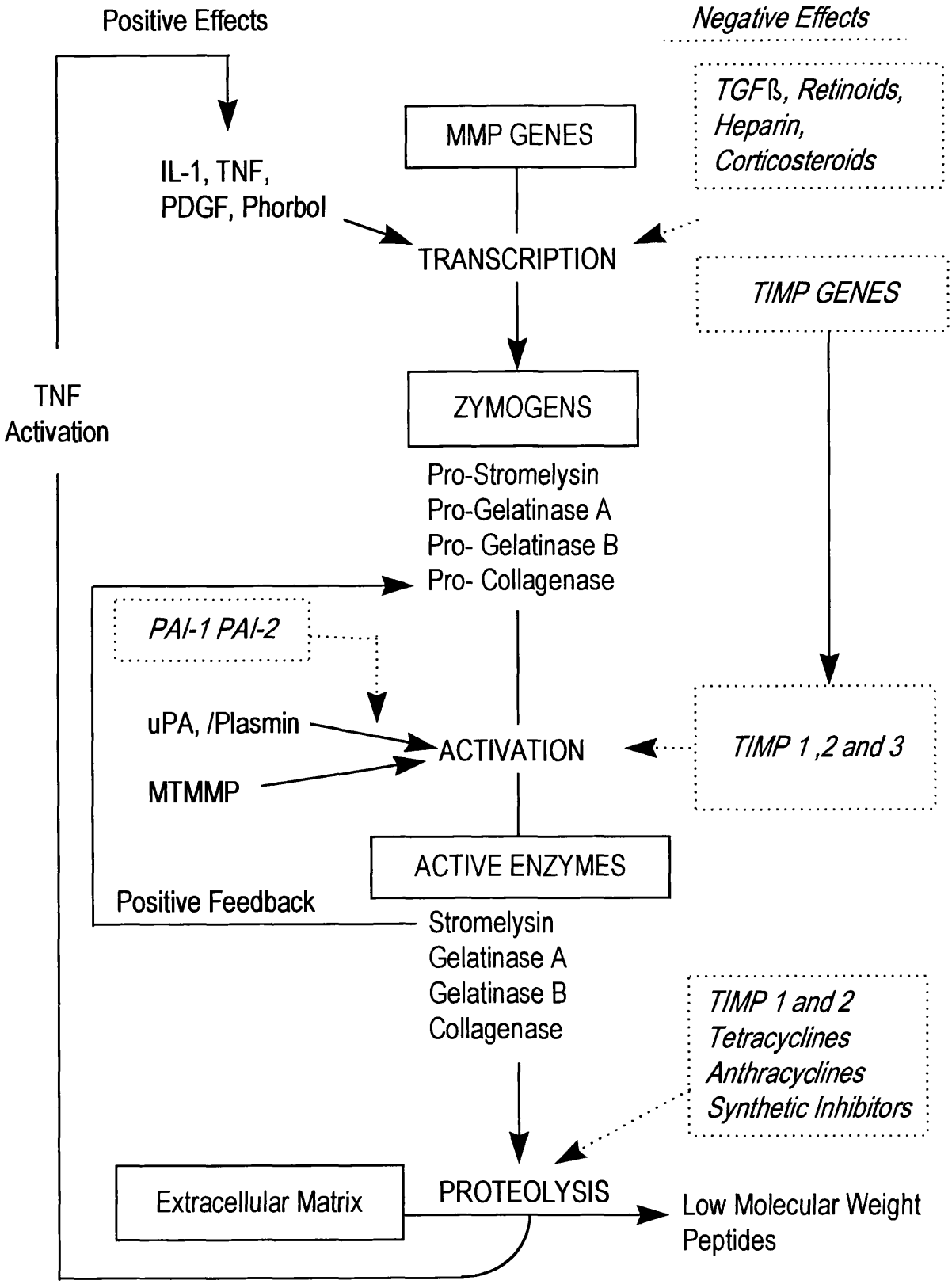


Figure 1 Regulation of the MMPs.

1.2.1 Transcriptional regulation

A number of cytokines and growth factors have been shown to induce or stimulate the synthesis of MMPs, including interleukin-1, platelet derived growth factor (PDGF) and tumour necrosis factor α (TNF-α), whilst others, such as transforming growth factor ß (TGFß), heparin, and corticosteroids have an inhibitory effect (Galis *et al.* 1995a; Galis *et al.* 1994a; Lee *et al.* 1996; Kenagy *et al.* 1994; Fabunmi *et al.* 1996). Studies of the Collagenase and Stromelysin gene promoters have identified a number of consensus sequences for nuclear binding proteins, including TPA-responsive elements and activator protein-1 (AP1) sites (Curran and Franza, 1988). In addition, the proto-oncogenes c-Fos and c-Jun act as transcription factors (Birkedal-Hansen *et al.* 1993). Recently two groups have demonstrated that TNF-α, is regulated by the MMPs. TNF-α a potent proinflammatory and immunoregulatory cytokine is synthesized as a zymogen which is cleaved to the active form (Black *et al.* 1997). It has been demonstrated that metalloproteinases inhibitors can prevent this post translational activation, and that the addition of Collagenase, Stromelysin, Matrilysin and the Gelatinases will restore its activity (McGeehan *et al.* 1994; Gearing *et al.* 1994). It is unlikely that MMPs are the major converting enzyme of TNFα but their ability to activate TNF may be important where focalized proteolysis is occurring at the cell surface (Basbaum and Werb, 1996; Black *et al.* 1996). The MMPs are, therefore, capable of regulating cytokine activity and may, at least in local areas of enhanced MMP activity, initiate cellular processes as well as participate in them.

1.2.2 Proenzyme activation

The second level of control of the MMPs is in the activation of latent proenzymes. Although some plasmin-independent pathways exist (Sperti *et al.* 1992), plasmin is a potent activator of most MMPs, promoting cleavage

of the latent propeptides to the active molecule (Nagase *et al.* 1990). There are similarities between this proteolytic system and the clotting cascade; for example while plasmin cleaves and therefore activates Stromelysin the resultant active enzyme can activate other proenzymes forming a positive feedback loop. This type of positive feedback also exists between the MMP enzymes themselves. When the Collagenases are cleaved at the carboxy terminal by Stromelysin there is a 5-8 fold increase in their proteolytic activity (Goldberg *et al.* 1992). The membrane type-1 MMP (MT1-MMP) is an integral membrane protein and an activator of pro-Gelatinase A (Cao *et al.* 1996). This combination allows localization of the proteolytic process to the cell surface and controls its activation, thus mirroring the plasminogen/plasmin activator system.

1.3 Inhibitors of MMPs- The TIMPs

Inhibitors of the MMPs include naturally occurring tissue inhibitors of metalloproteinases (TIMPs) 1,2,3 and 4, α2 macroglobulin, and exogenous substances such as heparin. The contribution of α2 macroglobulin is, however, limited by the large size of the molecule. The TIMPs are relatively small, heavily glycosylated, proteins which are classified based on their structural similarities and their ability to inhibit MMPs (Denhardt *et al.* 1993). See Table 2.

TIMP1 is synthesized by most types of connective tissue cells, as well as macrophages, and acts against all members of the Collagenase, Stromelysin, and Gelatinase classes of enzyme. It forms high-affinity irreversible (*in vivo*), noncovalent complexes with the active forms of the enzymes. It is highly expressed in actively resorbing tissues, and its role is to regulate enzyme activity tightly, at the level of both activation of MMPs from their latent form and their catalytic activity. TIMP2, however, has a dual inhibitory action binding to proGelatinase A and stabilizing this inactive form of the enzyme while the same 'stabilization site' in the active Gelatinase A molecule makes this form more susceptible to inhibition.

Independent of their anti-proteolytic properties the TIMPs also act as growth factors for erythroid precursors, but this function as yet has no known application to atherosclerotic processes (Hayakawa *et al.* 1992; Bertaux *et al.* 1991; Docherty *et al.* 1985; Hayakawa *et al.* 1994).

In general, TIMP1 is inducible in response to stimuli while TIMP-2 and 3 are constitutively expressed at low levels. To complicate the picture there are some situations in which this is reversed; for example, the responses of TIMP1 and TIMP2 to TGF-α are opposite in a numberof human cell types, that is TIMP1 is constitutive and TIMP2 inducible (Stetler Stevenson *et al.* 1996). These differences may be important in allowing divergent local regulation of proteolysis. In both rabbit and human vascular smooth muscle cells TIMPs 1 and 2 are constitutively expressed but appear unaltered by chemicals and cytokines such as Phorbol, TNFα, TGFß, IL1ß, and IL1ß (Galis *et al.* 1994a; Fabunmi *et al.* 1996). PDGF mildly increases TIMP1 but does not effect TIMP2. In contrast, TIMP3 is expressed at low levels in unstimulated cells and induced by PDGF and TGFß, which have a synergistic effect when added simultaneously (Fabunmi *et al.* 1996). Differential regulation of the different TIMPs also exists when smooth muscle cells are stimulated with FGF-2. TIMP1 is mildly increased, TIMP2 repressed, and TIMP3 unaffected (Pickering *et al.* 1997). TIMP1 is known to have AP-1 and polyoma enhancer A binding factor 3 (PEA-3) binding sites close to the major transcription sites of its gene, which are likely to be responsible for its basal expression (Clark *et al.* 1997). These motifs are also found close to the promoters of the inducible MMPs Collagenase and Stromelysin 1. There is, therefore, a potential molecular mechanism for simultaneous regulation of the MMPs and their inhibitors. The close relationship between TIMPs and MMPs is also seen in the interactions between Gelatinase A, MT1-MMP and TIMP2. Activated MT1-MMP acts as a cell surface receptor for TIMP2 and the resultant complex is a receptor for Gelatinase A, which is activated by MT1-MMP. TIMP1 does not inhibit MT1-MMP while it is susceptible to TIMP2 and TIMP3 (reviewed by (Basbaum and Werb, 1996)).

TIMPS	TIMP 1	TIMP 2	TIMP 3	TIMP 4
Protein	28 kD	21 kD	24 kD	23 kD
mRNA	0.9 kB	3.5 kB	4.5 kB	1.1 kB
Major Sites	Ovary, testis, bone	Lung, brain, testis	Kidney, decidua, lung	Heart, brain
Expression	Inducible	Largely constitutive	Cell cycle regulated	?

Table 2. The Tissue Inhibitors of Metalloproteinases.

The role of the most recently discovered TIMP, TIMP4, in the vasculature is largely unknown but it is known to be predominantly expressed in the heart (Greene *et al.* 1996). The MMPs and their inhibitors thus form a potentially linked cascade with a parallel and tightly regulated system, with numerous permutations, controlling the overall composition of the extracellular matrix.

1.4 MMPs and TIMPs In Cardiovascular Disease

1.4.1 Atherosclerosis And Aneurysm Formation

The importance of MMPs and their inhibitors in the vasculature was first identified in the study of atherosclerosis (Henney *et al.* 1991). Connective tissue remodeling is a key feature of the development of the atherosclerosis. One of the earliest events in the formation of the atherosclerotic plaque is the adherence of circulating monocytes to the vascular endothelium through which they gain entry to the sub-intimal tissue. Once there, a series of complex cell-cell interactions takes place over a long period, involving the secretion of a wide variety of growth factors and cytokines (Hansson *et al.* 1989). These include TNF-α, Interleukin 1 (IL-1), and PDGF, which are known to stimulate MMP synthesis in human aortic smooth muscle (Galis *et al.* 1994a; Yanagi *et al.* 1991). With time, the matrix of the vessel wall becomes modified through the migration and proliferation of cells and the deposition of extracellular matrix, eventually resulting in the formation of a plaque. The process of cellular migration has been studied *in vitro* using rat vascular smooth muscle cells, and confirms that 72 kD Gelatinase A is essential for cells to cross a basement membrane barrier (comprised of type IV collagen) (Pauly *et al.* 1994). Unless other cellular events can breach the basement membrane of the vessel wall this appears to be a limiting step in plaque formation which is governed by the MMPs.

Metalloproteinases are known to be expressed in human atherosclerotic plaques by both SMC and foam cells; this has been demonstrated by both *in situ* zymography and *in situ* hybridization (Henney *et al.* 1991; Galis *et al.* 1994b). Stromelysin mRNA transcripts were localized to both SMC and macrophages in frozen sections of both fibrous and lipid-rich atherosclerotic plaques. The accumulation of large numbers of macrophages and foam cells is not a feature of the normal vessel wall, and it is likely that the extensive synthesis of Stromelysin observed in these specimens is a pathological event. It is possible that cytokines, such as TNF-α, induce the production of Stromelysin and other MMPs in a localized area of mechanical stress, which then activates the proteolytic cascade described above and then results in plaque rupture and vascular occlusion.

Studies of degenerative aortic disease and aneurysm formation also suggest a role for the MMPs and TIMPs. Gelatinase A and Gelatinase B are both upregulated in aneurysmal aortic disease (Vine and Powell, 1991; Herron *et al.* 1991; McMillan *et al.* 1995; Knox *et al.* 1997; Freestone *et al.* 1995). Gelatinase B is detectable at significantly greater levels than in normal aortas and appears at highest levels in large aneurysms (McMillan *et al.* 1995; Freestone *et al.* 1995). While Gelatinase A was shown to be upregulated in small aneurysms by one study (Freestone *et al.* 1995). Another group showed an increase in MMP1,-3 and -2 in aneurysmal and atherosclerotic aortas compared with normal but no significant difference between the two disease groups (Knox *et al.* 1997). Studies of the TIMPs have also been made and these have shown direct and indirect evidence of high levels of TIMP activity in normal and diseased vessels (Knox *et al.* 1997; McMillan *et al.* 1995; Freestone *et al.* 1995). *In situ* zymography revealed a marked increase in the proteolytic activity in human aortic disease specimens compared with normal vessels, but was not able to detect any difference between aneurysmal and atherosclerotic disease (Knox *et al.* 1997). These studies point to the importance of proteolysis in these diseases but also emphasize the difficulty of interpreting results from

scarce human tissue, that may often be obtained only after the pathology is long standing.

1.4.2 Angioplasty Restenosis.

Coronary artery disease is the major cause of death in the United Kingdom and angina pectoris due to atheromatous coronary lesions is a cause of significant morbidity. Percutaneous transluminal coronary angioplasty (PTCA) has become a widely used treatment for angina in which the atheromatous narrowing is compressed into the vessel wall using a balloon catheter. Initially this procedure has a greater than 80% technical success rate, but the usefulness of angioplasty is limited by the fact that 25 -50% of patients have a recurrence of their symptoms within 6 months due to restenosis at the original site (Editorial, 1987; Landau *et al.* 1994). The process of restenosis has been the focus of research, as the appeal of angioplasty to both the patient and the physician are clear, but to date it has been resistant to multiple therapeutic interventions (reviewed by (Landau *et al.* 1994)).

Vascular injury initiates a combination of events. Initially, vascular smooth muscle cells (VSMC) from the media migrate into the neointima, and then rapid growth of these cells produces a characteristic lesion of fibrocellular intimal hyperplasia (Editorial, 1987; Waller *et al.* 1990). As the resultant lesion matures there is a greater preponderance of extracellular matrix. Animal models suggest that this process is regulated by growth factors, with basic fibroblast growth factor (bFGF) controlling smooth muscle cell replication and PDGF regulating cell migration (discussed in (Libby *et al.* 1992)). Despite the current state of catheter technology and clinical expertise, histology of the rare postmortem human coronary arteries after angioplasty reveal a significant injury to the vessel wall. Recent studies suggest that the injury and resultant repair processes are transmural, with clinical evidence from intravascular ultrasound demonstrating a change in total vessel dimensions after angioplasty (Glagov, 1994; Mintz *et al.*

1996). Laboratory studies of porcine coronary arteries have shown proliferative changes in the adventitia in response to medial balloon injury (Andersen *et al.* 1996). Restenosis probably represents an imbalance in the normal healing and repair processes within the blood vessel in response to injury, and raises the possibility that manipulation of the healing processes in the vessel wall could be used to prevent restenosis.

There is considerable evidence to suggest that a wide range of cellular components of both normal and diseased vessel wall such as smooth muscle cells, macrophages, and fibroblasts can degrade extracellular matrix (Galis *et al.* 1994a; Tyagi *et al.* 1997; Rajagopalan *et al.* 1996; Halpert *et al.* 1996). Vascular smooth muscle cells are capable of producing enzymes which will degrade both basement membranes and the extracellular matrix, in response to stimulation by many of the cytokines known to be released in response to vascular injury (Galis *et al.* 1994a; Yanagi *et al.* 1991; Fabunmi *et al.* 1996; Libby *et al.* 1992). Vascular smooth muscle cells have been shown to produce MMPs 1, 2, 3, and 9, three of the MT-MMPs (1-3), and TIMPs 1-3 (Galis *et al.* 1994a; Yanagi *et al.* 1991; Fabunmi *et al.* 1996; Shofuda *et al.* 1997). *In vitro* studies have demonstrated that mechanical injury can induce Collagenase and Stromelysin gene expression in vascular smooth muscle cells (James *et al.* 1993).

Animal models are now supplying further information. In the rat carotid model, 72 kD Gelatinase A is constitutively expressed with an increase in the active form of the enzyme four to five days after balloon injury (Bendeck *et al.* 1994; Zempo *et al.* 1994; Webb *et al.* 1997). 92 kD Gelatinase B was induced within six hours of injury and remained elevated for seven days (Bendeck *et al.* 1994; Zempo *et al.* 1994; Webb *et al.* 1997). It has been suggested that active Gelatinase B may, therefore, be controlling the migration of smooth muscle cells from the media to the intima. In support of this hypothesis the early *in vivo* migration of smooth muscle cells into the intima in the rat model was reduced by 97% by a metalloproteinase inhibitor (Bendeck *et al.* 1994). The pig has the same pattern of enzyme activity on zymography, with the 92 kD Gelatinase

induced at three days and the 72 kD Gelatinase at seven days. Both enzymes persist at elevated levels for up to 21 days (Southgate *et al.* 1996). In the rabbit model, however, no increase was observed in 92 kD Gelatinase but there was early induction of Gelatinase A which gradually decreased over twelve weeks (Strauss *et al.* 1996). Further evidence comes from studies of the plasminogen activator system post balloon injury in both the rat and rabbit models, which show acute upregulation of urokinase plasminogen activator (uPA) activity (Clowes *et al.* 1990; More *et al.* 1995) which could activate the MMP cascade.

The level of TIMPs in the vessel wall after injury will also clearly influence the overall proteolytic balance in the arterial wall. Two studies have shown evidence of upregulation of TIMP1 after vascular injury in rat and rabbit tissues (Webb *et al.* 1997; Wang *et al.* 1996). Another study in the rat model could not identify TIMP1 but found that plasminogen activator inhibitor (PAI-1) (6hours-3 days) and then TIMP2 (24 hours-7 days) were increased in response to injury (Hasenstab *et al.* 1997). It is clear that the smooth muscle cell can alter its surrounding matrix if stimulated by the appropriate cytokines. The ability of the medial smooth muscle cell to migrate into the intima and then proliferate appears to depend on the cells extricating themselves from the extracellular matrix. The MMPs, and in particular the early expression of Gelatinase B, are induced after balloon injury, and may be a molecular 'on-off switch' in the cascade of post-injury restenosis.

1.5 Animal Models of Vascular Injury.

As indicated in the examples above, a number of different animal models of vascular injury exist, and frequently display differences in their response to balloon injury. The ideal situation is to study the human disease, but this is particularly difficult as angioplasty fortunately has a low mortality rate (0-2%, reviewed in (Landau *et al.* 1994)). Human pathology specimens of

coronary arteries are thus scarce and may only be obtained long after the procedure.

The main animal models used are the rat carotid, the rabbit iliac, the pig coronary and carotid models and less frequently the dog coronary and primate saphenous or iliac models (reviewed in (Muller *et al.* 1992)). More recently, mouse vascular injury models have been developed for use in transgenics or knockouts (Carmeliet *et al.* 1997; Lindner *et al.* 1993). A healthy level of skepticism exists about many of these animal models following the success of angiotensin converting enzyme inhibitors in the rat model and their failure to prevent human restenosis (Powell *et al.* 1989; Anonymous1992). Each model has a particular suitability to investigate different aspects of atherosclerosis. Smooth muscle cell migration and proliferation, lipid rich unstable plaques and vascular remodeling are all important factors of the atherosclerotic and restenotic processes. In addition, the ability of the model to reflect clinically practical means of human local drug delivery is important, for example in gene transfer studies, and the model used will influence the distribution of drug (Feldman *et al.* 1995).

The rat model is perhaps the most commonly used and has the advantage of being extensively studied. Rat arteries are dissimilar to the human arteries in having no vasa vasorum, a smaller subintimal layer, and a low elastin content. Its main advantages are availability, low cost, and the ability to develop a rapid reproducible response to balloon injury. Proliferation reaches its peak approximately two days after injury, and migration can be assessed at four days (Clowes *et al.* 1983; Clowes and Clowes, 1986). Thus the lesion in this model is likely to equally reflect proliferation and migration (Clowes and Schwartz, 1985). Its disadvantages include the overestimation of the ability of pharmacological inhibitors to influence lesion development, which may partially be due the higher doses used in rodent studies. The model may also overemphasize the role of proliferation which peaks at two days after injury compared to sixteen days in man (Schwartz *et al.* 1996). The rat model also fails to reflect the

influences of lipids and vascular remodeling on vascular injury and does not result in an occlusive lesion. However, for many investigators the rat model is a starting point before moving to more complex models.

The rabbit iliac artery model can be studied in normocholesterolaemic rabbits, cholesterol fed rabbits, or Watanabe heritable hyperlipidaemic rabbits. The lesions in cholesterol fed rabbits are highly lipid rich, but may overestimate the role of lipids. The rabbit is suitable for double injury protocols which may more accurately reflect restenosis, where an occlusive narrowing develops at the site of ballooning of a preexisting lesion (Faxon *et al.* 1984). The rabbit iliac artery is of a similar size to the human coronary and can be used to test delivery devices (Willard *et al.* 1994). The rabbit models share the advantages of cost and reproducibility of the rat but have been principally used in studies of delivery of therapy rather than the biology of the restenotic process.

Dog models are resistant to the development of atherosclerosis and show medial rather than intimal thickening after balloon injury (Muller *et al.* 1992). In addition they have a highly active fibrinolytic system that is dissimilar to humans. Their main advantage is that the coronary vasculature can be studied but since the development of swine models their use has declined. For those studying gene therapy, canine cells also express high endogenous levels of ß-Galactosidase, making this marker gene unsuitable for use in this species (Lim *et al.* 1991). Non-human primate models show species differences but can develop lesions that mimic atherosclerosis on an atherogenic diet and seem to reflect recoil, intimal hyperplasia, and remodeling (Geary *et al.* 1996). Studies of the effect of heparin on restenosis suggest that the baboon model is a more accurate predictor than the rat (Clowes and Clowes, 1986; Clowes *et al.* 1991; Geary *et al.* 1995). The use of primates is limited by their expense and specific regulations which reflect ethical concerns and their poor response to captivity.

Swine models are most similar to humans in their cardiovascular morphology and susceptibility to atherosclerosis. The size of pig coronaries

corresponds closely to the human, and they can therefore be used to model therapeutic devices such as stents and gene delivery strategies (French *et al.* 1994; Post *et al.* 1997). Disadvantages, particularly in atherosclerotic swine, relate to the large cost of their maintenance and the difficulty in handling an animal that may grow to 400kg. Smooth muscle cell proliferation, migration, matrix accumulation and remodeling are all seen in pig coronary models (Post *et al.* 1997; Carter *et al.* 1994). However, they do not perfectly model human disease, for example, the peak proliferation in the pig coronary occurs 6 days after injury rather than 16 in the human coronary. Swine can be used to study occlusive lesions, although the degree of injury required to produce a stenosis is substantial and does not reflect the trauma occurring in human angioplasty (Andersen *et al.* 1996). The use of these so-called double injury strategies to create an initial lesion, which can then be ballooned using the same techniques that are applied clinically, may be the final testing ground for future therapies.

In the work described in this thesis the rat model was used because it is well characterized and reproducible. It was the aim of this study to study neointimal hyperplasia and smooth muscle cell migration in this model. Promising strategies would then be suitable for assessment in the pig coronary model.

1.6 MMP Inhibition

Possible mechanisms of MMP inhibition include: 1) increasing the levels of natural inhibitors (TIMPs) either by exogenous administration of recombinant TIMPs or increasing their local production; 2) administration of synthetic inhibitors and 3) decreasing the production of MMPs.

Exogenously administered TIMPs are liable to rapid metabolism, and denaturation. They tend to aggregate and adhere to surfaces therefore limiting tissue penetration. They have been used in mouse models of

arthritis but could not be shown conclusively to be effective (Carmichael *et al.* 1989). The upregulation of TIMPs might be achieved with administration of cytokines such as TGFß or retinoids, but it is likely that these compounds will have numerous side effects as they are active in many cellular processes. A number of synthetic inhibitors have been investigated, namely tetracycline-derived antibiotics, anthracyclins, and synthetic peptides (Greenwald, 1987; Karakiulakis *et al.* 1990). Inhibition of enzyme synthesis may be approached from both a conventional and a molecular standpoint. TGFß, retinoids, and corticosteroids all down-regulate MMP transcription. Corticosteroids have, however, already proven ineffective in the treatment of human restenosis (Pepine *et al.* 1990). Heparin has been shown to inhibit the production of Stromelysin, 92 kD Gelatinase and Collagenase and already has an established place in angioplasty protocols (Kenagy *et al.* 1994). It does not, however, eliminate the activity of these MMPs or influence the others and it does not prevent atherosclerosis or restenosis. At a molecular level the local application of antisense oligonucleotides to the MMP genes is possible but is unlikely to be successful where transcription is upregulated, for example, Gelatinase B after vascular injury. The antisense field remains controversial with problems of reproducibility, access of oligonucleotides to the cells and the specificity of its effects (Bennett and Schwartz, 1995).

The vascular system is a tempting target for MMP inhibitors (both molecular and pharmacological), because it offers opportunities for direct local transluminal delivery, which would eliminate many of the problems encountered in other disorders such as arthritis. Continued improvement in gene transfer techniques makes a molecular approach to overexpress the TIMP genes themselves more attractive than targeting common cellular processes. I have chosen to characterize and investigate both pharmacological and molecular approaches to the inhibition of MMPs in vascular injury.

1.7 Gene Transfer Approaches To MMP Inhibition

Gene transfer has been extensively studied as a means of modifying cardiovascular responses to therapy and disease. It may be used to achieve diverse aims, from the replacement of an absent or defective gene to restore cell function, to the transfer of a gene with a cytotoxic product to cause cell death. The clinical application of this technology has been termed "gene therapy" and may be defined as the introduction of foreign genetic material (usually Deoxyribonucleic acid, DNA) into somatic cells, in order to modify their genetic content and achieve a therapeutic end point. Gene therapy has developed rapidly because the technology to transfer genes has improved in parallel with the identification of new genes which have therapeutic potential. There are now more than 100 clinical protocols for human gene therapy approved in the United States (Anonymous 1997) including two clinical trials investigating plasmid-mediated gene transfer of vascular endothelial growth factor to promote angiogenesis and prevent restenosis (Isner *et al.* 1996a; Isner *et al.* 1996b).

1.7.1 Principles of Gene Transfer

Once a gene has been selected to be transferred, the DNA must be recombined into a form which is likely to be expressed in the target cell. To this end a number of different techniques have been developed to transport the DNA and these transport systems are called vectors. The entry of the DNA in the target cell is governed by the efficiency of delivery of the gene to the target cell, the physical properties of the vector and the time of exposure to the target. The vector carrying the DNA must first attach to and then cross the cell membrane, traverse the cytoplasm evading lysosomal digestion, and enter the nucleus where initiation of transcription of the DNA strand takes place. Current vectors used to aid DNA transport include those that make use of naturally occurring systems which insert foreign DNA into cells in disease states, i.e. the viral vectors, and man-made transfer systems such as the cationic liposomes (See Figure 2).

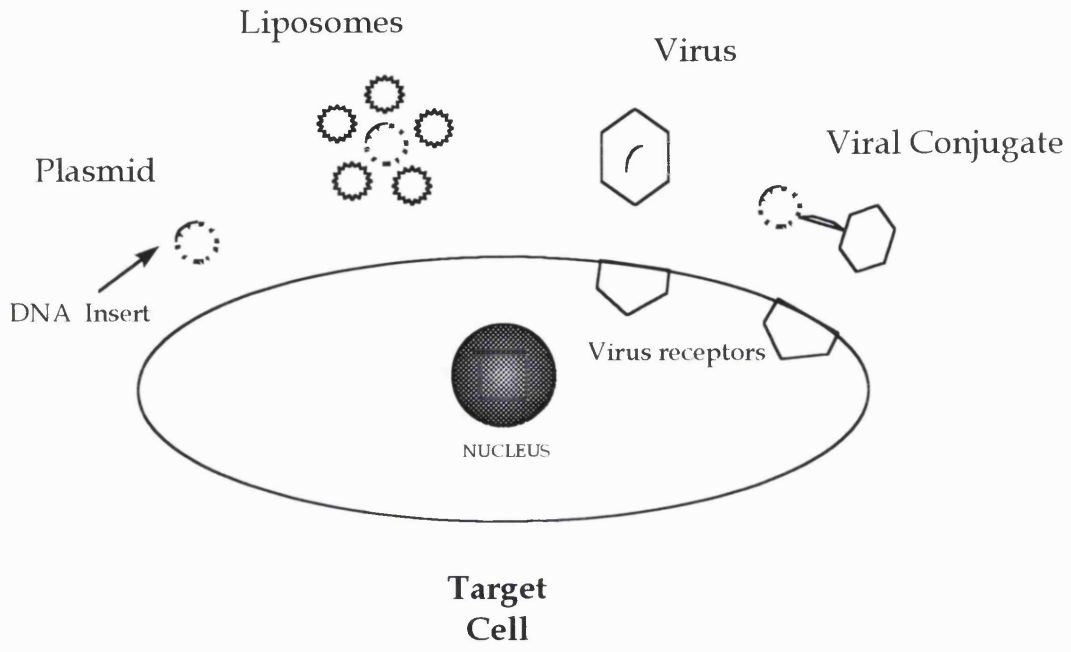


Figure 2 Schematic diagram of Gene Delivery Vectors

Vector	Gene Transfer efficiency	Duration of expression *in vivo*	Safety	Insertional mutagenesis risk	Clinical trials approved
Retrovirus	0.1	12 months	+ +	+ +	93
Adenovirus	100	2-4 weeks	+ +	+	18
Liposomes	1-5	2-4 weeks	+ + +	+	16
Plasmids	1	2-4 weeks	+ + +	+	5
Adeno associated virus	no comparative data	2-4 weeks	+ +	+	1

Table 3 Relative Advantages and Disadvantages of Gene Therapy Vectors.

The adenovirus, for example, has a specific receptor which enables entry into the cell, and viral coat proteins which disrupt the intracellular host defenses that normally cause destruction of the viral DNA and the gene of interest. Liposomes fuse with the cell membrane and release sufficient DNA into the cytoplasm, so that even without a protective transfer mechanism ample DNA reaches the nucleus. All viral vectors used in gene therapy have been rendered replication deficient so that they can infect the host cell but cannot then replicate. This is accomplished by cutting specific genes out of the viruses which are essential for their replication. The replication deficient vector is then grown in cells which have been modified to express the deficient genes, i.e. they are complementary to the deleted virus. This allows large amounts of the vector to be produced but once it is separated from the complementing cells it can no longer replicate. Once DNA reaches the nucleus it is only transcribed if it has the appropriate initiation signals. In addition, the gene of interest can be combined with a powerful promoter, so that a therapeutic effect may be achieved with lesser amounts of the vector and a potential reduction in toxicity. Tissue specific promoters are also being investigated, e.g. an actin promoter when targeting smooth muscle cells, but they often achieve specificity at the expense of efficiency. The duration of expression of a gene may also determine its uses, and is often governed by the vector used to administer it. Genes delivered using liposomes or adenoviruses do not integrate into the host genome and therefore have a limited life span, whilst retrovirally-administered genes are inserted into the genome and are, in theory, limited only by the life of the infected cell or its progeny. There is, however, no external control of the genomic site into which the DNA is inserted, raising the theoretical risk of insertional mutagenesis, where a native gene may be disrupted or separated from its normal control elements. The properties of some of the commonly used gene transfer vectors which have governed their usage are shown in Table 3. In addition to the vectors shown, viral conjugate vectors have been used, which aim to utilize the low toxicity of

liposomes with the efficiency of the viral vectors by linking these components using polylysine 'bridges'.

1.7.2 The Adenoviruses

Adenoviruses are the most widely used viral vector in vascular biology. The adenovirus genome is very well characterized and is relatively easy to manipulate. This has led to the development of a number of different adenoviral vectors which have been made replication incompetent by removal of essential genes (Graham and Prevec, 1991). Adenoviruses deleted in this way can accommodate the insertion of relatively large genes (8.3 kilobases), and high viral stocks can readily be grown in the laboratory (Brett *et al.* 1994). They also are efficient at infecting many different cell types in many species. An important advantage over the retroviruses is their ability to infect quiescent non-dividing cells. They are highly efficient in transferring genes due to their cell surface receptor and endosomal disrupter. Their efficiency is superior to retroviruses and liposomes in normal, uninjured and atherosclerotic blood vessels (Lemarchand *et al.* 1993; Guzman *et al.* 1993; Feldman *et al.* 1995). Adenoviruses also have a safety advantage over retroviral vectors in that they do not insert their DNA into the host chromosomes, and while this reduces their duration of action, it also reduces the chances of insertional mutagenesis. Insertion of viral genes into the host genome can occur but is rare (Ali *et al.* 1994).

Use of live adenoviruses as vaccines over some years has not revealed an increased frequency of malignancy and is safe even in immunocompromised subjects (Rhoads *et al.* 1991). The principal problem with adenoviruses is their ability to provoke a humoural and cellular immune response which may result in destruction of the infected cell. This limits expression of the transferred gene. In addition they cause production of circulating neutralizing antibodies which prevent successful repeat administrations (Wilson, 1996).

1.7.3 Making A Recombinant Adenovirus.

Adenoviruses are linear double stranded DNA viruses of about 36,000 base pairs (20-25 x 10^6 Daltons). The adenovirus virion is assembled from 13 viral polypeptides and the viral DNA genome, in an icosahedral structure (see Figure 3) about 70 nm in diameter, with an external capsid and inner core consisting of viral DNA and at least two basic proteins. Adenoviral infection of human cells can produce 1000-10,000 virus particles per cell (i.e. plaque forming units), with the virus remaining concentrated in the cell once maximal yield has been achieved, until the point of cell death and rupture due to overwhelming infection. There are two stages of expression of the adenoviral genome, 'early' 6-7 hours after infection before viral DNA replication has occurred and 'late'. The early genes (E1A, E1B, E2, E3 and E4) are transcribed by enzymes in the host cell and encode proteins important in viral DNA replication and regulation of transcription. Late genes encode the structural proteins of the virion. Wild type (replication competent) adenoviruses are commonly grown on He La cells.

43 adenoviral serotypes capable of infecting human cells have been described and the most extensively characterized include types 2, 5, and 12. These viruses have been rendered replication deficient by the removal by deletion or substitution of early genes essential for viral replication. To date the most widely used vectors have been the so called first generation adenoviruses which have E1a and E1b genes deleted to prevent replication (Graham and Prevec, 1991). Now, however, further research is being concentrated on second and third generation vectors. These newer vectors are rendered more genetically deficient by deletion of viral genes such as those encoding the adenoviral DNA polymerase or the DNA binding protein (Amalfitano *et al.* 1996; Engelhardt *et al.* 1994). These deletions further limit transcription of antigenic structural virion protein, ultimately minimizing the inflammatory response and improving the duration of transgene expression (Engelhardt *et al.* 1994).

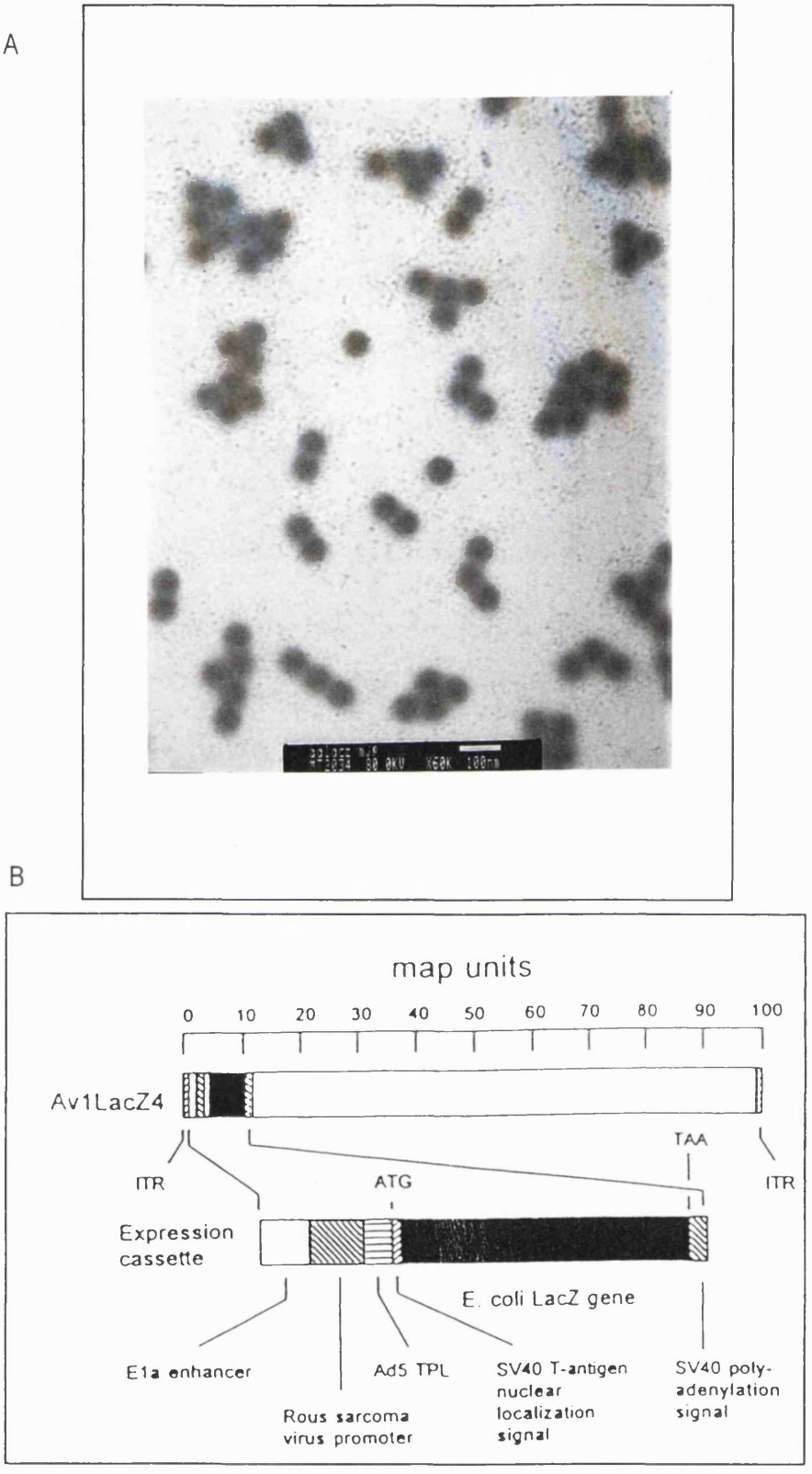


Figure 3 Av1.LacZ4 Vector.

Panel A shows an electron micrograph of the recombinant adenoviral vector containing the X-Gal gene encoding the enzyme ß- Galactosidase which is readily identifiable as a marker of gene transfer because it converts X-Gal substrate to a blue pigment.

Panel B shows the genomic organization of the Av1.LacZ4 vector, modified from Lee et al, 1993.

A first generation adenovirus was used in this study because of its availability, efficiency at infecting non-dividing smooth muscle cells, and its transient gene expression which becomes an advantage in modifying the response to a single procedural insult. The three regions which most readily accept DNA insertions or substitutions are in E1, E3, and a short region between E4 and the end of the genome (see Figure 3). The basic principles of generating a recombinant vector are outlined below:

the gene of interest e.g. TIMP1, is inserted into a shuttle plasmid, which is a bacterial plasmid already containing a subsegment of the viral genome. Usually this subsegment already contains the necessary early gene deletion to prevent replication. The resultant chimera is then cotransfected into cells, and homologous recombination occurs producing the desired recombinant virus. Any recombinants that contain an early gene e.g. E1a deletion, will be incapable of replication in normal mammalian cells and therefore a facultative cell line is used in which the relevant early gene has been stably overexpressed. For the E1A deleted virus used in this study 293 cells were used. These are human embryonic kidney cells that were transformed by exposure to sheared fragments of adenovirus type 5 DNA and express E1A (Graham *et al.* 1977). The products of recombination need to be distinguished from the original cotransfected plasmids, which can be achieved by a number of methods. One commonly used method involves the insertion of a 4.4 kb insert into a circularized piece of adenoviral DNA, modified to allow propagation in E.Coli and to encode ampicillin resistance. The resultant deleted adenoviral plasmid, pJM17, is then too large to be packaged into infectious virions. Thus the only infectious virions, produced from cotransfection of pJM17 with a shuttle plasmid, containing a gene of interest, will be successful recombinants. It is also possible to simply cotransfect the adenoviral DNA, containing appropriate deletions of replication genes, in linear or circularized form with the shuttle plasmid, under agar, and individually pick and screen the resultant plaques, as described in this study.

1.7.4 Other Viral Vectors

A number of other viruses are now being investigated for use in gene therapy. Herpes viruses have been used in the cardiovascular system in experimental animals (Mesri *et al.* 1995) and adeno associated virus has been approved for clinical trials but has not been used extensively in the heart or the vasculature (Wagner and Gardner, 1997; Flotte and Ferkol, 1997; Rubenstein *et al.* 1997).

Herpes virus vectors were originally developed to target the nervous system where they are able to remain latent for long periods (Coffin and Latchman, 1996). Herpes simplex 1 vectors can be divided into two types: disabled viruses and defective viruses or amplicons. The former follow the principles described for adenoviral vectors. They have genes deleted to make them unable to replicate (either in all cells or in neurons) and are grown on complementing cell lines. The latter are short sequences of the HSV genome, encoding replication and packaging signaling sequences and the gene of interest, but little else. They cannot replicate unless a helper virus is present.

Herpes simplex virus 1 (HSV1) is a neurotrophic virus but can infect a wide variety of cell types. Use of herpes viruses in myocytes has been limited to skeletal muscle (Huard *et al.* 1995). Disabled HSV vectors have been used to express a marker gene in cardiac myocytes in culture and *in vivo* (Coffin *et al.* 1996). Amplicon vectors encoding VEGF have been used *ex vivo* to infect fibroblasts in cultures. Infected cells were re-implanted *in vivo* where they elicited a brisk angiogenic response (Mesri *et al.* 1995). The reluctance to apply herpes vectors to the vasculature may reflect not only their development by those with specific interests in neuronal gene transfer but also concerns about possible associations between viral infection and atherogenesis (Raza-Ahmad *et al.* 1995; Datta *et al.* 1993; Kaner *et al.* 1993). I have studied the transfer of a marker gene using three novel disabled HSV vectors.

1.8 Gene Transfer To Prevent Angioplasty Restenosis.

A number of different strategies briefly outlined below have been used to target the processes which are activated by vascular injury. Current results suggest that adenoviral vectors are the most efficient gene transfer vectors, 20 to 100 fold better than liposomes, while liposomes are likely to be the safest (French *et al.* 1994). The most efficient gene delivery devices for smooth muscle cells in the media of an artery are those which cause the most injury to the artery, but multiple strategies are being adopted to over come this. There are also differences in susceptibility to gene transfer between normal and atherosclerotic arteries and between injured and uninjured arteries. In general, regardless of the vector used, it is easier, although not essential, to transfer genes after arterial injury to actively dividing cells such as smooth muscle cells (Li *et al.* 1995). Atherosclerotic vessels are relatively resistant to gene transfer unless this is accompanied by balloon injury (Feldman *et al.* 1995).

The trigger event in angioplasty restenosis is the procedural insult, which then gives rise to a cascade of growth factor release, smooth muscle cell migration and proliferation, extracellular matrix deposition and finally reendothelialization. There seems little doubt, however, that cell proliferation has some role to play, and this has led several groups to target the common pathways of cell cycle regulation and DNA replication (Chang *et al.* 1995a; Simari *et al.* 1996; Chang *et al.* 1995b).

1.8.1 Gene Transfer To Inhibit Cell Proliferation.

Guzman *et al* used gene transfer of a prodrug activator to kill all proliferating cells expressing the transgene. They incorporated the gene for herpes simplex thymidine kinase (HSV-tk) in an adenoviral vector and delivered the gene to rat carotid arteries, porcine iliofemoral arteries and rabbit hyperlipidaemic arteries (Chang *et al.* 1995a; Simari *et al.* 1996; Guzman *et al.* 1994). The animals were then given ganciclovir systemically, which is harmless unless phosphorylated by HSV-tk. The

phosphorylation product, however, is incorporated into the DNA of replicating cells and causes DNA chain termination and cell death. The phosphorylation product is able to diffuse into adjacent dividing cells, therefore a bystander effect is seen which improves the efficiency of this approach. In all the species tested, neointimal formation was limited by the cytotoxic effect of gene transfer, by 50-87% in porcine and by 39-61% in atherosclerotic rabbit iliofemoral arteries.

A number of cytostatic approaches to inhibiting cell proliferation have been used. Chang *et al* investigated the retinoblastoma (Rb) gene product to inhibit cell proliferation (Chang *et al*. 1995b). This protein is usually phosphorylated in dividing cells, while the active nonphosphorylated form of the protein binds to cellular transcription factors, arresting the cell cycle. The gene for a constitutively active nonphosphorylatable form of the Rb gene product was incorporated into an adenoviral vector, and using the double balloon catheter, administered to porcine iliofemoral arteries immediately after angioplasty. This significantly reduced SMC proliferation and reduced neointimal hyperplasia by 40-50% (Chang *et al*. 1995b). The same group, using an adenoviral vector, has subsequently overexpressed p21, which is an inhibitor of cyclin/cyclin-dependent kinase (Yang *et al*. 1996). The cyclin/cyclin-dependent kinase complex is thought to be essential for the phosphorylation of the Rb gene product, and therefore its inhibition will cause cell cycle arrest. The p21 protein also binds to PCNA to inhibit cell cycle progression. Transfer of the gene encoding p21 after balloon injury in the rat carotid model inhibited neointimal formation by 46% (Yang *et al*. 1996). Diverse methods from antisense to both cytotoxic and cytostatic gene transfer strategies have all managed to achieve similar degrees of inhibition of restenosis (Chang *et al*. 1995b; Yang *et al*. 1996; Chang *et al*. 1995c; Bennett *et al*. 1994; Abe *et al*. 1994; Morishita *et al*. 1994; Simons *et al*. 1994; Chang *et al*. 1995a; Simari *et al*. 1996; Guzman *et al*. 1994).

An interesting new manipulation of the molecular biology of the vasculature has been the use of a DNA decoy. Morishita *et al* have investigated inhibiting the transcription factor E2F, by introducing double stranded DNA containing the E2F binding site which can then decoy E2F and prevent it interacting with other genes (Morishita *et al.* 1995). E2F is pivotal in the activation of genes such as c-*myc*, *cdc2*, and the gene encoding PCNA. A conjugate of haemagglutinating virus of Japan and liposomes (HVJ-liposome complex) has been used to introduce the double stranded DNA into injured rat carotid arteries. Mean intimal/medial ratio was reduced from 1.12 to 0.29 and the effect was maintained 8 weeks after the single administration. This technique has drawn on our knowledge of transfer of single stranded antisense oligonucleotides and gene transfer vector developments, but unlike the other gene transfer experiments discussed here there is no requirement for the DNA encoding an mRNA and producing a protein product.

1.8.2 Gene Transfer To Prevent Thrombosis.

An alternative strategy has been to target local thrombotic mechanisms. The gene encoding human cyclooxygenase 1 has been incorporated in a first generation adenoviral vector and instilled into previously injured porcine carotid arteries, in order to increase prostacyclin synthesis and thus reduce thrombosis (Zoldhelyi *et al.* 1996). Recombinant hirudin, a highly potent and specific inhibitor of thrombin, has also been transferred into cells and into injured rat carotid arteries using an adenoviral vector. Increased hirudin production was seen in the arteries and a 35% reduction in neointimal formation was detected (Rade *et al.* 1996). This may reflect the known ability of thrombin to cause VSMC migration and proliferation via the release of growth factors from the extracellular matrix. These approaches, which appear to target thrombosis in fact have multiple effects on the arterial wall.

1.8.3 Gene Transfer To Restore Endothelial Function.

Both accelerating the reendothelialization of the vessel after balloon injury and restoring its function by replacing nitric oxide have been used to ameliorate restenosis. VEGF is a potent and specific mitogen for endothelial cells. The recombinant VEGF protein has been applied to rat carotid arteries after injury and been shown to reduce neointimal formation and accelerate the restoration of endothelial continuity (Asahara *et al.* 1995). Transfer of the gene encoding VEGF has not however been used in this setting, but the results obtained in the rat model have contributed to the gene transfer work with VEGF currently being applied to peripheral vascular disease both in angiogenesis and restenosis (Isner *et al.* 1996a; Isner *et al.* 1996b). Restenosis is likely to be triggered by the early release of mitogens and chemotactic factors after endothelial denudation. It has been suggested that under normal conditions the endothelium may supply inhibitory factors to keep this process in check (von der Leyden *et al.* 1995). The ability of nitric oxide (NO) to inhibit platelet aggregation and VSMC migration and proliferation *in vitro* implicates it in this process. The first demonstration of ability to transfer genes with potential therapeutic use to the vasculature used the HVJ-liposome complex of the gene for endothelial cell nitric oxide synthase (ecNOS) to overexpress nitric oxide synthase and hence nitric oxide after balloon injury in the rat (von der Leyden *et al.* 1995). The investigators were able to demonstrate restored NO production and vascular reactivity, in addition to a 70% reduction in neointimal formation.

Both antisense and gene transfer techniques have been used with success to suppress neointimal formation in animal models of angioplasty restenosis in normal and atherosclerotic vessels. After the failure of pharmacological agents such as angiotensin converting enzyme inhibitors to alter restenosis in man, despite favorable results in small animals, more groups are now testing gene transfer methods in porcine peripheral and coronary models. At present, no one gene or antisense molecule has been shown to be

superior to another, and apart from marker gene studies comparing vectors or delivery devices there is a paucity of comparative data. It seems possible that a combination of several strategies may be needed to overcome the considerable biological redundancy in the restenotic cascade. I have therefore chosen to investigate whether containment of smooth muscle cells and growth factors within the matrix of the arterial media by the overexpression of TIMP1 will alter the response to vascular injury.

CHAPTER 2. AIMS OF THIS THESIS.

Matrix metalloproteinase enzymes are known to be upregulated after vascular injury and may be an essential step in liberating smooth muscle cells from the arterial media to migrate and proliferate forming a neointima and narrowing the vessel. The aim of this project was to study the effects of inhibiting matrix metalloproteinase activity *in vitro* in smooth muscle cells and *in vivo* at the site of balloon catheter injury (utilizing a rat model of angioplasty) by systemic administration of a pharmacological inhibitor and by local overexpression of tissue inhibitor of metalloproteinases 1 (TIMP1) using a replication deficient adenoviral vector. These studies were expected to give insights into the possible physiological functions of the MMPs and TIMPs *in vivo* in the arterial wall. The principal aim was to investigate the potential for MMP inhibition, via effects on migration and proliferation of smooth muscle cells, to prevent the development of a neointimal lesion after vascular injury.

2.1 HYPOTHESIS

The hypothesis investigated in this thesis is that inhibition of MMP activity by pharmacological means or by gene transfer of human TIMP1 will inhibit intimal hyperplasia after vascular injury.

2.2 SPECIFIC AIMS

1) Assessment of the pharmacokinetics and effects on lesion development of a pharmacological inhibitor of the metalloproteinases *in vivo* in the rat carotid balloon injury model.
2) Assessment of the efficiency of gene transfer using a first generation adenoviral vector encoding a marker gene, ß-Galactosidase, in primary

and SV40 transformed smooth muscle cells and optimization of *in vivo* gene delivery techniques.

3) Assessment of the efficiency of gene transfer using four herpes virus vectors, encoding ß-Galactosidase, in primary and SV40 transformed smooth muscle cells and investigation of the effects of *in vivo* gene delivery of one of the vectors.

4) Development of a novel gene transfer vector encoding human tissue inhibitor of metalloproteinase 1 (Av1.TIMP1) and assessment of its ability to produce functional protein *in vitro*.

5) Investigation of the effect of overexpression of TIMP1 using the vector, Av1.TIMP1, on smooth muscle cell migration and proliferation *in vitro*.

6) Investigation of the ability of the vector Av1.TIMP1 to express TIMP1 *in vivo* in the injured rat carotid and assessment of its effects on neointimal development, smooth muscle cell proliferation and migration, medial cell numbers, and matrix composition in comparison with a vector encoding ß-Galactosidase.

PART 1. PHARMACOLOGICAL INHIBITION OF THE MMPs.

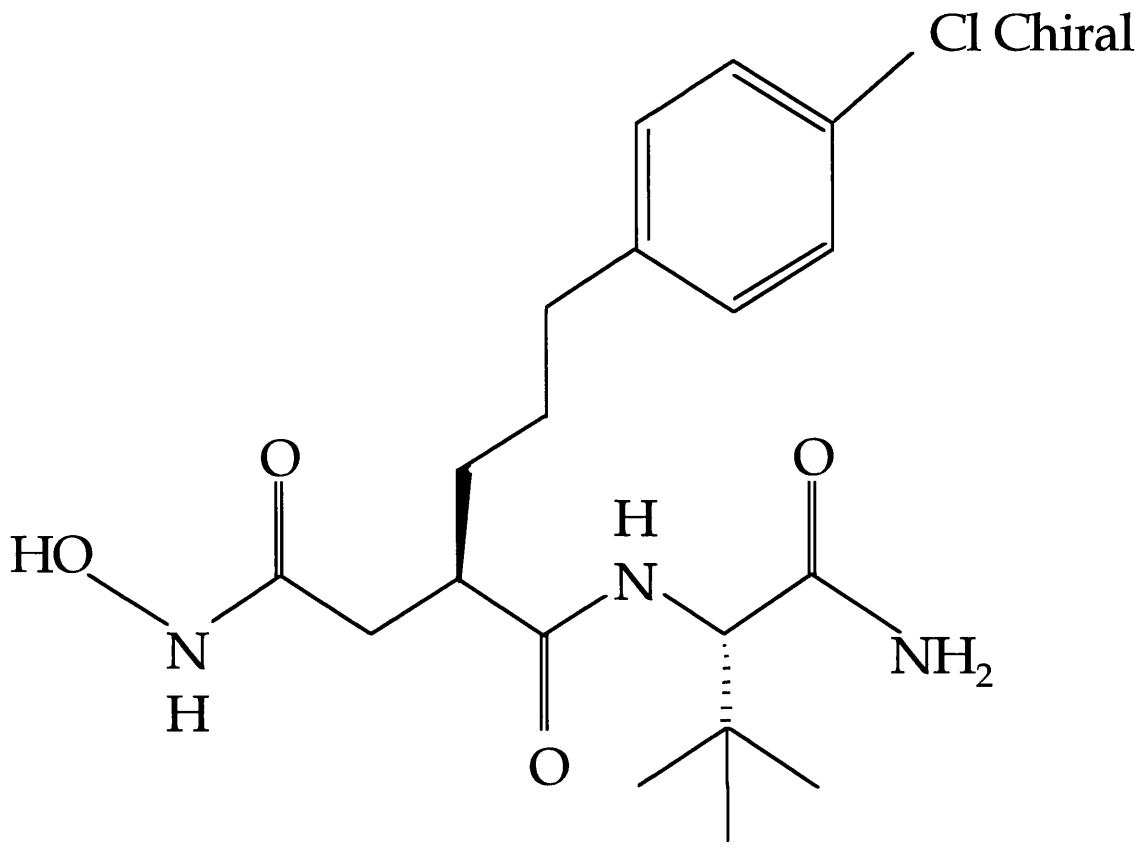
CHAPTER 3. PHARMACOLOGICAL INHIBITION OF THE MMPS

3.1 INTRODUCTION

Pharmacological inhibitors of the MMPs have been developed principally for use in the prevention of tumour metastasis where they may act to contain the tumour cells and exert an antiangiogenic effect on the tissue around the tumour (Talbot and Brown, 1996). A number of different inhibitors have been evaluated from antibiotic derivatives, such as tetracyclines, to synthetic peptides (Karakiulakis *et al.* 1990; Anderson *et al.* 1996; Greenwald, 1987). The action of the tetracyclines is unrelated to their antimicrobial activity and may be due to their action as zinc chelators (Greenwald, 1994). While the tetracyclines are moderately potent inhibitors of Collagenase *in vitro* their action in animal models is less impressive (Vincenti *et al.* 1994). Clinical trials in arthritis have failed to show a benefit when compared with placebo (Greenwald, 1987). This chain of events from initial promise *in vitro* to a lack of clinical effect will be familiar to cardiologists with an interest in angioplasty restenosis. Several synthetic peptides are being investigated by different groups particularly in the prevention of tumour metastasis (Wang *et al.* 1994; Watson *et al.* 1995; Watson *et al.* 1996; Eccles *et al.* 1996; Chirivi *et al.* 1994; Anderson *et al.* 1996). *In vitro* they are the most potent inhibitors identified to date (Brown, 1994; Anderson *et al.* 1996). Some are hampered in clinical use by poor bioavailability, rapid hepatic and renal metabolism and possible toxic systemic effects (An *et al.* 1997; Stetler Stevenson *et al.* 1996).

CT1746, N1-[2-(s)-3,33-dimethylbytanamidyl)-]-N4-hydroxy-2- (R) -[3-(4-chlorophenyl)-propyl]succinamide, (kind gift of Celltech Ltd.) is a synthetic peptide inhibitor of the matrix metalloproteinases with broad substrate specificity for all the MMPs (see Figure 4). It incorporates a zinc chelating hydroxamic acid group within a structure that resembles a peptide substrate. The principle of its action is that it has a peptide framework which will bind to the substrate binding site of the matrix metalloproteinase

and the ion chelating group will inactive the zinc dependent enzyme. CT1746 has *Ki's* against human Gelatinase A, Gelatinase B, Stromelysin 1, Collagenase, and Matrilysin of 0.04, 0.17, 10.9, 122, and 136nM respectively (Anderson *et al.* 1996). It has negligible activity against other metalloproteinases such as angiotensin converting enzyme. In work undertaken by Dr. Karen Webb prior to the start of this project the effective concentration of CT1746 against rat Gelatinases was determined. Gelatinases from rat vascular smooth muscle cells were assayed by zymography with pretreatment with 1, 10, and 100nM CT1746. 10, and 100 nM CT1746 (0.004-0.04 μg/ml) reduced the Gelatinase activity to a negligible level, closely paralleling the results in mice (Anderson *et al.* 1996). The use of CT1746 was developed principally in mice and therefore it was necessary to characterize its pharmacokinetics in the rat (An *et al.* 1997; Anderson *et al.* 1996). In order to establish optimal dosage and plasma levels were measured after single doses of CT1746 using 3 formulations and 4 doses.



$C_{19}H_{28}ClN_3O_4$

Figure 4 Chemical Structure of CT1746

The effect of CT1746 administration on the formation of a neointimal lesion after balloon injury in the rat carotid artery was then assessed using the optimal formulation. In initial experiments CT1746 was given by oral gavage and applied locally to the carotid artery in a pluronic gel and subsequently it was administered by continuous subcutaneous infusion into the carotid artery bed.

3.2 Materials and Methods

3.2.1 Pharmacokinetics

Animal surgery

Male Wistar rats (300-350g) were used throughout. For short studies of 0-12 hours animals were anaesthetized using intraperitoneal fentanyl/fluanisone (Hypnorm Jansson) 0.025 mg per 100g/0.8 mg per 100 g respectively, and midazolam (Hypnovel, Roche) 0.42 mg/ 100g. Oral administration of CT1746 was carried out by gavage. The femoral vein of the rat was canulated and used for serial sampling. An incision was made over the right femoral vein. A short segment of vein was isolated with 2 silk ligatures and a small incision made. Fine curved forceps were introduced into the vein and a 2 French gauge cannula inserted between the blades of the forceps. The cannula was secured with two sutures and connected to a syringe to allow aspiration of blood samples. The wound was then partially sutured and the heavily sedated animal placed in a restraining cage for the duration of the experiment. For time points greater then 12 hours animals were gavaged while conscious and at the appropriate time points anaesthetized with halothane to allow percutaneous tail vein blood samples to be taken.

Gavage protocols

Three formulations were tested as below:

a) Formulation 1: CT1746 15mg/ml in propylene glycol (PG)

b) Formulation 2: CT1746 10 mg/ml in hydroxypropylmethylcellulose (HPMC)

c) Formulation 3: CT1746 30 mg/ml in propylene glycol

Protocols were as follows:

a) Experiment 1: Gavage 100 mg/kg Formulation 1. (6 rats)

b) Experiment 2: Gavage 100 mg/kg and 30mg/kg Formulation 2. (4 rats)

c) Experiment 3: Gavage 100 mg/kg Formulation 3. (2 rats)

Venous sampling

Samples were taken initially at 5, 10, 15, and 30 minutes post gavage and then at 1, 2, and 3 hours thereafter samples were taken alternately from pairs of rats at hourly intervals up to 8 hours to give a maximal number of time points. At later time points samples were taken by tail vein puncture as described above. 300 µl of venous blood was taken for each sample into a tube containing 20 units (4µl of 5000 units/ml) of heparin and 15 µl of 1M potassium fluoride. Samples were kept at 4°C until being spun at 5000 rpm in a microfuge for 2 minutes. Plasma was then collected from the tube and stored at -20°C until assayed by high pressure liquid chromatography. All assays for CT1746 were kindly carried out by Ola Epemolu at Celltech Ltd. Validation studies carried out by Celltech showed a linear range for this assay of 0-20 µg/ml with a minimum quantifiable limit of 200ng/ml.

3.2.2 Balloon Catheter Injury Of The Carotid Artery.

Male Wistar rats weighing 300-350g were anaesthetized with an intraperitoneal injection of fentanyl/fluanisone (Hypnorm Jansson) 0.025 mg per 100g/0.8 mg per 100 g respectively, and midazolam (Hypnovel, Roche) 0.42 mg/ 100g. The left common carotid, external and internal carotid arteries were exposed by blunt dissection at the bifurcation of the common carotid (The branches of the common carotid artery are illustrated in Figure 5.

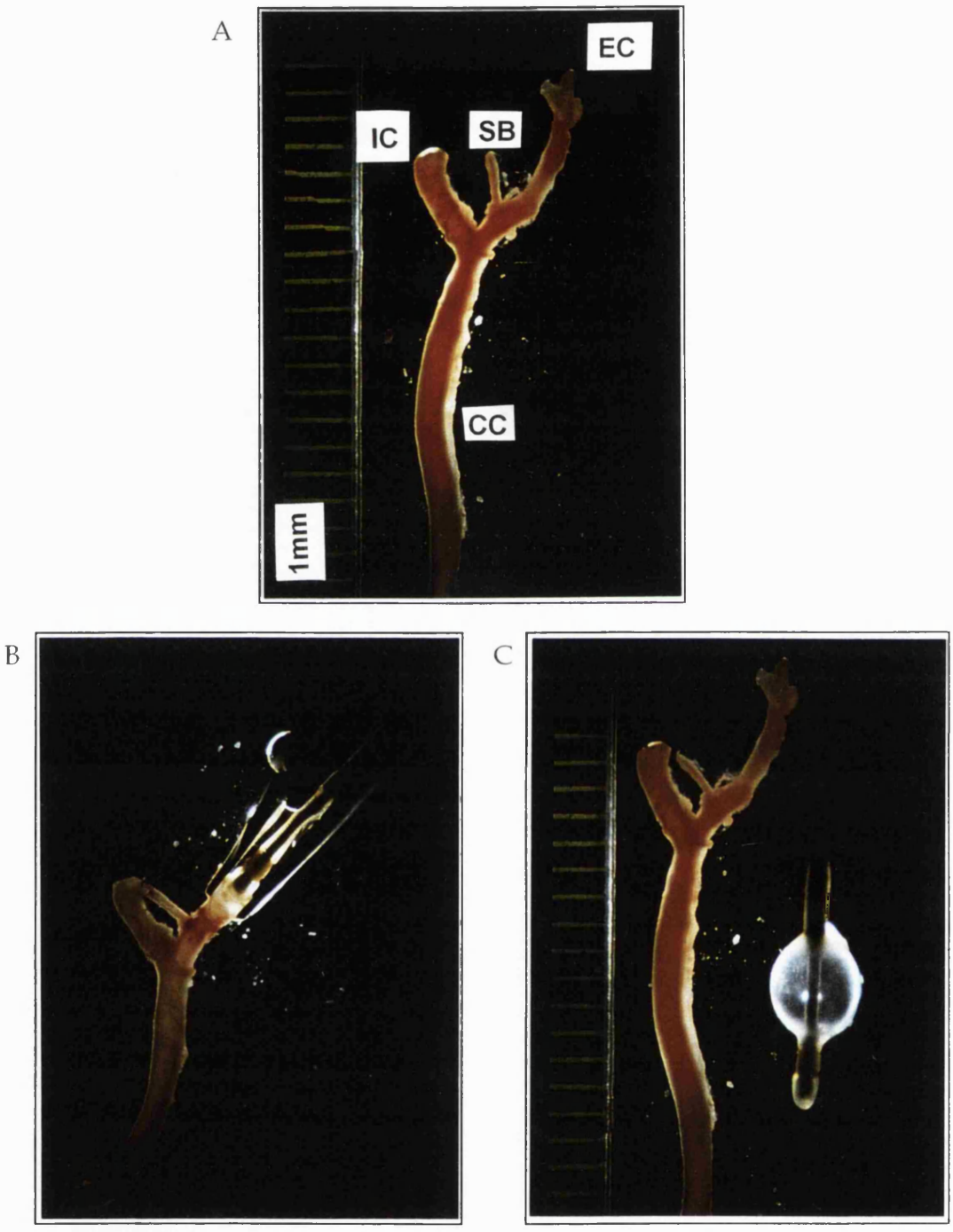


Figure 5 Rat Carotid Artery Balloon Injury Technique.

Panel A shows a rat carotid artery removed from the animal and photographed under dark ground microscopy to illustrate the branches of the common carotid artery (EC external carotid, CC common carotid, IC internal carotid, and SB side branch). Panel B shows the tips of fine curved forceps inserted into an arteriotomy in the external carotid, an embolectomy catheter is being introduced into the external carotid by passing it between the blades of the forceps. The balloon will then be inflated within the lumen of the common carotid artery. Panel C illustrates the nature of the balloon injury delivered to the common carotid artery peroperatively by showing the embolectomy balloon inflated adjacent to the common carotid artery.

A loose 3-O silk sling (Davies and Geck) was placed proximally around the common carotid artery and a suture loosely secured around the external carotid artery as close to the bifurcation as possible. The external carotid artery was then ligated at the most distal extent of the dissection and the artery place under slight tension by weighting the distal external carotid suture. A Diffenbach arterial clip was then placed on the external carotid artery immediately distal to the bifurcation to occlude flow. Iris scissors were used to make a small arteriotomy in the external carotid artery. The blades of a pair of fine curved forceps were then introduced into the arteriotomy and a Fogarty Balloon Embolectomy catheter (2 French gauge) (Baxter Healthcare) was passed between the blades of the forceps into the lumen (see Figure 5).The catheter was then passed down the common carotid artery into the Aorta. The proximal external carotid suture was loosely tightened to prevent hemorrhage at the arteriotomy site. The embolectomy balloon was then inflated with 0.03 ml of sterile saline (0.9% w/v), and pulled up the common carotid artery with a small twisting motion, in order to denude the endothelium and stretch the media of the arterial wall. This procedure was repeated twice, and the catheter was then removed, having secured haemostasis by reapplying the Diffenbach arterial clip. The external carotid artery was ligated proximal to the arteriotomy. Pluronic gel delivery was then performed as described below and the wound closed using surgical clips.

3.2.3 CT1746 Administration By Oral Gavage And Pluronic Gel Administration

In each group 10 rats received oral gavage with the vehicle/diluent only and 10 rats received CT1746. In each case the volume of gavage fluid administered was the same in the control and the CT1746 groups.

Gavage dosing regime:

Day 0 nocte Gavage 1ml formulation 3 i.e. 100 mg/kg/ control

Day 1 mane Carotid injury surgery

nocte Gavage 1ml formulation 3 i.e. 100 mg/kg/ control

Day 2-14 mane Gavage 1ml formulation 3 i.e. 100 mg/kg/ control

nocte Gavage 1ml formulation 3 i.e. 100 mg/kg/ control

In order to ensure that the local concentration of CT1746 was optimal throughout the oral gavage study, and at high local concentration immediately after injury, CT1746 was also applied locally to the carotid artery bed using a pluronic gel during the surgical procedure. Pluronic F127 polyol (BASF Corporation) was used in a 20% solution with CT1746 25 mg/ml (filter sterilized). At this concentration Pluronic F127 is a mildly viscous liquid at room temperature and rapidly forms a gel at 37°C. 200 µL of pluronic gel was applied along the exposed length of common carotid artery using a sterile tipped Gilson pipette. A gel was seen to form and the animal's neck was closed with surgical clips.

3.2.4 CT1746 Administration By Minipump.

3.2.4.1 Experimental Protocol

Thirteen rats were treated with CT1746 and thirteen with vehicle only. All rats were killed and tissues harvested fourteen days after surgery.

3.2.4.2 Preparation And Insertion Of Minipumps.

CT1746 25 mg/ml in 50% v/v DMSO (Sigma) and 33% 2-Hydroxypropyl-ß-cyclodextrin (Sigma) or vehicle were sterilized by autoclaving. 2ml osmotic minipumps (Charles River, Alzet model 2ML2) were prepared in a sterile field and, under strict aseptic conditions, filled with either CT1746 or vehicle alone (control group). The pumps were incubated in sterile saline at 37°C for 4 hours prior to insertion to ensure immediate delivery of the contents after insertion. The carotid balloon injury procedure was performed as described above. Under the same anesthetic, the ventral and dorsal aspects of the rat's neck were cleaned with 70% ethanol and an

incision made in the dorsum of the rat. A 5-7 cm length of plastic cannula was attached to the minipump dispensing port. A clamp was then inserted in the subcutaneous tissue of the rat from the original ventral neck incision. The clamp was advanced using blunt dissection through the subcuticulum until its tips protruded from the dorsal incision. The distal end of the minipump cannula was then drawn through the resulting track until its tip reached the carotid bed in the ventral aspect of the neck. The cannula was sutured to underlying muscle anteriorly and posteriorly. The two wounds were then closed with surgical clips.

3.2.4.2 BLOOD SAMPLING FOR CT1746 LEVELS.

Samples of arterial blood were taken from all rats receiving CT1746 and 2 control rats at time of sacrifice and analyzed for plasma CT1746 level as described above.

3.2.5 Fixation

14 days after the initial injury rats were anaesthetized with intraperitoneal injection of fentanyl/fluanisone (Hypnorm Jansson) 0.025 mg per 100g/0.8 mg per 100 g respectively, and midazolam (Hypnovel, Roche) 0.42 mg/ 100g. The descending aorta exposed by blunt dissection via a midline abdominal incision. The aorta was then retrogradely canulated with a 20G cannula (Vigon Venflon) secured with a silk suture. The rats were exsanguinated (5-10 ml of blood) and the blood reserved for further analysis. 1% w/v paraformaldehyde and 2% w/v glutaraldehyde fixative solution was infused retrogradely, through the aortic cannula, at a constant pressure of 120 mm Hg (using a pressure bag). Fixation was continued *in situ* for 10 minutes.

3.2.6 Processing For Morphometry

5 mm sections of artery were taken from the mid portion of the right and left carotid arteries and placed in the fixative solution described above

overnight at 4°C. The sections of artery were then washed in 2 changes of 0.1M phosphate buffer pH 7.4 and left overnight at 4°C. They were then fixed in 1% w/v osmium tetroxide (Johnson Matthey) in 0.1M phosphate buffer pH 7.4 for 2 hours at 4°C. The samples were washed in phosphate buffer and dehydrated sequentially for 15 minutes in each of the following solutions: 25%, 50%, 70%, 90%, and 100% ethanol. The samples were then washed in 4 changes of propylene oxide (BDH analar) for 10 minutes each and placed in a mixture of 40% v/v propylene oxide and 60%v/v epoxy resin overnight with agitation.

The epoxy resin was prepared with:

47 parts araldite CYC12

47 parts dodecenyl succinate anhydride (DDSA)

8 parts dibutyl phthalate (Dpth)

2 parts benzyldimethylamine (BDMA)

(all Agar Scientific)

The samples were placed in 100% epoxy resin for 8 hours, the resin solution was then changed and the samples left overnight with agitation. The samples were then transferred to fresh resin and placed in moulds in which they were baked at 60°C overnight.

Semithin (2μm) sections of each were cut with a microtome and dried onto uncoated slides on a hot plate. The sections were stained with 1% w/v toluidine blue (BDH)/1% w/v sodium tetraborate (BDH) in distilled water and heated for 30-60 seconds enabling the stain to penetrate the resin. The slide was then washed in distilled water, dried and mounted in DPX (Gurr).

3.2.7 Morphometry

Sections were viewed at x40 magnification and the images digitized using a JVC-MC280 TV camera connected to a Context Visual Gop-302 image analysis system. The lumen area, area within the internal elastic lamina, and area within the external elastic lamina were traced , and the intimal and medial areas were calculated by subtraction.

Schedule of animal experiments

1) CT1746 Pharmacokinetics See Page 52

2) CT1746 Administration By Oral Gavage And Pluronic Gel Administration.

10 rats were included in each group.

3) CT1746 Administration By Minipump.

13 rats were included in each group.

3.3 RESULTS

3.3.1 Pharmacokinetics

All animals tolerated the surgical and gavage procedures well. Experiment 1 showed adequate plasma concentrations of CT1746 in the majority of animals, which were maintained for up to 24 hours (see Figure 6).

The volumes of CT1746 and propylene glycol administered in order to reach this dose were approximately 2 ml per rat (as a single dose). If this volume of propylene glycol were administered daily over a 2 week period it is likely to be toxic to rodents. Formulation 2 was therefore evaluated using a less toxic vehicle, HPMC, in 2 doses, 100 mg/kg and 30 mg/ kg. This resulted in widely varying blood levels with 1 animal at each dose showing no measurable CT1746 in the plasma. The 30mg/kg dose showed diminishing levels at six and eight hours, while the higher dose showed no drug present in one animal and 2.94 μg/ml in the other. This high result was confirmed by repeat testing in the analysis laboratory. The drug levels achieved with this compound were highly unpredictable. (see Figure 6)

This formulation was, therefore, unsuitable for use in the animal model and formulation 3 was investigated. This used PG as a vehicle for the CT1746, but the concentration of CT1746 was 30 mg/ ml allowing only half the volume of PG to be administered to achieve similar plasma concentrations of CT1746 to formulation 1. The results are shown in Figure 6 and show sustained blood levels with some variability.

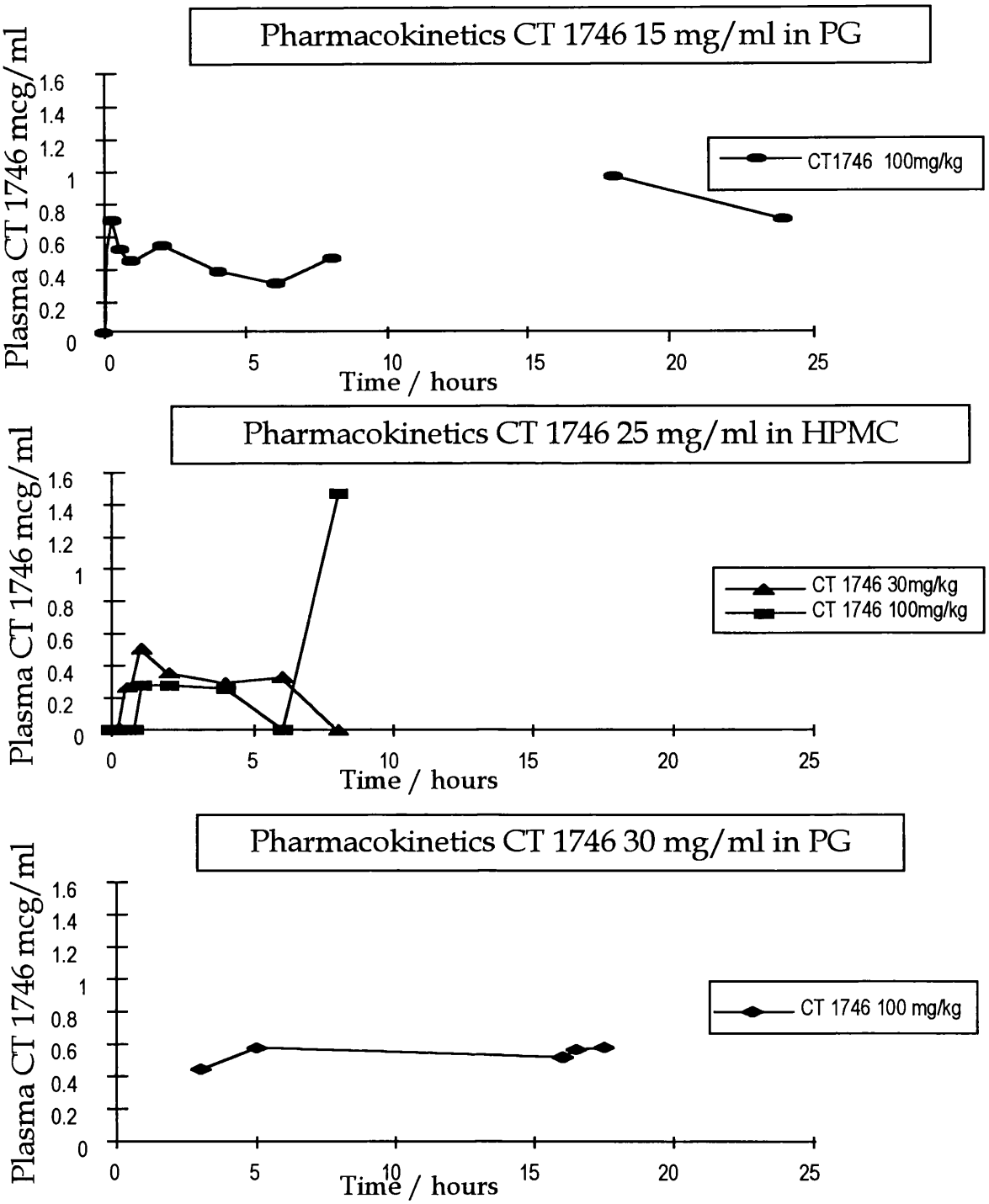


Figure 6 Pharmacokinetics of CT1746

The graphs above show the mean plasma levels of CT 1746 from two rats at each time point gavaged at time 0 with the indicated formulation of CT1746. 0.2mcg/ml is the minimum measurable inhibitory concentration of CT 1746 using this assay. The two formulations in PG (propylene glycol) show the most consistent plasma levels while the formulation in HPMC shows variable results. The results shown in the lower panel show the best plasma levels to allow once daily oral gavage with a minimum quantity of potentially toxic vehicle.

3.3.2 The effect of CT1746 administered by oral gavage and pluronic gel on neointimal hyperplasia in the rat carotid model.

4 rats (2 CT1746 and 2 control) died within 24 hours of operation. The 2 CT1746 group rats and one control rat became asystolic peroperatively, one control rat died overnight after operation, probably as a complication of anesthesia. 4 animals required repair of their neck wounds. 1 at 5 hours post procedure (CT1746 group), 2 at 24 hours post procedure (1 CT1746 group, 1 control group) and 1 at 72 hours. The requirement for neck repair was unusually high, but was evenly distributed between the CT1746 and control groups.

In Figure 7 the mean intimal area +/- 1 standard error and mean intimal/medial ratio +/- 1 standard error are shown. While both the intimal area and the intimal medial ratio indicated a greater degree of neointimal response in the CT1746 treated group than the controls, this difference did not achieve statistical significance in the intimal medial ratio measurement ($p = 0.06$ and 0.16 respectively). In the analysis of mean intimal medial ratio the CT1746 group showed a mean of 1.27+/-0.25, while the control group had 0.72 +/- 0.27. The lack of statistical significance in this data reflects the wide degree of variability in the degree of intimal hyperplasia, particularly in the control group, where 3 rats had no neointima detected at all.

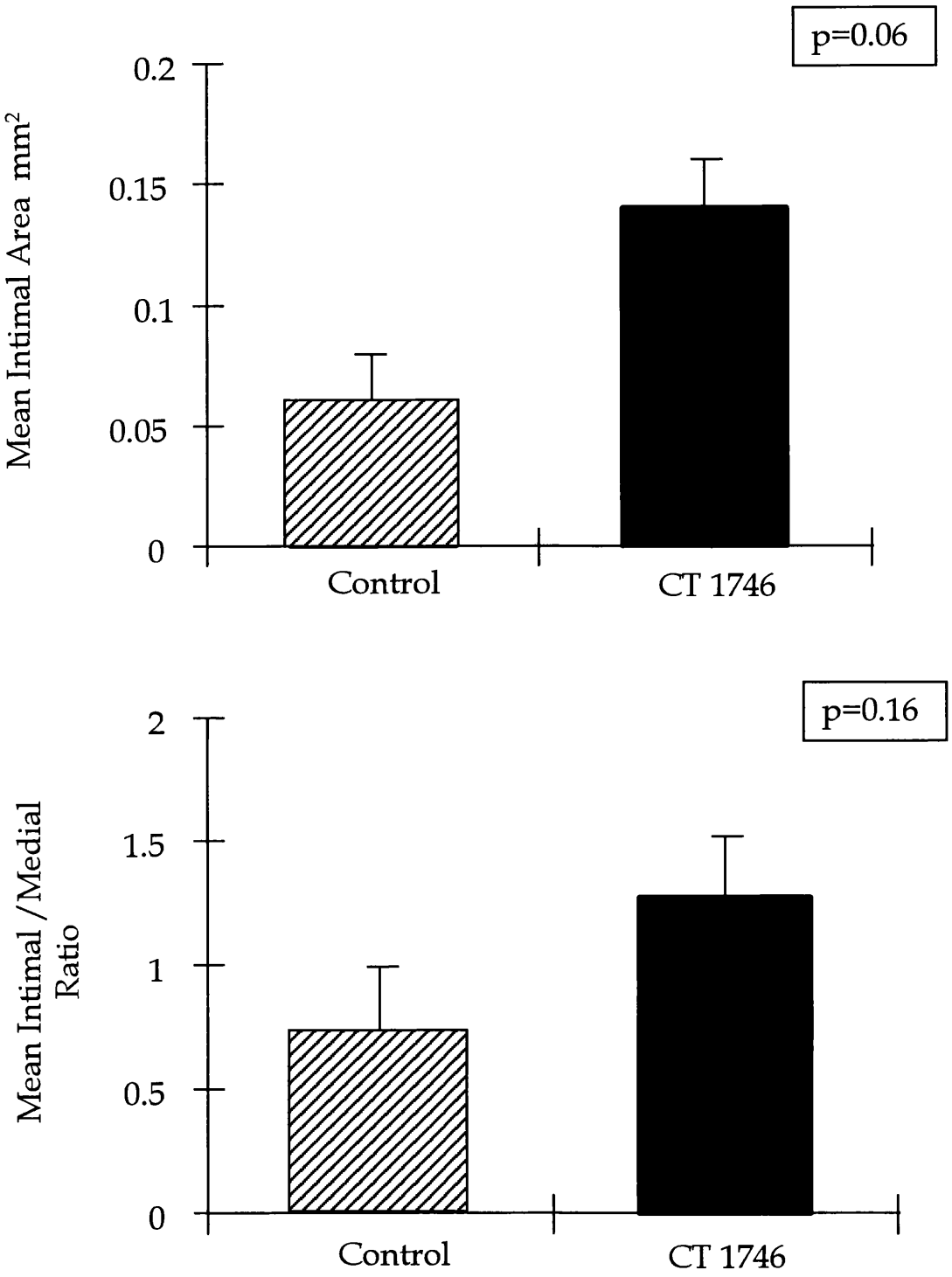


Figure 7 The Effect of CT1746 Orally and by Pluronic Gel on Neointimal Hyperplasia 14 Days After Balloon Injury.

8 rats received CT 1746 (100mg/kg per day) by oral gavage and a local application of CT1746 in pluronic gel peroperatively while the 8 control rats were gavaged with vehicle only and received pluronic gel with no additive. All animals were killed 14 days after surgery and the carotid arteries fixed at arterial pressure. The Mean Intimal area and Intimal Medial ratios (+ SEM) for sections of the arteries are shown above and show no significant difference between intimal medial ratio but an increase in the intimal area in the CT1746 treated group. 4 rats died 24 hours post operatively , 2 from each group.

3.3.3 The effect of CT1746 by subcutaneous minipump on neointima formation in the rat carotid artery model of neointima formation after balloon injury.

There were no specific complications of surgery. In 3 rats receiving CT1746 a yellow/white semisolid substance was seen at the tip of the cannula in the anterior aspect of the neck. In one animal it was also seen at the exit point from the minipump. Samples of this substance were cultured but showed no sign of bacterial growth. Crystals were visualized by microscopy, suggesting that the CT1746 had precipitated on discharge from the pump in these animals. Plasma CT1746 was measurable in 5 rats following the local delivery to the carotid bed. Levels were 0.68, 0.25, 0.57, 0.25, and 0.46 μg/ml in five animals and undetectable in all the others.

Mean intimal area was reduced from 0.11 +/-0.01 mm^2 to 0.08 +/-0.01 mm^2 but this trend did not achieve statistical significance ($p = 0.12$) (Figure 8). When the intimal area was normalized to the size of the artery, i.e. the medial area and expressed as intimal/medial ratio, the results showed a significant 27% reduction in intimal medial ratio from 1.1 +/-0.18 to 0.8 +/-0.11 ($p = 0.03$)(Figure 8). Medial areas were 0.11 +/-0.004 and 0.10 +/-0.004 mm^2 in CT1746 treated and control animals respectively and showed no significant difference ($p = 0.15$). The area within the external elastic lamina was, however, significantly different in the two groups (CT1746 0.31 +/-0.02, and control 0.25 +/-0.02, $p = 0.04$), suggesting expansive remodeling.

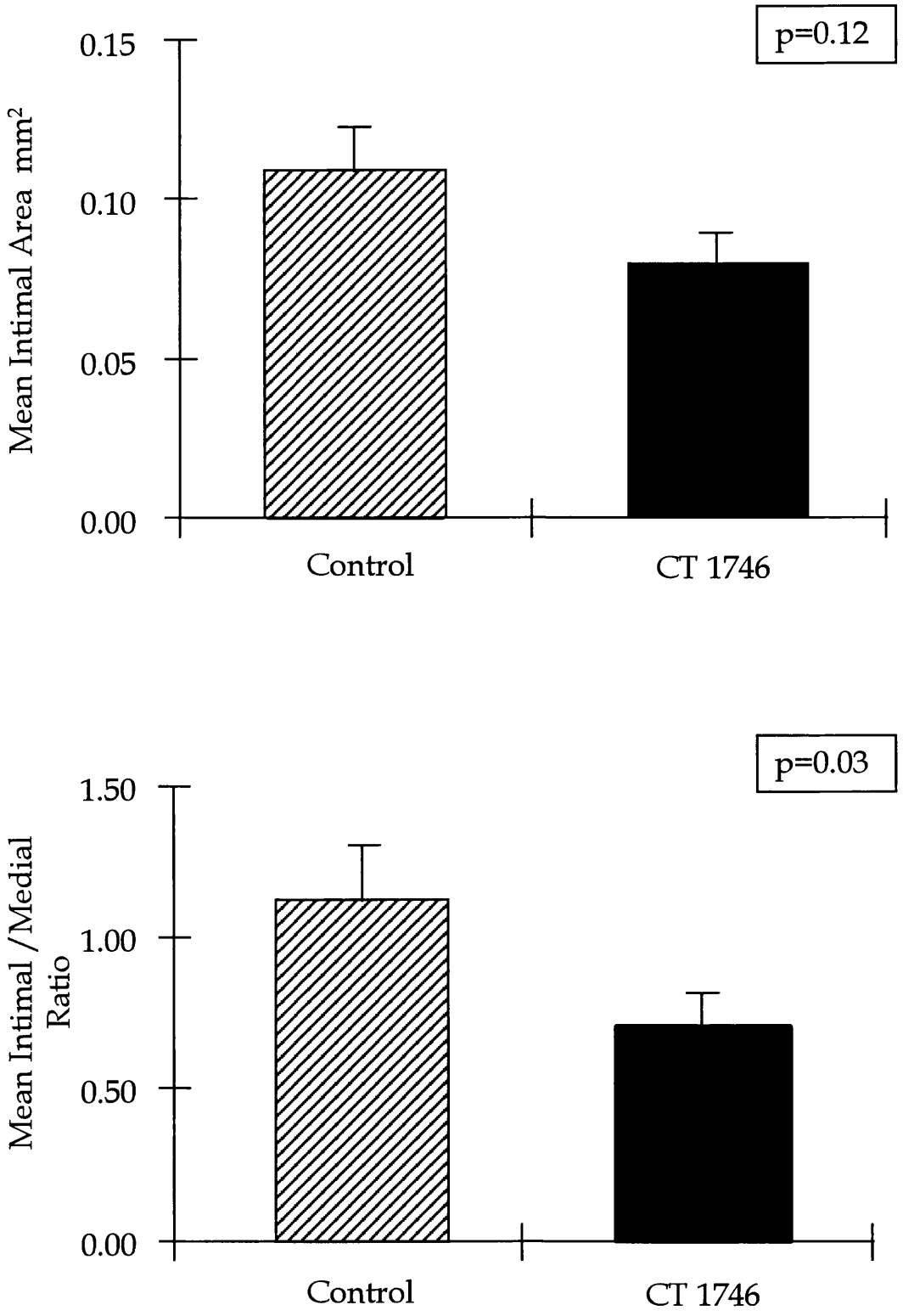


Figure 8 The Effect of CT1746 by Minipump on Neointimal Hyperplasia 14 Days After Balloon Injury.

13 rats in each group received either CT1746 (50mg over 2 weeks) or vehicle by minipump locally into the carotid bed following balloon injury. All animals were killed and fixed at arterial pressure 14 days after balloon injury and histomorphometric analysis carried out. The graphs above show the mean and SEM for Intimal area and Intimal Medial ratio for each group.

3.4 Discussion

3.4.1 Pharmacokinetics

The results of initial pharmacokinetic experiments in this species showed inadequate or unreliable blood levels of drug in the first 2 formulations tested. These studies were limited by the minimum quantifiable limit of the HPLC assay for CT1746 which is 200ng/ml. The preliminary studies described above had shown that CT1746 can inhibit Gelatinases in rat vascular smooth muscle cells at a concentration of 40 ng/ml. Undetectable levels of CT1746 could therefore still have had a biological effect. Variability of the results is unlikely to be due to failure of drug administration, as rats which appeared to poorly tolerate the gavage procedure or regurgitate the drug were not sampled. Sampling of blood from a large vein such as the femoral vein is likely to be subject to fewer errors than peripheral vein sampling. The third formulation achieved adequate levels and was, therefore, used in the subsequent gavage and pluronic gel experiments.

3.4.2 The Effect Of CT1746 Administered By Oral Gavage And Pluronic Gel On Neointimal Hyperplasia In The Rat Carotid Model

In the gavage experiment the four perioperative deaths were unexpected and unusual. They may reflect aspiration of gavage fluid, following the administration of a general anesthetic to a rat that has had a gavage procedure performed twice within 24 hours of anesthesia. The rat's airway is unprotected during surgery, and for this reason the gavage on the morning of surgery was omitted and pluronic gel administered. Post mortem did not reveal overt evidence of regurgitation or aspiration of the viscous gavage solution but this cannot be excluded. When gavaged fully conscious the rats intermittently regurgitated a small amount of gavage

fluid. The high incidence of neck wound breakdown in this group of rats is unlikely to have been due to any alteration in wound healing associated with the administration of the inhibitor CT1746. It was evenly distributed between the active treatment and control groups. It was probably due to the mechanical trauma to the neck caused by 'scruffing' the rats in order to perform the gavage procedure.

There was a non-significant trend towards a greater degree of intimal hyperplasia in the CT1746 group than in the control group. This result may indicate that CT1746 has no effect on intimal hyperplasia. It is consistent with the findings of two other groups using another pharmacological inhibitor of the MMPs, Galardin (GM6001) (Bendeck *et al.* 1996; Strauss *et al.* 1996). Bendeck *et al* showed in the rat model that while smooth muscle cell migration was profoundly inhibited (97% reduction) by Galardin there was a late phase of 'catch up' growth which removed any early difference in neointimal area between control and inhibitor groups. The inhibition of smooth muscle cell migration by MMP inhibitors has also been demonstrated by Kenagy *et al* (using batimastat) in a baboon aortic explant model (Kenagy *et al.* 1996). Studies of the alterations in collagen synthesis, degradation, and content 7 days after balloon injury have been carried out in the rabbit iliac artery model of balloon injury with and without Galardin (Strauss *et al.* 1996). They demonstrated a significant reduction in collagen synthesis and content in response to Galardin and a nonsignificant trend towards reduction in collagen breakdown. These changes were associated with a 25% reduction in neointimal area in the Galardin treated group which did not achieve statistical significance. At this relatively early time point this result may have reflected a change in smooth muscle cell migration as demonstrated in the rat model.

The trend towards increased neointimal area seen in the current study may indicate an effect that is not demonstrable using this number of animals ($n=8$ in each group). It might also indicate a lack of reproducibility in the drug administration. The pharmacokinetic data from experiment 3 suggested that while the drug was still detectable at 12 hours following

gavage there was a variation in drug level. This formulation represented the most effective oral formulation that could be given over a sustained period without significant toxicity. The oral effect of this compound may be limited by its poor solubility and unreliable absorption in this species and therefore in further experiments different delivery methods were explored.

3.4.3 The Effect Of CT1746 By Subcutaneous Minipump On Neointima Formation In The Rat Carotid Artery Model Of Neointima Formation After Balloon Injury.

Subcutaneous administration of drugs using osmotically regulated minipumps can be achieved with constant rates of subcutaneous infusion over periods of 2 weeks in rats. The volume of infusate that can be accommodated in a subcutaneous pump in small mammals such as rats is limited (2ml), and the poor solubility of CT1746 limits the concentration of solution that can be administered. The administration of CT1746 by minipump was therefore localized to the carotid artery, by placement of a cannula from the pump site on the dorsum of the rat into the carotid artery bed. A larger number of rats was studied to facilitate the detection of a statistically significant difference between the two groups.

The results showed no significant difference between the intimal area in the treated and control animals, but did show a significant reduction in neointima to medial ratio in response to CT1746 administration. Methodological problems in drug delivery were again encountered with this compound. The presence of precipitate in the neck of 3 of the treated animals is likely to be due to the poor solubility of CT1746, this may have lead to a failure of adequate delivery. It is possible, however, that the precipitate reflected a high local concentration of CT1746 in the carotid bed.

The results are only partially consistent with the findings of Bendeck *et al* who saw no difference in neointimal area 14 days after injury using a Galardin in the same model (Bendeck *et al*. 1996). They worked with a

different but also nonspecific inhibitor of MMPs which was administered systemically by daily intraperitoneal injection, blood levels confirmed systemic absorption in a subset of animals.

The results of this study of CT1746 showed a lack of effect on neointimal area but a reduction in intimal medial ratio at 14 days. This reflects a difference in the external size of the artery in the two groups. The statistical analysis suggested that this difference was unlikely to have occurred by chance. A change in the external elastic lamina was unlikely to be accounted for by the mechanisms investigated by Bendeck *et al*, although it is probable that reduced migration and compensatory proliferation occurred in the rats in this experiment, as the inhibitors have similar profiles (Bendeck *et al*. 1996).

Overall change in arterial size is usually accounted for by vascular remodeling, which has been highlighted by studies using intravascular ultrasound in patients undergoing angiography (Mintz *et al*. 1994). It was, however, originally highlighted by animal studies such as those of Lafont *et al* and Post *et al* (Post *et al*. 1994; Lafont *et al*. 1995). A change in favour of expansion of the external elastic lamina in response to MMP inhibition is unexpected. Studies of aneurysm development suggest that MMPs are upregulated in aneurysmal tissue (Vine and Powell, 1991; Herron *et al*. 1991). The differences may, however, reflect the animal model used in our study, which does not involve restriction of flow or reduction in the final lumen size after injury. The rat carotid balloon injury model is generally regarded as a model principally involving proliferation and migration of smooth muscle cells (Schwartz *et al*. 1996; Muller *et al*. 1992). One recent publication has confirmed a negative proteolytic balance in aneurysmal human tissues compared with normal tissues, despite upregulation of both the TIMPs and the MMPs (Knox *et al*. 1997). The technique of *in situ* zymography was used by Knox *et al* in this study but they were unable to identify a difference between occlusive and aneurysmal disease (Knox *et al*. 1997).

It is difficult to explain the decrease in neointimal medial ratio observed in this study as it occurred independently of neointimal area, and cannot be be attributed to inhibition of smooth muscle cell migration.

In the initial studies of orally administered CT1746 the control group showed a lack of reproducibility of neointima formation suggesting poor reproducibility of the injury to the vessel wall. This will have reduced the power of the study to identify any difference between the two groups. A formal study of the reproducibility of the technique would have addressed these questions. There was also, however, a lack of consistent absorption of the inhibitor with widely varying blood levels. In addition the assay of CT1746 hampers interpretation of the data because, as already discussed above, the lower limit of its detection is substantially greater than the concentration of compound required to inhibit gelatinases *in vitro*. As a result, many animals with undetectable blood levels may still be within the inhibitory range of the compound for the MMPs. In addition animals with measurable levels of the drug may be subject to non specific effects that are not related to its ability to inhibit MMPs.

Use of minipumps *in vivo* gives a more reliable delivery method for the drug but was also subject to substantial technical difficulty. As discussed on page 66 the formation of precipitates at the delivery sites of these pumps indicated inadequate delivery and was associated with oedema formation. It is unclear however if this resulted in low or high local levels of the compound making it impossible to attribute differences observed between the groups to inhibition of MMP activity.

The poor solubility and continued problems with reliable and reproducible delivery of CT1746 limited its usefulness in studying the effects of MMP inhibitors on intimal formation. More specific molecular techniques allowing increased expression of one naturally occurring inhibitor of the MMPs, TIMP1, were therefore employed.

PART 2 MARKER GENE TRANSFER

CHAPTER 4. *IN VITRO* CHARACTERIZATION OF AV1.LACZ4: METHODS FOR ADENOVIRUS PROPAGATION AND PURIFICATION AND CHARACTERIZATION IN VASCULAR SMOOTH MUSCLE CELLS.

4.1 INTRODUCTION

The adenovirus used in initial studies was a replication deficient E1-, E3-deletion mutant expressing a nuclear-targeted ß-Galactosidase reporter under the control of the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter (Lee *et al.* 1993; Yei *et al.* 1994). The virus was constructed by Genetic Therapy Incorporated.

Figure 3 shows the structure of the recombinant vector as described by Lee *et al* (Lee *et al.* 1993). The pAvS6nLacZ plasmid was linearized and cotransfected into human embryonic kidney-derived 293 cells with the 35kb *Cla I* fragment of Ad-dl327. The use of this cotransfection technique promotes homologous recombination between the regions of each molecule corresponding to Adenovirus type 5 sequences 3328-6241, with resultant generation of infectious virus. Recombinant plaques were picked and propagated in 293 cells. The recombination events were confirmed using Southern analysis. Aliquots of the resultant virus were the kind gift of GTI, and were subsequently propagated and purified as described below.

In order to assess the success of gene transfer by a recombinant adenoviral vector, and identify how much virus can be used before significant inflammation or cytopathic effect outweighs the benefit of gene transfer, it is essential to be able to assess the infectivity or titre of the virus. An objective method of virus concentration can be obtained by

assessing the number of virions in a fixed volume using electron microscopy. It may be possible to identify incomplete virions if they do not form full icosahedrons, but the particle number, while accurate and reproducible, cannot determine how many of the observed virions are capable of infecting cells. In this study two methods were used to assess infectivity. The first, the end-point cytopathic effect assay, was used to approximately assess the success of any one batch of virus while the second, the plaque assay is a more classical virological technique to quantitate infectivity.

The plaque assay was originally developed for use with type two adenovirus (Green *et al.* 1967). A plaque is a cluster of infected nonviable cells produced by infection of a single cell by one virus particle with secondary infection of surrounding cells by progeny virus. If a plate is stained with neutral red the uninfected viable cells around a plaque will stain red, and there will be a zone of clearing in the plaque area. In order to prevent dissemination of virus when infected cells lyse, plaque assays are performed under a layer of agar which localizes any released virus to the cells contiguous with the original infected cells thus allowing a plaque or cluster of dead cells to be seen. The plaquing efficiency of purified virus varies with adenoviral serotype as does the size and time of appearance of the plaques. Adenovirus 5 plaques are 2-3 mm across and appear from 8-14 days after infection. The end-point cytopathic effect assay is based on the appearance of a cytopathic effect visible on a cell monolayer at 3-4 days after infection.

Stocks of virus titred by these methods can then be used to test the ability of this vector to transfer genes. In order to determine a reproducible relationship between 'dose' or titre of virus and duration of exposure, and the success of gene transfer in rat vascular smooth muscle cells, initial experiments were carried out *in vitro*. In initial experiments a wide range of multiplicities of infection of Av1.LacZ4 were used to approximate the amount of transgene expression, but more importantly to demonstrate toxicity, and therefore identify a dose range of virus to use in future

experiments. An SV40 transformed rat aortic smooth muscle cell was initially employed, and subsequently primary cultured rat aortic vascular smooth muscle cells at passage 4-6 were studied.

4.2 Methods

4.2.1 Materials And Methods For Adenovirus Propagation And Purification.

4.2.1.1 293 Cells: Transformed Human Embryonic Kidney Cells

1) High passage number cells (passage 50+). The cells increase their rate of division with increasing passage number but do not appear to lose their permissiveness for adenovirus i.e. lose Ela. These cells are appropriate for propagation of virus where rapid expansion of cell numbers is required but not for recombination and plaque assays. Higher passage number is likely to be associated with an increased chance of spontaneous recombination resulting in a 'replication deficient' recombinant reacquiring the E1a gene from the host cell and becoming replication competent (Lochmuller *et al.* 1994). This is still a very rare event but all viral stocks grown on high passage cells were screened for wild type contamination or reversion prior to further use.

2) ATCC CRL-1573:Passage 32 293 cells, slow growing, suitable for plaque assays and recombinations.

4.2.1.2 Media

Complete media: Dulbecco's Modified Eagle's Medium with 0.11 M Na Pyruvate (Gibco), 10% Foetal Bovine Serum (50 ml in 500 ml), 1% Penicillin/Streptomycin (2.5 ml in 500 ml)

Infection media: Dulbecco's Modified Eagle's Medium with 0.11 M Na Pyruvate (Gibco), 2% Foetal Bovine Serum (10 ml in 500 ml), 1% Penicillin/Streptomycin (2.5 ml in 500 ml)

Cytopathic effect assay media: Dulbecco's Modified Eagle's Medium with 0.11 M Na Pyruvate (Gibco), 1% Foetal Bovine Serum (5 ml in 500 ml), 1% Penicillin/Streptomycin (2.5 ml in 500 ml)

4.2.1.3 Cell Care

293 cells are delicate and poorly adherent. Media and wash solutions were added to the side of the dish, with the dish tilted, and mechanical manipulation minimized. All media and solutions were prewarmed to 37°C.

4.2.1.4 Cell Growth for Propagation

Up to 200 10 cm plates were handled without loss of cells due to dehydration etc.

Cells were propagated in 10 cm dishes for ease of harvesting cells.

Cells were trypsinized and diluted 1 in 10 in complete medium, 72 hours before infection. Cells were split by removing the covering full growth media and discarding it into Virkon containing solution. The cells were washed carefully with sterile phosphate buffered saline which was then discarded, and 1 ml of Trypsin Versene, pre warmed to 37°C, was added to each plate and the plate tilted to fully cover the cells with liquid. The plates were then left to incubate at 37°C for approximately 3 minutes or until cells began to round up and detach. 2 ml of complete medium was added to each plate to inactivate the trypsin, and the media and cells were removed and pooled in a 50 ml sterile tube. The cells were briefly vortexed and then resuspended in complete medium to dilute the cells 1 in 10 from the original volume (i.e. 100 ml of media per 10 cm plate in the original stock). 10 ml of media was then dispensed to each 10 cm dish and the

dish tipped gently to cover the surface evenly with the cell solution. Cells were placed in 37°C, CO_2 5%, incubator until cells were 90% confluent. It was rarely necessary to feed the cells during this period and this was avoided where possible, due to the potential for damage to the monolayer.

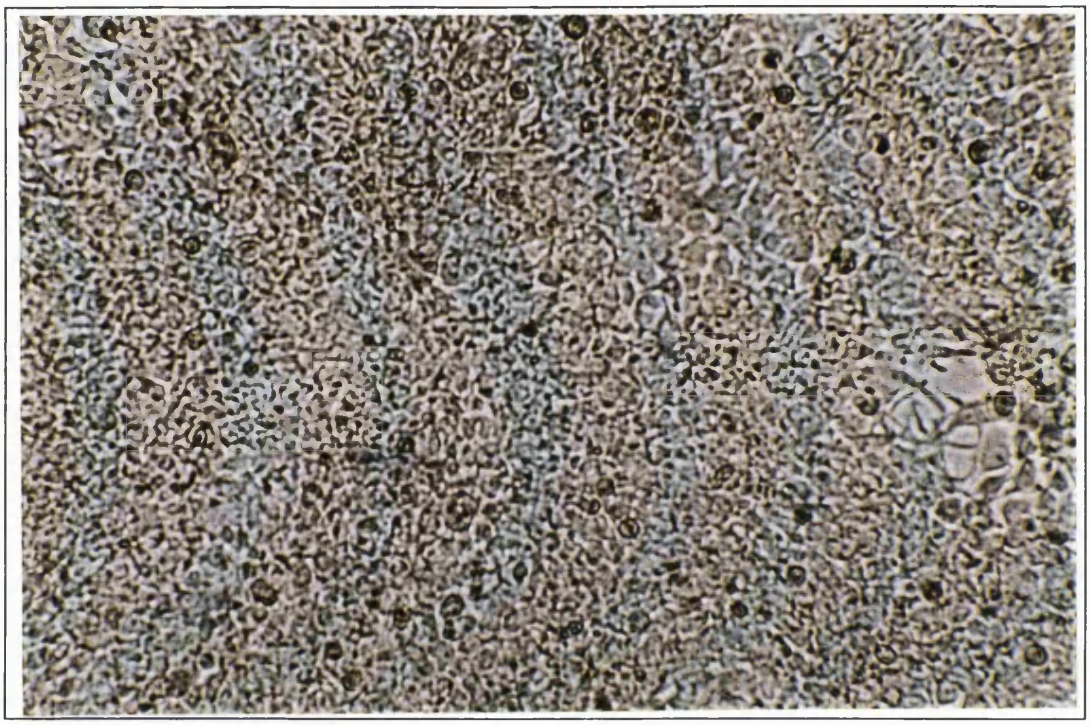
4.2.1.5 Preparation Of Virus For Infection

Virus stocks were stored at -70°C and rapidly thawed in a 37°C water bath and then placed on ice until required. All virus stocks were kept in small aliquots (e.g. 100 µl dependent on titre) to avoid loss of titre due to recurrent freeze thawing. Each 10 cm dish was infected at an approximate multiplicity of infection (MOI i.e. plaque forming units per cell) of 10. Cell counts using a haemocytometer showed that 10 cm dishes usually contain 2-5 x 10^7 cells. The total volume of infection medium required to infect each plate with 10 ml of infection medium containing 2-5 x10^7 virions per ml was made up to ensure even distribution of virus and that all plates would show a cytopathic effect at similar time points.

4.2.1.6 Infection Procedure

Complete growth media was aspirated from the dishes to be infected. 10 ml of virus containing infection media was added to each plate. One plate was used as a control plate and infection medium (i.e. 2% FCS) without any virus was added because prolonged serum deprivation can result in detachment of cells and rounding up which may be mistaken for a cytopathic effect (CPE). Dishes were incubated at 37°C for 48-72 hours. The cells were inspected twice a day for cytopathic effect, with typical 'bunch of grapes' appearance, but were usually ready to be harvested at 48-56 hours (see Figure 9).

A



B

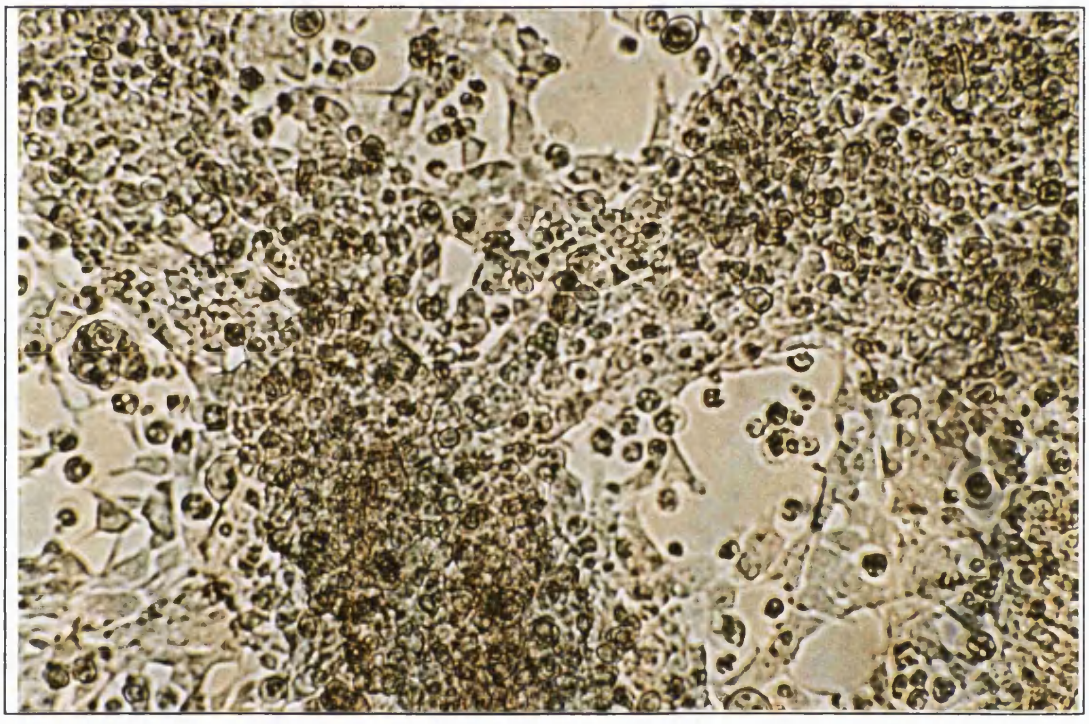


Figure 9 Photomicrographs of 293 Cells Showing Typical Cytopathic Effect.

Panel A shows a confluent monolayer of 293 (transformed human embryonic kidney cells expressing the E1A fragment of the adenoviral genome immediately prior to infection with Av1.LacZ4. Panel B shows cells 48 hours after infection illustrating the typical 'bunch of grapes' cytopathic effect.

4.2.1.7 Cell Harvest

Cells were harvested when most of the monolayer was beginning to show CPE and some cells had detached. Adenovirus is produced in the nucleus and stored intracellularly. The aim of harvesting is therefore to minimize cell trauma and to harvest before the cells have lysed due to on-going infection. The cells were harvested by gently pipetting the media across the monolayer of each dish until all the cells had detached. Cells showing significant cytopathic effect readily detached from the tissue culture dish with minimal manipulation. The cell suspension was centrifuged at 1500 rpm, 4°C, for 10 minutes and the supernatant discarded into a Virkon containing waste beaker. Cell pellets containing virus were resuspended in 1.5-2 ml of resuspension solution dependent on the time of planned centrifugation:

Resuspension solutions:

1) Supernatant from cells (this can be stored at -20°C until ready for use but this should only be stored for 2-3 days).

2) PBS containing 10% glycerol (Can be stored at -70°C until required)

3) 10 mM Tris-Cl, pH 8. (Can be stored at -70°C until required)

4.2.1.8 Crude Viral Lysate

The virus/cell suspension was exposed to three cycles of freezing and thawing in liquid nitrogen and a 37°C water bath. The cells were vigorously vortexed between each freeze thaw cycle. This process lyses the cells and liberates the virus into the surrounding liquid. The resultant crude viral lysate was placed in Beckman ultracentrifuge tubes which had previously been sterilized by spraying with 70% ethanol and allowing the tubes to dry completely. These were centrifuged in a precooled rotor for 5 minutes at 7000 rpm 4°C. The supernatant was retained and Virkon added to the cell pellet prior to disposal.

4.2.1.9 PURIFICATION OF CRUDE VIRAL LYSATE

The crude viral lysate was purified to remove empty capsids by centrifugation on two cesium chloride gradients.

Cesium chloride solutions:

1.25 g/ml buoyant density, Add 100 ml PBS to 36.2 g CsCl.

1.26 1.35 g/ml buoyant density, Add 100 ml PBS to 51.2 g CsCl

1.27 1.4 g/ml buoyant density, Add 100 ml PBS to 62.0 g CsCl

These solutions were autoclaved prior to use.

Ultraclear ultracentrifuge tubes (Beckman) were presterilized with 70 % ethanol and allowed to dry fully in a tissue culture hood prior to use. 3 ml of 1.25 g/ml CsCl was added to each tube. 3 ml of 1.4 g/ml CsCl was then 'underlaid' with a 5 ml pipette without disturbing the interface. 6 ml of crude viral lysate was then layered on the preformed gradient and

centrifuged in an ultracentrifuge at 30,000 rpm, 20 °C, for 1.5 hours.

A tube was then clamped vertically to a retort stand above a beaker of Virkon solution. A band of viral material was visible at the interface between the two densities of cesium chloride. This band was harvested using a 1 ml syringe and 20 G needle. The needle was slowly inserted 3 mm below the band using a rotational motion to 'drill' through the side of the tube without disturbing the gradient. The band was harvested by aspirating the syringe, and the needle was then slowly withdrawn so that any solution dripping from the tube fell into the Virkon solution.

The collected band was overlaid onto a centrifuge tube containing 10 ml of 1.35g/ml CsCl and spun at 40,000 rpm, 20°C, for 18 hours. Under these conditions a continuous gradient forms in the cesium solution and a band will be visible. When centrifugation was complete the lower opalescent band was harvested as described above in as small a volume as possible of cesium chloride and glycerol was added to a final concentration of 10%.

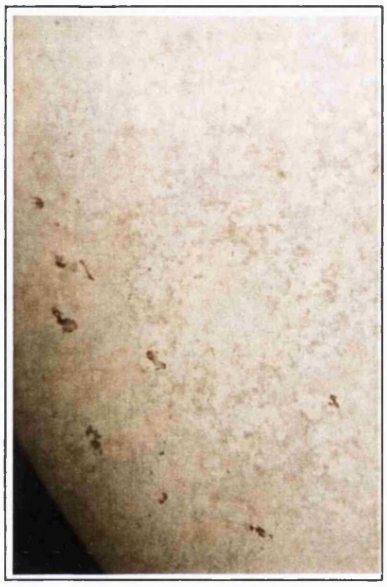
4.2.1.10 Dialysis

The cesium chloride/virus solution was then dialyzed to remove the cesium chloride which would be cytopathic. Dialysis cassettes were used according to the manufacturers instructions (Slide-A-Lyser dialysis kit). The solution was dialyzed at 4°C for 1 hour against 1 l of dialyzate (10% glycerol, 10 mM Tris Chloride pH 7.4, 1mM Mg Cl_2, in sterile phosphate buffered saline) and this was repeated three times allowing the last dialysis cycle to continue overnight. The virus was recovered from the cassette and frozen at -70°C in 100µl aliquots until required.

4.2.1.11 Plaque Assay

Plaque assays were carried out in 6 well plates. Low passage (< passage 40) 293 cells were seeded into these plates in complete medium until they reached 80-90% confluence. The medium was then aspirated and the cells washed with PBS. Serial 10 fold dilutions of virus samples were made in infection medium and added in triplicate to the 6 well plates (1ml per well commencing with 1µl of virus per ml). The cells were exposed to the infection medium for 1 hour after which the medium was removed and 1.5 ml of agar overlay was added. Overlay was prepared immediately prior to use. Overlay contained: 10% 10X DMEM (Gibco), 2% sodium bicarbonate (Gibco), 2% fetal calf serum (Gibco), 1% Penicillin, 1% stretomycin, 1% amphotericin, and 0.5% agar (2% agar Gibco) in sterile water. The 2% agar was heated until melted and allowed to equilibrate with the overlay at 44°C. The overlay was allowed to harden at room temperature prior to return of the plates to the incubator. On day five an additional 0.5 ml of overlay per well was added. On day seven 1ml of overlay containing 1ml of 0.2% w/v neutral red (filter sterilized) per 100 ml of overlay was added to each well. Plaques were visible on day 8-11 (See Figure 10).

A



B

Figure 10 Plaque Assay.

Panels A and B show plaques in monolayers of 293 Cells stained with neutral red. Panel A shows a photograph taken using a dissecting microscope with a lucent area in the middle of the field where cells have become cytopathic and died giving the appearance of a plaque. Panel B shows the plaque photographed at higher power (x40).

4.2.1.12 End-Point Cytopathic Effect (CPE) Assay

100µl aliquots of DMEM with 0.5 % fetal calf serum were placed in the wells of a 96 well tissue culture plate. 10 fold dilutions of virus were added in triplicate, and control wells prepared that received no virus. 100µl of 293 cells in DMEM with 0.5 % fetal calf serum were added at a concentration of 3 x 10^5 cells/ml to each well and incubated for 3 days. Microscopy was used to identify those wells that showed a 50% cytopathic effect, and an estimate of viral titre was obtained.

4.2.1.13 Screening For Replication Competent Adenovirus

There is a theoretical chance of a recombinant adenoviral vector reacquiring the E1A region of the adenoviral genome by homologous recombination with wild-type adenovirus, or the E1A region expressed in 293 cells. In practice this is a rare event, but stocks may also become contaminated with wild type adenovirus from the environment. All stocks were therefore screened by plaque assay with 10 fold dilutions on HeLa cells which support the growth of wild type but not replication deficient adenoviruses. The protocol used was identical to that used to titrate the virus as above (4.2.1.11 Plaque Assay) but HeLa cells were used in place of 293 cells. HeLa cells are grown and passaged under the same conditions and in the same media as 293 cells. Wild type adenovirus was used as a positive control in these experiments.

4.2.1.14 Adenoviral Stocks

A single stock of Av1.LacZ4 with a titre of 1.3 x10^{10} pfu/ml was used for all experiments described in this chapter.

4.2.2 Smooth Muscle Cell Line Culture

SV40 transfected rat abdominal aortic smooth muscle cells SV40LT-SMC Clone HEP-SA (ATCC No 2018) were used. Cells were cultured in Dulbeccos modified Eagle's medium, (DMEM) with sodium pyruvate and 1000g/l glucose (Gibco No 31885-03) supplemented with 10% foetal calf serum (Gibco) and 200 μg/ml geneticin (G418, Gibco 066-01811B) and 50mg/ml penicillin with 50 mg/ml streptomycin. Flasks were seeded at 3.5 x 10^6 cells/ml. Culture fluid was renewed 2-3 times per week. Once cells reached confluence when viewed by light microscopy they were subcultured as follows. The medium was aspirated and the monolayer washed with 5ml of phosphate buffered saline. This was removed and 3 ml of trypsin:EDTA (Gibco No 45300-019) added and the flask incubated at 37°C until the cells detached (5-10 minutes). Fresh medium was then added, aspirated, and the cell suspension dispensed to new flasks with dilution of 1:3 to 1:6.

4.2.3 Primary Vascular Smooth Muscle Cell Culture

Primary rat aortic smooth muscle cells were cultured using a modification of the method of Rennick *et al* (Rennick *et al*. 1988). 300-350g male Wistar rats were killed by CO_2 asphyxiation and their throats cut to allow partial exsanguination. A midline abdominal incision was then made using sterile technique and the aorta was exposed by blunt dissection from the renal arteries to the aortic arch. The adherent fat was removed from the aorta, which was then excised and placed in RPMI (Gibco) supplemented with 1% Penicillin/streptomycin and 1% transferrin (serum free RPMI). The aorta was then transported to a primary culture laboratory, and placed in a sterile petrie dish, on a ground glass plate, and covered with RPMI supplemented as above. Using a Prior S2000 dissecting microscope (Prior Scientific Instruments) and iridectomy scissors, the aorta was cut longitudinally, so that it rested intimal side uppermost. A sterile cotton bud was used to wipe the aorta to remove any endothelium. Using

watchmakers forceps (size 7), the media of the artery was removed in thin horizontal strips, by securing the edge of the vessel with a second pair of forceps. Strips were placed in a container of RPMI as above. The RPMI was then aspirated from the pooled strips of media from three arteries using a narrow bore pipette, and the tissue placed in 3ml of 3mg/ml Collagenase (Sigma C0130) in serum free RPMI (i.e. 1 ml per aorta) at 37°C for 1 hour. This was carried out in a 37°C oven or water bath with gentle shaking to aid digestion. The Collagenase solution was then removed and the tissue resuspended in 3ml of elastase 1mg/ml (Sigma type III elastase E1250) in serum free RPMI and incubated at 37°C with gentle shaking for 1 hour. 1ml of fresh Collagenase solution was added to the elastase solution, and incubation with shaking continued until all the tissue had dispersed into single cells (1-2 hours). The cell suspension was then centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The cell pellet was resuspended in RPMI supplemented with 5% fetal calf serum (Gibco) and 1% penicillin/streptomycin, at a concentration of 1-3 x 10^5 cells/ml. The cell concentration was determined using a haemocytometer after initial resuspension in 4ml of RPMI supplemented as above. The cells were placed in a humidified incubator at 37°C, 5% CO_2 and handling avoided for 72 hours. The media was then changed and renewed at 72 hourly intervals.

Cells were passaged when found under light microscopy to be confluent. The medium was aspirated and the monolayer washed with 5ml of phosphate buffered saline. This was removed and 3 ml of trypsin:EDTA (Gibco No 45300-019) and the flask incubated at 37°C until the cells detached (5-10 minutes). Fresh RPMI medium was then added, aspirated to resuspend the cells, and the cell suspension was dispensed to new flasks with dilution of 1:3 to 1:6.

Each batch of smooth muscle cells were tested at 72 hours or more to ensure that 98% of cells present stained positively for anti α smooth muscle actin (Sigma A2547).

4.2.4 Media

4.2.4.1 SV40LT-SMC CLONE HEP-SA (ATCC NO 2018)

Culture Media: Dulbeccos modified Eagle's medium, (DMEM) with sodium pyruvate and 1000g/l glucose (Gibco No 31885-03) supplemented with 10% foetal calf serum (Gibco) and 200 μg/ml geneticin (G418, Gibco 066-01811B) and 50mg/ml penicillin with 50 mg/ml streptomycin (Gibco).

Infection/serum free Media: Dulbeccos modified Eagle's medium (DMEM) with sodium pyruvate and 1000g/l glucose (Gibco No 31885-03, 200 μg/ml geneticin (G418, Gibco 066-01811B), and 50mg/ml penicillin with 50 mg/ml streptomycin (Gibco).

4.2.4.2 PRIMARY RAT AORTIC VASCULAR SMOOTH MUSCLE CELLS:

Culture Media: RPMI supplemented with 5% fetal calf serum and 1% penicillin/streptomycin (all Gibco).

Infection/Serum Free Media: RPMI supplemented with 1% transferrin, and 1% penicillin/streptomycin (all Gibco)

4.2.5 Immunohistochemistry For α Smooth Muscle Actin

In order to confirm that primary cultures contained 98% smooth muscle cells, immunohistochemistry for α smooth muscle actin was carried out. Initially a range of concentrations of primary antibody was tested using the protocol below in order to optimize the protocol for use on all batches of cells.

Primary vascular smooth muscle cells were cultured in 8 well chamber slides (Nunc No 177445) with one well containing the SV40 transformed SMCs as a positive control. Cells were fixed for 10 minutes with ice cold methanol. This was aspirated and replaced with 0.3% hydrogen peroxide in methanol for 30 minutes to quench endogenous peroxidase activity. The cells were then rinsed twice in PBS for 10 minutes. All incubation steps

after this point were carried out in a humidification chamber. 2.5 % horse serum in PBS, a source of nonspecific blocking antibody, was added and the cells incubated for 20 minutes. The primary antibody, in this case anti α smooth muscle actin (Sigma A2547), was added in dilutions of 1:100, 1:200, 1:400, 1:500, and 1:1000 in PBS containing 2% bovine serum albumin (BSA) and incubated for 30 minutes. The cells were washed twice in PBS containing 1:100 triton x-100 and once in PBS (10 minutes each). The cells were then incubated for 20 minutes in second antibody; biotinylated goat anti-mouse immunoglobulins (Sigma Mouse extravidin peroxidase staining kit) were diluted 1:100 in PBS containing 2.5% horse serum to reduce background staining. The cells were washed twice in PBS-triton and once in PBS as previously. Extravidin peroxidase (Sigma Mouse extravidin peroxidase staining kit) was then diluted 1:50 in PBS with 2% BSA and incubated with the cells for 20 minutes. The cells were then washed three times in PBS. The chromagen 3-amino-9-ethyl carbazole (AEC) was used to localize the peroxidase. 20 mg of AEC (sigma A-6926) was added to 2.5 ml of dimethylformamide (Sigma D-4254) and the solution retained as a stock stored at 4°C. 0.2 ml of this stock solution were then added to 3.8 ml of 0.05 M acetate buffer (pH 5.0) (Sigma 325-4) and 20 μl of hydrogen peroxide added immediately before use. The cells were incubated with this chromagen for approximately 5 minutes or until red staining was visible under light microscopy.

The lowest concentration of primary antibody at which readily detectable staining occurred was 1:500 (see Figure 11), and therefore this dilution was used for all other experiments.

Figure 11. Primary Cultured Rat Vascular Smooth Muscle Cells After Immunohistochemical Staining With Anti-Smooth Muscle Actin Primary Antibody 1:500.

Photomicrograph of primary cultured rat aortic vascular smooth muscle cells fixed with methanol and immunohistochemically stained using an antibody to smooth muscle actin. The chromagen is 3-amino-9-ethyl-carbazole (AEC) giving a red/brown colour to the positive cells. In all cultures greater than 95% of cells stained positively for smooth muscle actin.

4.2.6 Assessment Of Gene Transfer Efficiency By 5-Bromo-4-Chloro-3-Indoyl-ß-D-Galactopyranoside (X-Gal) Staining.

The efficiency of gene transfer following virus infection was estimated by staining transduced cells with 5-bromo-4-chloro-3-indoyl-ß-D-Galactopyranoside (X-Gal). This chromagen changes from a yellow to a blue colour in the presence of ß-Galactosidase allowing localization of the transgene.

Tissue culture media was removed from the cells to be stained and the monolayer was washed with PBS. The cells were then fixed in 0.05% Glutaraldehyde in PBS for 10 minutes. X-Gal buffer was prepared as follows:

X-Gal buffer: 0.07g Potassium ferrocyanide $K_4Fe(CN)_6.3H_2O$

0.05g Potassium ferricyanide $K_3Fe(CN)_6$.

1.852 ml 4M Sodium chloride

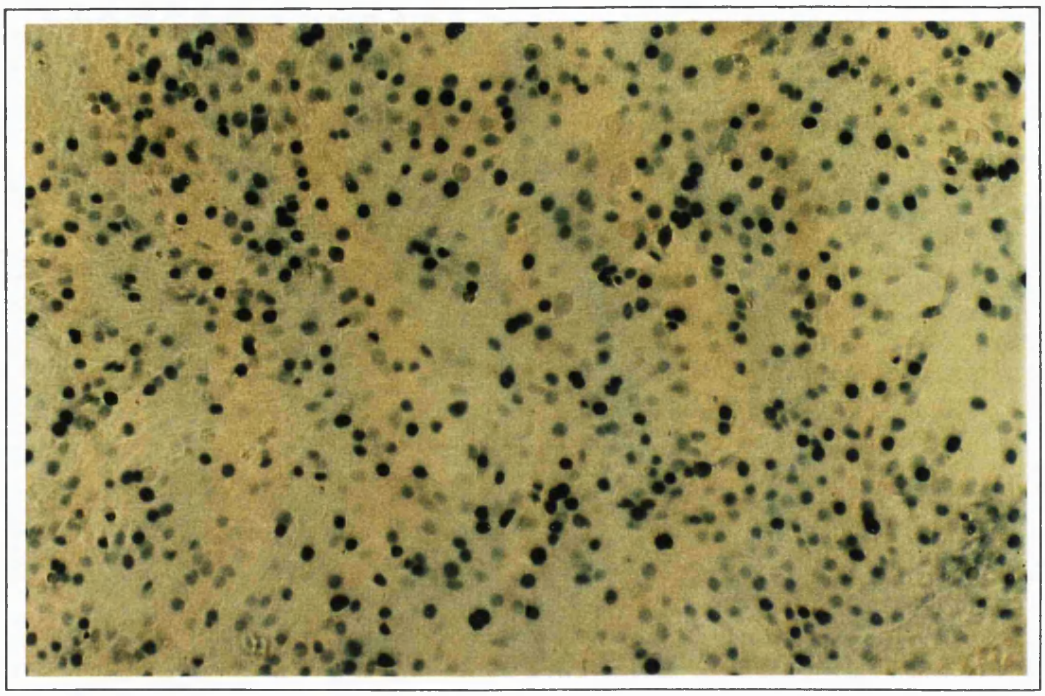
50µl 1M Magnesium chloride

made up to 50 ml with PBS.

X-Gal buffer was then heated to 37°C in a water bath. 25µl of 150mg/ml 5-bromo-4-chloro-3-indoyl-ß-D-Galactopyranoside (X-Gal) in dimethyl sulphoxide (DMSO) was added per 1 ml of X-Gal buffer, and then immediately applied to the cell monolayer. All reagents were warmed to 37°C to ensure minimal precipitation of X-Gal but X-Gal solution was filtered prior to application to the cells. The cells were incubated in the X-Gal solution for 2-4 hours, monitoring the colour change using a light microscope, and the reaction terminated by removal of the X-Gal solution, washing in PBS and storage under 70% glycerol. This allowed a visual assessment of gene transfer efficiency. Because visualization of vascular smooth muscle cell nuclei not expressing the transgene was difficult, and may allow selection bias on the part of the observer, trypsinized cells were also assessed. If cells were incubated for long periods in X-Gal endogenous ß-Galactosidase could react with the X-Gal, causing a colour change

unrelated to gene transfer (see Figure 12). Short incubation periods of 2-4 hours in cells, or 18 hours in arterial tissue, did not give rise to background or endogenous staining. Control samples not exposed to Av1.LacZ4 were used in X-Gal incubation procedures.

A



B

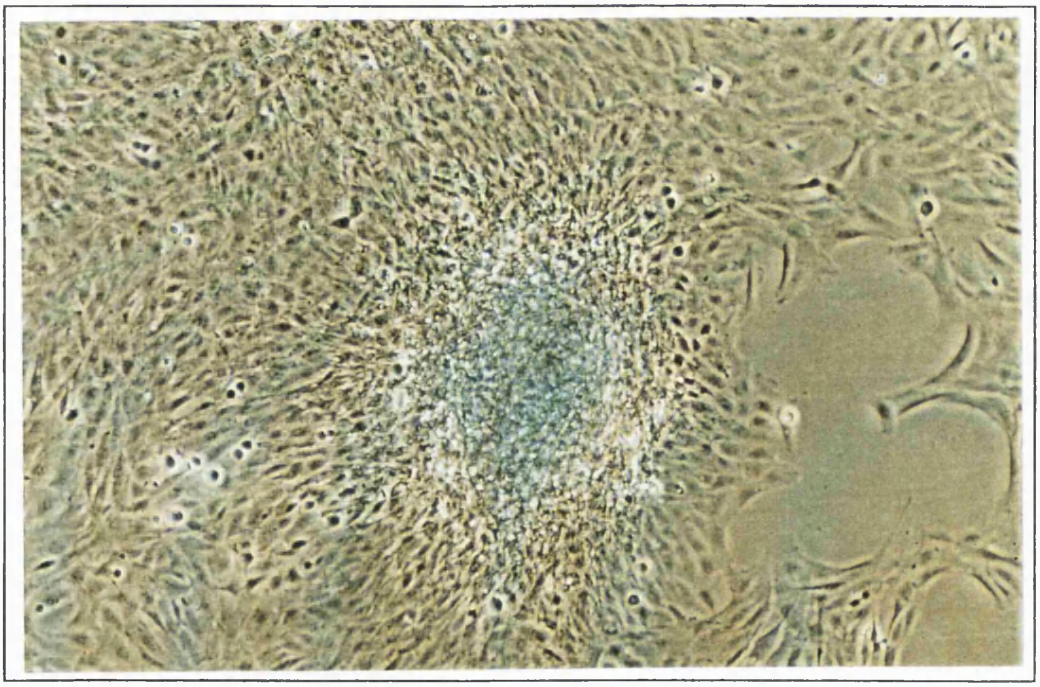


Figure 12 X- Gal Staining Of Vascular Smooth Muscle Cells.

Panel A shows blue cells 48 hours after infection with Av1.LacZ4 encoding nuclear localized beta Galactosidase. The cells have been fixed and incubated for 2 hours with X-Gal chromagen. Panel B illustrates the diffuse cytoplasmic staining due to endogenous beta Galactosidase activity in a clump of cells on a control plate. Successful gene transfer and endogenous activity of beta galactosidase can clearly be differentiated.

4.2.7 Quantitation Of Gene Transfer

Selection bias was eliminated using trypsinized cells (Guzman *et al.* 1993). Prior to X-Gal staining smooth muscle cells were detached from the culture plate or chamber slide by incubating with trypsin:EDTA (Gibco No 45300-019) for approximately 5 minutes at 37°C. An equal volume of 0.1M glutaraldehyde was then added to each trypsin/cell suspension and the cells fixed for 5 minutes prior to centrifugation at 1000 rpm for 5 minutes. The cell pellet was then resuspended in X-Gal solution for 3-4 hours. Stained cell suspensions were loaded into a haemocytometer and those in the counting chamber only were counted. Total cells and cells with nuclear localized blue staining were counted.

4.2.8 Assessment Of Toxicity And Transgene Expression In VSMC Line Infected With Av1.LacZ4

SV40 transformed VSMC s were grown to confluence and then placed in infection media for 24 hours prior to viral infection. Virus stocks were diluted in fresh infection media to infect cells at MOIs of 3 $x10^3$, 3 x 10^2, 30, 3, and a control well was not infected. A further well was infected with Av1.null virus at MOI $3x10^3$, a replication deficient adenovirus lacking the ß-Galactosidase expression cassette, to establish that viral infection itself did not cause ß-Galactosidase up-regulation. Infection solutions were incubated with the cells at 37°C for 1 hour. The cells were then returned to fresh serum free media, and fixed and stained with X-Gal 48 hours after infection as described on page 86. Sections were then photographed using phase contrast microscopy at a magnification of x10. Toxicity was indicated by disruption of the cell monolayer and detachment of cells.

4.2.9 Gene Transfer Efficiency Of Av1.LacZ4 In Smooth Muscle Cell Line And Primary Vascular Smooth Muscle Cells : Dose-Response

The relationship between transgene expression and multiplicity of infection was explored in primary vascular smooth muscle cells at passage 4-6 and in the SV40 transformed smooth muscle cells. VSMCs were grown to confluence and then placed in infection media for 24 hours prior to viral infection. Trypsinisation of confluent monolayers and counting in a haemocytometer showed 3×10^4 cells per well of an 8 well chamber slide and 2.5×10^5 cells per well in a 24 well plate. Virus stocks were diluted in infection media to infect cells at MOIs of 6.25, 12.5, 25, 50, 100, and 200. In 8 well chamber slides a volume of infection solution of 0.15 ml was used per well, and in 24 well plates 0.5 ml per well. Infection solutions were incubated with the cells at 37°C for 1 hour. The cells were then returned to fresh serum free media and fixed and stained with X-Gal 48 hours after infection as described on pages 86 and 89. Gene transfer efficiency was expressed as the percentage of the total cell number that showed nuclear blue staining. Cytotoxic effect was assessed by inspection of monolayers under light microscopy.

4.2.10 Gene Transfer Efficiency Of Av1.LacZ4 In Smooth Muscle Cell Line: Time Course

Primary and SV40 transformed VSMCs were grown to confluence and then placed in infection medium for 24 hours prior to viral infection. Virus stocks were diluted in infection medium to infect cells at an MOI of 100. Infection solutions were incubated with the cells at 37°C for 0 (control), 5, 15, 30, 45, 60 and 120 minutes. The cells were then returned to serum free medium, and fixed and stained with X-Gal, 48 hours after infection as described on pages 86 and 89. Gene transfer efficiency was expressed as the percentage of the total cell number that showed nuclear blue staining.

4.2.11 Duration Of Transgene Expression After Primary Smooth Muscle Cell Infection

Primary VSMC s were grown to confluence and then placed in serum free media for 24 hours prior to viral infection. Virus stocks were diluted in infection media to infect cells at an MOI of 100 for 1 hour. The cells were then returned to serum free media and the medium was changed every four days. Three wells were fixed and stained with X-Gal 48 hours, 14 days and 28 days after infection as described on page 86. Wells were photographed under a light microscope at x 100 magnification.

4.3. RESULTS

4.3.1 In Vitro Toxicity Of Adenovirus In VSMCs

Figure 13 shows the photomicrograph of the cells infected at MOI s of 3 $x10^3$, 3 x 10^2, 30, 3, and control. Significant cellular toxicity was observed at MOI of 3 x 10^3, with disruption of the monolayer and vacuolation of the cells when viewed at higher power. Transgene expression was seen to increase with increasing MOI. In some cells the production of chromagen was sufficient to cause leakage into the cytoplasm. No blue staining was seen with control cells or cells infected with a null virus. The null virus was used to infect cells at MOI 3 x 10^3, and showed a similar degree of toxicity for the smooth muscle cells to the ß-Galactosidase expressing virus.

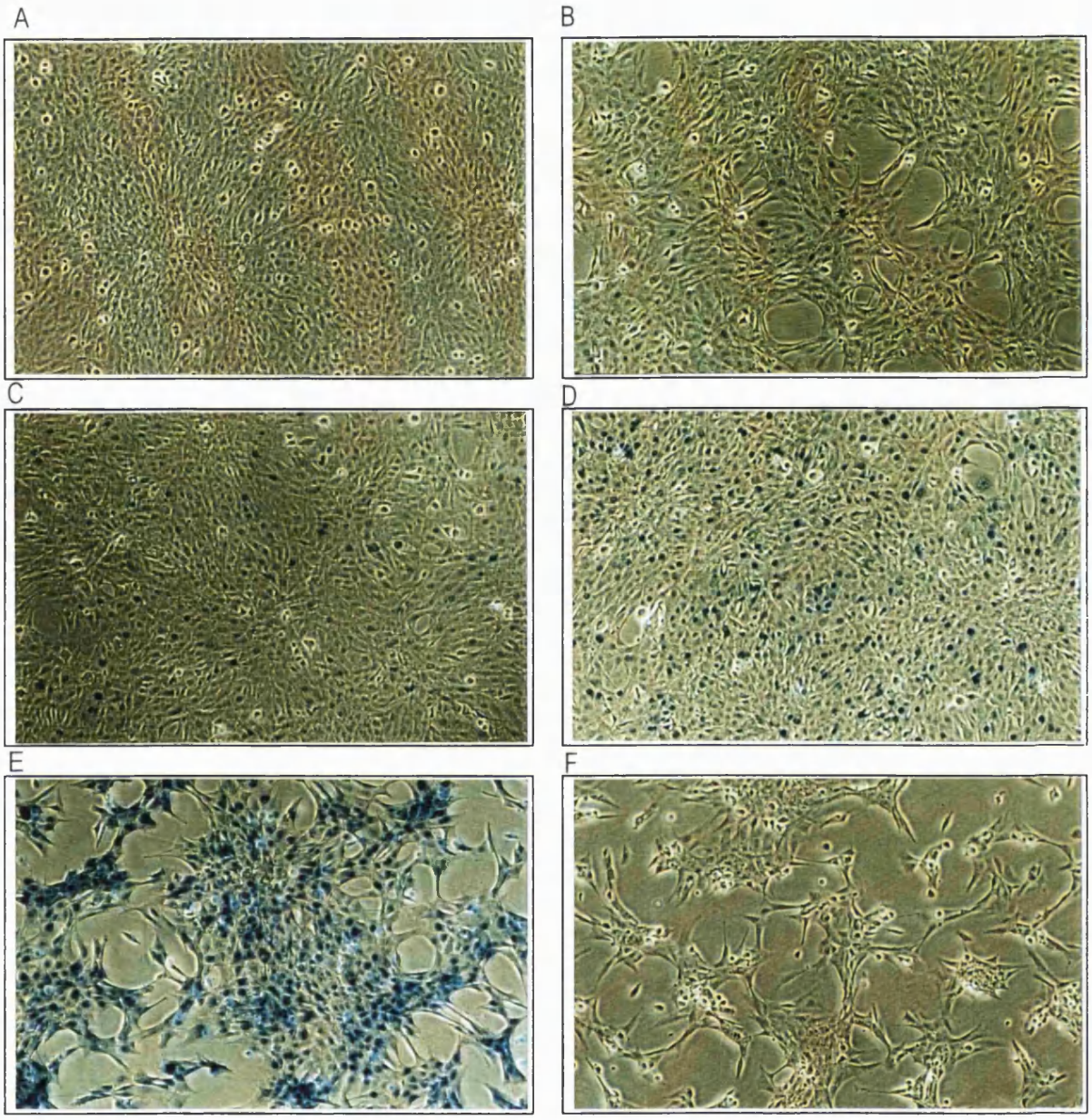


Figure 13 Dose Response/ Toxicity After Infection With Av1.LacZ4.

The photomicrographs above show (A) Control, uninfected cells, and (B)-(E) cells infected with Av1.LacZ4 at MOIs of 3, 30, $3x10^2$, and $3x10^3$, and (F) Cells infected with Av1.null at MOI $3x10^3$. Cells were fixed and stained with X-Gal chromagen 48 hours after infection. The higher multiplicities of infection clearly demonstrate toxic effects such as rounding up of the cells and detachment from the plate.

4.3.2 Relationship Between Transgene Expression And Multiplicity Of Infection.

Figure 14 and Figure 15 show the percentage transgene expression in cells infected at different multiplicities of infection in the SV40 transformed smooth muscle cells and the primary cells. The number of blue cells counted as a percentage of the total cells sampled was used to quantify transgene expression. The mean of three experiments is shown, and the standard error of the observations is shown in each case. There is a clear titre to response relationship between transgene expression and multiplicity of infection. The greatest increase in blue cells appeared from an MOI of 50 to 100 in the smooth muscle cell line. In the primary cultures a substantial increase in transgene expression was seen at MOI's greater than 6.25, but results at MOI 12.5 were highly variable. In the primary cells there was a highly variable response to infection at MOI 12.5 but a progressive increase in transgene expression with increasing MOI thereafter, with 80% of the cells expressing the transgene at MOI of 200. Photomicrographs of the cell monolayers did not reveal overt toxicity but some increase in vacuolation of the cells was seen at MOI of 200 (see Figure 16 and Figure 17).

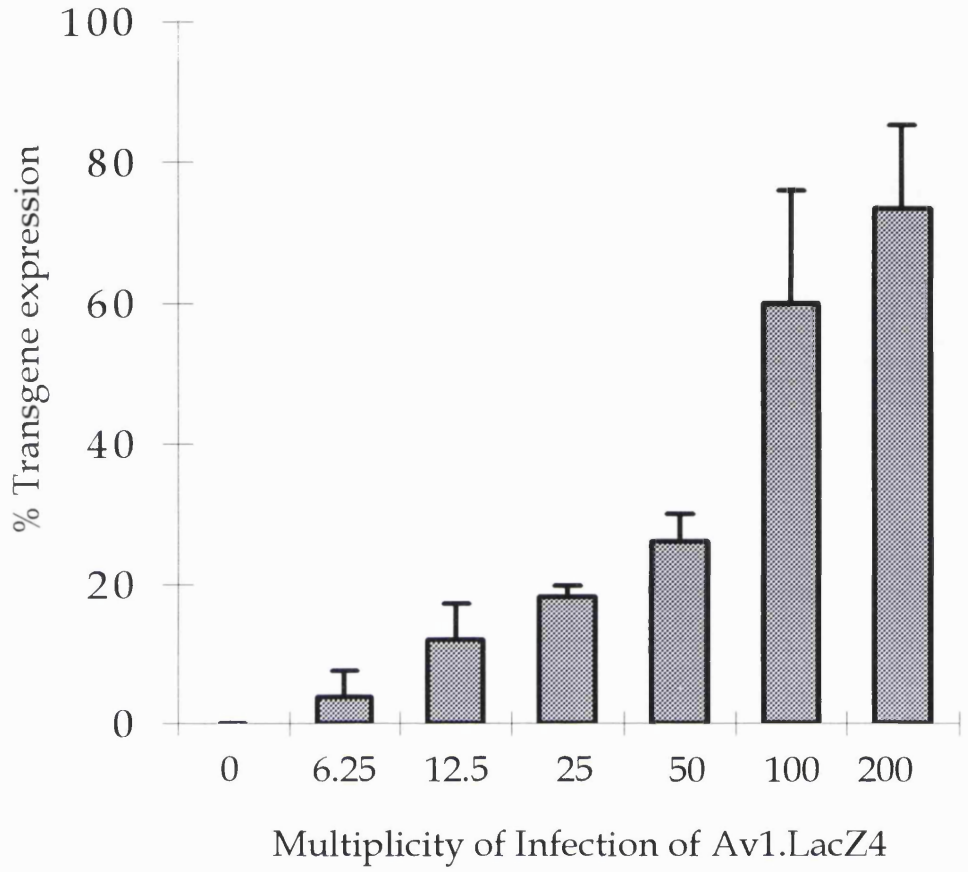


Figure 14 Transgene Expression With Av1.LacZ4 in SV40 Transformed VSMC Line After Infection at MOI 0-200.

The mean +SEM of three experiments are shown. Cells were infected with the concentration of virus indicated and fixed and stained with the chromagen X-Gal 48 hours later.

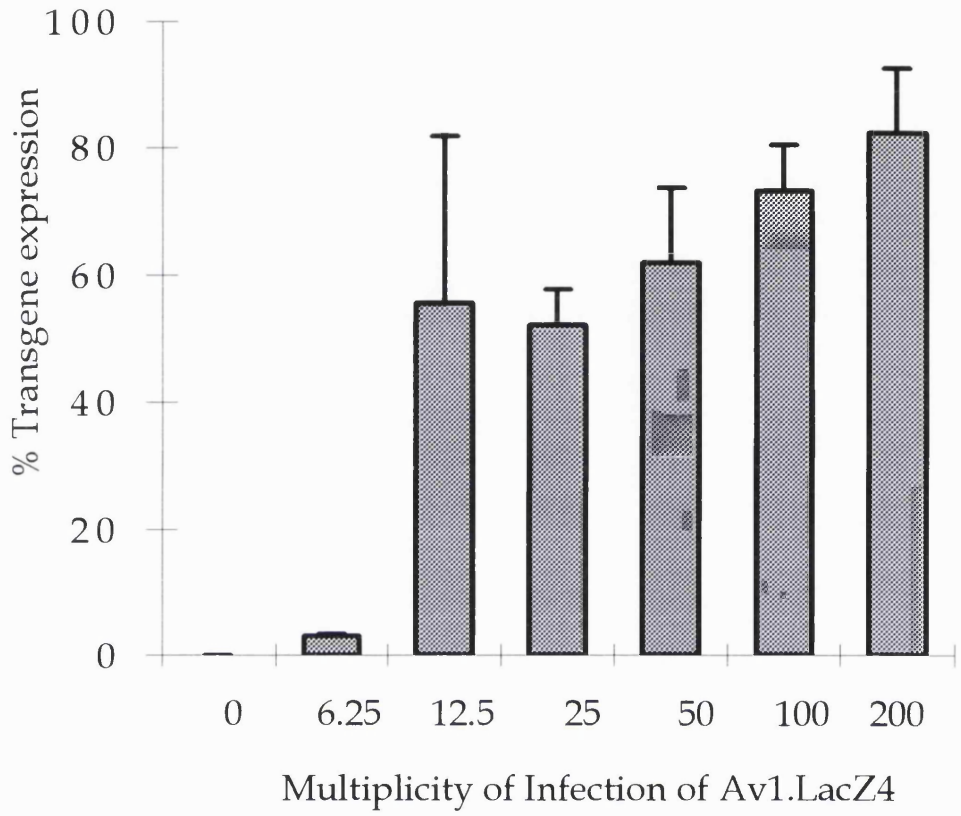


Figure 15 Transgene Expression With Av1.LacZ4 in Primary Cultured Vascular Smooth Muscle Cells After Infection at MOI 0-200.

The mean +SEM of three experiments are shown. Cells were infected with the concentration of virus indicated and fixed and stained with the chromagen X-Gal 48 hours later.

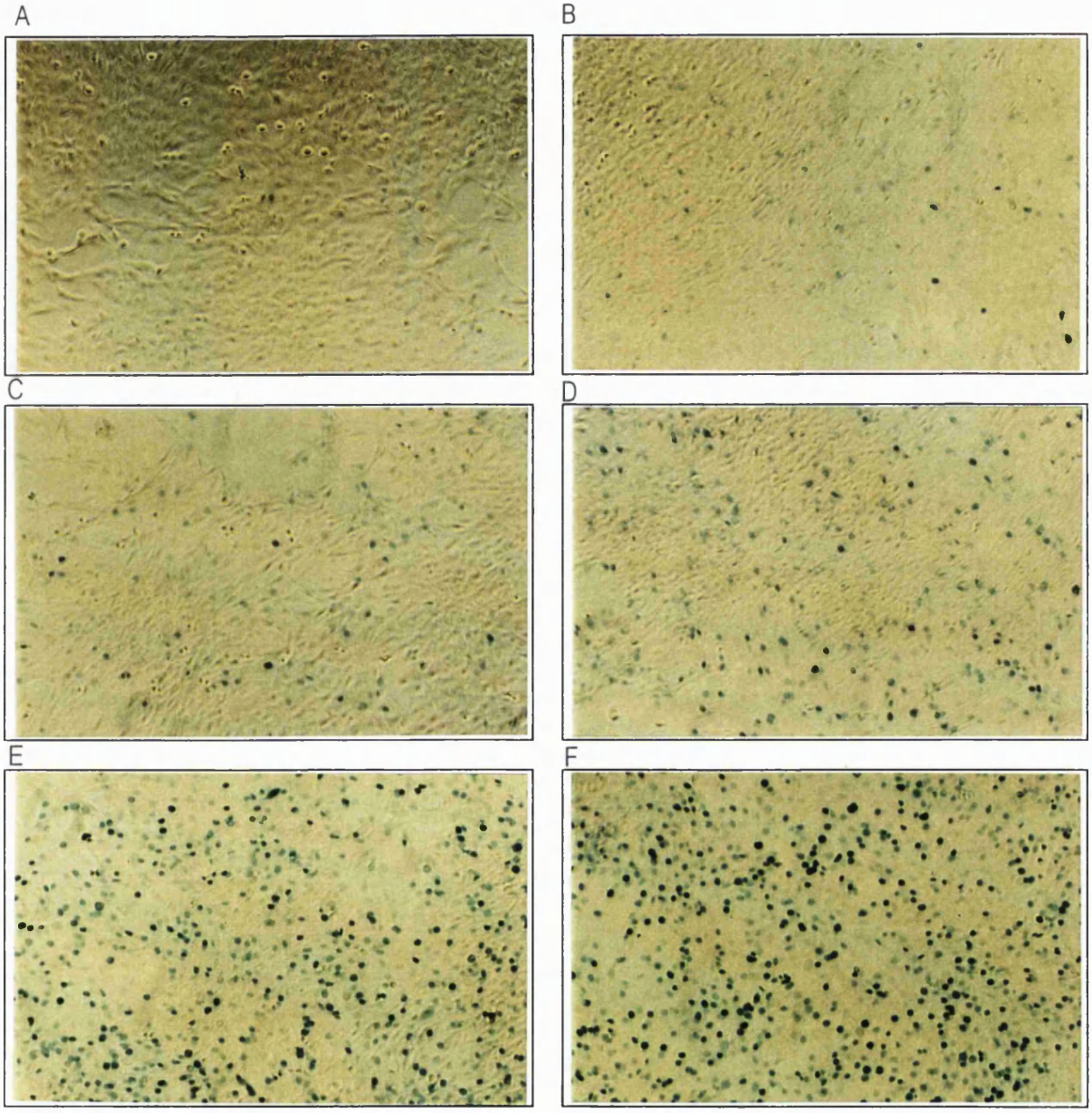


Figure 16 Photomicrographs of SV40 Transformed Rat VSMC Line Infected With Av1.LacZ4 at MOI 6.25-200.

The photomicrographs above show (A)-(F) cells infected with Av1.LacZ4 at MOIs of 6.25, 12.5, 25, 50, 100, and 200 respectively. Cells were fixed and stained with X-Gal chromagen 48 hours after infection. (x40)

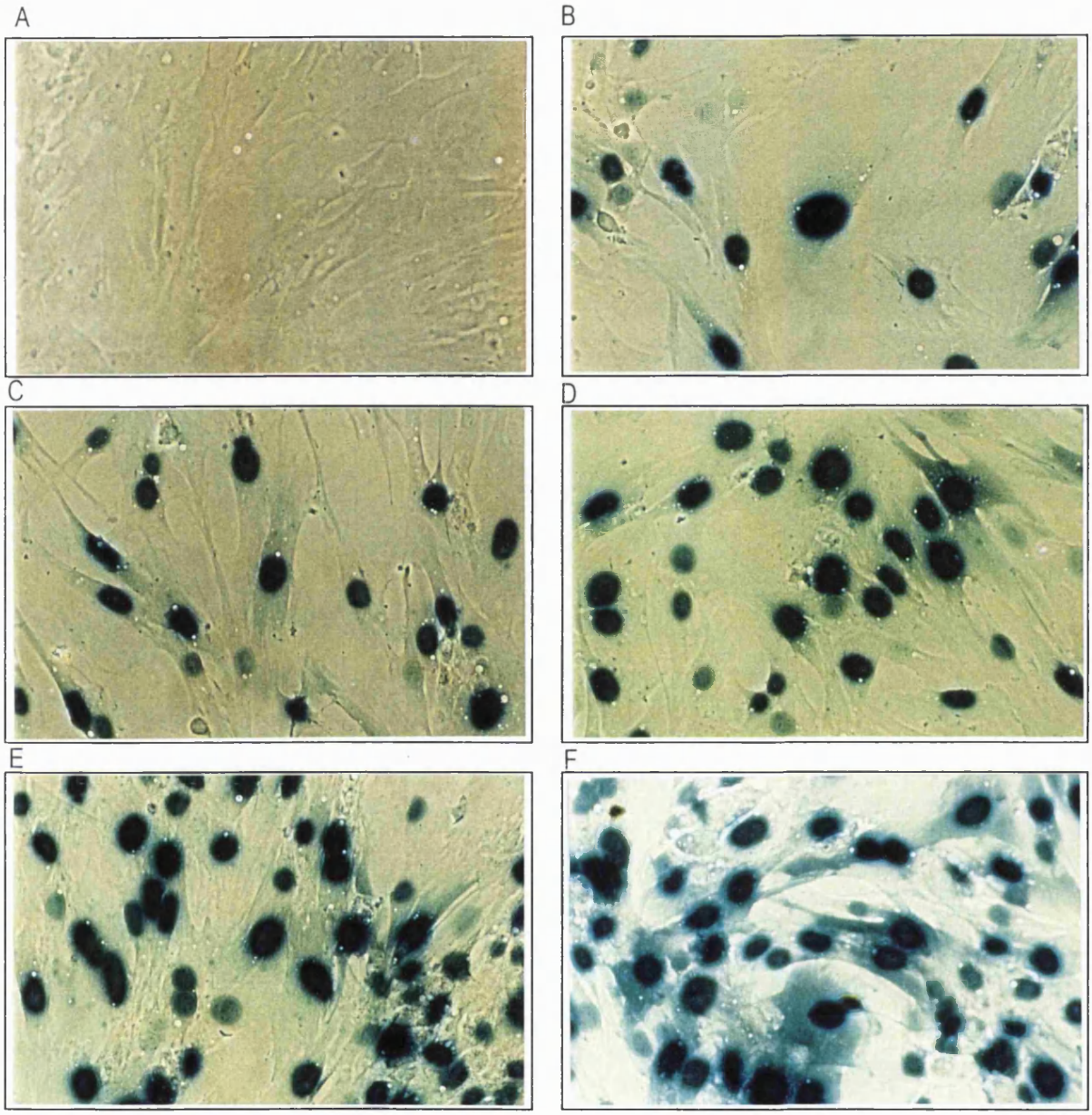


Figure 17 Photomicrographs of Primary Cultured Rat VSMCs Infected With Av1.LacZ4 at MOI 0-200.

The photomicrographs above show (A)-(F) cells infected with Av1.LacZ4 at MOIs of 6.25, 12.5, 25, 50, 100, and 200 respectively. Cells were fixed and stained with X-Gal chromagen 48 hours after infection. At higher MOIs the pigmented product of the X-Gal reaction is seen to leak out of the nucleus into the cytoplasm of the cells. (x100)

4.3.3 Relationship Between Transgene Expression And Time Of Exposure To Virus

The mean percentage transgene expression and time of exposure to virus are shown in Figure 18 and Figure 19. Figure 20 shows photomicrographs of the infected primary cells. A progressive increase in mean percentage transgene expression was seen with time, and no toxicity was observed in the monolayers. Near maximal transgene expression was seen at all times of 30 minutes or greater. In both cell types a greater percentage of blue cells was seen with 30 minutes of virus infection time than at 45 or 60 minutes.

4.3.4 Duration Of Transgene Expression After Optimized Virus Infection

The results are shown in Figure 21. Transgene expression was consistent with the results above at 48 hours but fell progressively with time, until approximately 5-10% of cells were expressing the transgene at 28 days.

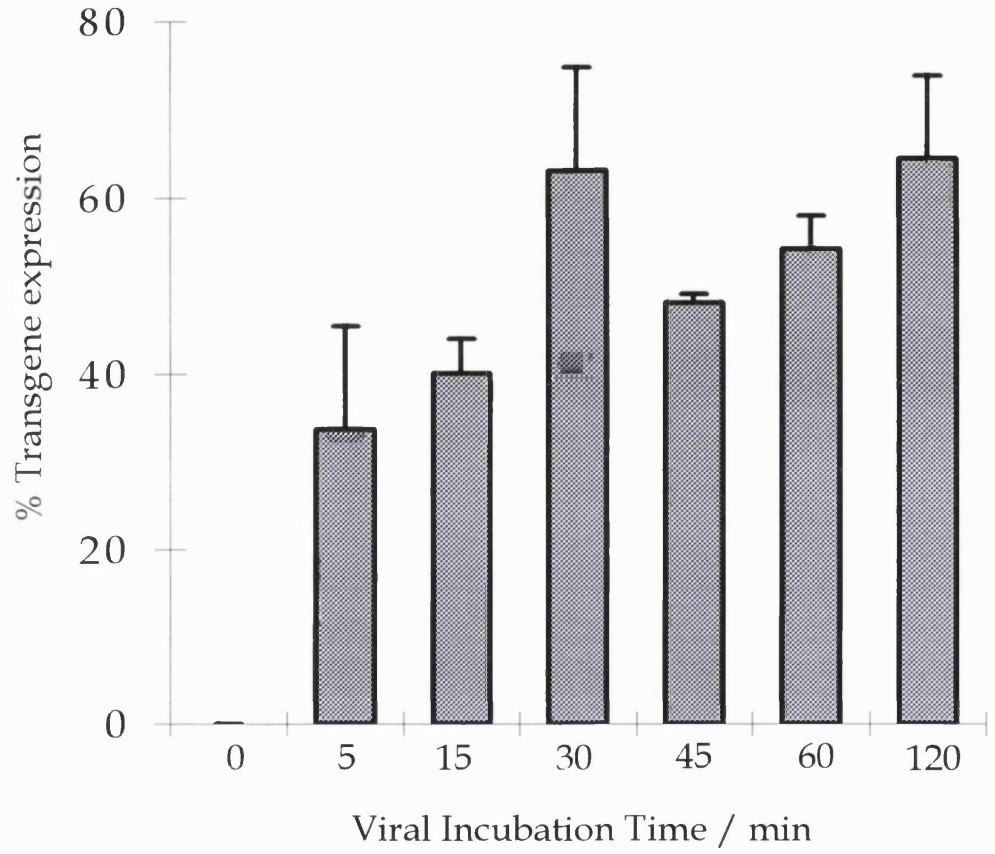


Figure 18 Transgene Expression With Av1.LacZ4 in SV40 Transformed VSMC Line After Viral Incubation for 0-120 Minutes

The mean +SEM of three experiments are shown. Cells were incubated with Av1.LacZ4 for the times indicated and 48 hours later, stained with the chromagen X-Gal.

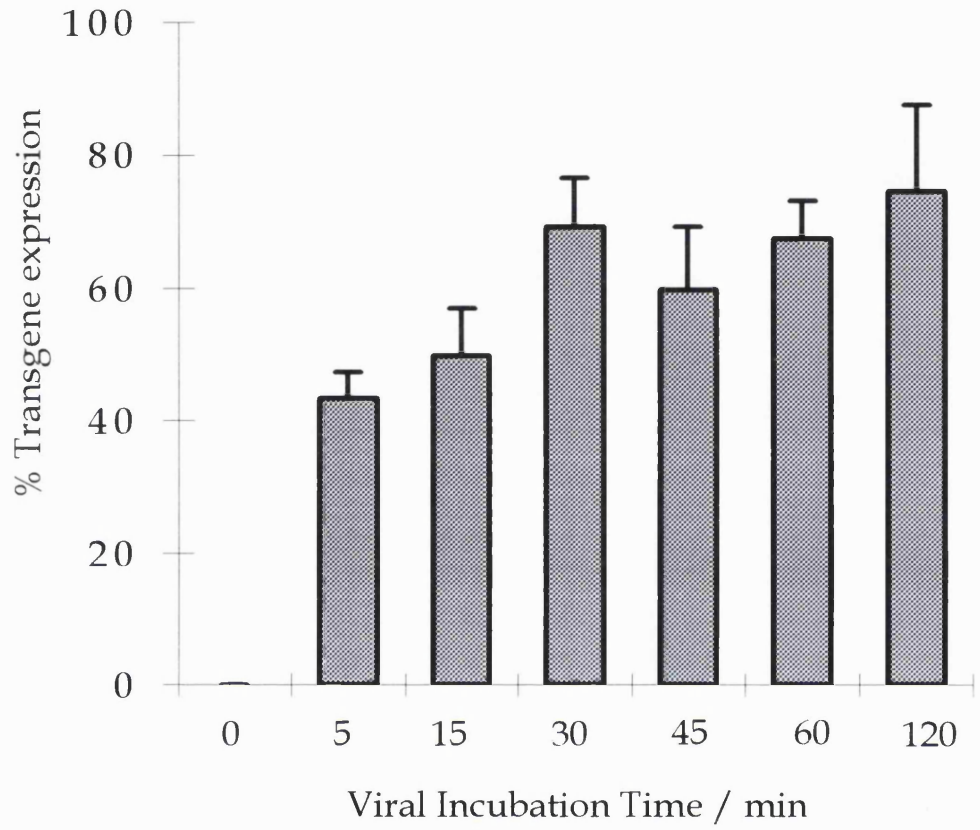


Figure 19 Transgene Expression With Av1.LacZ4 in Primary Cultured VSMCs After Viral Incubation for 0-120 Minutes

The mean +SEM of three experiments are shown. Cells were incubated with Av1.LacZ4 for the times indicated and 48 hours later, stained with the chromagen X-Gal.

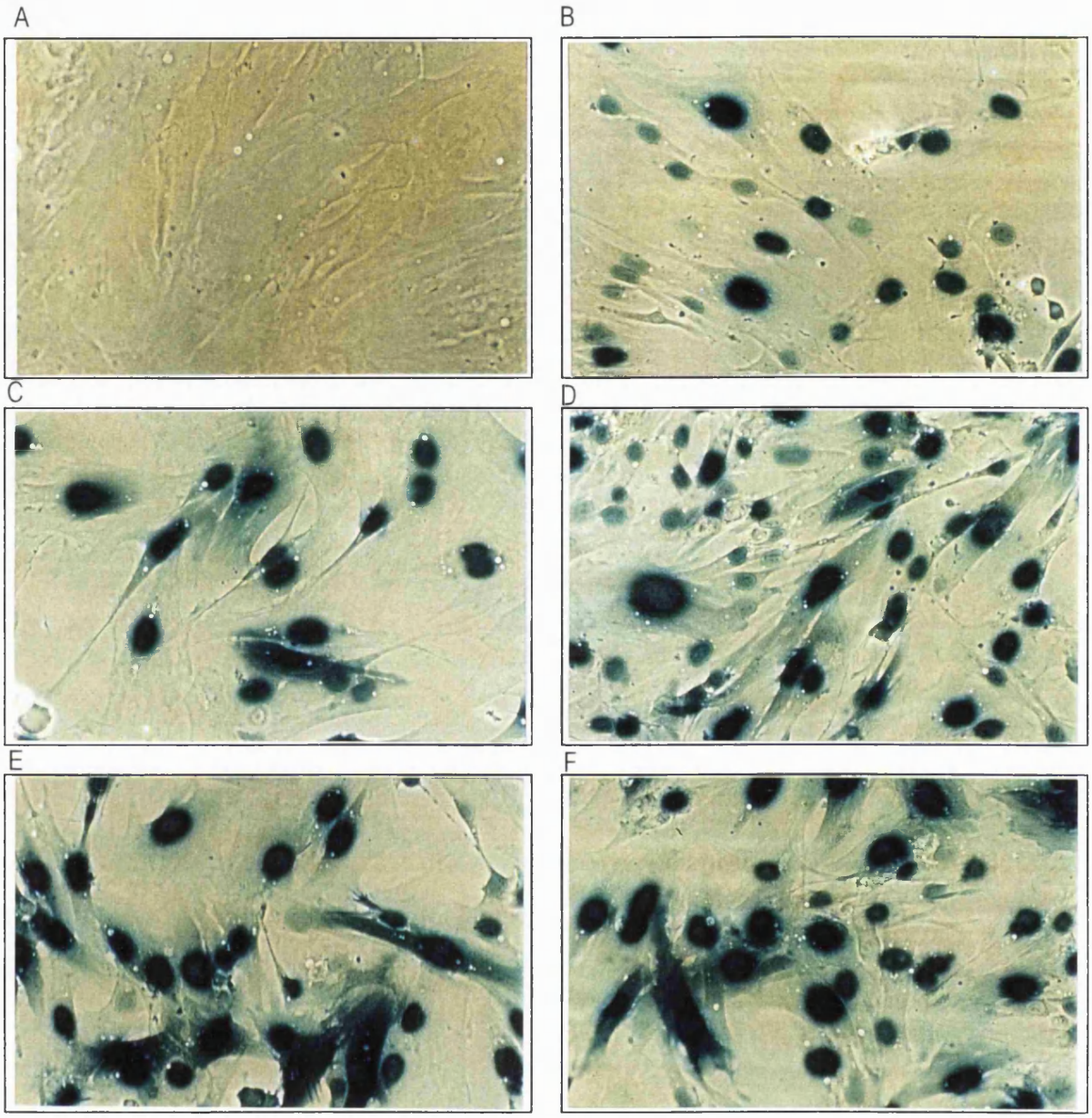


Figure 20 Photomicrographs of Primary Cultured Vascular Smooth Muscle Cells After Incubation With Av1.LacZ4 for 0-120 Minutes.

The photomicrographs above show (A)-(F) cells incubated with Av1.LacZ4 for 0, 5, 15, 30, 45, 60, and 120 minutes respectively. Cells were fixed and stained with X-Gal chromagen 48 hours after infection. (x100)

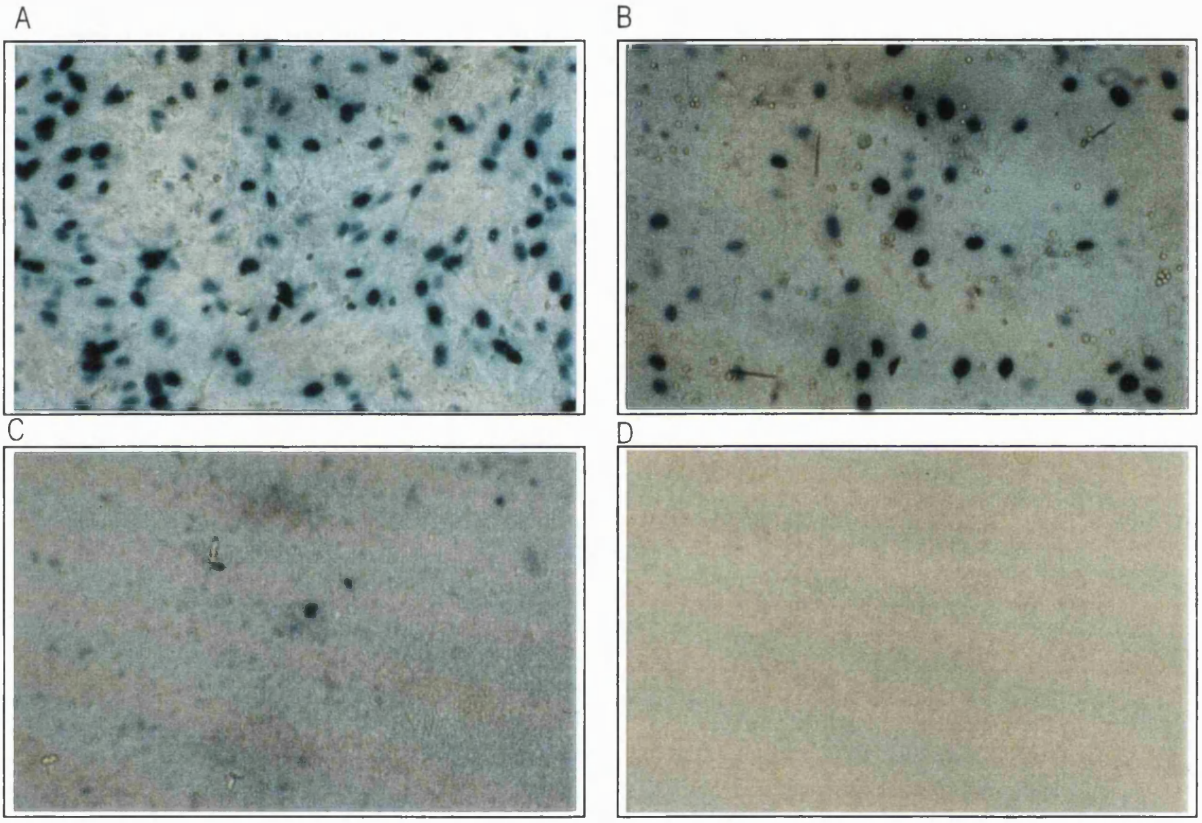


Figure 21 Photomicrographs Showing The Duration Of Transgene Expression 2, 14, And 28 Days After Infection With Av1.LacZ4

The photomicrographs (A)-(C) above show cells infected with Av1.LacZ4 at MOIs of 100 for 30 minutes. Cells were fixed and stained with X-Gal chromagen two days (A), 14 days (B), and 28 days after infection. Panel D shows cells 28 days after sham infection procedure with a solution containing no virus. (x40)

4.4 Discussion

4.4.1 Adenoviral Propagation And Purification

The techniques described allow propagation and purification of high titre stocks of virus. The plaque assays were reproducible within the triplicates in any one assay but showed a significant degree of variability between assays on the same batch of virus. This may be to be due to the adherence of virus to plastic ware, such as pipette tips which can vary with the speed of the operator. Despite standardizing pipetting techniques this variability could not be completely eliminated and may reflect the complex biological events required for plaque formation. The difficulties of accurate plaque titration of viruses has been noted by others (Mittereder *et al.* 1996). For all subsequent experiments a large volume of virus was grown. Where comparisons were made the control virus and active virus were assayed together in a single experiment. A single stock of virus was then used for all the experiments.

4.4.2 In Vitro Toxicity Of Adenovirus In VSMCs

In this preliminary experiment MOIs of 3 to $3x10^3$ were used. An MOI of $3x10^3$ and above caused toxicity in smooth muscle cells. This toxicity occurred at slightly lower MOI than published by another group using the same virus and primary smooth muscle cells (Lee *et al.* 1993). In their study an MOI of $5x10^4$ caused toxicity while maximal (approximately 80%) transgene expression was seen at an MOI of 5×10^3 . The difference observed may reflect the different cell types (primary compared with an SV40 transformed cell line), but the results also highlight the difficulty of accurate titration of adenoviruses. Plaque assays most accurately reflect infectivity of the virus, but results from identical protocols can vary up to ten fold, when titration of the same virus is carried out by different

individuals or at different times. Spectrometry, to determine DNA content, or electron microscopy, to count particles, are alternatives but neither reflect the activity of the virus.

The work of Clesham *et al* has shown that comparing viruses with different promoters is problematic, as variations may reflect the properties of the promoter rather than the vector or receiving cell type (Clesham *et al.* 1996). They studied the expression of ß Galactosidase in VSMC following adenoviral gene transfer using a vector containing the human cytomegalovirus immediate early promoter (CMV-IEP), and another with the RSV- long terminal repeat (RSV-LTR) (Clesham *et al.* 1996). Both the RSV-LTR and CMV-IEP are regarded as being constitutively active in many cell types because they use a number of different cellular transcription factors. It has been shown, however, that stimulation of enhancer elements within the CMV-IEP can increase transgene expression after infection with a CMV-ß-Galactosdase adenoviral vector, suggesting that there is limited constitutive expression from this promoter (Clesham *et al.* 1996). In these studies it has been suggested that low level transgene expression could, therefore, reflect failure of transcription rather then gene transfer. Comparison of results is likely to be further hampered by the possibility that adenoviral infection itself may enhance the CMV-IEP activity, via an NF-kB dependent mechanism. RSV-LTR dependent transgene expression is not influenced by phorbol or forskolin in VSMCs which stimulate NF-kB and CREB respectively (Clesham *et al.* 1996). Clesham *et al* found that constitutive expression from the RSV-LTR was lower than with the CMV-IEP and have, therefore, hypothesized that it may also have limited activity in vascular smooth muscle cells. To date there is no evidence to suggest that the activity of the RSV-LTR is altered by viral infection.

No endogenous ß-Galactosidase activity was seen in the control cells in this experiment or those infected with the null virus, suggesting that all blue colour seen was due to transgene expression. Despite the nuclear localization signal used to aid differentiation of the transgene from endogenous ß-Galactosidase, known to exist in smooth muscle cells

(Guzman *et al.* 1993; Lim *et al.* 1991; Flugelman *et al.* 1992),some cytoplasmic staining in the bluest cells was seen. The cytoplasmic staining is likely to represent leakage of the blue pigment out of the nucleus along a concentration gradient, when multiple copies of the transgene are expressed in a single cell infected by more than one virion. A maximum MOI of 200 was used in all subsequent experiments because of the toxic response observed at high titre.

4.4.3 Relationship Between Transgene Expression And Multiplicity Of Infection

These results suggest that 60% of cells in this smooth muscle cell line will express the transgene after 1 hour of incubation with 100 plaque forming units of virus per cell. Observing the monolayers under light microscopy suggests that the transgene expression may in fact be slightly higher than this, but as stated, this technique is subject to selection bias and the non-blue nuclei are difficult to quantify even under high power magnification. Using the trypsinisation technique, blue cells were seen to adhere to plastics such as pipette tips when they were resuspended prior to X-Gal incubation and transferred to the haemocytometer. It is thus also possible that selective loss of transgene positive cells during pipetting may have caused the discrepancy between the photomicrograph appearance and the quantification technique. It is unlikely, however, that non-blue cells would have been visible aggregating in a pipette tip, so the apparent loss of blue cells may represent selection bias. Overall these results suggest, that an MOI of 100 is the optimal 'dose' of virus in these SV40 transformed cells to achieve maximal transgene expression and minimal toxicity.

Transgene expression was greater in primary cultured cells than in the vascular smooth muscle cell line, particularly at lower MOIs. These results were the mean of three separate experiments on different cell preparations. Using a Student's t test the results at MOI 100-200 did not achieve a

statistically significant difference between primary and SV40 transformed cells.

These results can be compared with the study of Lee *et al* who, using primary cultured VSMCs, found only 30.4 +/- 5.6% of cells stained positively for ß-Galactosidase at an MOI of 5 x 10^3 (Lee *et al*. 1993). A number of differences may explain this lower level of transgene expression. Lee *et al* used the same trypsinisation technique to quantify their results, but infected their cells at an MOI of 5 x 10^3 for 90 minutes in media containing 2% fetal calf serum (Lee *et al*. 1993). It is thus possible that some virions were inactivated by antibodies present in the serum. The cells used by Lee *et al* were never deprived of serum to render them quiescent while the cells in this study were quiescent. Guzman *et al*, however, used a first generation RSV-ß-Gal virus to infect smooth muscle cells and saw no difference between quiescent and proliferating cells (Guzman *et al*. 1993). Interestingly, their results more closely parallel those presented here, with 70% positive cells using an MOI of 100 and an incubation time of 60 minutes (Guzman *et al*. 1993).

The results presented in this thesis showed lower levels of transgene expression in the experiments where the trypsinisation technique was used for quantitation than might be expected from the photomicrographs. In one recent report where transgene expression was quantified by microscopy, a first generation adenovirus with a CMV-LacZ expression cassette caused 100% positive cells at an MOI of 200 (Baker *et al*. 1996). Two other techniques of quantifying transgene expression have been adopted. Enzyme-linked immunosorbent assays have shown 610 pg or 40 µU of ß-Galactosidase per 10^3 transduced cells (Lee *et al*. 1993). Chemiluminescent assays of ß-Galactosidase activity are also available, but this method, while accurate, only reflects total blue pigment produced by ß-Galactosidase and does not give any information about the amount of pigment in each cell.

In both SV40 transformed and primary cells, high levels of transgene expression were achieved with MOIs of 100 without significant toxicity, and therefore this titre was used in future experiments.

4.4.4 Relationship Between Transgene Expression And Time Of Exposure To Virus

The time of exposure of a cell to virus may limit the entry of the virus into the cell if it is unable to bind to its receptor, or if only one virion is bound rather than a cell importing multiple virions. The uptake of virions, which is one determinant of the transgene expression, is likely to be subject to fewer limitations *in vitro* than *in vivo,* because antibodies in the serum and other cellular immune responses are not present. In addition, transgene expression *in vivo* may require times of exposure to virus solutions at least as long as those which are optimal *in vitro*.

In this study, percentage transgene expression (estimated as number of blue cells) was 35% and 45% after virus exposure of five minutes in the cell line and primary cells respectively. Optimal transgene expression was seen from 30 minutes of virus exposure onwards in both cell types, with approximately 60% and 70% maximal transgene expression at that time. These results are consistent with other groups (Guzman *et al.* 1993), who showed 80% of maximal transgene expression after a 30 minute incubation time and maximal values at 120 minutes. In one study of human umbilical vein endothelial cells (Lemarchand *et al.* 1993), an adenoviral vector containing an RSV ß-Gal expression cassette gave 75% percent positive cells at 120 minutes and 95% positive cells at 24 hours (MOI 100). Most investigators have not examined the effects of incubation periods longer than 120 minutes as these are unlikely to be practical in the arterial system *in vivo*. The results suggest that 30 minutes is optimal for achieving gene transfer in these cell types.

4.4.5 Duration Of Transgene Expression

The duration of transgene expression is likely to limit the effectiveness of a gene transfer-based therapy. In restenosis, the time of triggering of the pathological response can be accurately pinpointed. The aim of gene transfer is to transiently alter the normal cellular response to injury until re-endothelialization and other cellular events have returned the artery to its physiological state. In the rat model of vascular injury this is likely to take approximately 2 weeks. The duration of transgene expression *in vivo* is governed by the host immune response to both the vector used and the foreign transgene itself (Forough *et al.* 1996a). Duration studies *in vitro*, therefore, offer a limited preliminary insight into transgene duration of expression.

The duration of transgene expression was studied at 2 days, 2 weeks, and 4 weeks. The results suggested that transgene expression could be maintained for 2 weeks at a relatively high level but decreases substantially after that time. This is consistent with the episomal nature of adenoviral gene transfer. The smooth muscle cells were kept for a protracted period in serum free medium without visible change in the condition of the monolayer. Loss of cells due to prolonged serum deprivation cannot be excluded.

CHAPTER 5: *IN VIVO* CHARACTERIZATION OF TRANSGENE EXPRESSION BY AV1.LACZ4 IN THE RAT CAROTID MODEL OF VASCULAR INJURY.

5.1 INTRODUCTION

A number of different gene transfer techniques have been used *in vivo* to deliver genes to smooth muscle cells in the arterial wall. Some have employed delivery devices that deliver the vector under pressure while others isolated segments of the lumen to allow a prolonged dwell time of the virus with the smooth muscle cells. In the rat carotid model, the small size of the artery has limited delivery techniques to luminal dwell in surgically isolated segments of artery, and adventitial applications in pluronic gel or collars that enclose the artery. In the studies described in this thesis the luminal dwell technique was employed. The experiments described below were used both to optimize gene delivery and eliminate unwanted side effects of the delivery procedure such as arterial thrombosis. The effect of the components of the optimized technique on lesion size in this model were assessed and the reproducibility of the lesion confirmed. The techniques described have been published, with minor variations, by a number of authors but were new to this laboratory at the start of this project (Lee *et al.* 1993; Guzman *et al.* 1993). It was the intention of these experiments to validate these techniques in my hands, rather than to develop novel delivery procedures for gene delivery, which would have necessitated usage of larger numbers of animals.

5.2. METHODS

5.2.1 Intraluminal Delivery Of Av1.LacZ4

The left carotid artery of 300-350g male Wistar rats was balloon injured as described on page 52 and illustrated in Figure 5. After balloon injury, a segment of artery approximately 1.5 cm long was isolated using 2 arterial clamps, one placed on the proximal common carotid artery and the other on the proximal internal carotid artery (see Figure 22). A Portex cannula (2 French gauge) was inserted into the external carotid artery using the same arteriotomy that was used to insert the balloon catheter. The artery was flushed with saline to remove any remaining blood. For each rat a volume of 100 µl of infection solution was instilled into the artery, using a catheter inserted in the artery via the external carotid arteriotomy. 50µl of Av1.LacZ4 containing 5 x 10^8 pfu and 50 ml of RPMI medium was used in three rats and 100 µl of RPMI used in the control rat. The catheter was secured using a 3-0 silk suture and the artery was distended by the infusate for the duration of the instillation. 20 minutes of luminal dwell time was used, after which the vector-containing solution was aspirated and the external carotid artery ligated. The arterial clamps were then removed and the circulation restored through the common and internal carotid arteries. The animal was then allowed to recover and the tissue harvested 48 hours later.

The rats were anaesthetized prior to exsanguination and fixation using the retrograde perfusion technique described on page 56. 2% formaldehyde, 0.2% glutaraldehyde in PBS was used as a fixative and after a 10 minute *in situ* perfusion period the artery was washed extensively in PBS and placed in X-Gal solution overnight at 32°C. The artery was then transferred to 2% formaldehyde, 0.2% glutaraldehyde in PBS for 18-24 hours to complete fixation. It was then paraffin embedded, sections taken

at 100μm intervals through the entire artery, and stained with nuclear fast red (see Appendix 1 Histological Staining Protocols.)

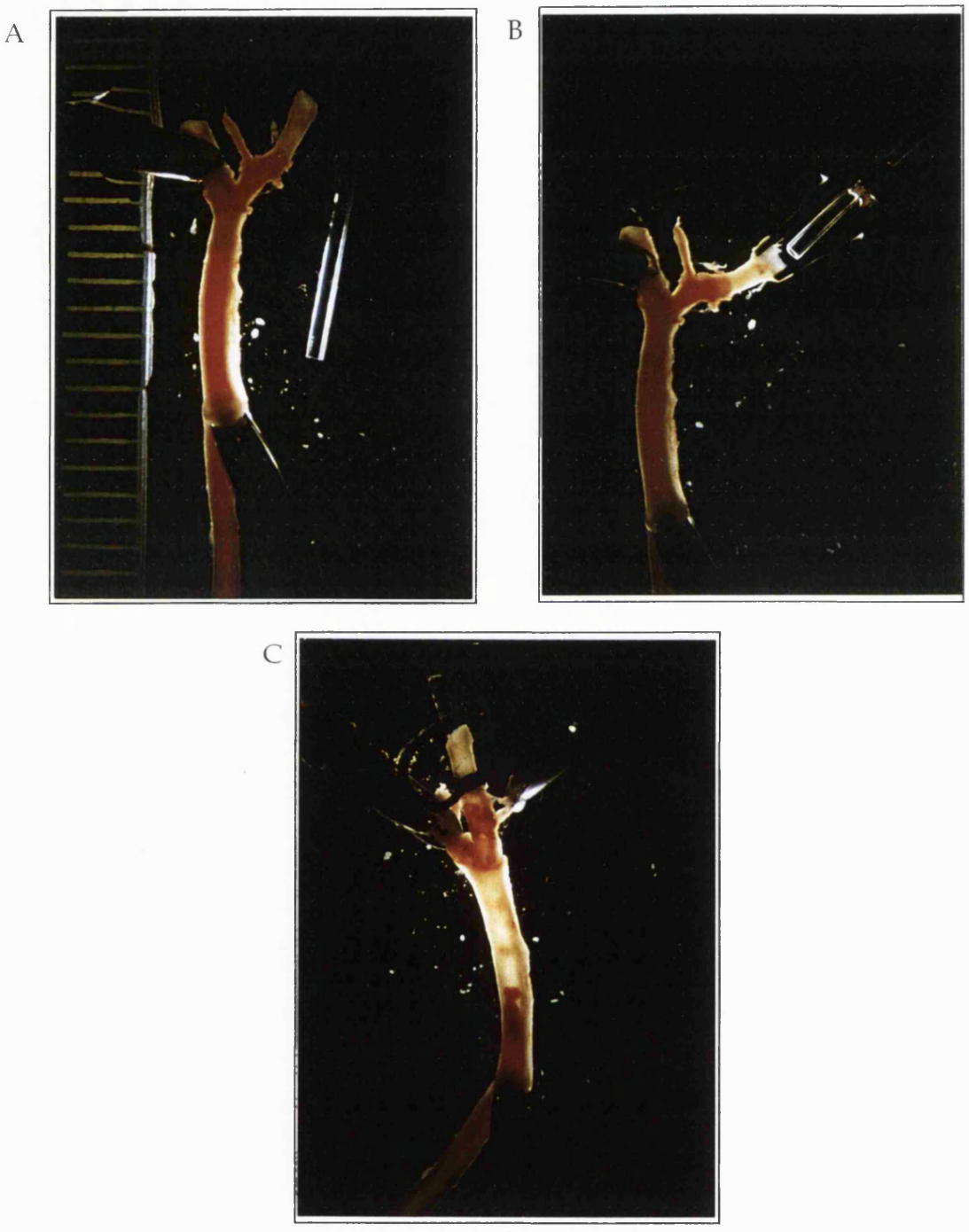


Figure 22 Rat Carotid Artery Virus Delivery Technique.

Panel A shows a rat carotid artery removed from the animal and photographed under dark ground microscopy. Clamps have been placed on the internal carotid artery distally (top) and 1 cm below on the common carotid. Panel B shows a Portex Cannula being introduced into the isolated segment of common carotid artery via the external carotid between the the tips of fine curved forceps inserted into an arteriotomy in the external carotid. Panel C illustrates the cannula secured in position with all side branches ligated. Virus or control solution has been instilled into the isolated segment of the common carotid artery as shown. It is allowed to dwell for 20 minutes within the lumen before being aspirated and the external carotid ligated before restoring flow in the common carotid artery.

5.2.2 Optimization Of Viral Titre And Delivery Procedure.

In five rats the rat common carotid arteries were balloon injured as described in the previous method. After balloon injury of the artery and before the luminal dwell of virus/control solution a systemic dose of anticoagulant was administered. The right internal jugular vein was exposed by blunt dissection, a distal suture was placed around the vein and tied off and a loose suture placed proximally. A small incision was made in the vein and a Portex cannula (2 French gauge) inserted. Heparin 100 iu/kg in 100µl of normal saline was given and the cannula was withdrawn. The vein was then ligated proximally and the virus or control solution instilled into the common carotid artery as described above. Two rats received 5x 10^8 pfu of Av1.LacZ4 and two received 5x 10^7 pfu of Av1.LacZ4, in both cases the virus was diluted to a final volume of 100µl with RPMI. One rat received 100 µl of RPMI only. Tissue was harvested and prepared as described in the previous section.

5.2.3 Reproducibility Of Carotid Injury After Balloon, Balloon And Systemic Heparinisation And Sham Intraluminal Delivery

Five rats were used in each reproducibility experiment. In the first group simple balloon injury as described above (page 52) was carried out. This is shown schematically in Figure 23 panels 1-3. In the second group a systemic dose of anticoagulant was administered via the internal jugular vein, and 100µl of RPMI instilled into the common carotid artery using the luminal dwell technique (Figure 23, panels 4-6). Arteries were harvested 14 days after surgery and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS by retrograde perfusion at 120 mm Hg. In the balloon only rats from group one, 5 mm of the mid section of the common carotid artery was embedded and sectioned. Figure 23 shows the sampling sites in the sham delivery group and in the balloon plus heparin group.

A sample was taken from the distal section which had been ballooned and exposed to the sham delivery procedure. A second sample was taken in the proximal portion of the artery 5mm distal to the aortic arch, i.e. not within the segment of artery that had been distended with RPMI. Sections were cut from each block and stained with Haematoxylin and Eosin prior to morphometry.

5.2.4 Duration Of Gene Expression In Vivo

In order to assess the duration of gene delivery *in vivo* four rats were subjected to balloon injury followed by adenoviral gene delivery of 5 x 10^8pfu of Av1.LacZ4 using the twenty minute luminal dwell technique. All received peroperative systemic heparin via the internal jugular vein. Tissue from two rats was harvested at two days after injury and gene delivery and from the others fourteen days after gene delivery. The tissue was incubated in X-Gal prior to sectioning as described above.

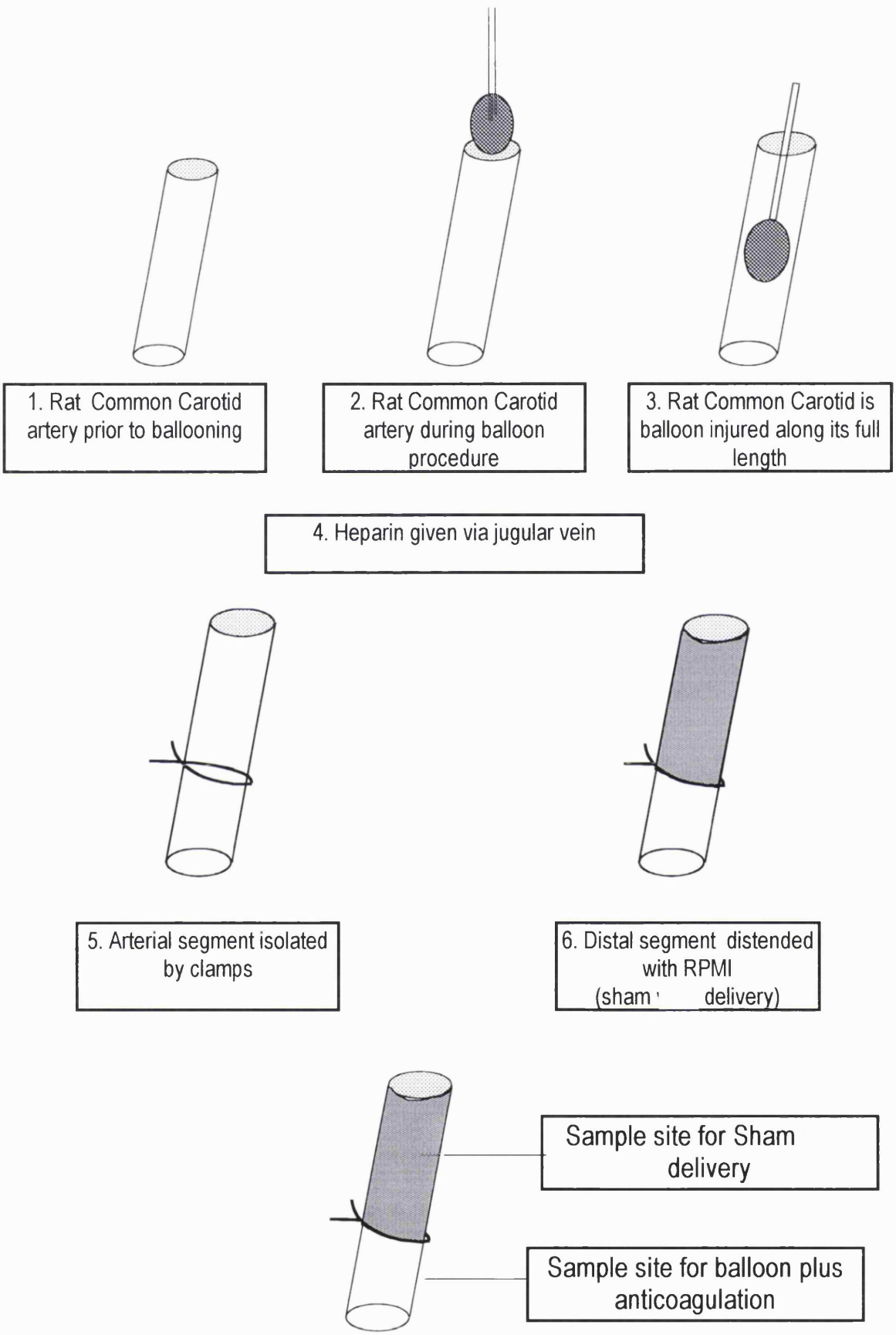


Figure 23 Schematic Representation Of Methods And Sample Sites For Reproducibility Experiment

5.3 RESULTS

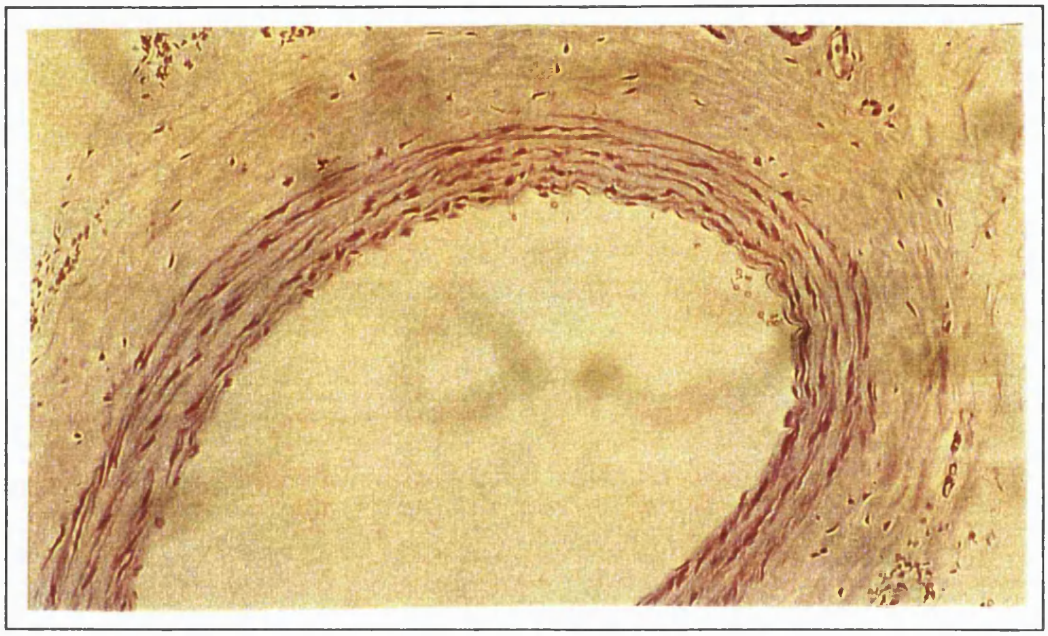
5.3.1 In Vivo Gene Delivery Of By Av1.LacZ4 Using Luminal Dwell.

Transgene expression was seen in the arteries of all rats that received virus and none was seen in the control artery (see Figure 24). Isolated islands of blue staining cells were seen under light microscopy. All left carotid arteries had thrombosed while the right carotid arteries remained patent.

5.3.2 Optimization Of Viral Titre And Delivery Procedure

Using this technique no arteries thrombosed. One rat had a small haematoma observed in the anterior aspect of the neck at the time of collection of the arteries, but no significant haemorrhage occurred. X-Gal staining results in whole arteries and sections are shown for one animal which received 5x 10^8 pfu of Av1.LacZ4 and one which received 5x 10^7 pfu of Av1.LacZ4 in Figure 25. No blue cells were seen in the rat which received RPMI only while there was an improvement in gene transfer with 5x10^8 pfu of virus compared with 5x10^7 pfu of at least 10 fold. At both doses, results were similar in the duplicate animals.

B



A

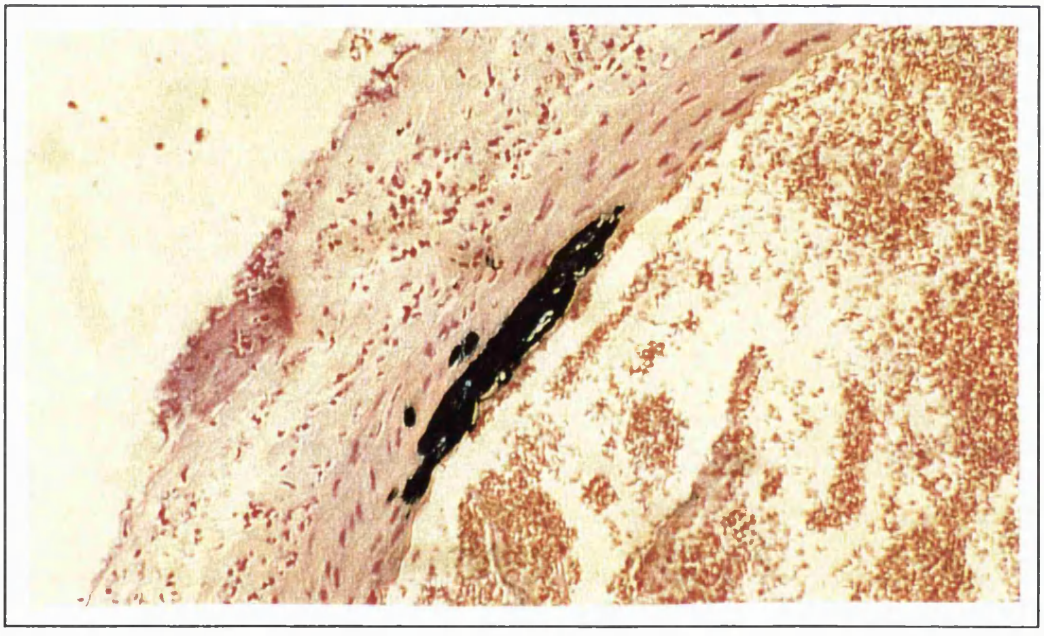


Figure 24 *In Vivo* Gene Transfer to the Rat Carotid Artery.

Panel A shows a section of rat carotid 2 days after balloon injury and *in vivo* delivery of Av1.LacZ4. The artery has been fixed and stained in X-Gal. Blue staining is seen in the media of the artery indicating successful transgene expression but extensive thrombus is seen in the lumen. Panel B shows a control artery which received RPMI in place of the viral solution.

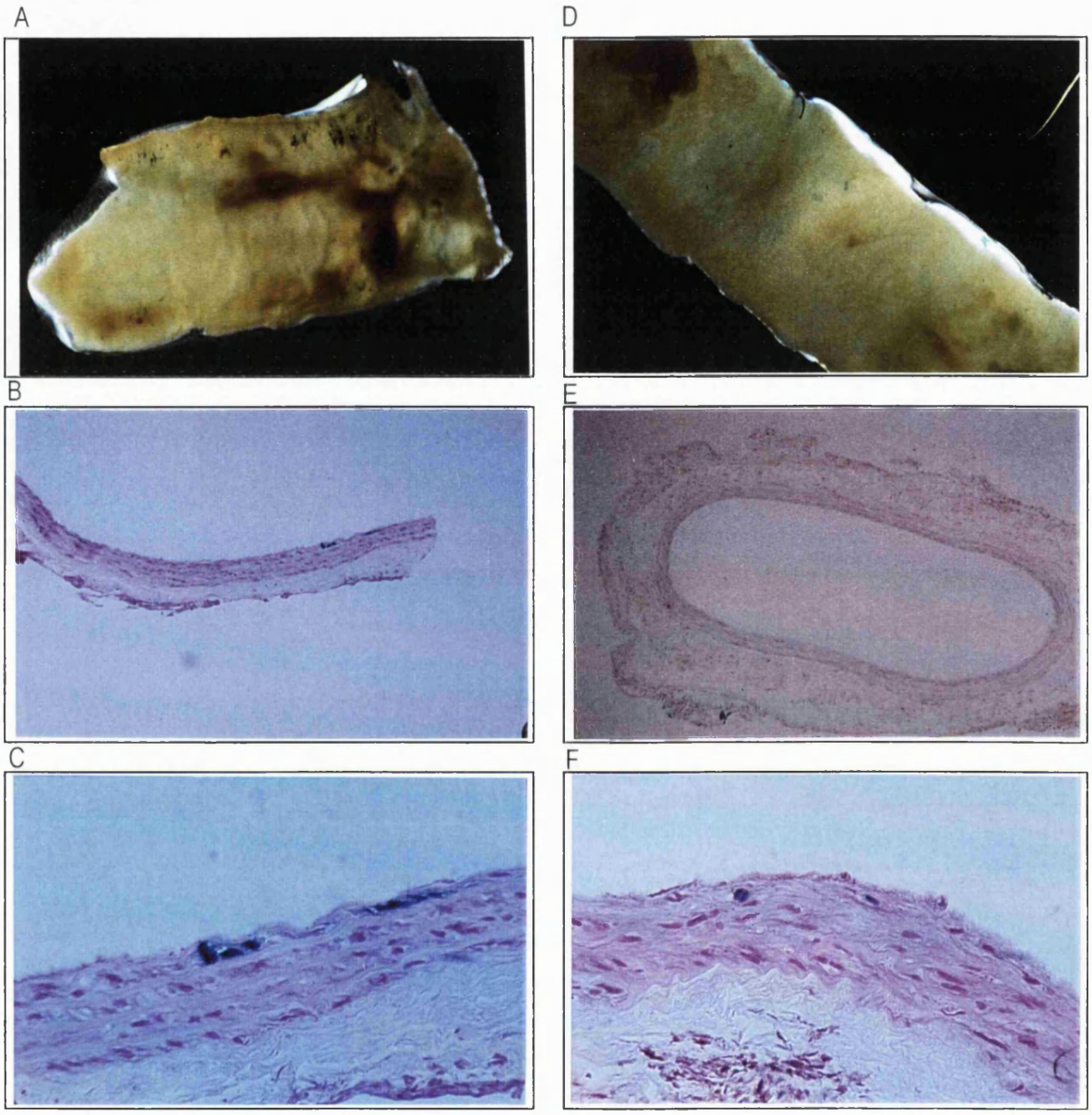


Figure 25 *In Vivo* Av1.LacZ4 Dose Response.

Panels A-C show a rat carotid artery which received $5X10^8$pfu of Av1.LacZ4 virus after balloon injury photographed under a dissecting microscope, and at magnifications of x40 and x200 respectively. The artery was harvested 48 hours later and fixed and stained in X-Gal. Panels D-E show comparative photographs of an artery from an animal which received $5X10^7$pfu of Av1.LacZ4. Transgene expression is clearly greater using the higher titre of virus.

5.3.3 Reproducibility Of Carotid Injury After Balloon, Balloon And Systemic Heparinisation And Sham Intraluminal Delivery Of Virus

The mean intimal area mm^2 and the mean intimal/medial ratio are shown in Figure 26. The intimal area was significantly higher in the balloon injury only group (0.21 +/-0.02) than in the group receiving heparin and balloon injury or the sham delivery procedure (0.13 +/-0.02 and 0.12 +/-0.04 respectively, p = 0.03 and 0.02 respectively when each group was compared with balloon only). The intimal medial ratios showed the same effect, with significant reductions in the heparin-treated animals compared with balloon only (p = 0.03 and 0.02 respectively). There was no significant difference between the sections of the arteries which received sham delivery and those which were only exposed to the heparin and balloon aspects of the procedure.

5.3.4 Duration Of Gene Expression.

The blue cells stained per section was qualitatively equivalent to the previous experiments at four days. At fourteen days staining was still visible in sections taken along the length of the artery but showed more isolated positive cells and fewer large plaques of transgene expressing cells

The number of blue cells per section ranged from 0 to 20 (approximately 0-6% of the cells).

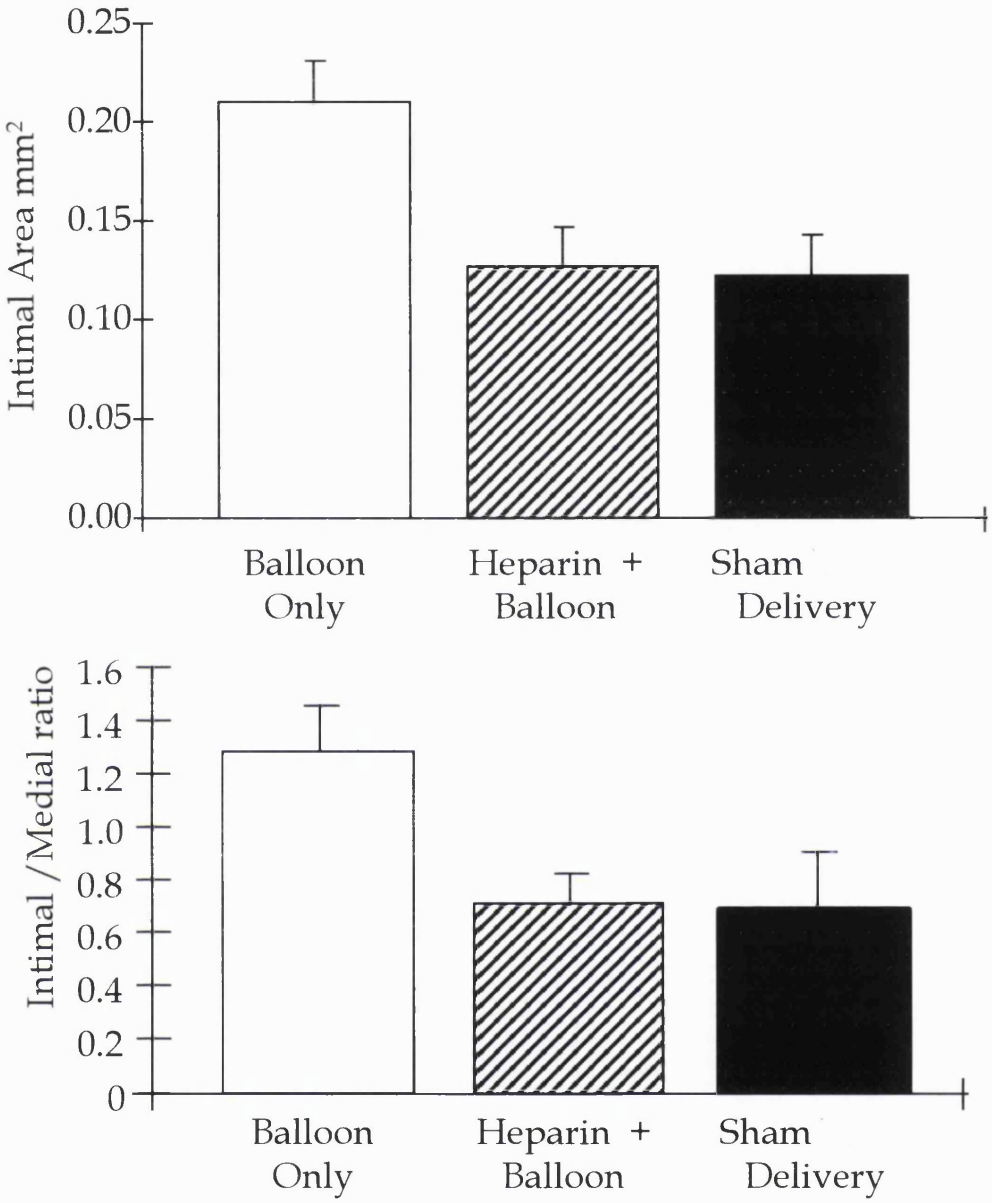


Figure 26 Reproducibility Study.

The graphs above show the mean and SEM of histomorphometric measurements of sections from 5 animals in each group. The upper panel shows the intimal area and the lower panel intimal medial ratio, 14 days after balloon injury. On each graph animals that had progressively more of the steps involved in the gene transfer technique are shown. The balloon only animals had a simple carotid balloon injury procedure, the heparin and balloon animals had an intravenous dose of heparin after balloon injury but the sample shown was taken from an arterial segment that did not undergo the sham delivery technique. The final group of animals demonstrate the effects of balloon, heparin and arterial distention for 20 minutes in the sham virus delivery technique.

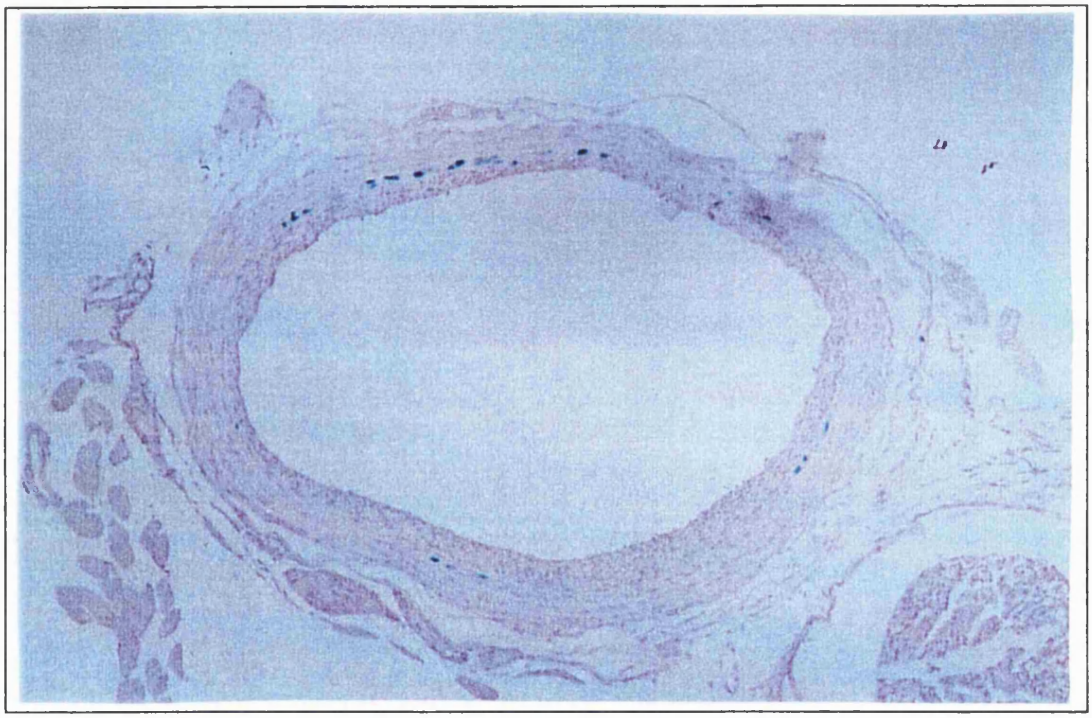


Figure 27. Rat Carotid Artery 14 Days After Gene Transfer With Av1.LacZ4.
Photomicrograph of a section of a rat carotid artery 14 days after arterial gene transfer using $5X10^8$pfu of Av1.LacZ4. The artery was fixed and incubated with X-Gal prior to embedding and sectioning. Transgene expression is indicated by blue nuclei and is seen only in the media of the artery. The level of transgene expression has reduced slightly compared to arteries harvested at 2 days.

5.4 Discussion

5.4.1 In Vivo Gene Delivery Of By Av1.LacZ4 Using Luminal Dwell.

Successful *in vivo* gene transfer was achieved using this technique which is a modification of that described by Lee *et al* using the same viral vector (Lee *et al.* 1993). Semi quantitative analysis of the results were comparable but further quantitation was not undertaken because the luminal dwell technique used was associated with an unacceptably high incidence of thrombosis. Thrombosis effected the control animals as well as those given viral solutions and therefore was not associated with a systemic inflammatory response to virus delivery. It did not effect the right carotid artery, and had not occurred in studies using the balloon injury technique alone.

Thrombosis appeared to be associated with the prolonged haemostasis, occurring during 20 minutes of interruption of flow, which is an integral part of this technique. The ideal gene delivery technique for these studies would offer minimal haemostasis and maximal gene delivery. In larger animals gene transfer devices have been used to maintain perfusion in all but the isolated arterial segment but the small size of the target artery in the rat prevented their use in these studies (Willard *et al.* 1994; Ohno *et al.* 1994; Nabel *et al.* 1990; Feldman *et al.* 1995; French *et al.* 1994). Comparative studies in rabbits also support the use of luminal dwell techniques despite the possible thrombotic risk. They have shown that luminal dwell techniques are a more efficient means of delivering adenoviral vectors than double balloon catheters, perforated catheters, and hydrogel-coated catheters (Willard *et al.* 1994).

It was not, therefore, possible to substantially alter the delivery technique to prevent thrombosis. *In vitro* studies described in this thesis suggested that a significant loss of gene transfer might occur if the luminal dwell time was reduced. This has since been confirmed by Feldman *et al* who used an

adenoviral vector encoding luciferase in the rat carotid and found a 75% reduction in luciferase activity if the dwell time was reduced from twenty minutes to ten minutes (Feldman *et al.* 1997). The use of anticoagulation with a single dose of heparin was, therefore, adopted.

5.4.2 Optimization Of Viral Titre And Delivery Procedure

The administration of a single dose of intravenous heparin successfully prevented thrombosis of the left common carotid artery. No bleeding complications resulted. The results of the X-Gal staining for ß-Galactosidase are consistent with published reports, showing the number of positive cells per section of about 2-10 at a viral titre of 5×10^8 pfu (Feldman *et al.* 1997). Some authors have quantitated their findings by examining arterial extracts using an ELISA to detect ß-Galactosidase or a chemiluminescent assay for luciferase (Schulick *et al.* 1995; Li *et al.* 1995; Lee *et al.* 1993; Feldman *et al.* 1997). The most detailed studies of the relationship between viral titre and transgene expression have shown an optimal dose of 5×10^8 pfu per artery (1×10^{10} pfu/ml) with variable low levels of expression at $5x\ 10^7$ pfu (Lee *et al.* 1993; Schulick *et al.* 1995). It was also shown that the toxicity produced by adenoviral infection at 5×10^9 pfu per vessel ($1x\ 10^{11}$ pfu/ml) reduced transgene expression, and caused a loss of smooth muscle cells in the neointima (Schulick *et al.* 1995). This smooth muscle cell loss was accompanied by a neutrophil infiltrate. The authors attributed the loss of transgene expression to selective toxicity and cell death in the infected cells.

Despite efforts to reliably quantitate results of *in vivo* gene delivery, different investigators have found varying results (Li *et al.* 1995; Schulick *et al.* 1995; Guzman *et al.* 1993). For example, two early studies in the rat have shown that lower transgene expression is seen if gene delivery is combined with a balloon injury procedure than if the gene transfer is delayed by seven days (Lee *et al.* 1993; Guzman *et al.* 1993). Another study in the rabbit showed no difference in transgene expression with

delayed gene transfer (Li *et al.* 1995). As suggested by Guzman *et al* it seems likely that the physical barriers to the virus in the arterial wall, in particular the internal elastic lamina, underlie many of the variations in transgene expression (Guzman *et al.* 1993). It is therefore critical to vascular injury gene delivery experiments that the amount of injury to the arterial wall is reproducible as this may determine the efficiency of subsequent gene delivery. It is also possible that minor differences in titration protocols for the adenoviruses may explain differences in 'dose-response' results.

5.4.3 Reproducibility Of Carotid Injury After Balloon, Balloon, Systemic Heparinisation And Sham Intraluminal Delivery Of Virus

This study validated the reproducibility of the model used, but does highlight the disadvantage of the 20 minute luminal dwell technique requiring the administration of heparin. The results showed little difference in lesion size or reproducibility between the arterial segments exposed to the sham virus delivery (including heparin) and those exposed to balloon injury and systemic heparin. There was, however, a 39% reduction in intimal area or intimal medial ratio in those animals receiving heparin compared to those that had no anticoagulant. The use of heparin in clinical angioplasty, often given by both the intracoronary and intravenous routes, is standard. Other authors, whose results were published while these protocols were being refined, have incorporated use of a similar dose of heparin (Schulick *et al.* 1995).

Heparin is, however, known to reduce neointimal hyperplasia in the rat model (Au *et al.* 1993; Clowes *et al.* 1992; Clowes *et al.* 1991; Clowes and Clowes, 1986; Clowes and Clowes, 1985). Prevention of migration and proliferation of smooth muscle cells in the arterial wall is the likely mechanism of this change in lesion size (Clowes and Clowes, 1986; Clowes and Clowes, 1985). Heparin has also been shown to inhibit the induction of Stromelysin, Gelatinase B, and Collagenase in smooth muscle

cells in culture (Kenagy *et al.* 1994) and the expression of tissue plasminogen activator in the rat carotid artery *in vivo*. It does not, however, inhibit intimal hyperplasia in all animal models, for example the baboon model shows no change in lesion size (Geary *et al.* 1995).

The use of heparin in these studies is likely to have less effect than the continuous infusion of heparin over 7-14 days in the studies mentioned above (Clowes and Clowes, 1986; Clowes and Clowes, 1985; Clowes *et al.* 1992; Au *et al.* 1993; Clowes *et al.* 1991). It is likely that the lesions studied will be 30% smaller than arterial lesions resulting from the same protocol without heparin. In addition, the effects of the metalloproteinase enzymes may be diminished. The use of heparin did not, however, alter the reproducibility of the lesion. Control groups, which also receive heparin, were included in all the studies relating to TIMP1 gene transfer which are described in Part 3 of this thesis. Brief anticoagulation should, if anything, mask the effects of a metalloproteinase inhibitor but it is unlikely to produce false positive data. In the studies of TIMP1 gene transfer, therefore, a single dose of intravenous heparin was used, and the overall amount of neointima was reduced by this part of the technique.

5.4.4 Duration Of Gene Expression In Vivo

The persistence of gene expression at a reduced level at 14 days after injury is consistent with the findings of Lee *et al* (Lee *et al.* 1993). They documented 210 mU/mg total protein of ß-Galactosidase activity in rat carotid arteries 3 days after gene transfer, which was sustained to 7 days but fell to 3.9 mU/mg by 14 days. This duration of gene expression has, however, been accompanied by a successful effect of the transgene on neointimal development in both the rat and other species (Yang *et al.* 1996; Chang *et al.* 1995c; Chang *et al.* 1995a). In studying TIMP1 gene transfer an early effect on migration of smooth muscle cells, without prolonged inhibition of matrix breakdown in the second week after angioplasty, would be desirable.

CHAPTER 6. *IN VIVO* AND *IN VITRO* ASSESSMENT OF HERPES VIRUS GENE DELIVERY TO VASCULAR SMOOTH MUSCLE CELLS AND THE RAT CAROTID ARTERY.

6.1 INTRODUCTION

This study explores the use of 3 herpes viruses which have mutations which render them replication deficient in vascular smooth muscle cells. This work was done in collaboration with Professor D Latchman, whose post doctoral fellows supplied the viruses and invaluable advice on their usage. The most frequently used method of rendering these viruses replication deficient is deletion of the immediate early genes (e.g. ICP27), which are responsible for viral replication soon after infection. Inactivation of the VMW65 gene, which transactivates immediate early genes after infection, will also prevent replication (reviewed by (Ali *et al.* 1994) and (Coffin and Latchman, 1996). In addition, removal of the ICP34.5 gene prevents replication in some fully differentiated cell types (such as neurons and cardiocytes). The herpes virus genome and position of the deletions in the viruses used in this study are shown schematically in Figure 28.

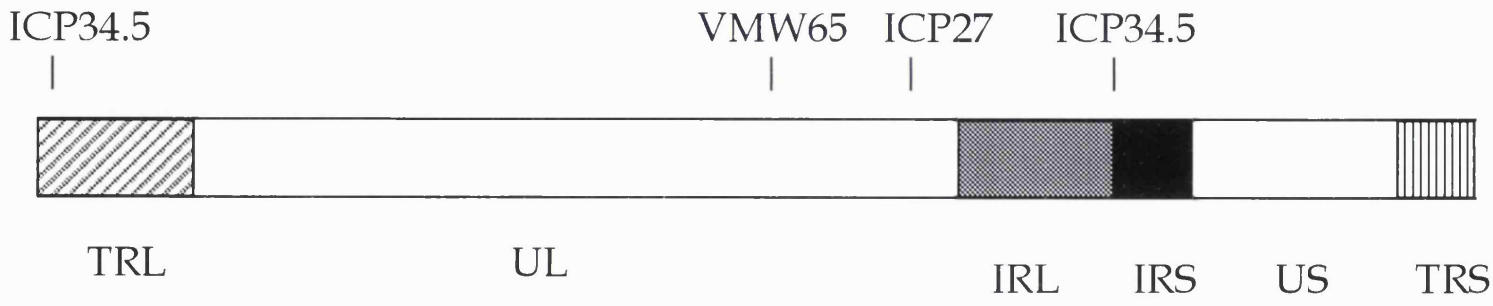


Figure 28. Schematic representation of the HSV1 genome.

The positions of the long and short repeat elements are shown as internal (IRS, IRL) and terminal (TRL, TRS). UL and US denote the long and short unique regions respectively. The positions of the genes deleted or modified in the viruses used are also shown. ICP34.5 deletion prevents viral replication in terminally differentiated cells. ICP27 is an immediate early gene essential to all viral replication. VMW65 encodes an essential structural protein of the virus but can be inactivated by a small insertion which selectively removes its ability to transactivate immediate early genes.

The disabled viruses used in the studies described in this thesis had been modified to express the marker gene ß-Galactosidase. Two viruses were deleted for both copies of ICP34.5 (1716/LacZ and 1764/LacZ). In addition, 1764/LacZ had an insertional mutation in the gene for VMW65. An expression cassette, containing the chimaeric herpes latently active transcript (LAT)- Moloney murine leukaemia virus long terminal repeat (MoMLVLTR) promoter controlling the LacZ gene, was inserted in the UL43 region of the HSV1 genome in both these viruses. No nuclear localization signal was incorporated in this vector. In the 27-LacZ virus an ICP6 promoter/LacZ insertion was put into the gene encoding the essential immediate early protein ICP27. A non disabled virus with a LacZ insertion was used as a positive control (BE8). For *in vivo* experiments only the 27-LacZ virus was used.

6.2 Herpes Gene Delivery To Vascular Smooth Muscle Cells.

6.2.1 Methods

SV40 transformed vascular smooth muscle cells and primary cultured rat aortic vascular smooth muscle cells were prepared and infected with herpes virus using the same protocol described on page 90. Cells were exposed to infection media for one hour. In initial experiments MOI 0, 1, 2.5 and 5 of the 27-LacZ virus was used to determine toxicity. An MOI of 5 was used in subsequent experiments using all four viruses (BE8, 1716/LacZ, 1764/LacZ, and 27-LacZ) and control uninfected cells of both cell types. Fixation and X-Gal staining was performed 24 hours after infection as described on page 86. Gene transfer efficiency was determined by qualitative examination by light microscopy.

6.2.2 Results

In the SV40 transformed cells a small cluster of blue cells was seen in the replicating positive control virus (BE8), which may reflect infection of a single cell followed by viral replication. No blue cells were seen in the SV40 transformed cells with any of the other viruses, regardless of the MOI applied (see Figure 29).

The primary rat aortic smooth muscle cells were more readily infected. They showed an increase in transgene expression in response to increasing MOI of the 27-LacZ virus in initial experiments (see Figure 30). However, even at an MOI of 5, only about 1% of cells were expressing the transgene and toxicity was visible in a small number of cells, which became rounded and detached from the plate. When the different viruses were compared less than 1% of cells showed transgene expression when infected with 1716-LacZ or 1764-LacZ. 27-LacZ gave the best results, while BE8 caused gene expression and toxicity (see Figure 31).

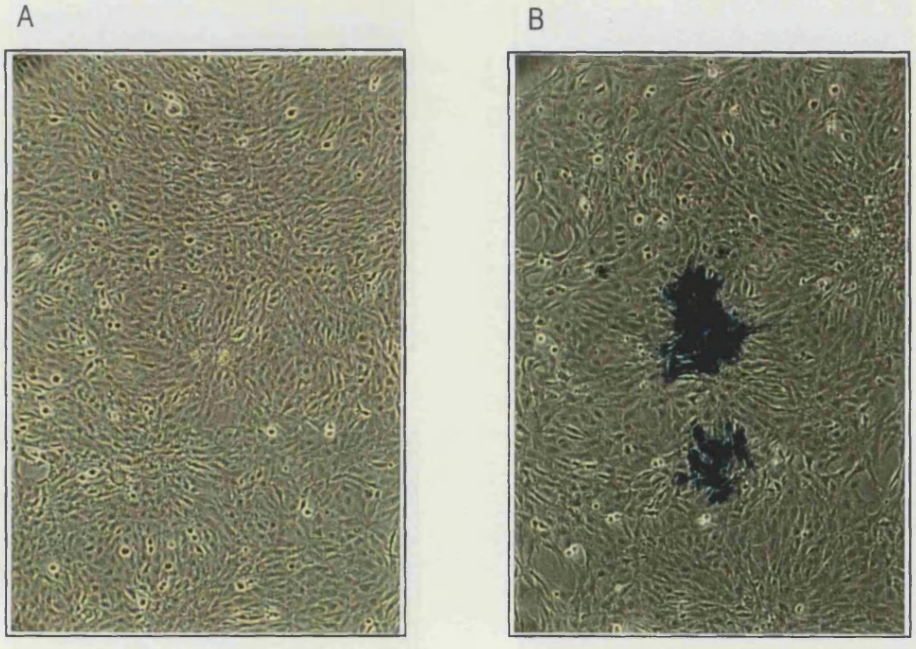


Figure 29. Photomicrographs of SV40 Transformed Rat VSMCs Infected With BE8 (Replication Competent) Herpes Virus (A) and ICP 27 Deletion Mutant (27-LacZ) (B).

Cells were infected with the virus indicated and fixed and stained with the chromagen X-Gal 48 hours later. No transgene was detected using the 27-LacZ, 1716-LacZ and 1764-LacZ viruses.(x40)

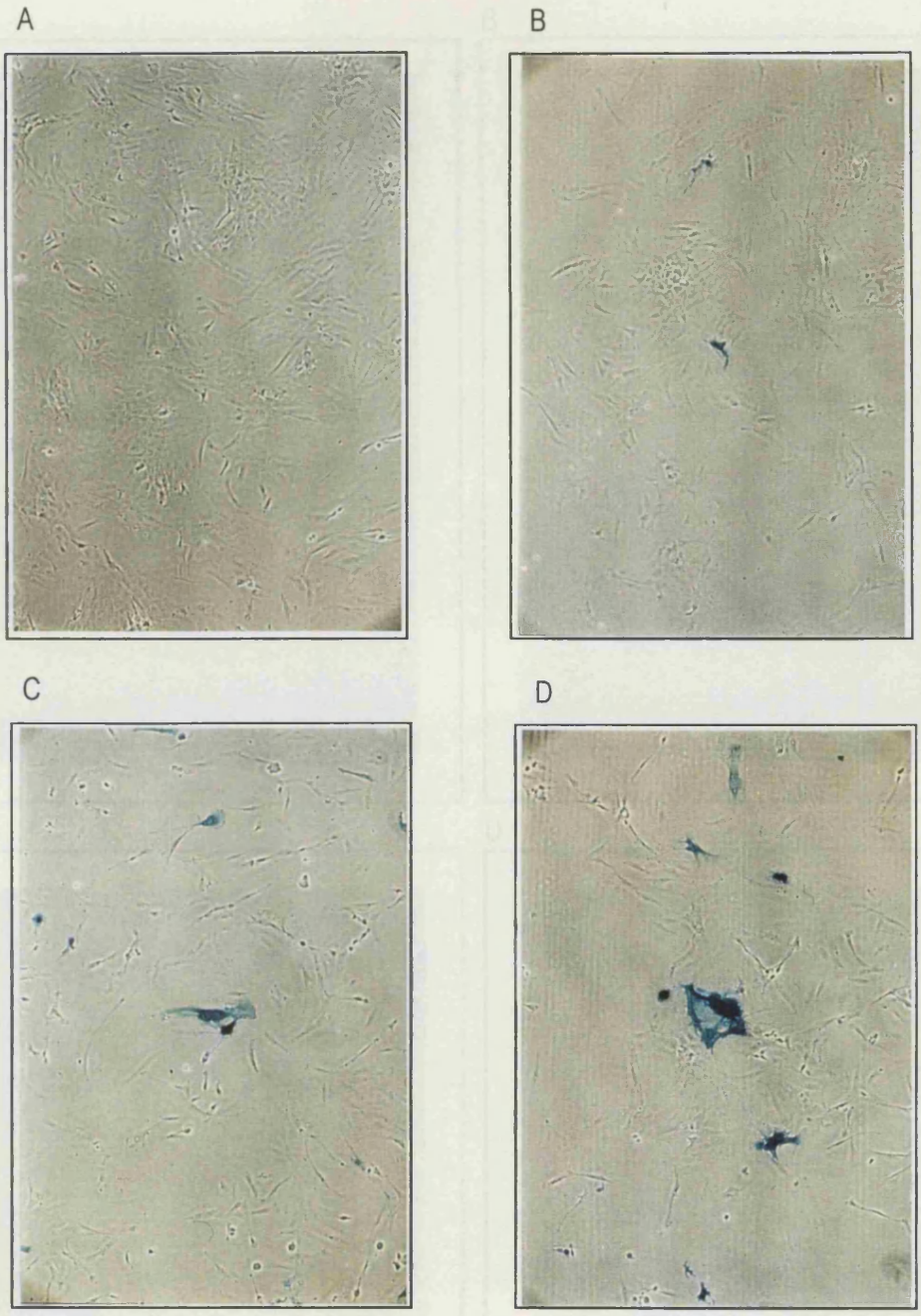


Figure 30. Photomicrographs of Primary Rat VSMCs Infected With 27-LacZ Herpes Viral Vector at MOIs of 0, 1, 2.5, And 5 (A-D respectively).

Cells were infected with the concentration of virus indicated and fixed and stained with the chromagen X-Gal 48 hours later. A progressive increase in transgene expression was seen with increasing viral concentration with a small number of cells detaching from the plate at an MOI of 5, indicating viral toxicity (x40)

Figure 31. Photomicrographs of Primary Rat VSMCs Infected With 1716-LacZ, 1764-LacZ, 27-LacZ, and BE8 (Replication Competent Virus) at an MOI of 5(A-D respectively). Cells were infected with the virus indicated and fixed and stained with the chromagen X-Gal 48 hours later. (x40)

6.2.3 Discussion

The herpes viruses tested showed no transgene expression in the SV40 transformed cell line and very low levels of expression in the primary smooth muscle cells. The difference in infectivity between the cell line and primary cells is not unexpected as it is known that primary vascular smooth muscle cells from rats of different ages may show widely differing responses to herpes viridiae (Kaner *et al.* 1993). The multiplicities of infection used were lower than those employed with the adenoviral vectors, but mild toxicity was seen at an MOI of 5 preventing further increases. The levels of transgene expression were poor when compared with cardiomyocytes infected with the same viruses (Coffin *et al.* 1996). In these experiments the promoter controlling the transgene was different to that used in the adenoviral constructs (RSV), which prevents direct comparison of the relative efficiency of the different components of the viruses. The toxicity and efficiency of gene delivery of these herpes virus vectors *in vitro* was poor when compared to the adenoviral vectors tested. The 27-LacZ vector showed the greatest transgene expression among the herpes vectors, and was therefore tested in a limited pilot study *in vivo*.

6.3 HERPES GENE DELIVERY TO THE RAT CAROTID ARTERY *IN VIVO*

6.3.1 Introduction

The *in vitro* efficiency of gene delivery to vascular myocytes of these herpes viruses was substantially less than the adenovirus Av1.LacZ4, although the MOI of virus used was lower due to greater toxicity of the herpes viruses. These results precluded use of the 1764-, and 1716-LacZ viruses *in vivo.* The 27 -LacZ virus was tested *in vivo* in the pilot study described below.

6.3.2 Methods

The rat model of vascular injury described in section was used. Briefly 2 rats were anaesthetized and the left carotid artery balloon injured. The animals were then heparinized and the 27-LacZ virus administered using the 20 minute luminal dwell procedure already described. In one rat a titre of $2x10^7$ pfu was used, and in the second $2x10^8$ pfu. 48 hours after gene transfer the arteries were perfusion fixed *in situ* and stained with X-Gal. The arteries were then examined under a dissecting microscope and fixed. Sections were cut at 100μm intervals through the artery and counterstained with nuclear fast red (see page 263).

6.3.3 Results

Figure 32 shows the macroscopic results of herpes virus infection. 7 clusters of blue cells were seen in the 1.5 cm of artery infected with $2x10^8$ pfu and none in the artery infected with $2x10^7$ pfu. Microscopic examination of the sections taken revealed no evidence of transgene expression at either titre of virus.

6.3.4 Discussion

The efficiency of gene delivery using this virus and promoter-LacZ cassette was very poor. Microscopy did not reveal any transgene expression in either artery, but the small areas identified macroscopically may not have been sampled with the 100μm intervals studied. In addition it is known that use of the X-Gal technique to identify the transgene product of LacZ underestimates gene transfer when compared with immunohistochemical techniques using beta galactosidase antibodies (Couffinhal *et al.* 1997). *In vivo* gene delivery to the arterial wall using herpes virus vectors has not previously been published and therefore these results could not be compared with other studies. The efficiency of gene transfer and expression using the herpes vector was very poor when compared with

Av1.LacZ4. The use of these herpes virus vectors for *in vivo* gene delivery to the arterial wall is inappropriate and the limited pilot study was not extended.

A

B

Figure 32. Rat Carotid Artery 2 Days After Gene Transfer With Replication Deficient Herpes Virus Vector 27- LacZ Using $2X10^7$ and $2X10^8$ pfu (A and B respectively).

The artery was fixed and incubated with X-Gal and then cut longitudinally. The luminal surface has been exposed and photographed using darkground illumination under a dissecting microscope. Transgene expression, indicated by small islands of blue staining, is seen only at the higher titre (B).

PART 3: GENE TRANSFER OF TIMP1

CHAPTER 7: SCREENING AND *IN VITRO* ASSESSMENT OF AN ADENOVIRAL VECTOR FOR TRANSFER OF HUMAN TIMP1 (AV1.TIMP1).

7.1 INTRODUCTION

In this study adenoviral gene transfer was employed to inhibit matrix metalloproteinases to gain the advantages of a specific and local effect and to overcome the difficulties associated with the currently available pharmacological inhibitors.

The basis of the generation of the adenoviral vector was cloning of the TIMP1 cDNA with the Rous Sarcoma Virus (RSV) promoter and polyadenylation signals into a shuttle plasmid (pAVS6A) downstream of the extreme left hand end of the adenoviral genome. The viral sequences comprise an intermediate terminal repeat (ITR), package signal and the E1A enhancer (Ad5 ITR-Pkg) see Figure 3. The shuttle plasmid is then cotransfected onto 293 cells with gel-purified Adenovirus dl327 large Cla I fragment (this modified adenovirus lacks E3). Recombinant DNA is then replicated and packaged into infectious particles which form plaques under agar and are then picked as outlined below (plaque purification page 152).

The human TIMP1 cDNA was ligated into a shuttle vector pAVS6A.MAP from the bluescriptKS plasmid by Dr. Karen Webb using Bam HI and Hind III restriction sites to remove the TIMP1 cDNA from the bluescript plasmid (both plasmids are shown schematically in Figure 33). These restriction sites were blunt ended with Klenow and the TIMP1 run on a low melting point gel to obtain the correct fragment. A double blunt ended ligation was then performed into pAVS6A which had been linearized with EcoRV resulting in pAVS6A.TIMP1 (shown schematically in Figure 34). 1 μg of DNA from pAVS6A and PAVS6ATIMP1were then digested using BamHI,

NCOI, Pst I, Ava II, and Ava I to confirm correct insertion and orientation. The pAVS6ATIMP1 shuttle vector was cotransfected on 293 cells with the large Cla I adenoviral fragment to form 32 potentially recombinant plaques (shown schematically in Figure 35). This work was carried out by Dr. Sue Stevenson and Ms Michelle Rollence under the guidance of Dr. Alan McClelland at Genetic Therapy Incorporated. The 32 plaques were then picked and used to infect 293 cells (i.e. a limited propagation) and the cells were harvested and frozen for transfer to the author for screening.

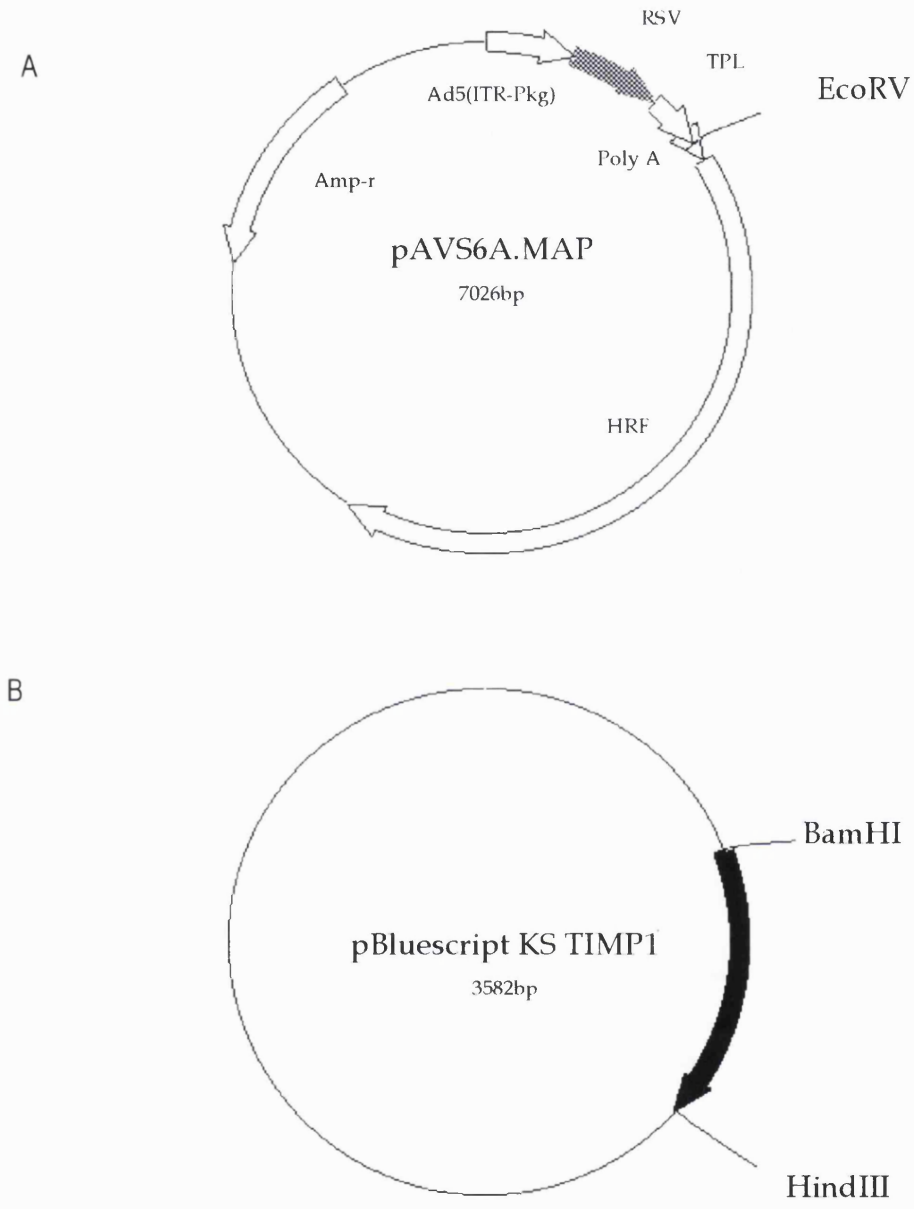


Figure 33. Schematic Representations of the Adenoviral Shuttle Vector (PAVS6A.MAP) and Bluescript KS Plasmid From Which TIMP1 Was Removed.

A: Adenoviral shuttle vector containing adenoviral intermediate terminal repeat and packaging signal (Ad5ITR-Pkg), the Rous S arcoma Virus promoter (RSV), Terminal polylinker (TPL), Polyadenylation signal (Poly A), Fragment of the adenoviral genome to promote homologous recombination (HRF), and the ampicillin resistance gene (Amp-r). The EcoRV restriction enzyme site is illustrated, this site was used to linearise the shuttle vector prior double blunt ended ligation with the TIMP1 cDNA excised from the bluescript plasmid shown in panel B.

B: Bluescript plasmid containing the cDNA for human TIMP1 with the restriction sites used to excise the TIMP1 cDNA illustrated.

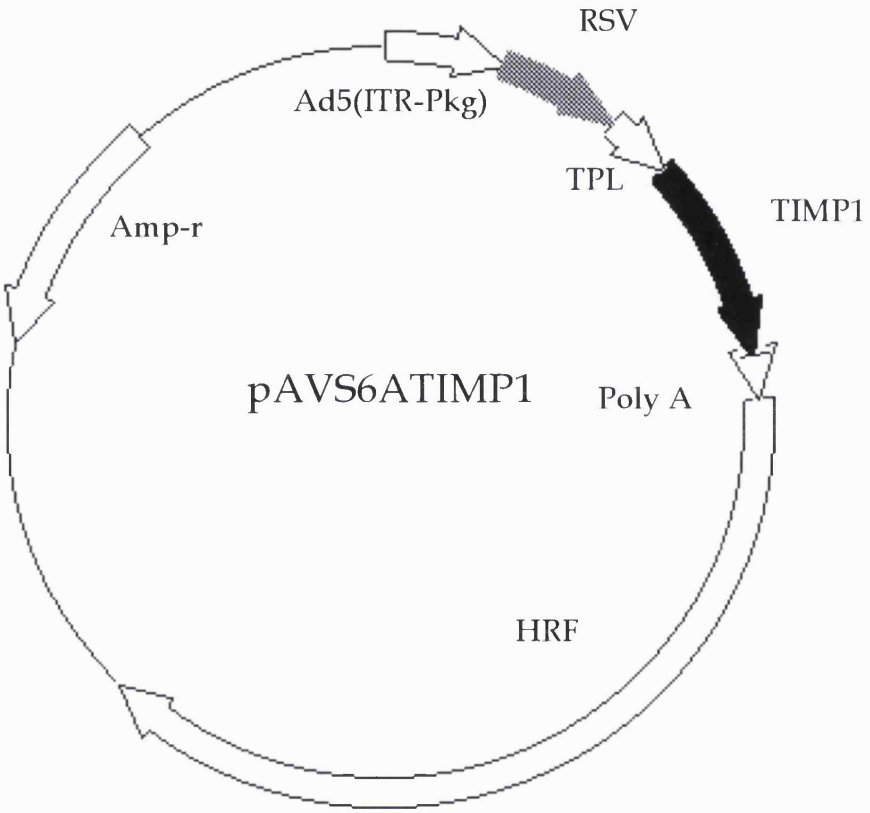


Figure 34. Schematic Representations of the Adenoviral Shuttle Vector Containing the Human TIMP1 cDNA, PAVS6A.TIMP1.

Adenoviral shuttle vector contains the adenoviral intermediate terminal repeat and packaging signal (Ad5ITR-Pkg), the Rous Srarcoma Virus promoter (RSV), Terminal polylinker (TPL), Polyadenylation signal (Poly A), Fragment of the adenoviral genome to promote homologous recombination (HRF), and the ampicillin resistance gene (Amp-r) as shown in figure 33. The TIMP1 cDNA has been ligated into the shuttle vector upstream of the homologous recombination fragment.

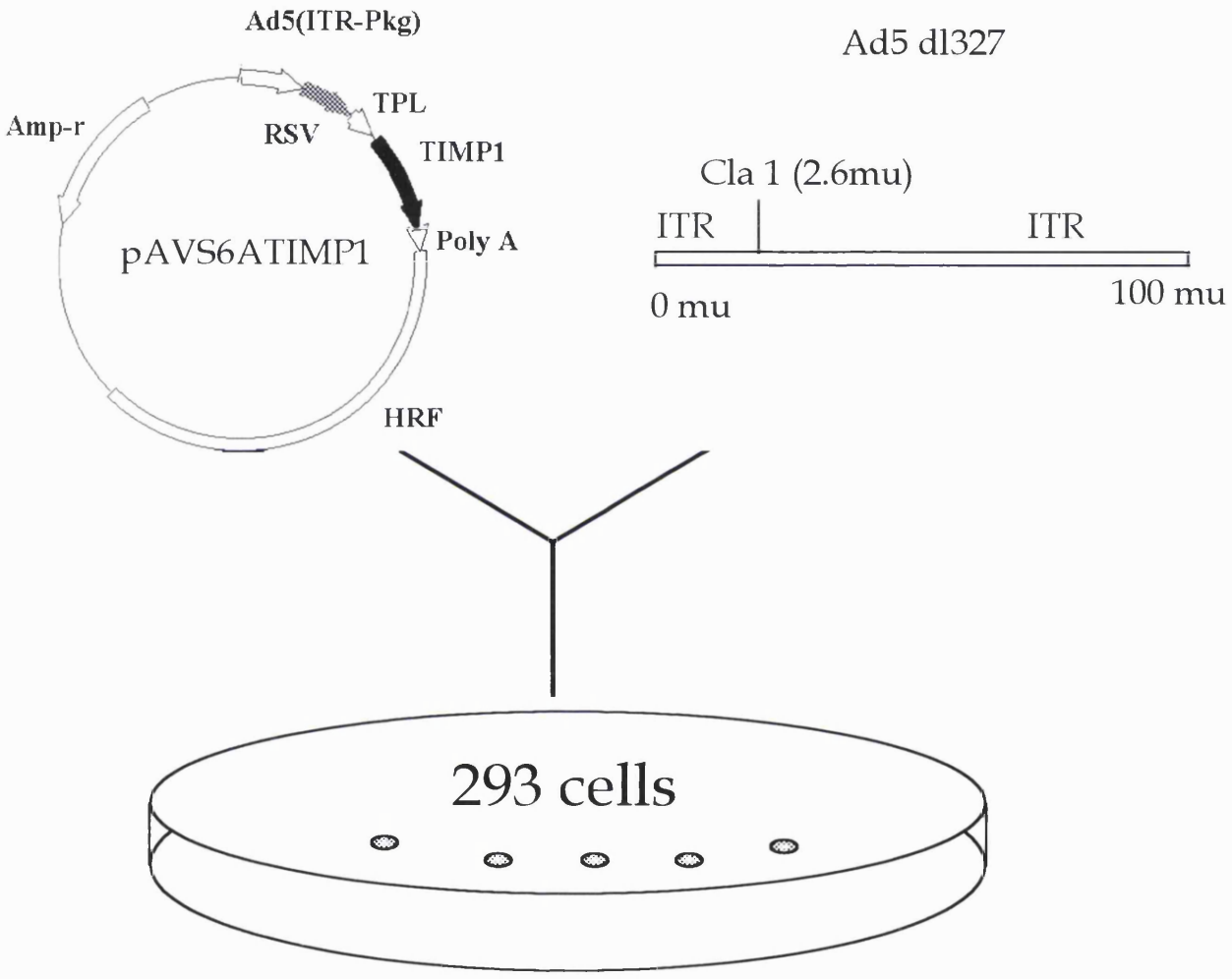


Figure 35. Schematic Representations of the Calcium Phosphate Cotransfection of the Adenoviral Shuttle Vector Containing the Human TIMP1 cDNA, PAVS6A.TIMP1 and the Large Cla1 Fragment of the E1A and E3 Deleted Type 5 Adenovirus (Ad5dl327) .

The adenoviral shuttle vector containing the TIMP1 cDNA is cotransfected with the large Cla1 fragment of the deleted adenovirus into 293 cells using calcium phosphate. The 293 cells are human embryonic kidney cells stably expressing the E1A fragment of the adenoviral genome which allows replication of viruses deleted in this region. A layer of agar gel a culture medium is put on the cell monolayer to localize the cytolytic effects of replication of individual virions. Homologous recombination then occurs and plaques appear over 7-14 days. A plaque indicates the presence of replicating virus which may be a successful recombinant which has incorporated the TIMP1 cDNA or be due to the Ad5dl327 Cla1 fragment.

This section of this thesis deals with four specific aims of the project. Firstly to identify an adenoviral vector containing the TIMP1 cDNA, secondly to confirm that the vector could accomplish expression of an immunoreactive transgene product, thirdly to confirm that this protein product of the transgene was functionally active, and lastly to investigate its biological effects.

Screening of candidate viral plaques at DNA and protein level, was accomplished by Southern and western analysis respectively. The resultant virus incorporating the cDNA for human TIMP1 and the rous sarcoma virus LTR was plaque purified and is referred to as Av1.TIMP1.

In order to determine a reproducible relationship between multiplicity of virus infection, the amount of TIMP1 protein produced in response to infection with Av1.TIMP1, and its biological activity initial experiments were carried out *in vitro*. SDS polyacrylamide gel electrophoresis was used to identify secreted MMP's and TIMP's from conditioned media of smooth muscle cells. Western analysis was used to confirm immunoreactivity of the transgene product. To identify rat Gelatinase A and B and TIMP's 1-3, to which antibodies are not readily available, substrate gels which assay biological activity were employed. Investigation of the effect of adenoviral infection and overexpression of TIMP1 on smooth muscle cell migration and proliferation utilized primary cultured smooth muscle cells.

7.2 Methods

7.2.1 Screening Recombinant Plaques

Candidate recombinant plaques were screened for presence of the human TIMP1 cDNA as outlined below by limited propagation on 293 cells, Southern analysis and subsequent western analysis for production of protein from vascular smooth muscle cells.

7.2.1.1 Infection Of 293 Cells To Prepare Fresh Crude Viral Lysate.

293 Cells were grown to 50% confluence in 6 well plates. 50 µl of the candidate recombinant crude viral lysates were freeze thawed three times to release the virus and diluted in 550µL of infection medium and one lysate was used to infect each well. In addition to the candidate recombinants one well was infected with Ad5dl327 and one well was not infected to serve as negative controls. The infection medium was removed after 90 minutes and replaced with fresh complete medium. Cytopathic effect was observed 36 hours after infection and the cells were harvested in their media. An aliquot of the cell suspension was then spun slowly for five minutes until a pellet formed. The supernatant was aspirated and the pellet stored at -70°C.

7.2.1.2 Extraction Of Genomic DNA.

Each cell pellet was resuspended in 0.5 mg/ml proteinase K, 0.5% SDS, in 10mM Tris, 1mM EDTA, pH 7.5 (TE) and incubated overnight at 37°C. The DNA was extracted with 250µl of water equilibrated phenol and the tube was vortexed. The tube was then spun for five minutes at 5,000 rpm and the top aqueous layer removed for further extraction. This process was repeated extracting with 250µl of phenol-chloroform and then 200µl of chloroform. To the final extract 1/10 volume 3M sodium acetate and 2.5 volumes ethanol was added, mixed, and allowed to stand for five minutes. The samples were centrifuged for ten minutes at 5000 rpm. A DNA pellet was then visible, the ethanol was removed, and the pellet briefly dried in a vacuum for ten minutes before being resuspended in 100 µl of TE and allowed to rehydrate overnight.

7.2.1.3 Spectrophotometric Evaluation Of Nucleic Acids

Quantification of DNA extracts was performed by spectrometry. Nucleic acids absorb light maximally at 260 nm. At this wavelength 50μg/ml of DNA has an absorbance of 1. The presence of contaminating protein can be measured at 280 nm. Calculation of the ratio of absorbence at 260 and 280 nm was then used to estimate sample purity with ideal ratios of 1.8 or more.

7.2.1.4 Restriction Digestion

Restriction enzyme PST I in React 2 buffer was used to digest 10μl of each genomic DNA sample overnight at 37°C and the bluescriptKS plasmid containing human TIMP1 was used as a positive control. This was expected to result in fragments of 29, 27, 121, 353, 430, 733, 767, and 5140 kb in viral recombinants and 39, 121, and 3432 kb in the bluescript plasmid (see Figure 36).

7.2.2 Southern Analysis

7.2.2.1 Sample Preparation

Samples of potential recombinant DNA and DNA from Ad5dl327 infected and uninfected 293 cells (negative controls) and bluescript KS TIMP1 (positive control) were digested as above. For each sample 10 μl of DNA was added to 2μl of 6x Gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% sucrose in dH_2O).

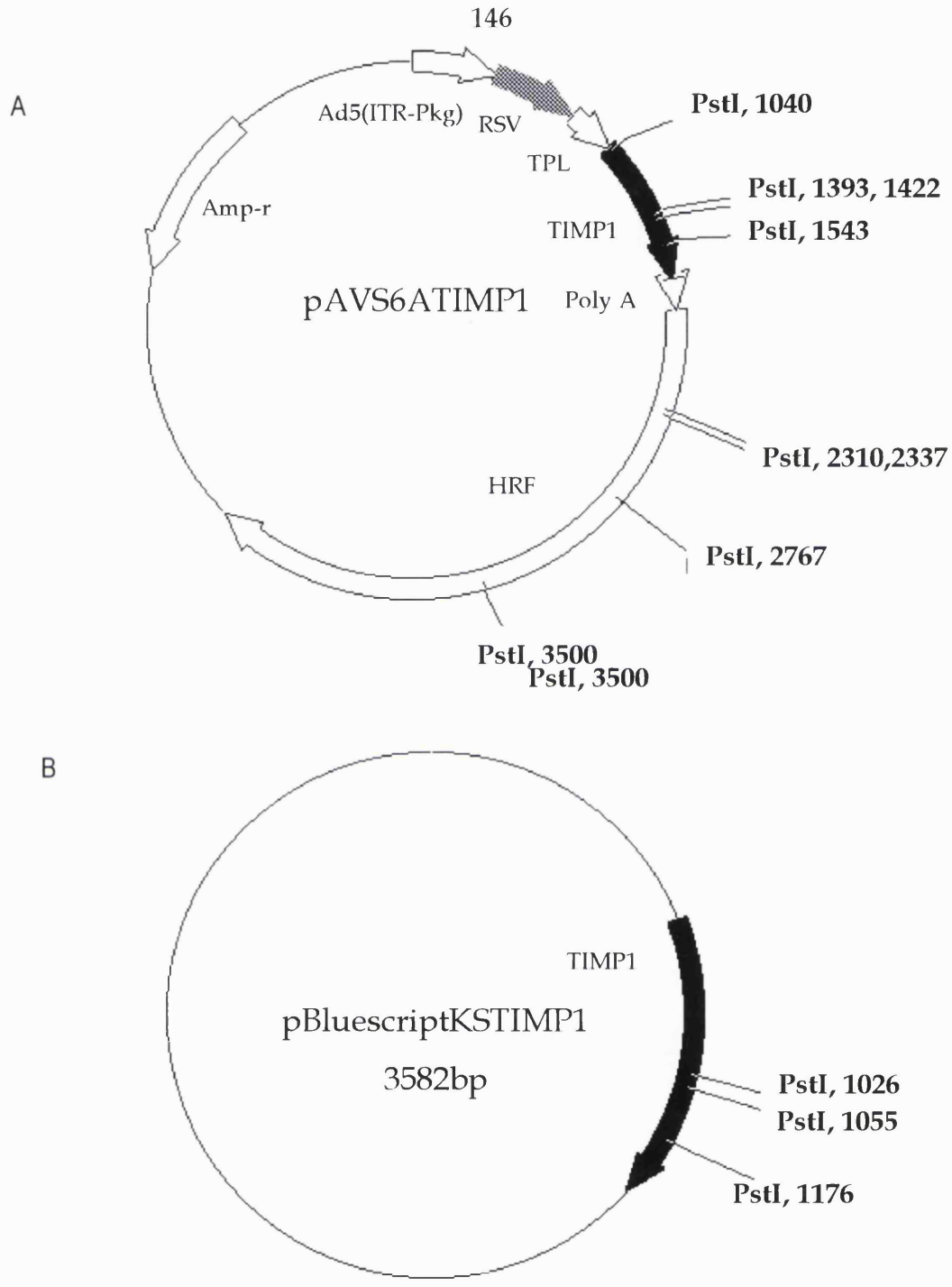


Figure 36. Schematic Representations of the Adenoviral Shuttle Vector Containing The TIMP1 cDNA (A)and Bluescript KS Plasmid (B) Illustrating the PstI Restriction Sites.

Digestion with the PstI restriction enzyme was expected to result in fragments of 29, 27, 121, 353, 430, 733, 767, and 5140 kb in viral recombinants and 39, 121, and 3432 kb in the bluescript plasmid

7.2.2.2 DNA Electrophoresis

A 1X TBE (0.045M Tris-borate, 0.001M EDTA), 1% agarose gel with 0.5 μg/ml of ethidium bromide was prepared by heating the TBE, agarose suspension in a microwave oven until the agarose had fully dissolved. Ethidium bromide was then added from a 10mg/ml stock and the gel allowed to cool to 60°C before pouring. The gel was then allowed to set for 30 minutes.

The gel was immersed in 1 x TBE, samples and DNA ladder loaded, and a 30 mV voltage applied overnight. The gel was photographed under an ultraviolet light transilluminator.

7.2.2.3 Capillary Blotting and Fixation

The DNA was denatured to allow binding of the radiolabeled probe to the single DNA strands by washing in 0.5M NaOH; 1.5M Na Cl for 90 minutes with gentle shaking. The gel was then neutralized by immersion in 1M Tris; 1.5M Na Cl pH 7.0 for one hour.

A capillary blot was set up to transfer the DNA to Hybond N membrane according to the manufacturers instructions. Four sheets of Whatmann 3MM filter paper were cut to the exact size of the gel and placed in a dish on top of an inverted gel casting tray. The contents of the dish were saturated with 20 X SSC (3M NaCl, 0.3M Na_3Citrate). The gel was gently slid into position on the filter paper. A sheet of clingfilm was then placed over the gel and a window the exact size of the gel cut in the film with a razor blade. A piece of Hybond N membrane was cut to the exact size of the gel, soaked in water and then transfer buffer for 5 minutes, and placed on top of the gel. A pipette was gently rolled across the surface to remove any air bubbles. Three pieces of Whatman 3MM filter paper the size of the gel were then wetted with blotting buffer and placed on top of the

membrane. A 5cm stack of absorbent towels was then placed on top of the filter paper and a 1kg weight placed on top of the stack.

Transfer was allowed to proceed overnight. The membrane was then removed having lightly marked the lanes in pencil on the side of the membrane to which DNA had been transferred. The membrane was briefly washed in 2X SSC. The membrane was then placed on a sheet of Whatmann filter paper and placed in an ultra violet transilluminator to cross link and therefore fix the DNA to the membrane (program AutoXlink).

7.2.2.4 Prehybridization

The membrane was placed in a hybridization tube with 25 ml of 5 X SSC, 5 X Denhardt's solution (100 X Denhardt's solution: 2% Bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone), 0.5% w/v SDS. 0.5 ml of 1mg/ml herring sperm (non homologous) DNA was denatured by heating to 100°C, chilled on ice, and added to the prehybridization solution which was incubated for 1 hour at 65°C.

7.2.2.5 Making A Radiolabeled Probe.

A radiolabeled probe which would hybridize selectively with part of the TIMP1 sequence was made as follows. The TIMP1 cDNA in the bluescript plasmid was used to generate a fragment of DNA to be radiolabeled. 1µl of the plasmid was digested for 1 hour with Ava I at 37°C and the digest was run on a 1% low melting point agarose gel containing 0.05 µg/ml ethidium bromide. The 587 kB fragment was cut out under brief exposure to ultraviolet light, weighed and diluted in 3 ml of distilled water per gram of gel slice (see Figure 37).

The mixture was then heated to 65°C for 2 minutes to melt the agarose. 25µl of the solution was prepared for labeling by heating to 100°C for 7 minutes and then incubation at 37 °C for 10 minutes, briefly centrifuged and allowed to cool to room temperature. The DNA was then labeled using

$\alpha^{32}P$ dCTP and the Ready.To.Go DNA labeling kit (Pharmacia Biotech) according to the manufacturers instructions. The principle behind this kit is the random labeling of denatured DNA. When the DNA is mixed with the random oligomers they anneal to the DNA and serve as primers for DNA synthesis by DNA polymerase. When radiolabeled nucleotides are present highly labeled DNA should result. A tube of reaction Mix was reconstituted with 20µl of dH_2O without mixing and placed on ice for 5 minutes. To this mixture was added 25µl of denatured DNA, and 5µl of [α-^{32}P] dCTP (3000 Ci/mmol) and the tube incubated at 37°C for 45 minutes.

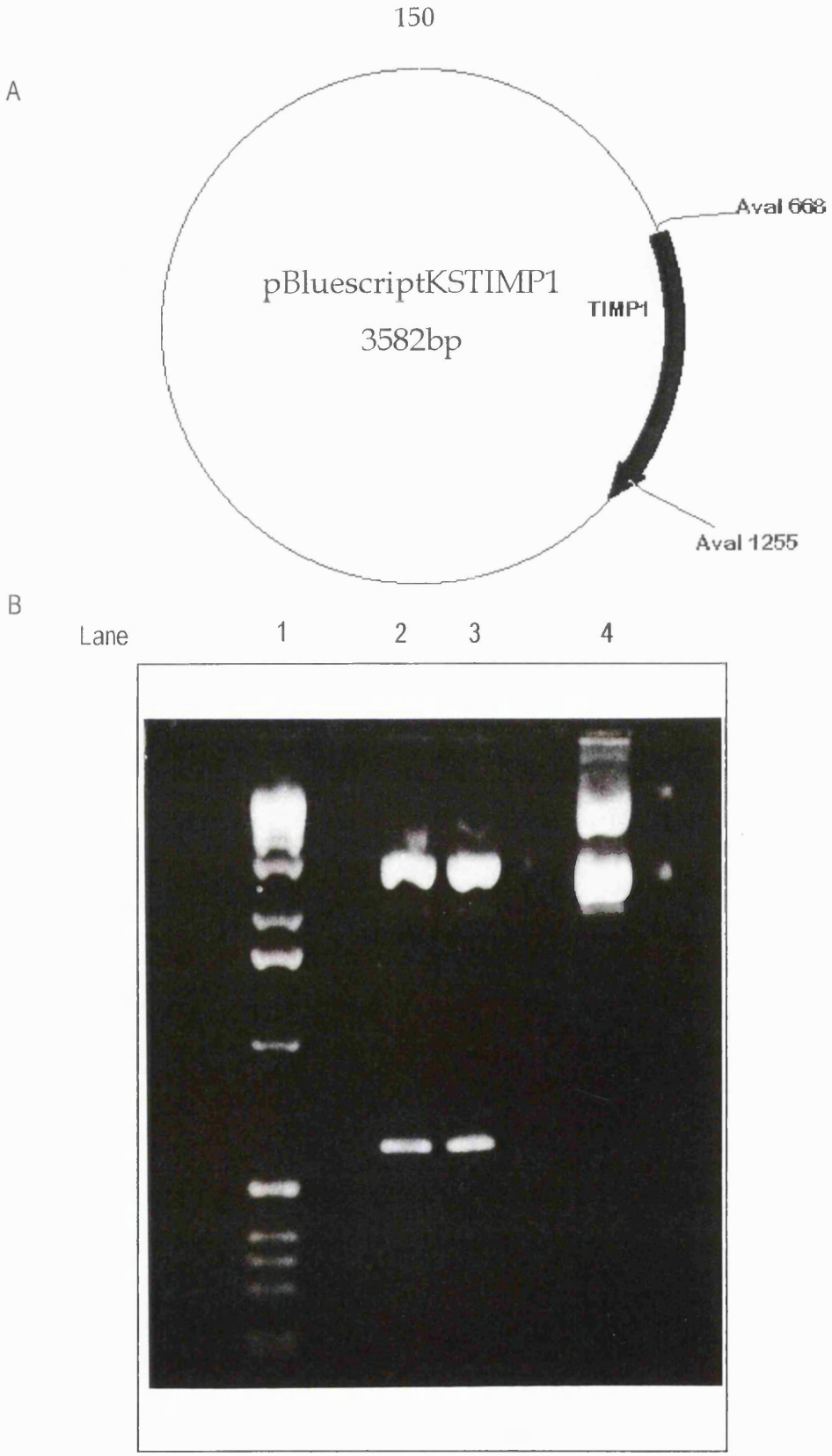


Figure 37. Making a Probe for Human TIMP1.

A: Schematic representation of the Bluescript KS Plasmid illustrating the AvaI restriction enzyme sites. B: Low melting point agar gel photographed under ultraviolet light: Lane 1 1kb ladder, Lane 2 and 3 Ava I restriction digest of Bluescript KS TIMP1, and Lane 4 undigested Bluescript KS TIMP1. The single TIMP1 fragment was excised from the gel and used to make a radiolabelled probe for southern analysis of candidate recombinant viral plaques.

In order to remove unincorporated nucleotides spun columns were made by filling 1ml syringe barrels with a glass wool bung in the neck and Sephadex G-50 DNA grade (Pharmacia Biotech) equilibrated in 1x TE buffer (10mM Tris Cl, pH8.0, 1mM EDTA, pH 8.0, and 100mM NaCl) and spinning the column for periods of 3 minutes at 1800rpm and refilling with Sephadex. The column was placed in a 15ml falcon tube with an eppendorf tube at the neck. 150µl of TE and the probe mixture was added to the column and spun at 1800rpm for 3 minutes. A Geiger counter was used at each stage to ensure that no radioactive spillage had occurred and to confirm that the majority of the activity had moved through the column to the eppendorf tube below.

7.2.2.6 HYBRIDIZATION

The radiolabeled probe was then denatured by heating to 100°C for 5 minutes, cooled on ice for 10 minutes and added to the prehybdridization buffer and incubated at 65°C overnight. After hybridisation the membrane was washed in 2 X SSC, 0.1% SDS twice at room temperature for 10 minutes, in 1 X SSC, 0.1% SDS at 65°C for 15 minutes, and then 0.1 X SSC, 0.1% SDS for 10 minutes. Autoradiography was then performed and filters exposed to film for 24 hours at -70°C.

7.2.3 Screening Viral Recombinants For Production Of TIMP1 Protein by Infection Of Vascular Smooth Muscle Cells With Crude Viral Lysate

The SV40 transformed vascular smooth muscle cell line was used to assess the ability of these cells to produce human TIMP1 in response to viral infection with Av1.TIMP1. VSMCs were grown to 90% confluence and rendered quiescent by placing them in serum free medium for 24 hours.

This medium was then removed and replaced with one of the following: 1 ml of fresh serum free medium plus 1 µl of crude viral lysate from 2 of the

positive plaque results from Southern analysis, 1µl crude viral lysate from Ad5dl327, 1µl of serum free medium. This media was replaced with fresh serum free medium after 1 hour of incubation at 37°C. The medium was harvested at 72 hours, and centrifuged at 1500rpm for 5 minutes to remove cell debris. Conditioned media were analyzed by western analysis as described below to confirm the presence of immunoreactive TIMP1 protein.

7.2.4 Plaque Purification

In order to ensure that the candidate plaques originally picked were not mixed a process of small scale viral amplification, plaquing under agar, picking plaques, reamplifying, and screening the amplified plaques must be undertaken. 2 cycles of plaque purification were carried out in order to ensure purity of the virus. A single virus was chosen that showed positive results on both the Southern and Western analysis and the crude viral lysate used to infect 293 cells under agar as described on page 78. When plaques become visible they are marked in ink on the under surface of the well. A wide bore 200 µl pipette tip is then inserted into the agar above the mark and a 50 µl agar plug aspirated including the cell monolayer but not disturbing surrounding cells. The tip of the pipette containing the agar plug was cut off using sterile scissors into 500µl of infection medium (see page 72) and kept at 4°C overnight. The medium was then aspirated and freeze thawed 3 times and used to infect a fresh 293 cell monolayer. Once cytopathic effect was visible Western analysis was used to screen the conditioned medium for human TIMP1 using uninfected cells as a negative control. The process was then repeated once more before large scale amplification. The resultant virus is referred to as Av1.TIMP1.

7.2.5 Assays of MMPs and TIMPs

7.2.5.1 Concentration Of Conditioned Media.

Samples of conditioned media were concentrated by centrifugation through filters using the centricon-3 system (Amicon cat no 4203) according to the manufacturers instructions. The concentration is achieved by ultrafiltration through an anisotropic membrane. Using this system all proteins above 3,000 Daltons are retained by the filter. The media were placed in the concentrators sample reservoir above the filtrate vial taking care not to touch the filter with the tip of the pipette. The apparatus was then spun at 7,500 rpm at 4°C for 3.5 hours. The filtrate was discarded and the contents of the sample reservoir were inverted into the retentate cup and spun for 3 minutes at 2000 rpm to collect the concentrated medium.

7.2.5.2 Bradford Assay.

Protein content of the samples was analyzed using the method of Bradford (Bradford, 1976). For each set of samples to be analyzed a set of standards was prepared diluting bovine serum albumin to give final concentrations of 0.2, 0.5, 1, 2, 3, 5, 7.5, and 10 μg/ml. A volume of sample diluent equal to that of the samples e.g. 5 μg RPMI medium was added to each of the standard solutions. Samples were prepared in a similar fashion and the total volume made up to 0.5 ml with distilled water. 0.5 ml of Bradford reagent, 0.06 % coomassie blue w/v in 3% perchloric acid, was added to each standard sample and the reaction allowed to proceed for 5 minutes prior to measurement of absorbance at 595 nm using a spectrophotometer. The standards were then used to create a standard curve and regression line from which the protein content of the samples was calculated. Sodium dodecyl sulphate and Triton X-100 can both interfere with this assay and therefore when it was used to analyze tissue extracts 1-2μl volumes of sample were used. All gels were run with sample volumes adjusted to equalize protein loading.

7.2.5.3. Sample Preparation For Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Equal samples of protein were prepared and diluted with dH_2O to a total volume of 10µl. To this was added 2X loading buffer containing 100µ M TrisCl (pH 6.8), 4% SDS, 0.1% bromophenol blue, 10% glycerol and 5% ß mercaptoethanol. All samples were heated to 100°C for 10 minutes prior to electrophoresis in order to denature the proteins and then spun at 3,000 rpm for 1 minute prior to loading.

7.2.5.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were separated on SDS-polyacrylamide gels according to the method of Laemmli (Laemmli, 1970). Polyacrylamide gels were prepared between clean glass plates using the Biorad Mini Gel system according to the manufacturers instructions. Running gels were made to a concentration of 10% and overlaid with water saturated butanol while setting. This was removed, the upper surface of the gel briefly washed with dH_2O, and stacking gels (3% acrylamide) were poured on top of the polymerized running gel to a depth of 25mm. Loading wells were formed using a 12 tooth comb inserted into the stacking gel prior to polymerization. Samples and molecular weight markers (Rainbow coloured protein molecular weight markers, Amersham) were loaded and the gel mounted in the electrophoresis apparatus. Tri-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine (pH 8.3), and 0.1% SDS) was then added to the top and bottom of the reservoirs. A current of 25 mA was then passed through the gel until the samples had entered the running gel when the current was increased to 55mA. All gels were run in a cold room at 4°C using pre-cooled electrophoresis buffer.

7.2.6 Western Analysis

Western blotting involves the transfer of proteins from SDS-polyacrylamide gels to solid supports and subsequent immunological detection of immobilized proteins. This technique was used to identify the transgene product human TIMP1 after viral infection using a specific anti human TIMP1 antibody (kind gift of CellTech Ltd.).

7.2.6.1 Western Blotting

Proteins were then transferred from the gels to nitrocellulose membranes (Hybond C, Amersham) using the Protean minigel system (Biorad). The blotting buffer used was 39mM glycine, 49 mM Tris base, 0.037% SDS, and 20% methanol. Two pieces of Whatman 3mm filter paper were cut to the size of the gel and one piece of Hybond C. The anode plate (Black) in the Protean minigel system was submerged in a dish containing blot buffer and the foam lining and first piece of filter paper placed on it. The gel was then place on the filter paper and the Hybond C, which had been briefly soaked in blot buffer, placed on top of it, followed by the second filter paper. A glass rod was rolled across the layers to remove air bubbles and the cathode plate was opposed to the second filter paper and the apparatus closed before transfer to the tank for blotting. Proteins were transferred for 1 hour at 4°C at 300mA. Transfer was confirmed by visualization of rainbow markers on the membrane.

7.2.6.2 Immunodetection of TIMP1

Non specific binding to the membrane was blocked by incubation with shaking for 1 hour at room temperature in 5% non-fat milk powder (Marvel) and 0.1% tween in PBS. Primary antibody (Celltech MAC15 1.76μg/ml) was diluted 1 in 1000 in blocking solution and incubated for 2 hours with shaking at room temperature. The membrane was then washed for 15 minutes in 1% non-fat milk powder (Marvel) and 0.1% tween in

PBS. An horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibody (Sigma) was diluted 1 in 1000 in blocking solution and incubated with the membrane for 1 hour. The membrane was then washed for 30 minutes in 1% non-fat milk powder (Marvel) and 0.1% tween in PBS. Recombinant human TIMP1 (kind gift of CellTech limited) was used to confirm the sensitivity of this technique, 1 ng of Human TIMP1 could be readily detected. 5ng of rTIMP1 was used as a positive control in all experiments.

7.2.6.3 Enhanced Chemiluminescence Detection (ECL)

Protein bands were then visualized using the ECL (Amersham) chemiluminescence system which employs a nonradioactive light emitting reaction. Luminol, is oxidized by the horseradish peroxidase covalently bound to the second antibody, in the presence of hydrogen peroxide. Oxidized luminol is excited and decays to a stable state via a light emitting pathway. Membranes were incubated with ECL reagents for 1 minute prior to wrapping in cling film.

The light emitting signal was detected by autoradiography using Hyperfilm ECL (Amersham, UK). Exposure times between one and fifteen minutes were used.

7.2.7 Zymography

To detect the Gelatinases gelatin zymograms identify proteolytic activity of the enzymes which have been separated in the polyacrylamide gels under non reducing conditions (Kleiner and Stetler Stevenson, 1994). Gelatin was copolymerized with the acrylamide during casting of the running gel and the enzymes are separated under denaturing but non-reducing conditions. 0.1 % sodium dodecyl sulfate (SDS), 10 % polyacrylamide gels were made (Sambrook *et al.* 1989) and 1mg /ml gelatin (sigma) was added prior to addition of polymerizing agents (10% ammonium persulfate and TEMED)

and heated in a water bath to 60°C for 10 minutes. After the gelatin had dissolved the solution was allowed to cool to room temperature before polymerization was initiated and the gel cast as described on page 154. Samples were loaded in 5X sample buffer consisting of 0.4 M Tris, pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromophenol blue without reducing agent (i.e. ß mercaptoethanol is omitted and samples are not heated). Electrophoresis was carried out as previously described but samples were not exposed to heat prior to electrophoresis. The gel was removed and washed in 2.5% triton X-100 for 30 minutes. Triton X-100 detergent was used to remove the denaturing agent, sodium dodecyl-sulfate. The gel was incubated for 18 hours in 50 mM Tris-Cl pH 7.5 and 5mM calcium chloride at 37°C with gentle shaking. Gels were stained with 2.5 mg/ml Coomassie brilliant blue R-250 in 10% acetic acid, 30% isopropanol, and for 3 hours and then destained in the above solution without coomassie blue until optimal contrast was achieved. The gel was then rehydrated in distilled water and dried between cellulose sheets using the Biorad gel drying system.

7.2.8 Reverse Zymography

The principle of reverse zymography is very similar to zymography except in this case the inhibitor is detected and therefore the basis of the bioassay is to combine both Gelatinase and gelatin in the same gel. The samples were electrophoresed at 4°C to minimize Gelatinase activity as the gel is running. The gel was incubated and stained as for zymograms. If an inhibitor is present the digestion of gelatin by Gelatinase will be inhibited in that area of the gel and therefore blue bands reflecting the amount of inhibitor present appear on a pale or clear gel.

Reverse zymography was carried out using Reverse zymography kit (UTI) (Leco *et al.* 1994). This supplied a source of TIMP1,2, and 3 positive controls and Gelatinase A conditioned medium from stably transfected BHK

cells. 0.1 % sodium dodecyl sulfate (SDS), 10 % polyacrylamide gels were made omitting 1ml of water per 15ml total volume and 1mg /ml gelatin (sigma) was added prior to addition of polymerizing agents (10% ammonium persulfate and TEMED) and heated in a water bath to 60°C for 10 minutes. After the gelatin had dissolved the solution was allowed to cool to room temperature. 1 ml of solution A (conditioned media from cells overexpressing Gelatinase A) per 15ml of total volume was added before polymerization was initiated and the gel cast. Samples were loaded and electrophoresis carried out as described above (see page 154 and page 156) The gel was then washed in 2.5% triton X-100, 50 mM Tris -Cl pH 7.5 and 5mM $CaCl_2$ for 30 minutes. It was incubated for 16 hours in 50 mM Tris-Cl pH 7.5 and 5mM calcium chloride at 37°C with gentle shaking. Staining and drying of the gel was carried out as described above (page156)

7.2.9 Densitometry

All zymograms, reverse zymograms and autoradiographs of western blots were quantified using densitometry and the results expressed as a percentage of the positive control in each case. For zymograms transmission rather than density was used. Gels and blots were scanned using a Biorad model 620 video densitometer and peak area integration carried out using Biorad 1D Analyst software.

7.2.10 In Vitro Assessment Of Av1.TIMP1 In SV40 Transformed Vascular Smooth Muscle Cells And Primary Vascular Smooth Muscle Cells.

SV40 transformed VSMCs and primary VSMCs at passage 4-6 were grown to confluence and then placed in serum free medium for 24 hours prior to viral infection. Virus stocks were diluted in serum free medium to infect cells with Av1.TIMP1 at MOIs of 0 (i.e. control) 0.5, 5, 50, and 100, and with Av1.LacZ4 (MOI 100) to control for the effects of viral infection.

Infection solutions were incubated with the cells at 37°C for 1 hour. The cells were then returned to serum free medium and the medium was harvested 48 hours after infection. The media was spun at 1200 rpm, 4°C, for 5 minutes and the supernatant removed for quantitation of transgene product and TIMP1 bioactivity by Western analysis and reverse zymography respectively. The supernatants from the primary cells were also analyzed by zymography and densitometry performed on the reverse zymography bands corresponding to the molecular weight of TIMP2 . Each experiment was performed in triplicate.

7.2.11 The Effect Of Infection With Av1.TIMP1 On Proliferation Of Vascular Smooth Muscle Cells

7.2.11.1 VSMC Infection And Stimulation.

Primary cultured vascular smooth muscle cells were infected according to the protocol above at MOIs of 0.5, 5 , 50, 100 (Av1.TIMP1) and control wells were either infected with Av1.LacZ4 at MOI 100 or not infected. 8 replicates of each well were studied. 12 control wells were used. 24 hours after infection 10 ng/ml of basic fibroblast growth factor (bFGF) was added to half the wells and 4 of the control wells had fetal calf serum added to a final concentration of 20% as a positive control. The experiment was carried out three times.

7.2.11.2 [^{3}H] Thymidine Labeling

48 hours after infection 1μCi of ^{3}H thymidine was added. The cells were incubated for 2 hours and then washed twice in PBS. The cells were left at -20°C for 2 hours in ice cold 10% trichloroacetic acid. All this solution was removed by initial aspiration with a fine tipped glass pipette and then tipping plates for 10 minutes and aspirating the remaining liquid. The cell

precipitate was dissolved in 200 µl of 0.5M NaOH (plates were shaken for 30 minutes).

7.2.11.3 Scintillation Counting

800 µl of distilled H_2O was added and after mixing a 500 µl sample from each well was added to 5ml of echoscint and the ^3H thymidine incorporation determined in a scintillation counter. A 200µl sample was used to determine the protein content of the well using the method of Bradford. 2 vials containing 200µl of 0.1M NaOH (i.e. blank) were counted in the scintillation counter.

7.2.11.4 Data Analysis

The mean counts measured from the two 'blanks' or negative controls was subtracted from the final scintillation count for each sample. The result for each sample was then normalized to the protein content of the well to allow for unequal seeding. Students t test was used to compare samples.

7.2.12 The Effect Of Infection With Av1.TIMP1 On Migration And Invasion Of Vascular Smooth Muscle Cells.

The use of chemotaxis cell culture inserts of the type described here was new to this laboratory. In initial experiments it was necessary to establish the optimal cell seeding density, concentration of chemoattractant required for migration and invasion and the time course of cell movement in order to determine sampling times in future studies. The migration and invasion of cells in conditioned media from cells infected with Av1.TIMP1 and of cells infected with Av1.TIMP1 was then studied.

7.2.12.1 Characterization Of The Migration And Invasion Of Vascular Smooth Muscle Cells Towards A Chemoattractant.

7.2.12.1.1 Insert preparation

Control (i.e. non matrigel coated) inserts of a Biocoat Matrigel invasion chamber (Becton Dickinson) were coated with 100μg/ml type 1 collagen and 5μg/ml fibronectin and air dried. The matrigel inserts were rehydrated with serum free RPMI for 2 hours prior to use according to the manufacturers instructions.

7.2.12.1.2 Cell Preparation

Primary vascular smooth muscle cells were trypsinized at 80% confluence and centrifuged at 1200 rpm for 5 minutes at 4°C. They were then used

to study the parameters outlined below:

7.2.12.1.3 Cell Density Studies

Cells were then resuspended in serum free RPMI at densities of $2X10^6$/ml or $2X10^5$/ml. The control (i.e. non-matrigel coated) inserts were then placed in 0.75 ml of 300ng/ml platelet derived growth factor -BB (PDGF-BB R&D systems) in a cell culture companion plate (24 wells) and 0.5 ml of the cell suspension was immediately added to the insert. Cells were incubated at 37°C for 24 hours before staining and quantitation as outlined below.

7.2.12.1.4 Chemoattractant Concentration

Cells were resuspended in serum free RPMI at $2X10^6$/ml. Control (i.e. non matrigel coated) inserts were placed in 0.75 ml of platelet derived growth factor -BB (PDGF-BB) in a cell culture companion plate (24 wells) at concentrations of 10 ng/ml, 30 ng/ml and 300 ng/ml. 0.5 ml of the cell suspension was immediately added to the insert. Cells were incubated at 37°C for 24 hours before staining and quantitated as outlined below.

7.2.12.1.5 Time course of migration/invasion:

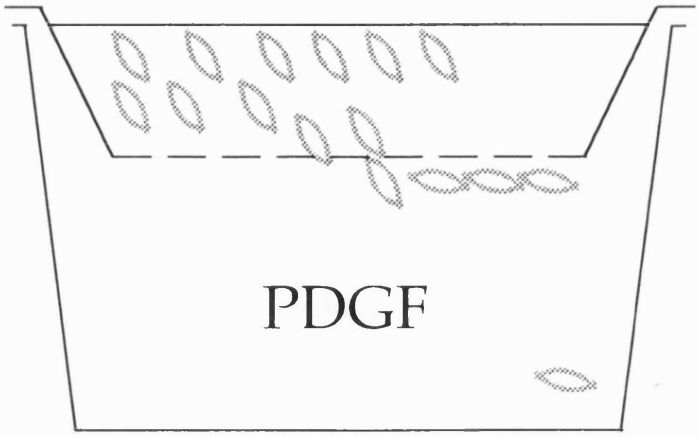
In this experiment both matrigel coated and uncoated wells were used. Cells were resuspended in serum free RPMI at $2X10^6$/ml. Control (i.e. non matrigel coated) inserts and matrigel inserts were placed in 0.75 ml of

300ng/ml platelet derived growth factor -BB (PDGF-BB) in serum free RPMI in a cell culture companion plate (24 wells). Cells were incubated at 37°C and stained and counted at the following times: 17, 20, 24, 25, 26, 28, 42, 48, 50, and 52 hours.

7.2.12.2 Quantitation Of Migration/Invasion

After incubation the cells adherent to the upper surface of the insert membrane were removed by firmly wiping the surface twice with a cotton wool swab. The membranes were then stained using the Prodiff kit (Baxter) allowed to dry and removed from the insert. The membranes were mounted using a water soluble mountant (Aqua Polymount). Cells were counted from the central area of the membrane. Sections were viewed at x40 magnification using a Labphot 2A microscope (Nikon) and the images digitized using a JVC-TK 1281 video camera connected to a Lucia M colour image processing system. Only cells in which the nuclei were visible were considered to have fully migrated. The image analysis program was then used to count the number of cells per high power field (see.

A



B

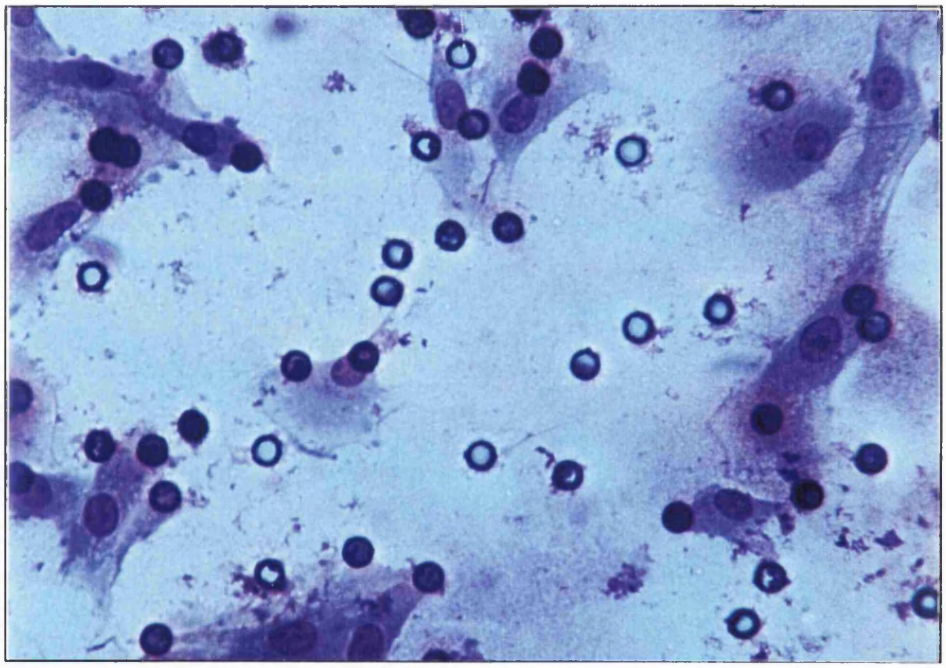


Figure 38. Assessment of Migration/Invasion of Smooth Muscle Cells.

The upper panel shows a schematic representation of the cell culture insert apparatus used in these experiments. Smooth muscle cells were seeded into the upper chamber and migrated across the microperforate membrane towards the chemoattractant PDGF. For invasion experiments inserts coated in a uniform layer of matrigel were used.

The lower panel shows a photomicrograph of a section of a membrane showing the smooth muscle cells that have successfully reached the lower surface of the membrane. The cells have been fixed with methanol and stained with Diff Quick reagents. All the cells on the upper surface have been removed with a cotton bud so that all stained cells are successful migrants (x40).

7.2.12.3 The Effect Of Conditioned Media On Migration/Invasion of VSMCs.

Primary VSMCs were infected with Av1.TIMP1 or Av1.LacZ4 (MOI 100) and control wells exposed to serum free media for one hour. 48 hours later the media were harvested and stored prior to use in the subsequent experiments. Primary VSMCs were resuspended in each conditioned medium at a density of $2x10^6$/ml and seeded into uncoated or matrigel coated cell culture inserts.

7.2.12.4 The Effect Of Prior Infection Of VSMCs With Av1.LacZ4 Or Av1.TIMP1 On Migration/Invasion.

Primary VSMCs were infected with Av1.TIMP1 or Av1.LacZ4 (MOI 100) and control wells were exposed to serum free media for one hour. 24 hours after infection the cells were trypsinized and resuspended in fresh serum free media at a density of $2X10^6$/ml.

Aliquots of the cell suspensions were placed in 24 well cell culture plates for 48 hours before fixation and X-Gal staining. The results were quantified microscopically by counting the number of blue cells with predominant nuclear staining in three high power fields.

The remaining resuspended cells were seeded into cell culture inserts in empty companion culture plates (i.e. they were not exposed to chemoattractant). The cells in inserts were allowed to adhere to the plates and condition the media in which they were suspended for 24 hours. The inserts were then moved to companion plates containing 0.75 ml of PDGF-BB 300 ng/ml.

7.2.12.5 Migration/Invasion Assay.

Cells were allowed to migrate towards 300ng/ml PDGF-BB for the 24 hours for uncoated wells and 48 hours for matrigel coated wells. The cells were then fixed and counted as described above.

7.3. RESULTS

7.3.1. Southern Analysis Of Candidate Plaques

Figure 39 shows the Pst1 restriction enzyme digest results for the candidate plaques. The autoradiograph in Figure 40 shows radiolabeling of the expected fragments of the positive control plasmid at 121 and 3432 bp in lane 3. In the lanes corresponding to the samples dense bands were seen at 353 and 767 bp in lanes 5, 8-15, 17 and 18. Hybridization with the 29 bp fragment was only seen in the positive control and with the 121 bp fragment in lane 8. Bands at higher molecular weights may represent nonspecific labeling in genomic DNA due to hybridization with short segments of the probe which is labeled throughout its length. The negative controls from cells infected with Ad5dl327 and uninfected cells in lanes 19 and 20 showed no bands. A similar pattern is seen in Lanes 6 and 7 suggesting that these plaques were due to Ad5dl327 rather than successful recombination. Incomplete digestion of the samples was seen in lanes 8 and 16. These results confirm that at least 11 of the candidate plaques show successful recombination to generate Av1.TIMP1.

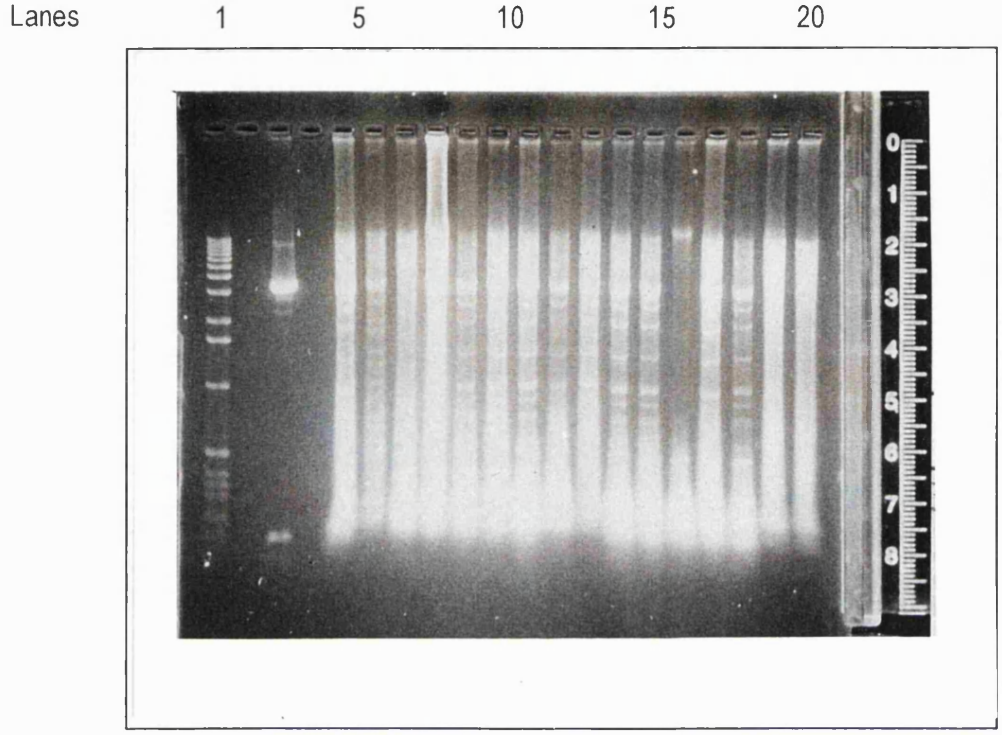


Figure 39. Restriction Digest of Genomic DNA Extracted From Rat Smooth Muscle Cells Infected With Candidate Recombinant Viral Plaques.

Agar gel photographed under ultraviolet light: Lane 1 1kb ladder, Lane 2 Empty. Lane 3 Pst1 restriction digest of Bluescript KS TIMP1, and Lane 4 Empty. Lanes 5-18 DNA extracted from cells infected with potential recombinant viral plaques and digested with Pst1. Lanes 19 and 20 show digests of DNA from smooth muscle cells infected with empty replication deficient adenovirus used in recombination (Ad5dl327) and uninfected cells respectively. Lanes 8 and 16 show only partial digestion by the restriction enzyme Pst1.

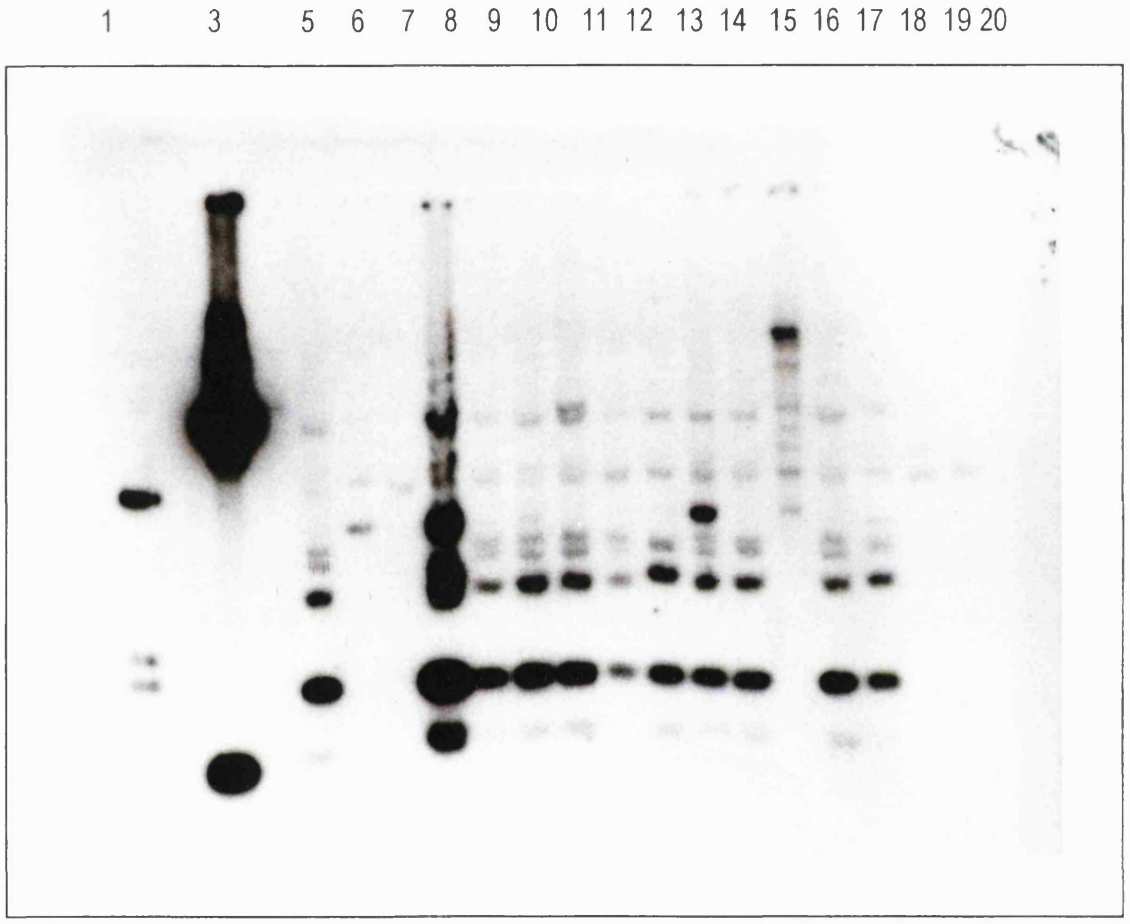


Figure 40. Autoradiograph of Southern Analysis for Human TIMP1 of DNA From Smooth Muscle Cells Infected With Candidate Plaques.

Lane 1 1kb ladder, Lane 2 Empty, Lane 3 Pst1 restriction digest of Bluescript KS TIMP1, and Lane 4 Empty. Lanes 5-18 DNA extracted from cells infected with potential recombinant viral plaques and digested with Pst1. Lanes 19 and 20 show digests of DNA from smooth muscle cells infected with empty replication deficient adenovirus used in recombination (Ad5dl327) and uninfected cells respectively.

This illustrates radiolabelling of the expected fragments of the positive control Bluescript KS TIMP1 (121kB and 3430 kB) in Lane 3. No TIMP1 is present in the negative controls (uninfected cells and Ad5dl327 infected cells) in lanes 19 and 20. The expected fragment size from TIMP1 adenoviruses detectable with this probe are 29, 121, 353, and 767. In the lanes corresponding to the possible TIMP1 viruses dense bands were seen at 353 and 767 bp in lanes 5, 8-15, 17 and 18. Hybridization with the 29 bp fragment was only seen in the positive control and with the 121 bp fragment in lane 8 which may reflect a larger amount of DNA loaded in those lanes. Lanes 8 and 16 showed only partial digestion by the restriction enzyme Pst1. This confirmed that at least 10 of the plaques tested contained viral recombinants that had incorporated the TIMP1 cDNA.

7.3.2 Western Analysis Of Candidate Plaques

The results are shown in Figure 41 with clear bands seen in the lanes corresponding to the positive control and candidate Av1.TIMP1 infected cells. No bands resulted from infection of VSMC's with the Ad5dl327 (Null virus).

7.3.3 In Vitro Assessment Of Av1.TIMP1 In SV40 Transformed Vascular Smooth Muscle Cells And Primary Vascular Smooth Muscle Cells.

Figure 42 shows examples of a western blot and a reverse zymogram performed on samples from the SV40 transformed cells. The mean TIMP1 band densities and standard errors from 3 experiments are shown in Figure 43. Western analysis using the anti human TIMP1 antibody demonstrates the transgene product only and therefore no TIMP1 was seen in the control or Av1.LacZ4 infected cells. A progressive increase in TIMP1 secretion was seen with near maximal levels reached at MOI of 50. This was confirmed using reverse zymography which detects the bioactivity of rat TIMP in addition to that of the human TIMP1 resulting from gene transfer (see Figure 43B).

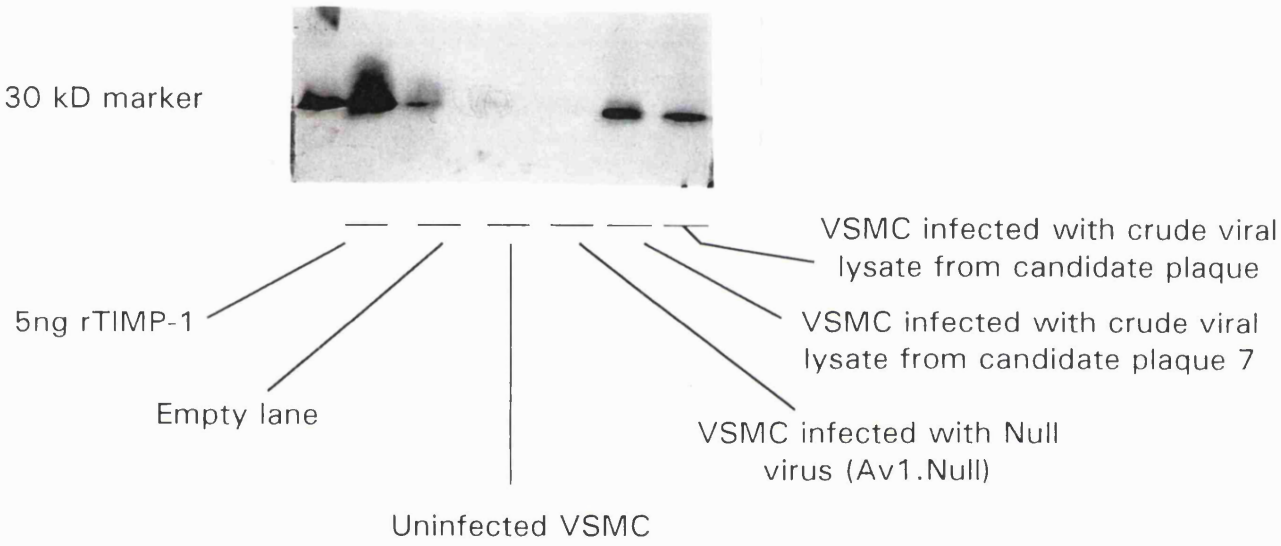


Figure 41. Western Analysis of Conditioned Media From Rat Vascular Smooth Muscle Cells Probed With Anti Human TIMP1 Antibody.

Lanes 1 and 2 show 30kD molecular weight marker and 5ng of rTIMP1 (positive control) respectively. Lanes 4-5 contain conditioned media from uninfected cells and cells infected with null virus and show that neither the conditions of the experiment or viral infection itself causes secretion of immunoreactive human TIMP1. Lanes 6 and 7 show contain media from cells infected with crude viral lysate from two plaques which contained TIMP1 by Southern analysis (7 and 37), both these lanes contain protein which reacts with human TIMP1 antibodies.

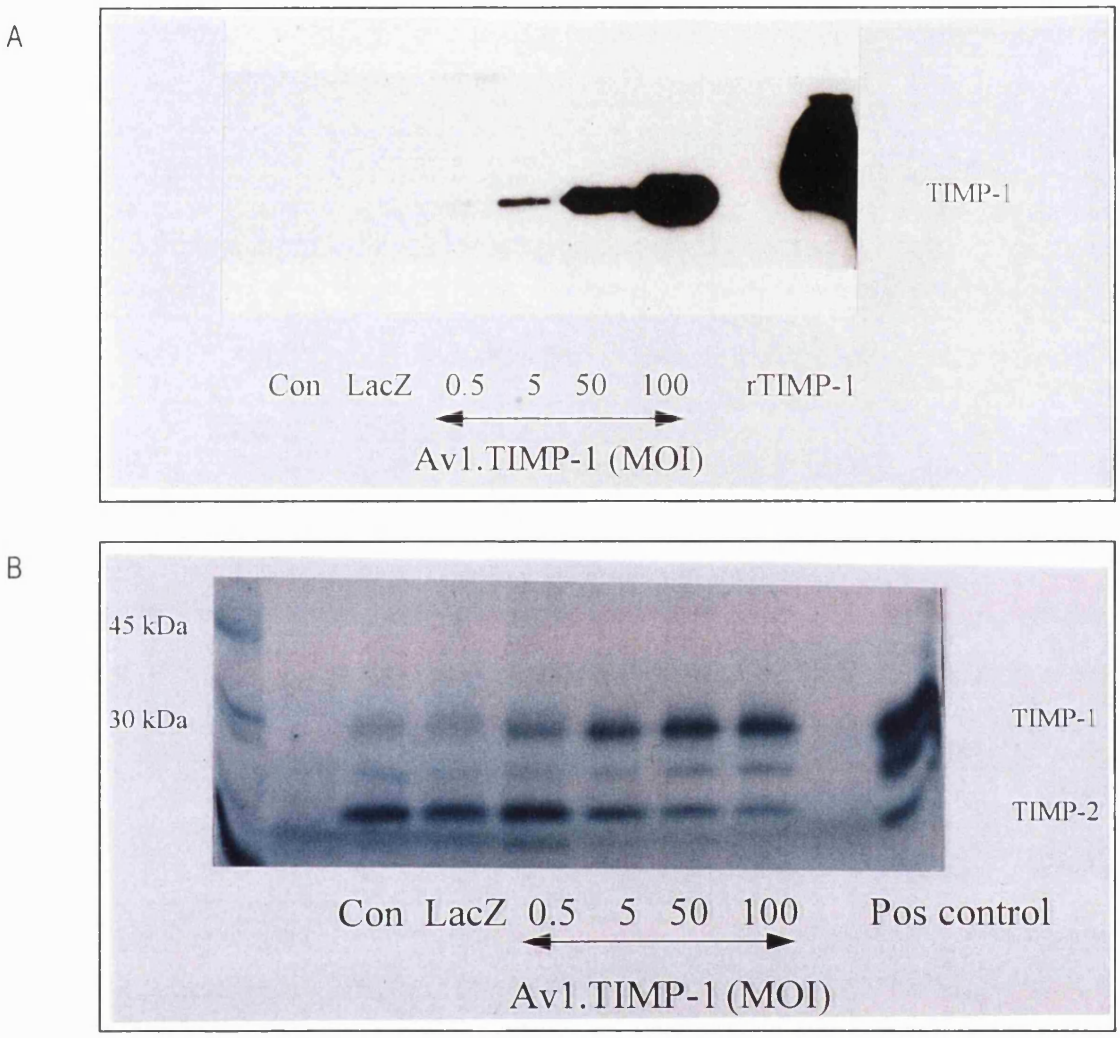


Figure 42. Sample (A) Western Blots and (B) Reverse Zymograms of Conditioned Media From SV40 Transformed Rat Vascular Smooth Muscle Cells 48 Hours After Exposure to Control Solutions or Increasing Amounts of TIMP1 Virus (Av1.TIMP1) (n=3).

Cells were infected with either increasing multiplicities of infection of Av1.TIMP1 (0.5-100), with control virus (LacZ), or with serum free media containing no virus (Con). 48 hours later conditioned media were harvested and concentrated. Western analysis using an antihuman TIMP1 antibody shows increasing amounts of the transgene product in response to higher viral titres but does not detect secretion of endogenous rat TIMP1. Recombinant TIMP1 was used as a positive control and to normalize results for densitometry (see figure 42). The bioassay, reverse zymography, illustrates that the transgene product maintains its biological activity and that activity is related to the amount of virus used and greater than in uninfected or Av1.LacZ4 infected cells. Blue bands on reverse zymograms reflect increased TIMP activity, the gelatinase in the gel has been inhibited where TIMP is present and is therefore unable to breakdown the gelatin which is stained with coomassie blue, giving the final colour. The upper band is TIMP1 and the lower TIMP2, the intermediate band is likely to represent a partially glycosylated form of TIMP1.

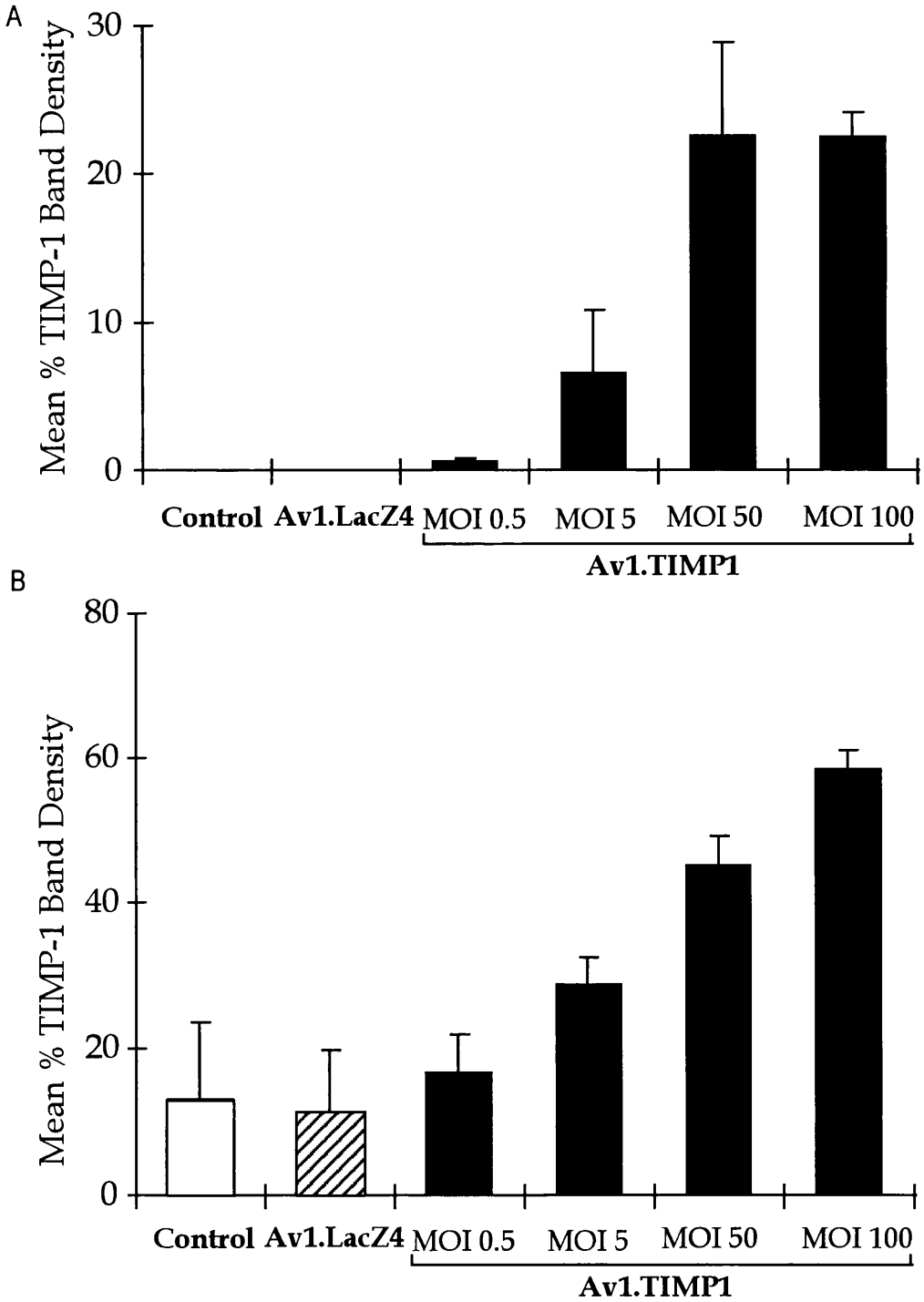


Figure 43. Densitometry of (A) Western Blots and (B) Reverse Zymograms of Conditioned Media From SV40 Transformed Rat Vascular Smooth Muscle Cells 48 Hours After Exposure to Control Solutions or Increasing Amounts of TIMP1 Virus (Av1.TIMP1) (Mean + SEM are shown, n=3).

Cells were infected with either increasing multiplicities of infection of Av1.TIMP1 (MOI 0.5-100), with control virus Av1.LacZ4, or with serum free media containing no virus (Control). 48 hours later conditioned media were harvested and concentrated. Western analysis using an antihuman TIMP1 antibody shows increasing amounts of the transgene product in response to higher viral titres but does not detect secretion of endogenous rat TIMP1. The bioassay, reverse zymography, illustrates that the transgene product maintains its biological activity and that activity is related to the amount of virus used and greater than in uninfected or Av1.LacZ4 infected cells.

Figure 44 shows examples of a western blot, a reverse zymogram, and a gelatin zymogram performed on samples from the primary VSMCs. These results confirmed the findings in the smooth muscle cell line, with an increase in immunoreactive protein and biological activity with increasing MOI of Av1.TIMP1Both western blots and reverse zymograms show an additional band at a lower molecular weight than expected for TIMP1 (28kD) which is also proportional to the multiplicity of infection. The mean density of the bands corresponding to TIMP1 in the western analysis and reverse zymograms were analyzed by densitometry and are shown in Figure 45. The mean bioactivity of TIMP-2 was not altered by infection with either virus (see Figure 46). Figure 47 shows the results of the mean transmission seen by zymography for each multiplicity of infection as a reflection of Gelatinase activity. Bands at the molecular weights corresponding to Gelatinase A and B were analyzed and plotted separately. There was no significant change in the levels of Gelatinase expression in response to infection with Av1.LacZ4 or Av1.TIMP1.

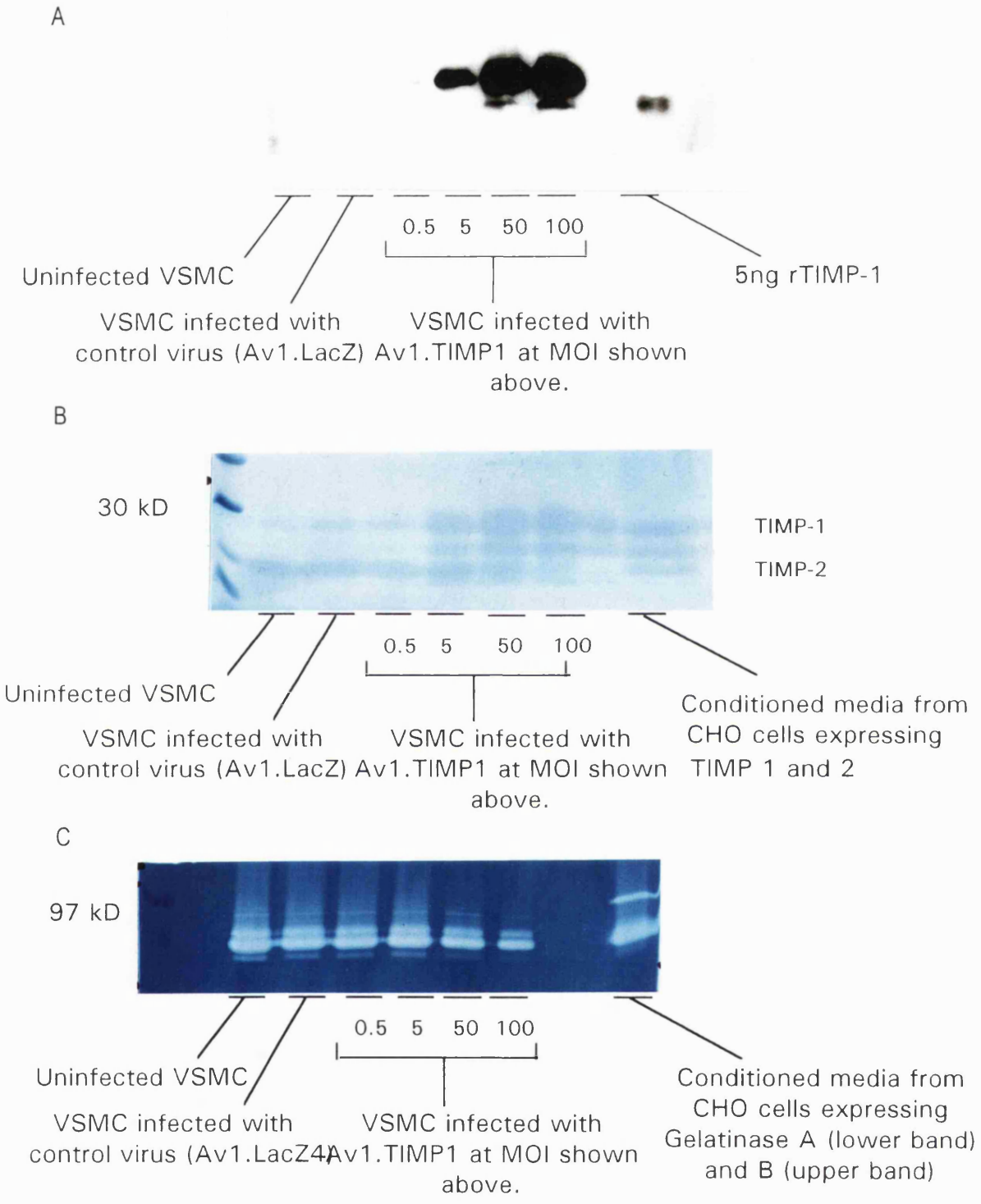


Figure 44. Sample (A) Western Blot, (B) Reverse Zymogram, and (C) Gelatin Zymogram of Conditioned Media From Primary Rat Vascular Smooth Muscle Cells 48 Hours After Exposure to Control Solutions or Increasing Amounts of TIMP1 Virus (Av1.TIMP1).

Western analysis using an antihuman TIMP1 antibody shows increasing amounts of the transgene product in response to higher viral titres. The bioassay, reverse zymography, also illustrates this 'dose response' effect. Blue bands on reverse zymograms reflect increased TIMP activity. The Gelatin zymogram shows lucent areas where the substrate has been digested by gelatinase in the conditioned media.

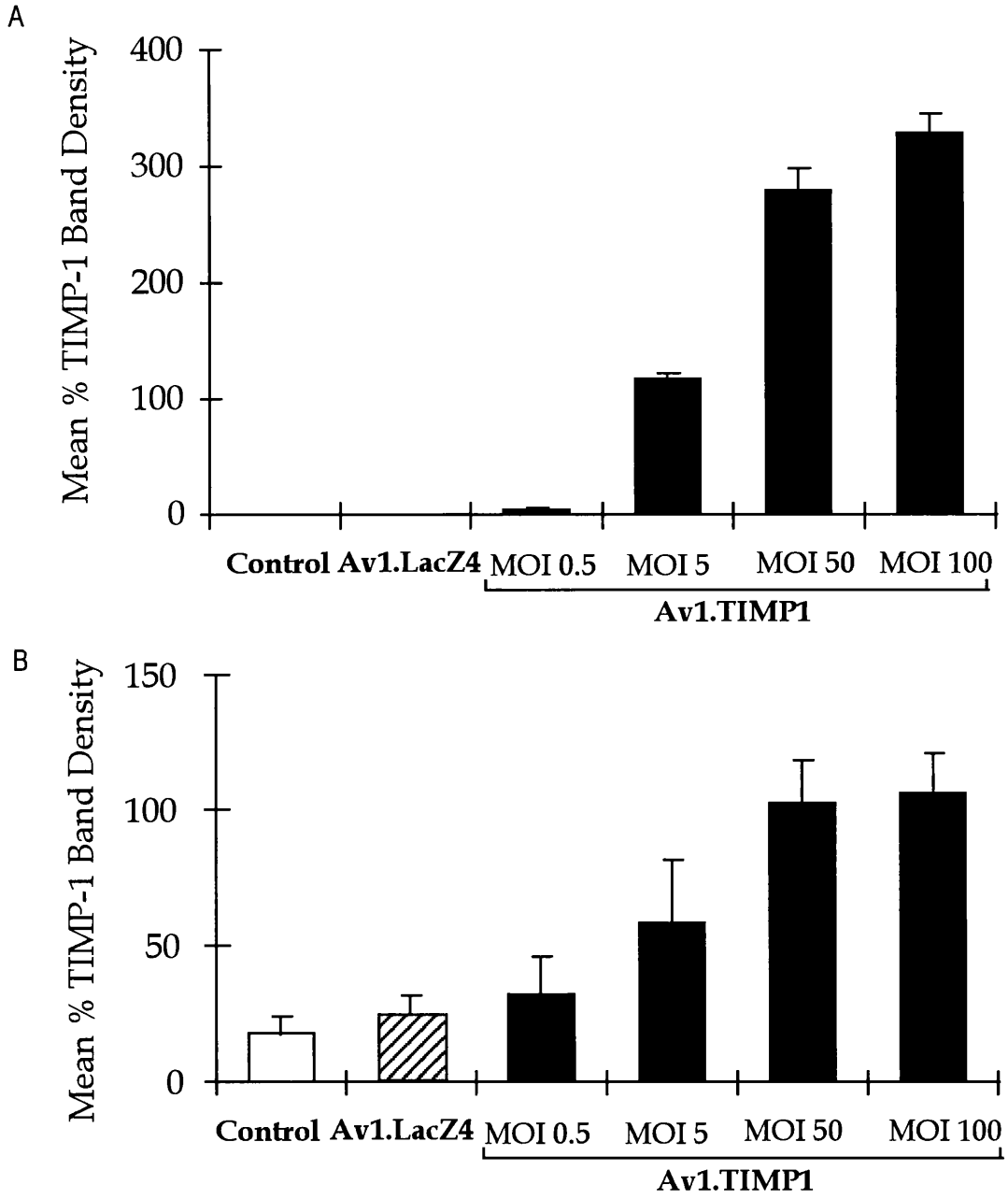


Figure 45. Densitometry of (A) Western Blots and (B) Reverse Zymograms of Conditioned Media From Primary Rat Vascular Smooth Muscle Cells 48 Hours After Exposure to Control Solutions or Increasing Amounts of TIMP1 Virus (Av1.TIMP1) (Mean +SEM are shown, n=3).

Cells were infected with either increasing multiplicities of infection of Av1.TIMP1 (MOI 0.5-100), with control virus Av1.LacZ4, or with serum free media containing no virus (Control). 48 hours later conditioned media were harvested and concentrated. All densitometry results are shown as a percentage of the positive control used. Western analysis using an antihuman TIMP1 antibody shows increasing amounts of the transgene product in response to higher viral titres but does not detect secretion of endogenous rat TIMP1. The bioassay, reverse zymography, illustrates that the transgene product maintains its biological activity and that activity is related to the amount of virus used and greater than in uninfected or Av1.LacZ4 infected cells.

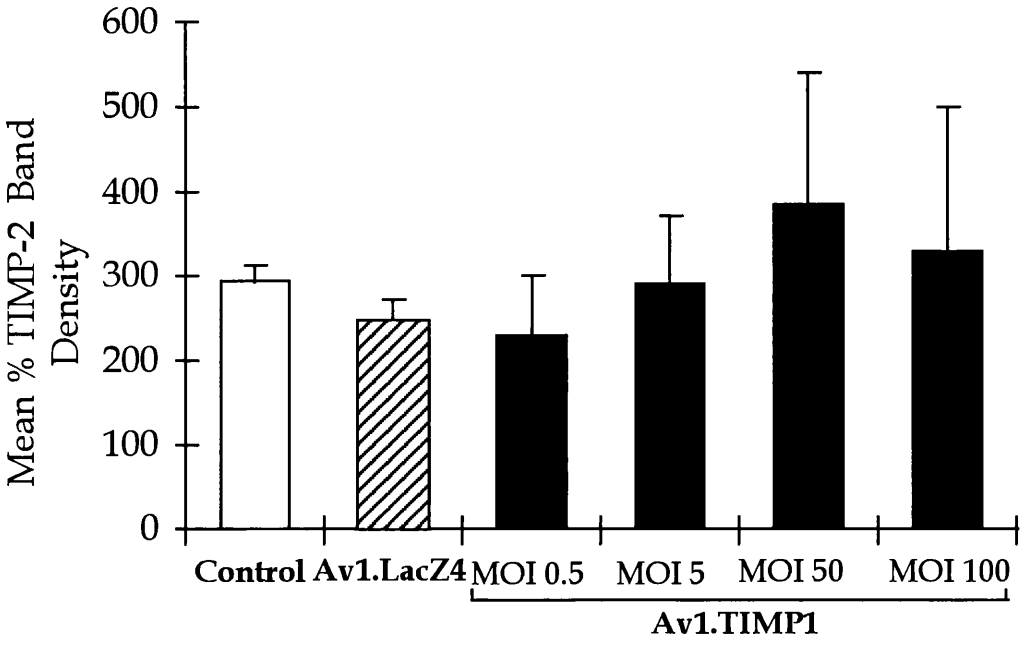


Figure 46. Densitometry of TIMP2 Band of Reverse Zymograms of Conditioned Media From Primary Rat Vascular Smooth Muscle Cells 48 Hours After Exposure to Control Solutions or Increasing Amounts of TIMP1 Virus (Av1.TIMP1) (Mean +SEM are shown, n=3).

Cells were infected with either increasing multiplicities of infection of Av1.TIMP1 (MOI 0.5-100), with control virus Av1.LacZ4, or with serum free media containing no virus (Control). 48 hours later conditioned media were harvested and concentrated.

Reverse zymography is a bioassay that will detect activity of any of the four TIMPs. Some reverse zymograms had suggested that there might be a compensatory decrease in TIMP2 in response to infection with Av1.TIMP1 and elevated levels of TIMP1. densitometric analysis of reverse zymograms shown above did not reveal an overall change in the TIMP2 bioactivity in primary cells.

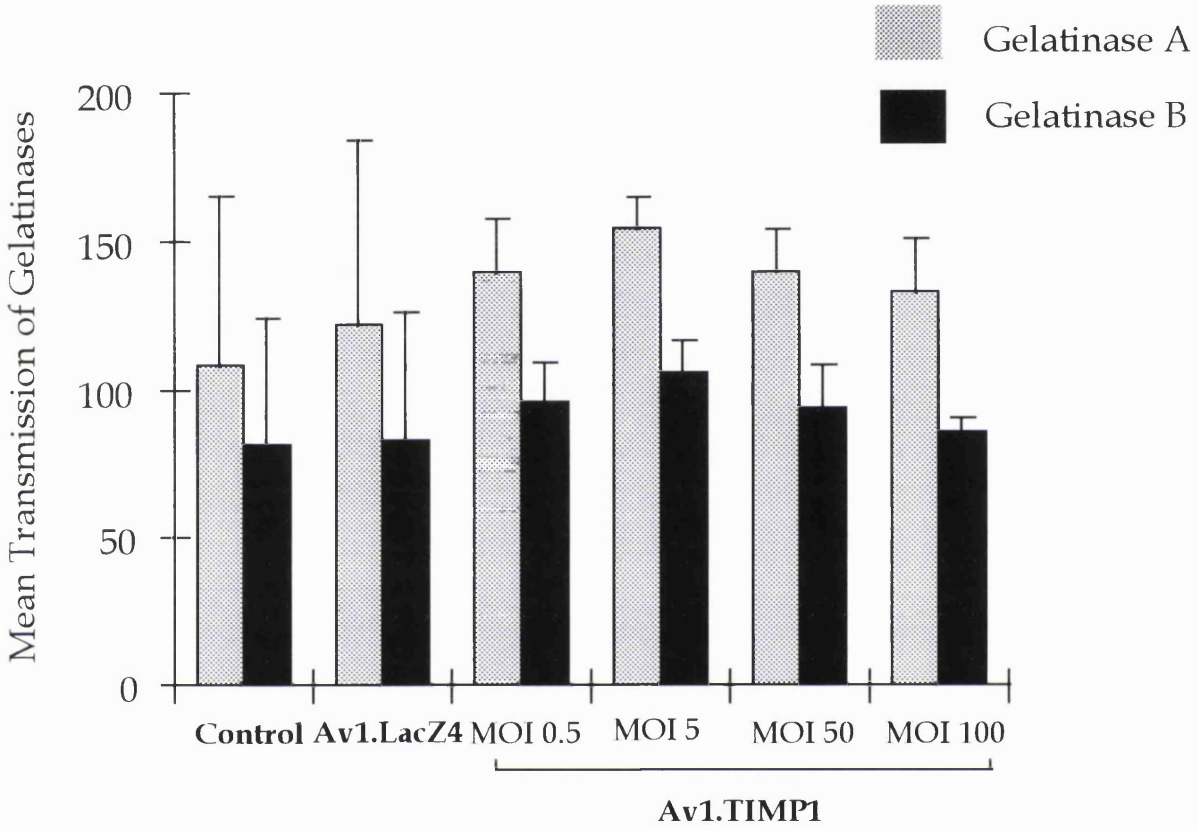


Figure 47. Densitometry of Gelatinase Activity on Zymograms of Conditioned Media From Primary Rat Vascular Smooth Muscle Cells 48 Hours After Exposure to Control Solutions or Increasing Amounts of TIMP1 Virus (Av1.TIMP1). (Mean +SEM Transmission Is Shown, n=3).

Cells were infected with either increasing multiplicities of infection of Av1.TIMP1 (MOI 0.5-100), with control virus Av1.LacZ4, or with serum free media containing no virus (Control). 48 hours later conditioned media were harvested and concentrated.

Bands at the molecular weights corresponding to Gelatinase A and B were analyzed and plotted separately. There was no significant change in the levels of Gelatinase expression, with low levels or absence of Gelatinase B and baseline expression of Gelatinase A. Active and inactive gelatinases were analyzed together but showed no change when separate densitometry was performed. Zymography of conditioned media separates TIMPs and Gelatinases and therefore is not influenced by the increased levels of TIMPs already demonstrated.

7.3.4 The Effect Of Infection With Av1.TIMP1 On Proliferation Of Vascular Smooth Muscle Cells

The mean [^{3}H] thymidine incorporation normalized to the protein content of each well and standard error of the mean are shown graphically in Figure 48. All cells responded to bFGF or serum with an increase in [^{3}H] thymidine incorporation of at least 50%. In quiescent cells no significant change in DNA synthesis was seen in Av1.TIMP1 infected cells compared with uninfected or Av1.LacZ4 infected cells (p all NS). There was a small reduction in proliferation in response to both Av1.LacZ4 and Av1.TIMP1 but this was not statistically significant

However cells stimulated to actively proliferate with bFGF and infected with higher multiplicities of infection of Av1.TIMP1 (5, 50, and 100) showed significantly greater proliferation compared with cells infected with Av1.LacZ4 (MOI 100)($p = 0.02$, 6×10^{-5}, and 0.03 respectively for MOIs 5, 50, and 100). Comparison of Av1.TIMP1 infected stimulated cells with uninfected cells showed no significant change in DNA synthesis.

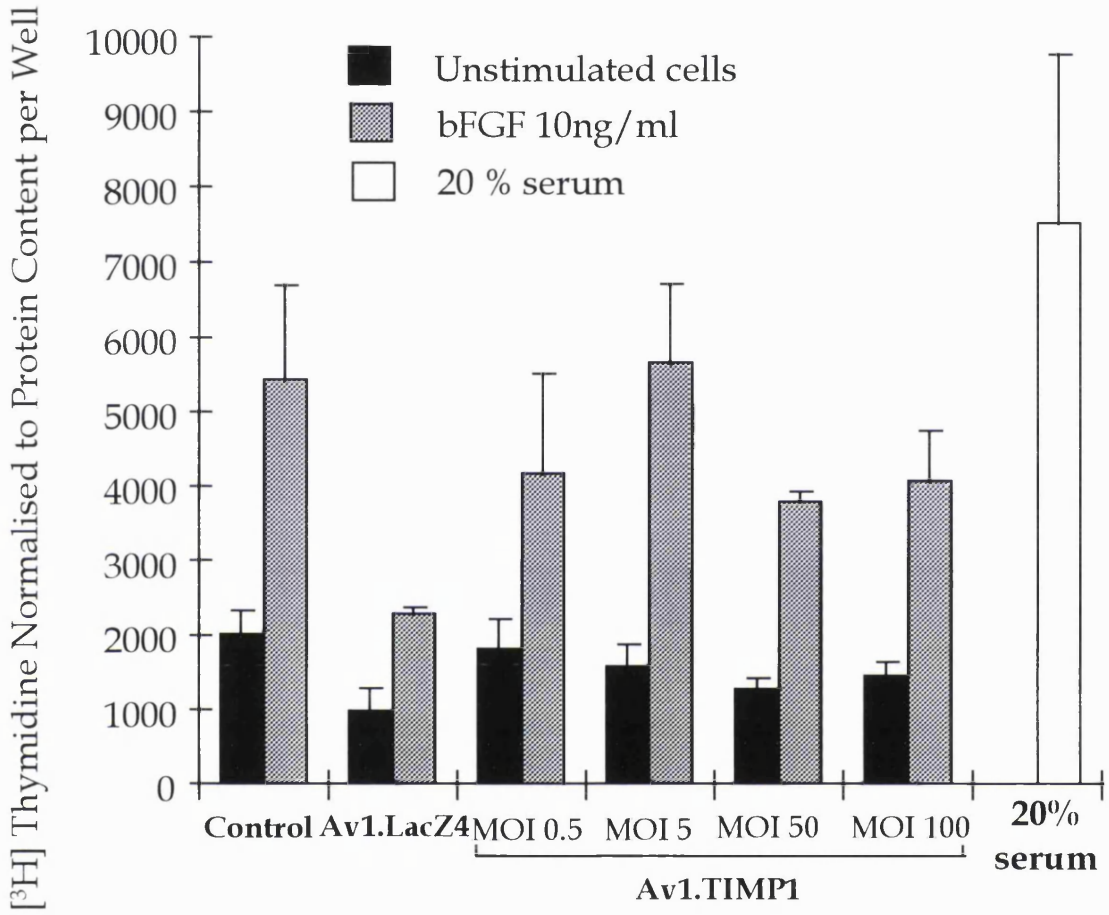


Figure 48: DNA Synthesis by Primary Smooth Muscle Cells After Infection With Av1.TIMP1. DNA synthesis is expressed as the uptake of [^{3}H] thymidine per well, normalized to the protein content of the cells within the well to remove any effect of unequal seeding (Mean +SEM are shown, n=4). Cells were infected with MOI 0.5-100 of Av1.TIMP1 or Av1.LacZ4 or exposed to serum free media with no added virus (control) and 24 hours later half the wells were stimulated to proliferate with bFGF 10 ng/ml. 48 hours after initial infection [^{3}H] thymidine was added and incorporation into the cells assayed by scintillation counting. The above graph shows inhibition of DNA synthesis by Av1.LacZ4. In contrast DNA synthesis after Av1.TIMP1 infection is not significantly different from uninfected cells. This may reflect an inhibitory effect of beta galactosidase on the cell cycle or a mild proliferative effect of TIMP1 which returns DNA synthesis to the baseline state.

7.3.5 The Effect Of Infection With Av1.TIMP1 On Migration and invasion Of Vascular Smooth Muscle Cells

7.3.5.1 Optimization of Migration/Invasion Assays

Table 4 shows the results of different cell densities, concentrations of chemoattractant, and times of migration. Cells were seen on the lower surface of the membranes only in the presence of PDGF-BB 300ng/ml. Optimal results occurred with a seeding density of $2x10^6$ cells/ml. Relatively long incubation times were required to observe migration/invasion (24 and 48 hours respectively).

	Migration, Number of cells/ high power field	
Cell density $2x10^6$/ml	118	95
Cell density $2X10^5$/ml	9	20
PDGF-BB 10 ng/ml	0	0
PDGF-BB 30 ng/ml	0	0
PDGF-BB 300 ng/ml	87	135

Time of Incubation, hours	Migration, Cells per high power field			Invasion, Cells per high power field			
17	21	0	15	0	0	0	0
20	43	66	58	0	0	0	0
24	214	170	190	0	0	0	0
25	177	190	230	0	0	0	0
26	227	225	241	0	0	0	0
28				1	0	0	0
42				2	5	0	0
48				50	53	60	48
50				22	35	30	40

Table 4 Optimization Of *In vitro* Migration Assay.

7.3.5.2 Confirmation of transgene expression in Migration/invasion studies

78% of cells showed blue staining for ß-Galactosidase after the trypsinisation and reattachment protocol used to assess migration in previously infected cells suggesting that continued transgene expression is possible under the experimental conditions used.

7.3.5.3 Migration/Invasion Results

Migration of VSMCs.

Cells resuspended in conditioned media showed a 45% reduction in migration when suspended in conditioned medium from Av1.LacZ4 infected cells compared with control but a 91 % reduction in those in conditioned medium from Av1.TIMP1 infected cells (see Figure 49A) ($n=9$). This comparison controls for the nonspecific effects of virus infection compared with the actions of the TIMP1 secreted after infection with the Av1.TIMP1 virus. In cells directly infected migration was reduced with Av1.LacZ4 (60% reduction) and Av1.TIMP1 (90% reduction)(see Figure 49B) ($n=9$). All these results showed a high degree of statistical significance ($p<0.01$ through out).

Invasion of VSMCs.

When cells suspended in conditioned media were allowed to invade through a basement membrane barrier there was, however, no significant effect of either virus ($p>>0.05$) (see Figure 50A) ($n=12$). In contrast cells directly infected with Av1.TIMP1 showed a 27% reduction in invasion but there was no effect of infection with Av1.LacZ4 (see Figure 50B) ($n=12$).

The chemoattractant used through out the chemotaxis experiments was PDGFBB.

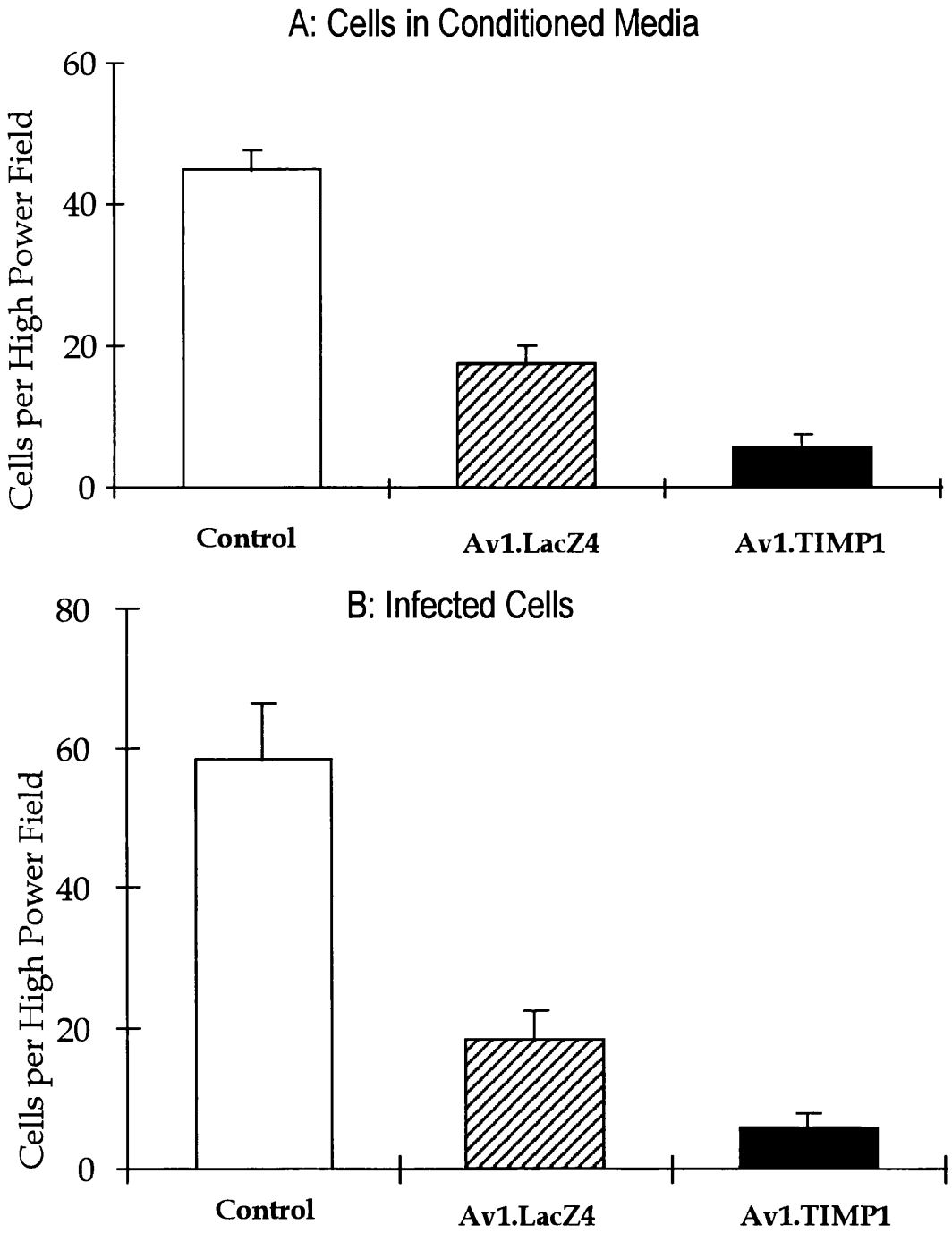


Figure 49 Migration of VSMCs.

A: Vascular smooth muscle cells were suspended in conditioned media from cells infected with Av1.TIMP1 or Av1.LacZ4 (MOI 100) or from uninfected control cells. They were then encouraged to migrate through collagen coated microperforate membranes by exposure to PDGF using a cell culture insert system. The cells on the lower side of the membrane were then stained and the number of cells per high power microscope field counted.

B: Vascular smooth muscle cells infected with Av1.TIMP1, Av1.LacZ4 and control uninfected cells were allowed to condition their own media for 24 hours prior to exposure to PDGF and migration (as above). Cells showed a 45% reduction in migration in response to Av1.LacZ4, which may represent a toxic or adhesive effect of the virus but a 90% reduction in response to Av1.TIMP1 suggesting that TIMP1 inhibits migration, results were the same in conditioned media or infected cells suggesting a paracrine effect for both TIMP1 and Av1.LacZ4. (Mean +SEM are shown, n=9)

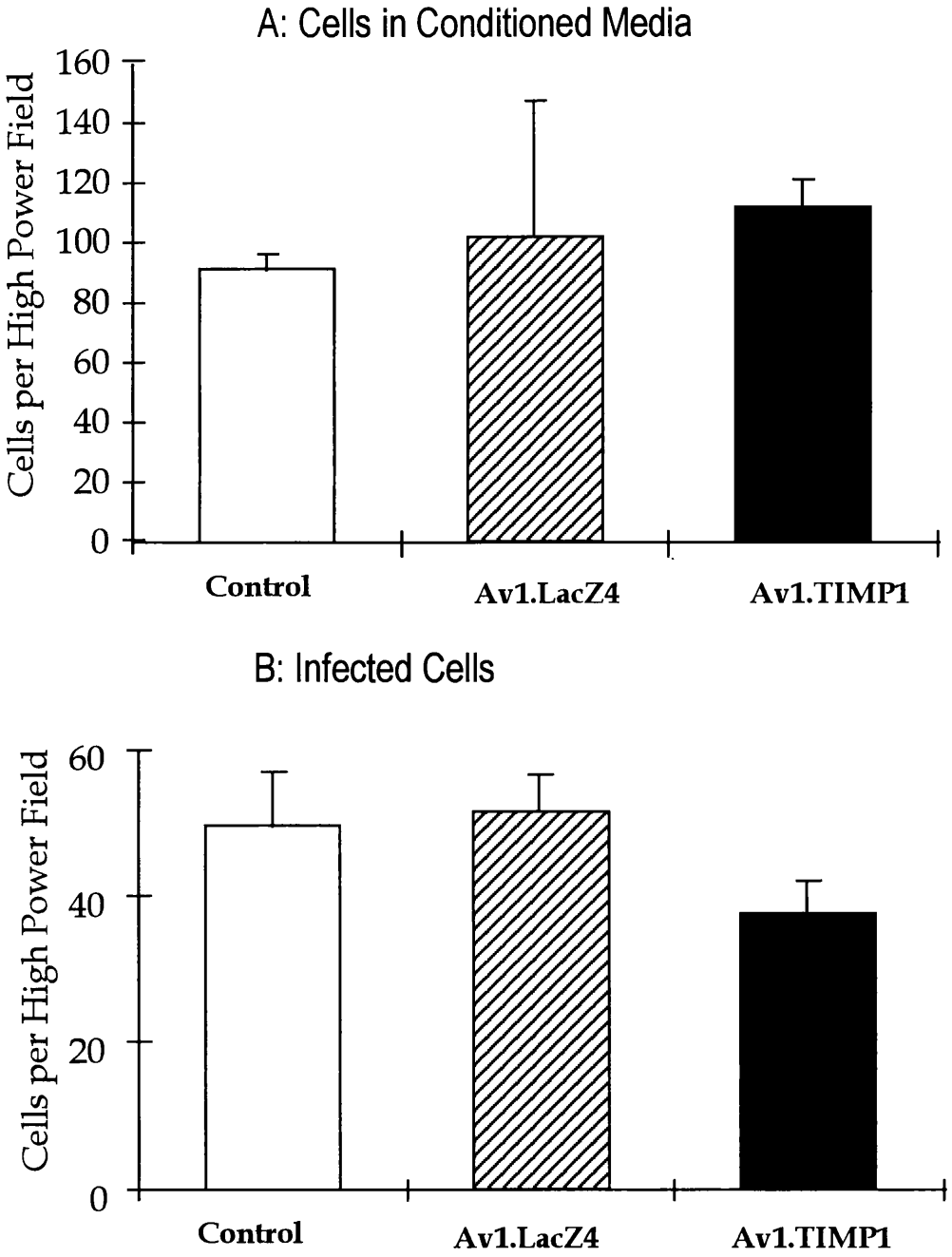


Figure 50 Invasion of VSMCs.

A: Vascular smooth muscle cells were suspended in conditioned media from VSMCs infected with Av1.TIMP1 or Av1.LacZ4 (MOI 100) or from uninfected control cells. They were then exposed to PDGF to promote invasion through matrigel coated microperforate membranes. The cells on the lower side of the membrane were then stained and the number of cells per high power microscope field counted.

B: Vascular smooth muscle cells infected with Av1.TIMP1, Av1.LacZ4 and control uninfected cells were allowed to condition their own media for 24 hours prior to exposure to PDGF and invasion of a matrigel barrier (as above).

Exposure to conditioned media from either virus did not alter invasion compared with media from untreated cells. In contrast cells directly infected with Av1.TIMP1 showed a 27% reduction in invasion compared with either control or Av1.LacZ4 infected cells. (Mean +SEM are shown, n=12).

7.4 Discussion

7.4.1 Screening Of Candidate Recombinant Plaques By Southern And Western Analysis

32 potential recombinant plaques resulted from the calcium phosphate cotransfection of the shuttle vector pAVS6ATIMP1 and the large ClaI fragment of the deleted type 5 adenovirus performed at Genetic Therapy Incorporated. 18 of these were screened for the presence of the TIMP1 cDNA by using crude viral lysate to infect VSMC's after limited propagation of each of the plaques. At least 11 of the plaques screened hybridized with the TIMP1 cDNA probe. Controls for presence of the TIMP1cDNA in 293 cells or the Ad5dl327 (deleted adenovirus used for recombination) showed no evidence of hybridization with the TIMP1 probe. The viruses were not screened at RNA level. It has been shown that the cDNA was present and resulted in production of immunoreactive protein. Plaque purification of one of the viruses studied by western analysis was therefore carried out and the resultant virus is referred to as Av1.TIMP1.

7.4.2 In Vitro Assessment Of Av1.TIMP1 In SV40 Transformed Vascular Smooth Muscle Cells And Primary Vascular Smooth Muscle Cells

In SV40 transformed and primary cultured vascular smooth muscle cells infection with Av1.TIMP1 results in secretion of immunoreactive human TIMP1 and this protein is biologically active. Reverse zymograms showed a major band of activity at 28 kD, the expected size for rat and human TIMP1, in cells infected with Av1.TIMP1 and also showed minor bands at 28 kD in media from uninfected or Av1.LacZ4 infected cells. The bands in the control lanes are likely to represent constitutively secreted rat TIMP1. These bands are absent in the western analysis because rat TIMP1 is not

detected by the anti human TIMP1 antibody used. Viral infection with Av1.LacZ4 (MOI 100) does not appear to effect TIMP1 bioactivity. The increase in TIMP1 seen using reverse zymography is similar to that seen by Western analysis. Comparison of the Western analysis and reverse zymography suggests that there has been little change in the baseline secretion of rat TIMP1 despite overexpression of human TIMP1 and that most of the human TIMP1 is biologically active. RNA analysis would be required to compare the true levels of expression of the human and rat TIMP1 but reverse zymography of conditioned media reflects the overall inhibition of MMP activity (Leco *et al.* 1994; Hawkes *et al.* 1996). At higher multiplicities of infection of Av1.TIMP1 an additional band was seen below the characteristic 28kD TIMP1 band. This band showed both immunoreactivity and biological activity proportional to viral titre, suggesting that it represents partially glycosylated biologically active TIMP1 produced by the transgene. This may indicate that at these levels of TIMP1 production the glycosylation pathway becomes saturated.

One of three gels suggested a compensatory decrease in TIMP-2 in response to TIMP1 overexpression but this was not born out by densitometric analysis of the triplicate experiments. Zymograms suggested that Gelatinase activity was unchanged by infection with Av1.TIMP1. Zymography is accurate to picogram levels and efficiently separates the Gelatinases from TIMP1 and 2 during electrophoresis of conditioned media (Kleiner and Stetler Stevenson, 1994).

Baker *et al* have published similar *in vitro* results to those above using an adenovirus containing a CMV-TIMP1 expression cassette to infect rabbit smooth muscle cells (Baker *et al.* 1996). In addition to studying bioactivity by reverse zymography they quantified secreted TIMP1 using an ELISA for total TIMP1(i.e. bound and free). They were able to achieve higher levels of TIMP1 by infecting cells at an MOI of 1000: 1243 ng/10^4cells/24 hours compared with 0.75 ng/10^4cells/24 hours at an MOI of 3. These data suggest that these cells can continue to increase the amount of TIMP1 produced up to this high level. However it is unlikely that such high titres

of virus would be applicable *in vivo* where toxicity of the vector may become the limiting factor.

In summary a recombinant adenovirus has been screened and characterized which expresses human TIMP1 at high levels in rat VSMC's producing a protein which is both immunoreactive and has biological activity.

7.4.3 The effect of infection with Av1.TIMP1 on proliferation of vascular smooth muscle cells

Previous studies have supplied evidence that MMP inhibition may inhibit proliferation while TIMPs may stimulate it (Southgate *et al.* 1992; Hayakawa *et al.* 1992; Bertaux *et al.* 1991; Hayakawa *et al.* 1994). It was the intention of these studies to address both questions by comparing baseline proliferation (which might be induced) and FGF stimulated proliferation (which might show some degree of inhibition). Pharmacological inhibitors of MMPs have been shown to inhibit proliferation assessed by [^{3}H] thymidine incorporation in a concentration dependent manner (Southgate *et al.* 1992). These results were obtained in a rabbit explant model where the extracellular matrix surrounding the cells may play a more important part in regulation of proliferation than in enzyme dispersal studies used here. It should be noted that this effect is not seen in all species (Kenagy *et al.* 1996).

TIMP1 is known to be a mitogen in some cell types, deriving its original name from its erythroid potentiating activity (Docherty *et al.* 1985; Bertaux *et al.* 1991; Hayakawa *et al.* 1992). The mechanism of this mitogenic effect appears to be distinct from the ability to inhibit MMPs but is poorly understood. The proliferative effect of TIMP2 has been shown to be due to activation of adenylate cyclase stimulating cAMP dependent protein kinase but this mechanism has not been demonstrated for TIMP1 (Corcoran and Stetler Stevenson, 1995). The most systematic study was undertaken in a Burkitt Lymphoma cell line. Free TIMP1 and inactive reductively alkylated

TIMP1 caused increased proliferation but TIMP1-ProGelatinase B complexes (which retain MMP inhibitory activity) did not (Hayakawa *et al.* 1994). Less detailed studies of human aortic smooth muscle cells have been undertaken but showed a relatively small proliferative response to 100ng/ml of TIMP1 compared with other cell types (Hayakawa *et al.* 1992).

A further consideration in these studies is the role of the adenoviral vector. Wild type adenoviruses are known to influence the proliferation of host cells both by inducing them to enter S phase and by blocking apoptosis (Debbas and White, 1993). These are properties usually associated with the E1A region of the adenoviral genome which interacts with a number of transcription factors including the retinoblastoma protein and E2F . This region is, however, deleted from the replication deficient viruses studied here. To confirm this the viruses used were screened for the ability to replicate by the absence of plaques on HeLa cells. It is clearly important to the study of proliferation that screening either by this method or by polymerase chain reaction for E1A be carried out (Zhang *et al.* 1995). In these studies of proliferation there was no evidence of an alteration in the DNA synthesis in quiescent cells in response to Av1.TIMP1 (p all NS) or Av1.LacZ4 ($p = 0.06$) infection ($n = 4$).

In cells stimulated to proliferate with bFGF a significant 58% reduction in DNA synthesis was seen in Av1.LacZ4 infected cells compared with uninfected control cells ($p = 0.045$). This reduction was not expected but has been described at higher multiplicities of infection in endothelial cells by other authors (Quax *et al.* 1996). The control vector was chosen because it has identical deletions to the TIMP1 vector and the gene expressed is under the control of the same promoter. This vector should therefore control for the effects of adenoviral infection, which are not related to TIMP1 gene expression. If the Av1.TIMP1 infected cells are compared with the viral control (Av1.LacZ4) a significant increase in DNA synthesis is seen at higher multiplicities of infection of Av1.TIMP1. The most relevant comparison is between the cells receiving MOI of 100 of

either Av1.LacZ4 or Av1.TIMP1 cells and a 45% increase in proliferation was seen ($p=0.03$). Clearly any proliferative effects of TIMP1 are undesirable in a gene therapy vector. An additional control vector containing a scrambled or reversed TIMP1 sequence would be of use in interpreting these results as it is unclear whether the most important effect here is of the Av1.LacZ4 virus to inhibit DNA synthesis or the secreted product of Av1.TIMP1 to stimulate it.

Quiescent cells were not stimulated to proliferate by Av1.TIMP1 but did respond to serum stimulation. Synergism between FGF and TIMP1 has not been described and could potentially be excluded by repeating this study with a different mitogen. The stimulation of differentiated cells to proliferate is, however, known to increase expression of Gelatinase A, TIMP2 and reduce TIMP1 (Pauly *et al.* 1994). The amount of TIMP1 complexed to proGelatinase B, or possibly to Gelatinase A, may be critical to any mitogenic effect. There was no change in proliferation in these experiments when Av1.TIMP1 infected cells were compared with uninfected cells.

In contrast to these results, which show constant or increased DNA synthesis, retroviral gene transfer of TIMP1 is associated with a reduction in cell number from 6 to 10 days after gene transfer compared to cells infected with a control retrovirus (Forough *et al.* 1996b). The difference may reflect the two parameters measured. [^{3}H] Thymidine incorporation measures entry of the cells into S phase of the cell cycle when DNA synthesis occurs while an increase in cell number reflects mitosis (Kirschenlohr *et al.* 1995). In studies of human VSMCs the two measures of proliferation appear to parallel each other, but this assumes that the conditions of study cause a constant loss of cells (Kirschenlohr *et al.* 1995). Cell number will increase as cells divide but could also be influenced by either necrosis or apoptosis. In this study DNA synthesis was measured. It was unchanged by Av1.TIMP1 infection in quiescent cells. In bFGF stimulated cells receiving the TIMP1 gene DNA synthesis was

increased when compared to adenovirus infected cells but not when compared to uninfected controls.

7.4.4 The Effect Of Infection With Av1.TIMP1 On Migration and invasion Of Vascular Smooth Muscle Cells

The hypothesis underlying this study is that neointimal hyperplasia may be inhibited by preventing the migration of smooth muscle cells into the neointima by the overexpression of TIMP1. Inhibition of both migration and invasion of a basement membrane barrier by TIMP1 gene transfer was demonstrated. Studies of pharmacological inhibitors of MMPs have revealed that they suppress invasion of smooth muscle cells *in vivo* (Bendeck *et al.* 1994; Zempo *et al.* 1996) and *in vitro* (Pauly *et al.* 1994). 72 kD Gelatinase has been shown to be essential for the migration of cells through a basement membrane barrier (Pauly *et al.* 1994). It has also been shown that phenotypic changes in vascular smooth muscle cells may alter their ability to migrate. Proliferating cells show enhanced migration compared to differentiated cells. It has therefore been suggested that one means of upregulating Gelatinase A activity after vascular injury may be the change of smooth muscle cells from a quiescent to a proliferating motile phenotype (Pauly *et al.* 1994).

The experiments described in this thesis show two unexpected results. Firstly migration of smooth muscle cells is nonspecifically inhibited by adenoviral infection. Secondly migration is inhibited by TIMP1 both in conditioned media and when cells were infected with Av1.TIMP1.

The inhibition of migration which accompanied adenoviral infection may represent a toxic effect of the virus but was only seen in the migration experiments not in the invasion studies. The effect was similar whether the cells were infected directly with the virus or had been resuspended in conditioned media. This raises the possibility that the effect could be mediated by a paracrine factor. Previous studies have shown that infection with Av1.LacZ4 does not alter the activities of the Gelatinases or TIMPs

suggesting that they do not have a role in this effect of Av1.LacZ4 on migration. The mechanism appears to be related to an interaction between the cells and the collagen coated filter as it was not seen when the cells were seeded on to matrigel. It is possible that the expression of leukocyte adhesion molecules in response to adenoviral infection may make the cells adhere firmly to the upper surface of the PET membranes and prevent migration (Newman *et al.* 1995). The induction of adhesion molecules by adenoviruses has, however, only been described *in vivo* and is usually attributed to infiltration of surrounding leukocytes rather than a direct effect on smooth muscle cells (Newman *et al.* 1995). Further investigation of this effect with lower viral titres, immunohistochemical staining for ICAM and VCAM, and different filter coatings might address these questions.

TIMP1 significantly reduces migration in its own right, whether it is compared with controls or cells infected with Av1.LacZ4. The ability of smooth muscle cells to invade a membrane barrier is clearly influenced by MMP inhibition (Forough *et al.* 1996b; Pauly *et al.* 1994). The mechanisms of inhibition of migration through plastic membranes are less obvious. Using peptide inhibitors and antisera Pauly *et al* have observed no difference in migration through a collagen coated membrane in response to MMP inhibition (Pauly *et al.* 1994). Results of experiments manipulating MMP activity using the TIMPs do not, however, always parallel those using pharmacological inhibitors. The experiments described in this thesis suggest that TIMP1 can inhibit migration. This has also been observed in studies of B16F10 cells transfected with wild type and deletion mutants of TIMP1 (Walther and Denhardt, 1996). Deletion mutants that retained anti collagenolytic activity were able to inhibit either migration or invasion of the cell line.

The mechanism of this effect is unclear. Interactions between MMPs and integrins may play a part. MMP 2 is known to block adhesion to vitronectin but not to laminin and its inhibition by TIMP1 might block adhesion rather than migration itself (Brooks *et al.* 1996). It seems probable that cell-cell

and cell-matrix interactions are important in the motility of smooth muscle cells and *in vitro* models that look only at the ability of a cell to digest a hole in a matrix layer are informative but overly simplistic.

A 30% reduction in invasion of a matrigel barrier was seen in the Av1.TIMP1 infected cells compared with control or Av1.LacZ4 infected cells. These results parallel those of Pauly and Forough (Forough *et al.* 1996b; Pauly *et al.* 1994). Pauly *et al* used a number of strategies to study this process *in vitro* including a comparison of inhibition of MMPs using MMP propeptides, thiol-based inhibitory peptides, and antisera to the MMPs (Pauly *et al.* 1994). They found that invasion through a matrigel barrier was reduced by 80% when Gelatinase A activity was inhibited. Recent results show that cleavage of laminin 5, a likely component of matrigel (Kleiner and Stetler Stevenson, 1994), by Gelatinase A stimulates migration (Giannelli *et al.* 1997). This may be an additional component in the results of Pauly *et al.* Forough *et al* showed a similar 75% reduction in invasion of retrovirally transfected smooth muscle cells overexpressing TIMP1 using a thick basement membrane barrier (Forough *et al.* 1996b). The greater degree of inhibition of invasion seen by these investigators compared with the results presented in this study is likely to be due to the presence of a higher concentration of TIMP1. The concentration of MMP inhibitors used in Pauly's study reduced the MMP activity in media by more than 99%. Retrovirally transduced cells used by Forough *et al* were selected to ensure 100% of the cells express the transgene TIMP1. In addition the transgene was under the control of the CMV promoter, known to give higher levels of transgene expression than RSV. It is likely, therefore, that a higher level of MMP inhibition explains the greater inhibition of invasion in the studies discussed above compared to the results presented in this thesis.

Invasion of VSMCs resuspended in conditioned media was the same in the three experimental groups. This may reflect the long time course of the invasion experiments (48 hours). It is possible that TIMP1 in conditioned media for 48 hours at 37°C may lose its biological activity. Forough *et al*

studied the effect of conditioned media from retrovirally transduced cells over four hours and showed a 60% inhibition of invasion (Forough *et al.* 1996b). These results were however obtained using media that had been concentrated ten fold and a matrigel layer substantially thinner than that in the inserts used in this thesis. The higher concentrations of TIMP1 which may occur with the retroviral selection system (discussed above) may also explain these divergent results.

Overall these results support the hypothesis that Av1.TIMP1 may prevent the invasion of smooth muscle cells through a basement membrane barrier and therefore reduce neointimal development.

CHAPTER 8. *IN VIVO* ASSESSMENT OF AV1.TIMP1

8.1 INTRODUCTION

The onset of a proteolytic state associated with increased MMP and plasminogen expression after vascular balloon injury appears to promote proliferation and migration of smooth muscle cells and the development of a neointima. The Av1.TIMP1 virus inhibits migration *in vitro* . This would suggest that local overexpression *in vivo* may reduce neointimal hyperplasia although *in vitro* there is little effect on proliferation of primary vascular smooth muscle cells. However there are difficulties with gene delivery *in vivo*, such as prolonged vessel occlusion and most importantly the systemic inflammatory response to viral infection. Newman *et al* have shown that infection with replication deficient adenoviral vectors causes both inflammation of the vessel wall and development of neointimal hyperplasia (Newman *et al.* 1995). Immune responses can also be provoked by the product of the transgene as well as the vector. This has been demonstrated by overexpression of baboon TIMP1 in the rat resulting in neutralizing antibody production which prevented any biological effect on second administration of the same transgene (Forough *et al.* 1996b). Therefore it is not possible to fully predict the success of a gene transfer strategy *in vivo* from *in vitro* results.

The results of *in vitro* experiments discussed above have demonstrated that overexpression of TIMP1 by adenoviral gene transfer can inhibit migration of smooth muscle cells without altering proliferation. In this study Av1.TIMP1 virus was used to express human TIMP1 *in vivo* in the injured rat carotid. Immunohistochemistry and western blotting have been used to demonstrate successful gene transfer and histomorphometry used to assess the effect on the lesion formed after balloon injury. Proliferation, migration and collagen staining were studied to investigate the effect of TIMP1 on the different components of the response to vascular injury.

The aim of this study was to examine the effect of TIMP1 expression, using the virus already characterized above, on the development of a neointima in the balloon injured rat carotid artery.

8.2 Methods

8.2.1 Animal Surgery.

300-350g male Wistar rats underwent balloon injury to the left carotid artery under general anesthesia as described on page 52. 100μl of Av1.TIMP1 diluted in RPMI medium (Gibco) was instilled into the carotid artery with a 20 minute luminal dwell period as described on page 123. Control rats received 100μl of diluted Av1.LacZ4. Tissue was harvested two, four, and fourteen days after injury.

8.2.2 Isolation Of Arterial Protein And Western Analysis For TIMP1.

Western analysis of protein extracted from the arteries after gene transfer allowed assessment of optimal dosing for morphometric experiments.
Surgery was carried out on 4 rats as described above. Two concentrations of virus were used for Av1.TIMP1 and Av1.LacZ4 (5μl and 50μl of Av1.TIMP1 ($1x10^{11}$ pfu/ml) and 5μl and 50μl of Av1.LacZ4 ($1x10^{10}$) each made up to 100μl total volume for gene delivery with RPMI. The arteries were harvested 2 days after injury and the fresh tissue snap frozen in liquid nitrogen and homogenized in 0.05 mol/l Tris (pH 7.6), 1% SDS, 100 μg/ml leupeptin, and 1μmol/l phenyl methyl sulphonyl fluoride (PMSF) (Webb *et al.* 1997). The homogenate was vigorously vortexed and centrifuged at 12000 rpm for 5 minute. The supernatants were then assayed for protein concentration using the method of Bradford and western analysis for human TIMP1 carried out.

8.2.3 Zymography of Arterial Extracts.

Aliquots of the extracts of the arterial tissue prepared for western analysis described above were used for gelatin zymography to assess the biological activity of the Gelatinases. The samples had equal protein content and zymography was carried out as described on page 156.

8.2.4 Immunohistochemical Demonstration Of In Vivo TIMP1 Gene Transfer.

Tissues harvested after pressure perfusion fixation at 120mmHg in 4% formaldehyde, 4 days after balloon injury, were embedded in paraffin. 5μm sections were cut onto poly-L-lysine coated microscope slides and heated at 60°C for one hour. The sections were deparaffinized in xylene for 3 minutes and rehydrated in a graded alcohol series as follows: 100 % ethanol 30 seconds, 95 % ethanol for 30 seconds, 75 % ethanol for 30 seconds and H_2O for 10 seconds. Immunohistochemistry for human TIMP1 was carried out as described on page 83 using monoclonal anti- human TIMP1 antibody at a dilution of 1:1000. Sections were lightly counterstained with Haematoxylin to delineate the areas which did not demonstrate positive immunostaining (see page 261).

8.2.5 Morphometry.

Fourteen days after surgery tissues were fixed by 10 minute retrograde perfusion with 2% formaldehyde, 0.2% glutaraldehyde at 120mmHg. The carotid arteries were harvested and paraffin embedded. 5μm serial histological sections were stained with Haematoxylin and Eosin and Verhoeff's -van Gieson and viewed at x40 magnification using a Labphot 2A microscope (Nikon) and the images digitized using a JVC-TK 1281 video camera and a Lucia M colour image processing system. The luminal area, area within the internal elastic lamina, and area within the external

elastic lamina were traced and the intimal and medial areas were calculated by subtraction.

8.2.6 In Vivo SMC Migration

Migration of vascular smooth muscle cells was assessed by counting the number of cells which had migrated beyond the internal elastic lamina in samples perfusion fixed four days following gene transfer. This method is based on observations that the cells within in the neointima at this time point are largely the result of migration and not proliferation (Clowes *et al.* 1989). 20 Haematoxylin and Eosin stained sections were assessed per artery. Serial sections were stained with Verhoeff's-van Gieson's stain to confirm the site of the internal elastic lamina.

8.2.7 Measuring DNA Synthesis And Cell Number.

Proliferation was assessed two days after injury in arterial sections perfusion fixed with 4% formaldehyde. Immunohistochemistry for the Ki 67 antigen using rabbit polyclonal anti Ki 67 antibody at a dilution of 1:25 (Dako) according to the principles described on page 83 was used. Prior to immunohistochemistry antigens were revealed by placing slides in 0.01M Citrate buffer (0.01M Citric Acid brought to pH 6.0 with 2M NaOH) at 60°C, covering the container and microwaving the slides at 20% power for 2 periods of 5 minutes. The level of citrate buffer in the container was replenished as required to keep the slides immersed. The container was then rapidly cooled to 40°C by addition of distilled water. Following immunohistochemistry the reaction was developed using diaminobenzidine (Sigma fast DAB). Sections were lightly counterstained with Haematoxylin to determine the percentage of positive nuclei.

The cell number within the media and neointima was quantified four days after injury by counting nuclei in three Haematoxylin and Eosin stained sections taken at 100μm intervals.

8.2.8 Picrosirius Red Staining For Collagen

Collagen studies used picrosirius red staining of paraffin embedded sections. Sections were dewaxed and rehydrated thoroughly. They were then incubated for two minutes in 0.2% phosphomolybdic acid prior to staining in a 0.1% solution of Sirius red F3BA in saturated aqueous picric acid pH2 for 90 minutes. Saturated aqueous picric acid was made by dissolving 6 g of picric acid in 200 ml dH_2O with heating. The PSR staining solution was saturated with picric acid such that crystals were visible. Sections were washed in 0.01M HCl for two minutes prior to dehydration and mounting. The image analysis system described above was used to compare this measure of collagen content by calculating the integral density of the staining in the media and neointima of each section (Coats, Jr. *et al.* 1997). Where comparative density measurements were required all slides for a single experiment (control and intervention) were stained together.

8.3 RESULTS.

8.3.1 Western Analysis For TIMP1

Figure 51 shows the western blot of the arterial extracts from the arteries infected with Av1.TIMP1. No human TIMP1 immunoreactivity was seen in the samples from right carotid arteries of animals infected with Av1.TIMP1 or those from Av1.LacZ4 infected arteries (not shown). The Av1.TIMP1 infected arteries showed a clear band with $5x10^8$ pfu but no band was seen when the higher concentration of virus ($5x10^9$pfu) was used.

8.3.2 Immunohistochemistry For TIMP1

Positive immunostaining was seen through out the media and adventitia of the left carotid arteries which received the Av1.TIMP1 virus with similar results at two and four days after injury (see Figure 52). No staining was seen in the Av1.LacZ4 left or right carotid arteries or in the right carotid arteries of the Av1.TIMP1 rats.

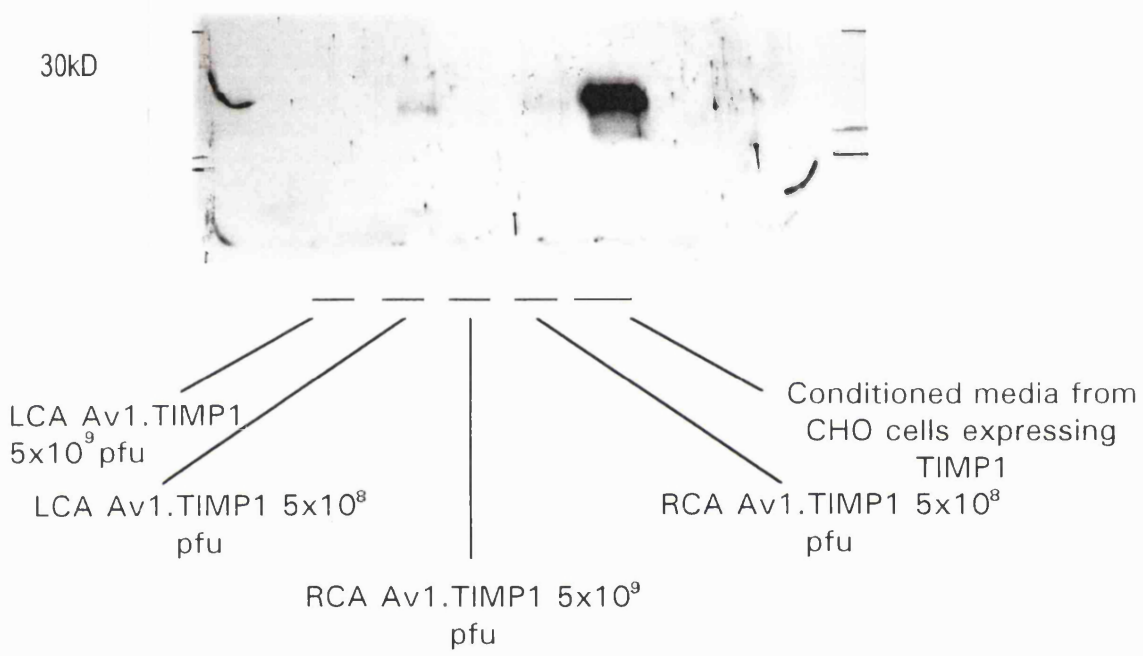


Figure 51. Western Analysis of Arterial Extracts Probed With Anti Human TIMP1 Antibody.

Lanes 1 shows the 30kD molecular weight marker. Lane 2 is empty. The other lanes contain the samples of protein extracted from rat arteries 2 days after balloon injury or media from cells known to stably overexpress human TIMP1 (positive control). In each case virus was administered to the left carotid artery (LCA) of the animal and the right carotid artery (RCA) is used as a control.

Immunoreactive protein is only seen in the lanes containing extracts from the LCA which received the lower concentration of virus, 5 x 10^8 pfu, and the positive control.

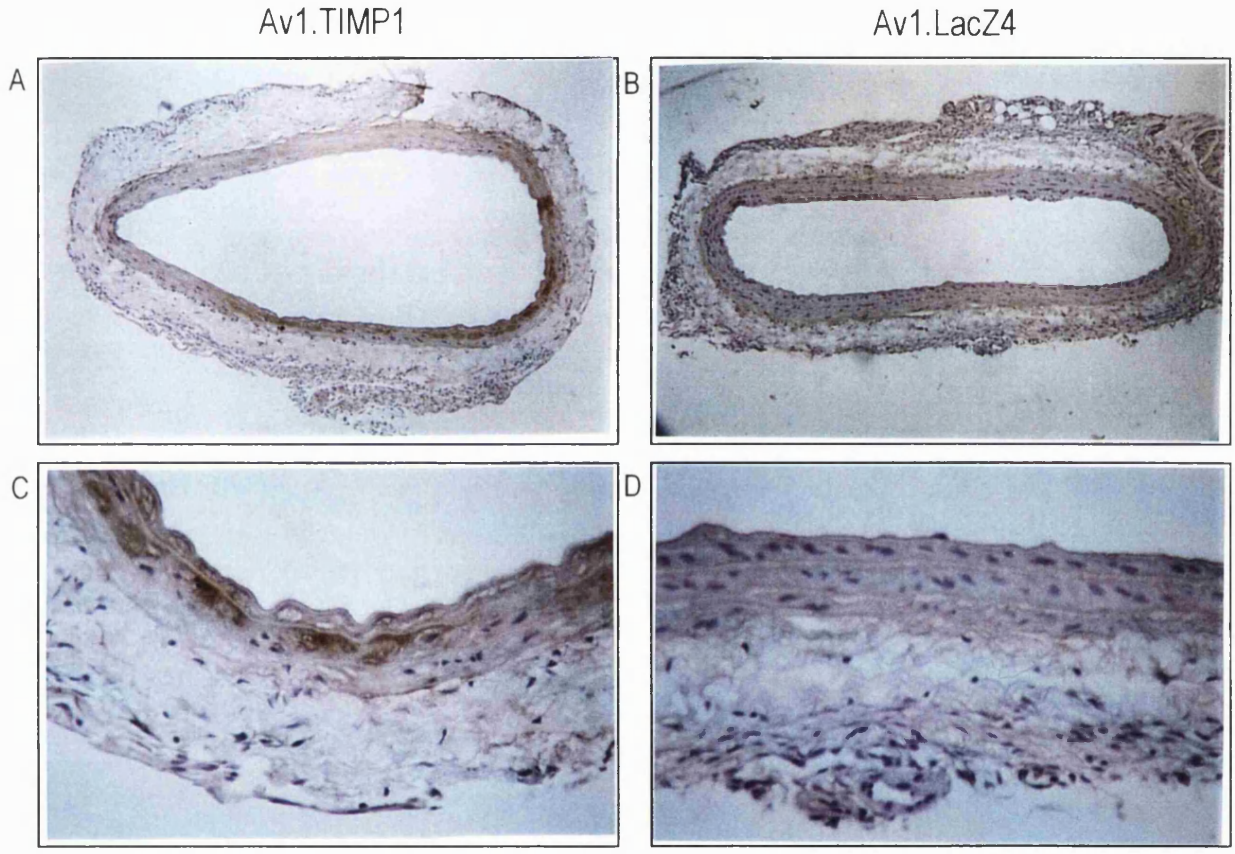


Figure 52 Photomicrographs of Anti-Human TIMP1 Immunostaining of Rat Carotid Arteries Two Days After Injury and Viral Gene Delivery.

Panels A and C show Av1.TIMP1 infected arteries at x40 and x400 magnifications and panels B and D show arteries exposed to Av1.LacZ4 at the same magnification. The tissue was perfusion fixed *in situ* with paraformaldehyde and immunostained with anti human TIMP1 antibody prior to light counterstaining with haematoxylin.

Immunohistochemistry of Av1.TIMP1 and Av1.LacZ4 infected arteries confirms the transmural distribution of human TIMP1 with the majority of immuostaining in the media. No staining was seen in the Av1.LacZ4 sections.

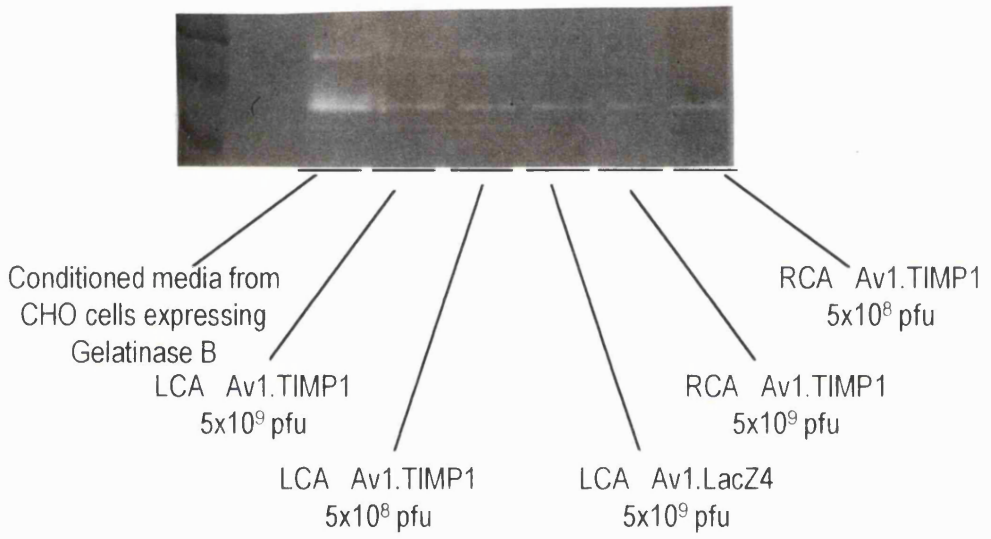


Figure 53. Zymogram of Arterial Extracts

Lanes 1 shows the molecular weight markers. Lane 2 is empty. The other lanes contain the samples of protein extracted from rat arteries 2 days after balloon injury or media from a cell line which constitutively expresses Gelatinase A and has been stably transfected to overexpress Gelatinase B (positive control). In each case balloon injury and virus administration has been carried out on the left carotid artery (LCA) of the animal and the right carotid artery (RCA) is used as an uninjured control.

Only the injured left carotid arteries show detectable Gelatinase B (92kD) which is known to be induced by vascular injury. No difference in the levels of Gelatinase A (72kD) and B was seen in the Av1.TIMP1 and Av1.LacZ4 treated arteries.

8.3.3 Zymography Of Arterial Extracts

Figure 53 shows the zymogram of the arterial extracts from the arteries infected with Av1.LacZ4 and Av1.TIMP1. All samples from left carotid arteries, which had been balloon injured, showed the presence of Gelatinase B with equal activity in both Av1.TIMP1 and Av1.LacZ4 treated arteries. No Gelatinase B was seen in samples from the right carotid arteries. Levels of Gelatinase A were equal in all the arteries.

8.3.4 Morphometry Of Av1.TIMP1 And Av1.LacZ4 Infected Arteries

To determine the effect of TIMP1 expression on neointimal development, histomorphometric analysis of arterial cross sections from 28 animals, 14 days after vascular injury and gene delivery was carried out. Three animals (one from the Av1.LacZ4 group and two from the Av1.TIMP1 group) had thrombosed left carotid arteries and were therefore excluded from the analysis. A 32% reduction in neointimal area in the Av1.TIMP1 infected arteries was seen compared with Av1.LacZ4 infected arteries. The 14 animals in the Av1.LacZ4 showed mean neointimal areas of 0.14 +/- 0.01 mm^2 compared to 0.09+/-0.01 mm^2 in the 14 Av1.TIMP1 infected arteries (p= 0.02, see Figure 54). Similarly, neointima to medial ratio was 27% less in the Av1.TIMP1 group compared with the Av1.LacZ4 group (p= 0.04) (see Figure 55). In order to assess overall vessel wall remodeling the area within the external elastic lamina in each group was also calculated. This showed a mean area of 0.448+/- 0.02 cm^2 in the Av.LacZ4 infected arteries and 0.403+/- 0.01 cm^2 in the Av1.TIMP1 infected arteries (p=NS). Figure 56 shows representative sections from the Av1.TIMP1 and Av1.LacZ4 treated groups stained with Haematoxylin and Eosin, Verhoeff's-van Gieson, and Picrosirius red.

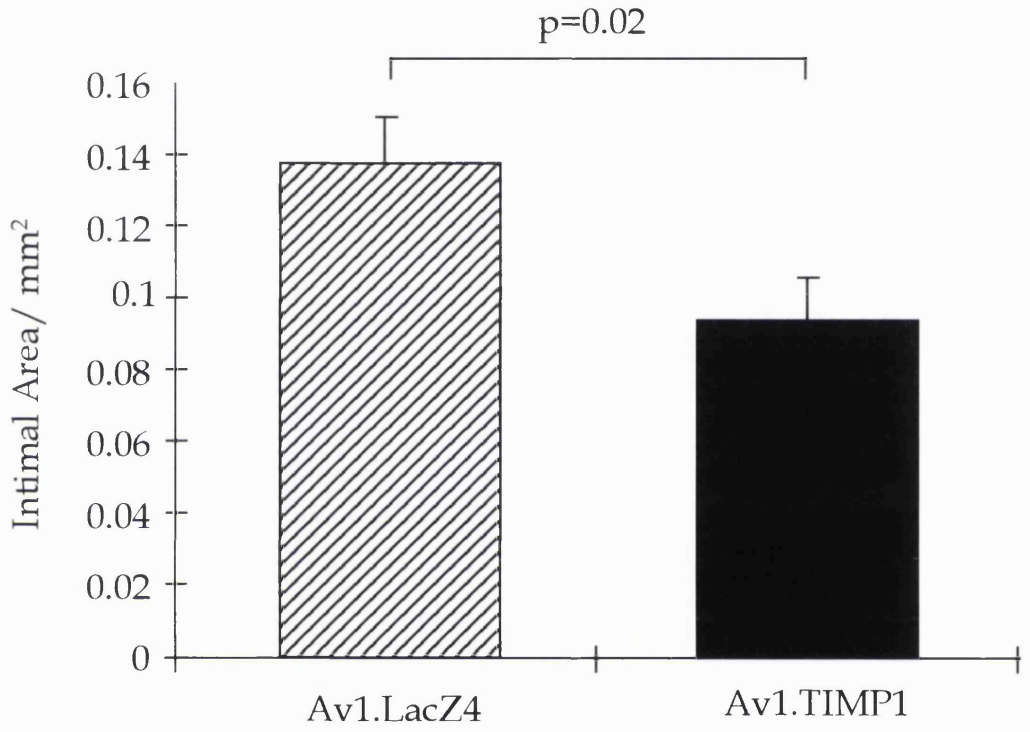


Figure 54 Intimal Area 14 Days After Gene Delivery.

Neointimal area in Av1.TIMP1 and Av1.LacZ4 infected arteries is shown, fourteen days after injury and gene delivery. Data shown are mean + SEM, (n=14). A 30 % reduction in the area of the neointima formed is seen in the Av1.TIMP1 treated arteries.

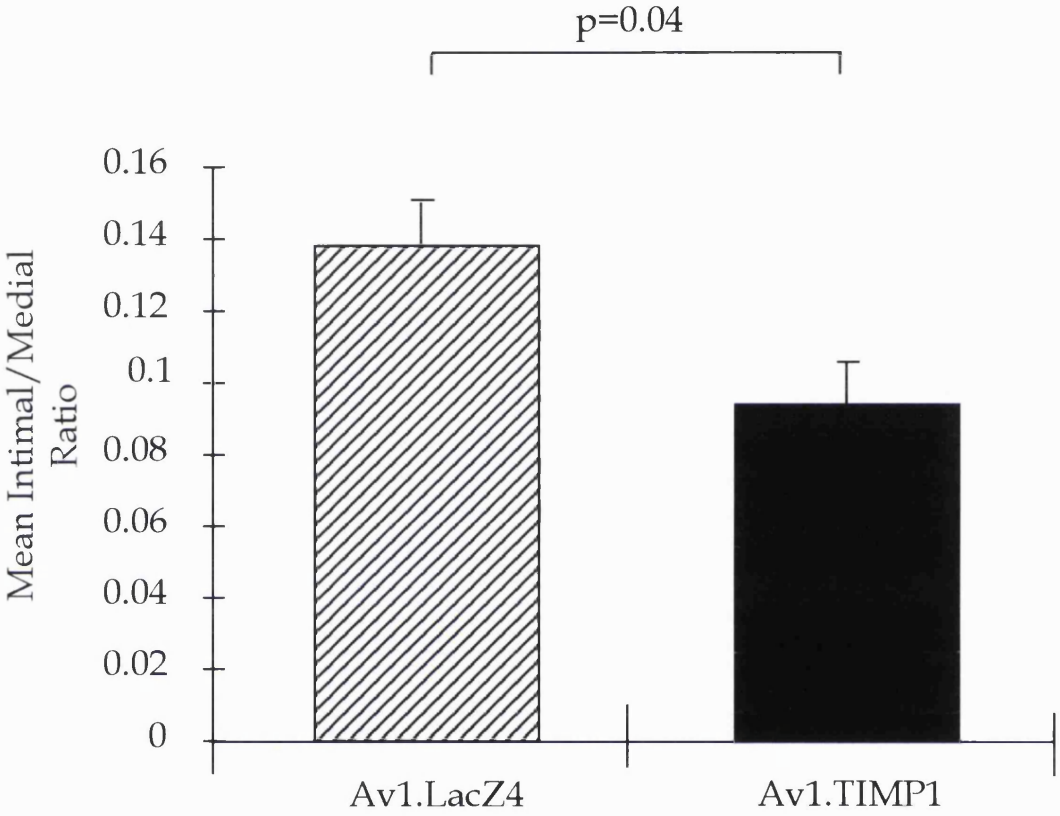


Figure 55 The Ratio of Intimal Area to Medial Area in Av1.TIMP1 and Av1.LacZ4 Infected Arteries, Fourteen Days After Injury and Gene Delivery.

Data shown are mean +SEM, (n=14). Intimal Medial ratio shows a 27% reduction in the Av1.TIMP1 group compared to the Av1.LacZ4 group. This method standardizes the size of the intima in each animal to the size of the media and therefore allows for variations in the original size of the artery.

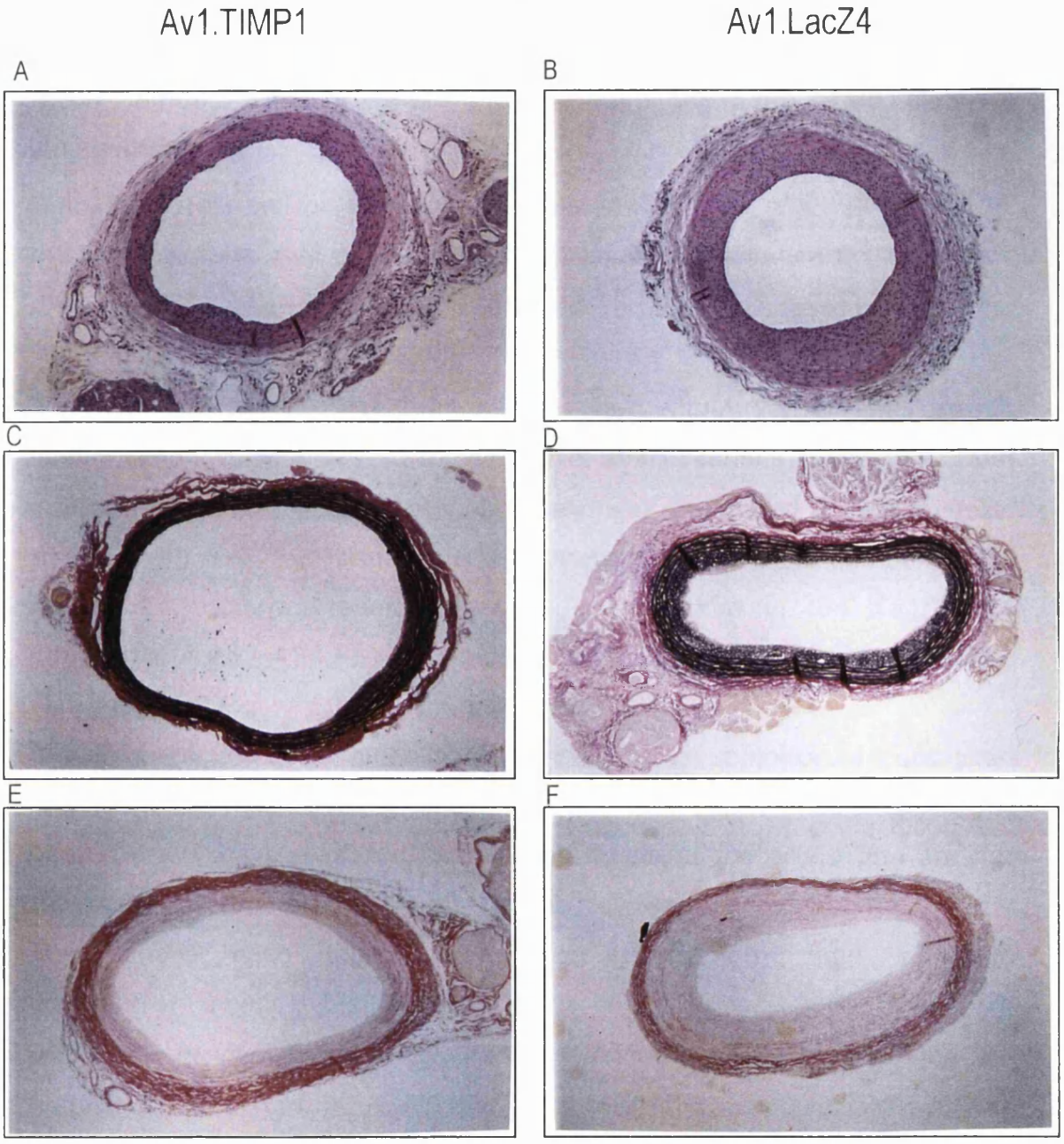


Figure 56 Photomicrographs of sections of Av1.TIMP1 and Av1.LacZ4 infected arteries fourteen days after injury, illustrating the reduction in neointimal thickening.

Panel A and B show Av1.TIMP1 and Av1.LacZ4 sections stained with Haematoxylin and Eosin Panel C and D show Av1.TIMP1 and Av1.LacZ4 stained with Verhoeff's-van Gieson which stains elastin black and Collagen I red. Panel E and F show Av1.TIMP1 and Av1.LacZ4 sections stained with Picrosirius Red which stains all collagen fibres red. (Magnification x40)

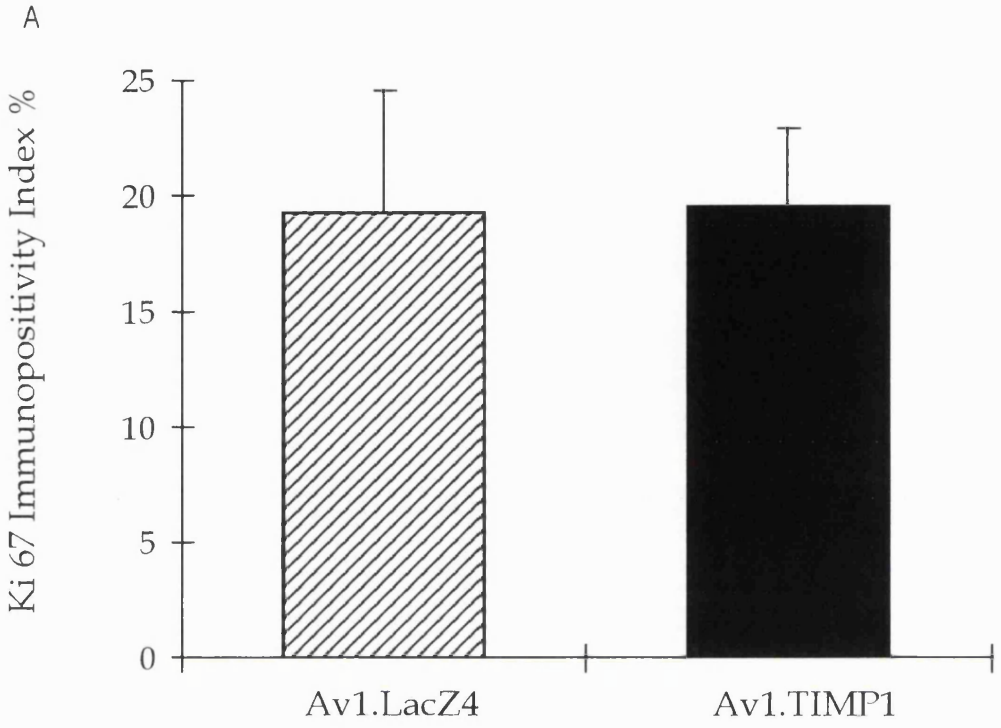
8.3.5 Smooth Muscle Cell Proliferation, Migration And Collagen Accumulation.

Smooth muscle cell proliferation was assessed using immunoreactivity to the Ki 67 antigen two days after balloon injury and showed no significant difference in the Av1.TIMP1 infected arteries compared with the Av1.LacZ4 (Ki 67 labeling index: 19.5 +/- 5.3 versus 19.2+/- 3.3, p= NS) (see Figure 57). The total cell number within the external elastic lamina, counted four days after injury, in three sections taken at 100μm intervals along the length of each artery was compared in five animals treated with Av1.TIMP1 and five with Av1.LacZ4. The cell number in the Av1.LacZ4 treated arteries was 40% greater than in the Av1.TIMP1 infected arteries (434+/- 56 compared with 260+/- 36, p= 0.048) (See Figure 58 and Figure 59).

It has been previously established that the great majority of cells seen within the internal elastic lamina four days after balloon injury of the rat carotid artery have migrated in from the media of the artery and are not the result of proliferation in the intima (Clowes and Schwartz, 1985). These have been quantitated using morphometric and *en face* immunohistochemical techniques (Clowes and Clowes, 1986; Jackson *et al.* 1993). *In vivo* migration was assessed in this study by counting the number of cells present within the internal elastic lamina of 20 sections taken at 100 μm intervals in each artery (See Figure 60). A highly significant difference was seen between the Av1.TIMP1 and Av1.LacZ4 arteries of 5.2+/- 0.5 versus 12.8+/-1.5 cells per section ($p= 5x10^{-6}$) (See Figure 61).

Collagen accumulation is seen in the late stages of the response to balloon injury, and collagen content might be increased after gene transfer of a matrix breakdown inhibitor. Collagen content was assessed by densitometric analysis of picrosirius red stained sections. The integral density of picrosirius red staining in the neointima and media of Av1.TIMP1

infected arteries was in fact significantly lower than in Av1.LacZ4 arteries (2483 +/- 171 versus 3161 +/- 187, p = 0.01) suggesting that no increase in total collagen content of the sections had occurred (see Figure 62). The apparent decrease in total collagen staining in the Av1.TIMP1 arteries is likely simply to reflect the smaller area of the arteries. When the PSR staining is expressed per unit area of artery measured the results are not significantly different (Av1.TIMP1 12900 +/- 780 versus Av1.LacZ4 13400 +/-490 densitometry units mm^{-2}).



B

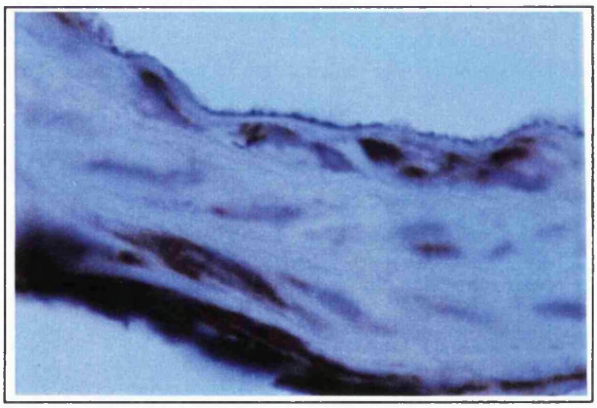


Figure 57 Proliferation of Smooth Muscle Cells *In Vivo*.

A: Smooth muscle cell proliferation assessed using immunoreactivity to the Ki 67 antigen two days after balloon injury and showed no significant difference in the Av1.TIMP1 infected arteries compared with the Av1.LacZ4 (Mean +SEM are shown, n=14). Panel B shows an example of a section stained with antiKi67 antibody showing positive immunostaining of nuclei that are actively proliferating. Ki 67 index indicates ratio of the number of stained nuclei to the total number of nuclei in the media and intima of the section. (Magnification x400)

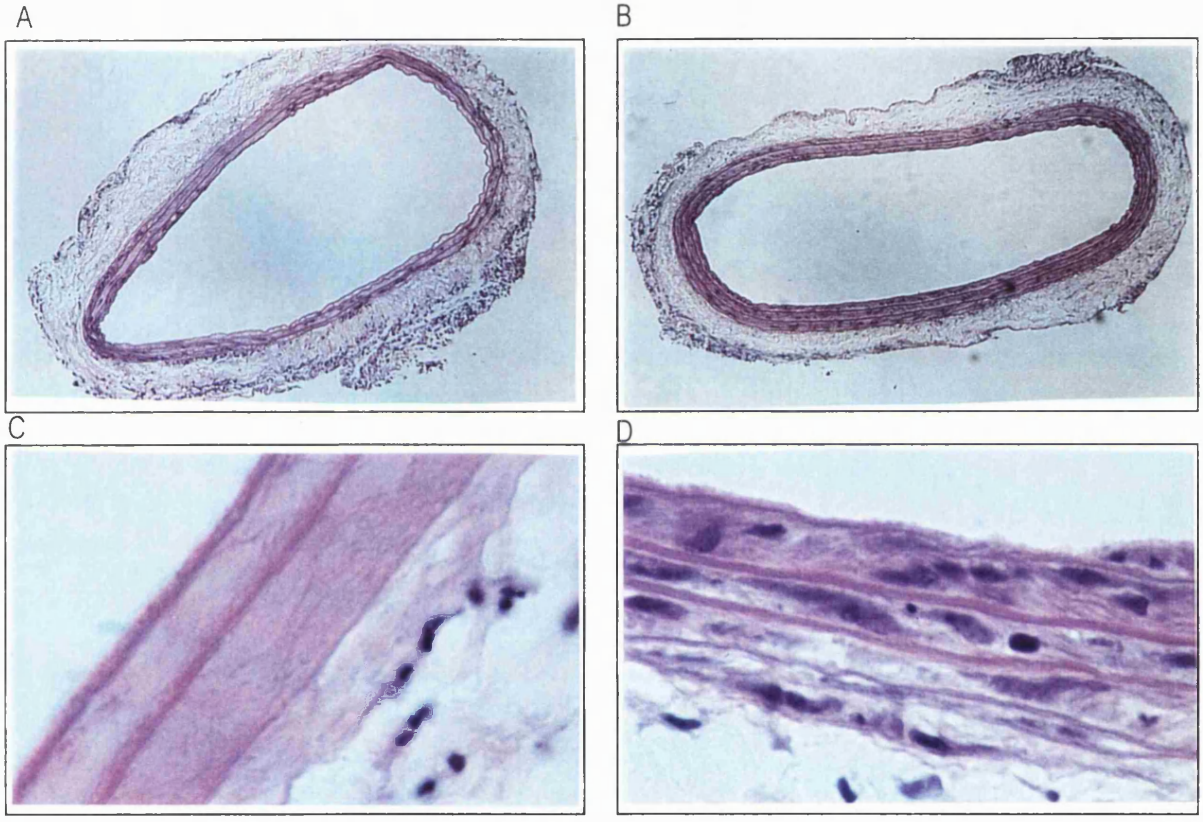


Figure 58 Photomicrographs Illustrating Cell Loss In Av1.TIMP1 Arteries Compared With Av1.LacZ4.

Panels A and B show Av1.TIMP1 and Av1.LacZ4 sections respectively, harvested four days after vascular injury and stained with Haematoxylin and Eosin (Magnification x40). Panel C and D show Av1.TIMP1 and Av1.LacZ4 sections respectively, photographed at higher magnification (x400).

The media of the Av1.TIMP1 infected arteries shows a 40% reduction in the cell number with large areas in the arterial wall devoid of nuclei.

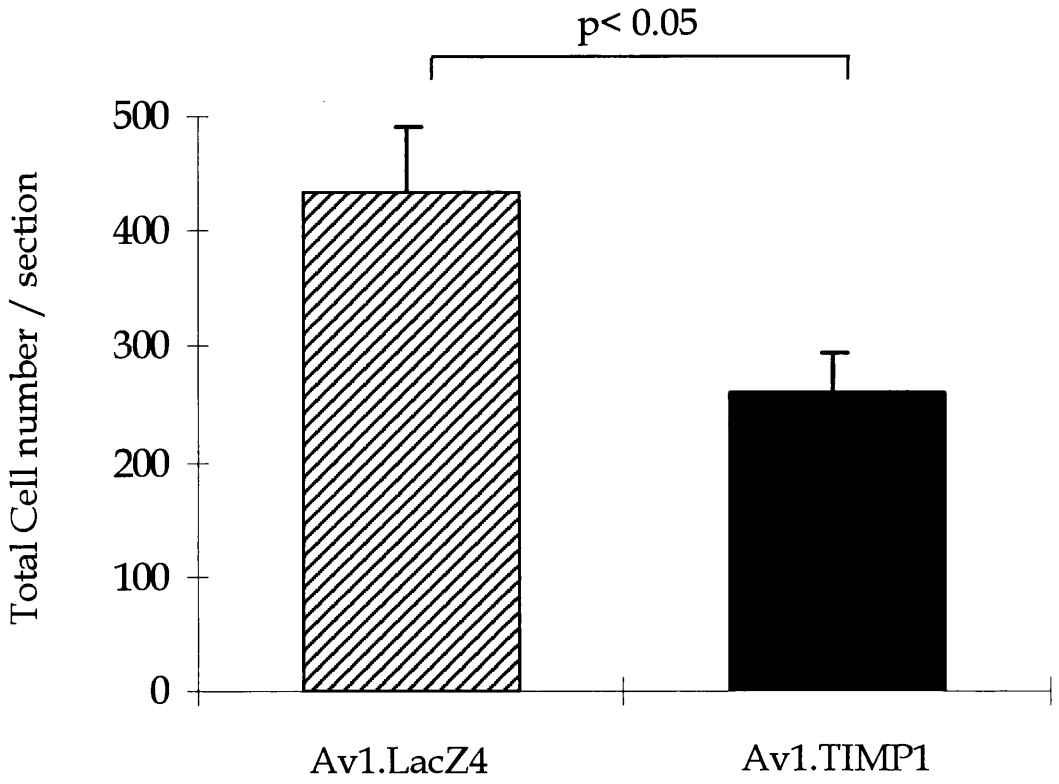


Figure 59 Medial And Intimal Cell Number Four Days After Arterial Injury And Gene Delivery.

Data shown are mean +SEM, (n=5). The total cell number within the external elastic lamina, was counted in arteries harvested four days after injury. The cell number in the Av1.LacZ4 treated arteries was 40% greater than in the Av1.TIMP1 infected arteries.

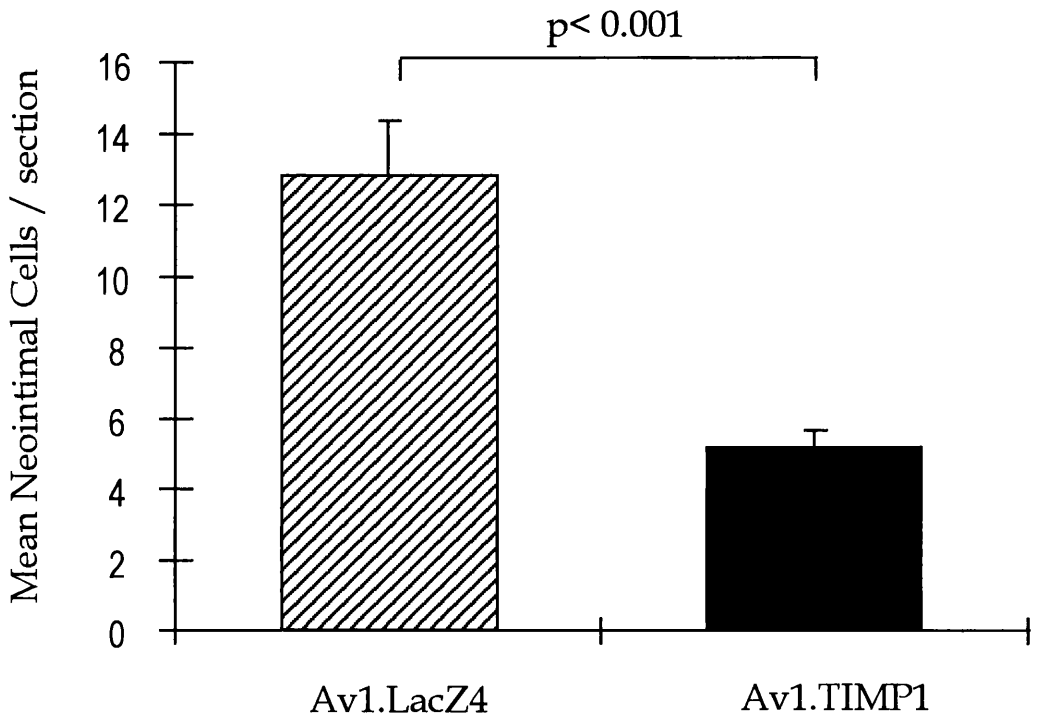


Figure 61 *In Vivo* Smooth Muscle Cell Migration.
Data shown are mean +SEM, (n=5). Migration of smooth muscle cells was assessed in Av1.TIMP1 and Av1.LacZ4 infected arteries, four days after injury, by counting cells within the internal elastic lamina in 20 sections from each artery. Five arteries were studied in each group and the mean and SEM are shown. A 60 % reduction in migration is seen in the Av1.TIMP1 treated group.

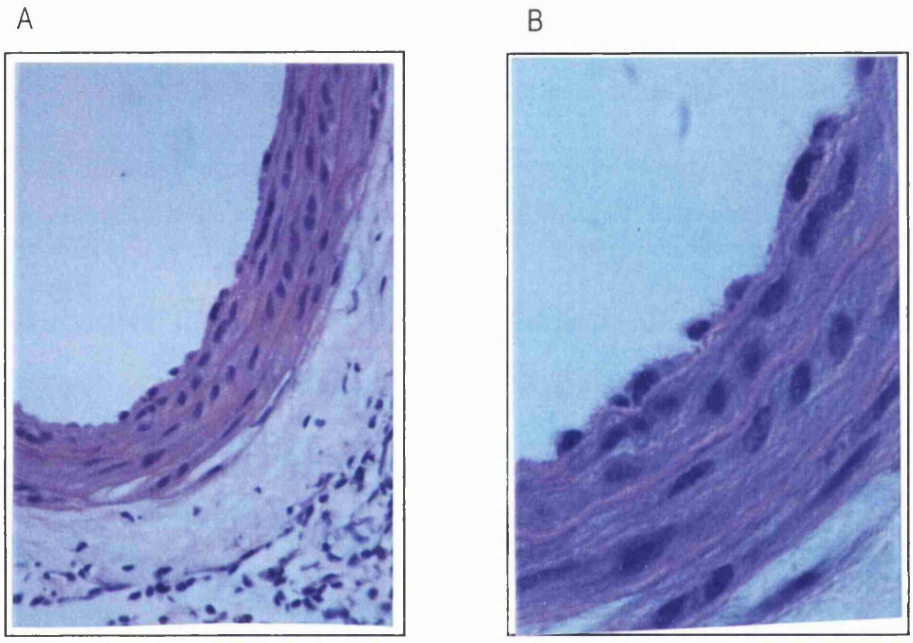


Figure 60 *In Vivo* Assessment of Migration of Smooth Muscle Cells.

Panels A and B show sections harvested four days after vascular injury and stained with Haematoxylin and Eosin at magnifications of x 100 and x 400 respectively. Both photomicrographs represent arteries exposed to Av1.LacZ4 at the time of vascular injury and show a number of cells which have migrated beyond the internal elastic lamina.

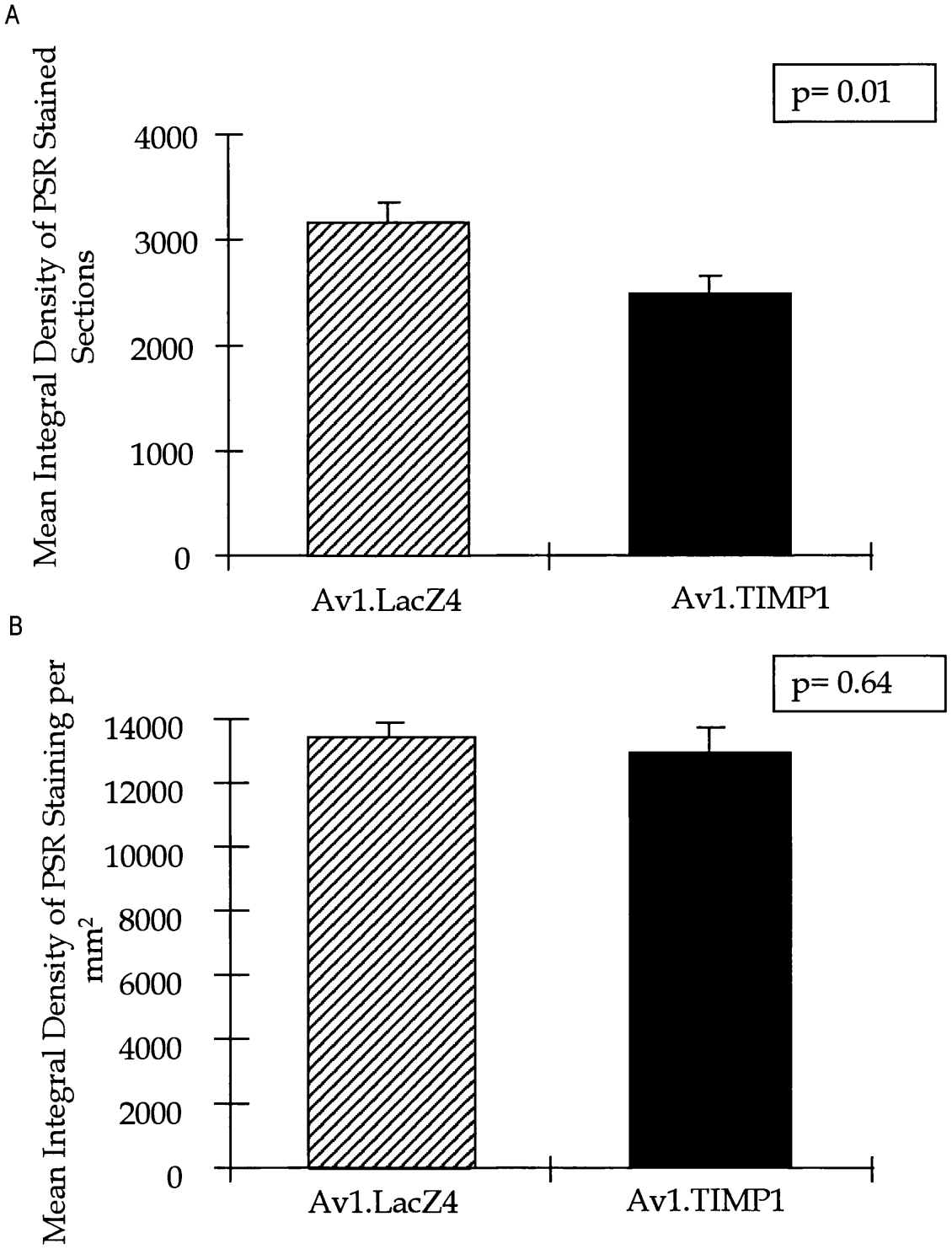


Figure 62 Collagen Content Assessed by Densitometric Analysis of Picrosirius Red Stained Sections.

A shows the mean and SEM of the integral density of picrosirius red staining in the neointima and media of Av1.TIMP1 infected arteries. This was significantly lower in the Av1.TIMP1 arteries than in Av1.LacZ4 arteries.

B shows the mean and SEM of the integral density of picrosirius red staining per unit area of the arterial wall, that is the data shown in panel A have been Normalised to allow for the difference in area of the arteries.

8.4 Discussion.

The results presented above represent the first demonstration that *in vivo* arterial gene transfer of TIMP1 attenuates the development of a neointima after vascular injury with a marked reduction in smooth muscle cell migration. *In vitro* experiments showed that the secretion of biologically active TIMP1 which follows Av1.TIMP1 infection inhibits the migration of vascular smooth muscle cells and this was confirmed by a 60% reduction in migration of smooth muscle cells *in vivo*. In this study both *in vivo* and *in vitro* analysis of DNA synthesis and proliferation showed no reduction in proliferation in response to TIMP1. This study confirms that the inhibition of matrix breakdown *in vivo* alters neointimal formation and presents the first direct *in vivo* evidence of attenuation of the response to vascular injury by altered smooth muscle cell migration (Forough *et al.* 1996b).

8.4.1 Transgene Expression After In Vivo Gene Delivery Of Av1.TIMP1.

Western analysis confirms the successful transfer of the human TIMP1 cDNA and production of the transgene at the expected molecular weight within two days of balloon injury. In addition the data from infection with Av1.LacZ4 and Av1.TIMP1 suggest that $5x10^8$pfu is the optimal amount of virus to infect a 1.5 cm segment of artery. The reduction in gene product at the higher dose of Av1.TIMP1 is likely to be explained by toxicity of the virus causing direct cytopathic effects and an increased immune response. This then results in the death of the infected cells and an overall reduction in TIMP1 protein. The toxic effects of adenoviral coat proteins are likely to be sufficient to cause cell death without infection and transgene expression when higher doses are used (Schulick *et al.* 1995). In all subsequent experiments $5x10^8$pfu of virus in 100µl of RPMI was used.

Immunohistochemistry revealed transmural immunoreactive human TIMP1 protein with the strongest immunostaining seen in the medial smooth

muscle cell layer. These results confirm successful gene transfer *in vivo* and demonstrate the 'bystander effect' which can be seen when the gene for a secreted protein is transferred. The previous studies, transferring a nuclear localized form of the Lac Z gene, showed a low transfer efficiency. The results above show diffusion of the TIMP1 protein throughout the arterial wall. Similar results were seen at two and four days after balloon injury.

8.4.2 Morphometry Of Av1.TIMP1 And Av1.LacZ4 Infected Arteries.

Neointimal area and neointimal medial ratio results both confirm that *in vivo* gene delivery of Av1.TIMP1 reduces the formation of a neointima after vascular balloon injury. A 32% reduction in neointimal area and a 27% reduction intimal medial ratio occurred in the Av1.TIMP1 infected arteries was seen compared with Av1.LacZ4 infected arteries. These results closely parallel those of Forough *et al* who saw a 40 % reduction in neointimal area 14 days after balloon injury in the same animal model when retrovirally transduced smooth muscle cells expressing TIMP1 were seeded into the carotid artery. The slightly greater reduction observed using retroviral gene transfer is unlikely to be significant as the variability in the retroviral gene transfer experiments was greater than that observed in this study (standard error 0.049mm compared with 0.01mm in the retroviral and adenoviral experiments respectively).

The results presented in this thesis support the hypotheses put forward in previous observational studies that the upregulation of matrix metalloproteinases after vascular injury indicates an important role for them in the formation of a neointima (Bendeck *et al.* 1994; Zempo *et al.* 1994; Webb *et al.* 1997).

8.4.3 Smooth Muscle Cell Migration, Proliferation And Collagen Accumulation

Migration of smooth muscle cells *in vivo* is difficult to quantify and methods which are based on *en face* immunohistochemistry and electron microscopy have been described (Jackson *et al.* 1993; Bendeck *et al.* 1994). Both techniques are based on the observation that smooth muscle cells first appear in the neointima 3-4 days after injury and that up to 96% of these cells appear to be the result of migration and not proliferation (Jackson *et al.* 1993; Clowes *et al.* 1989). The en face technique relies on an anti-histone antibody which identifies, nonspecifically, any cell within the internal elastic lamina. The *en face* technique benefits from the ability to observe the full length of an artery but is only reliable if no damage to the internal elastic lamina is sustained during preparation of the artery for immunohistochemistry and the absence of endothelial regrowth. In this thesis, rather than adopting the *en face* technique, intimal cells were counted in 20 transverse sections taken at 100μm intervals along the length of infected arteries. A 60% reduction in Av1.TIMP1 infected arteries compared to those infected with Av1.LacZ4 was observed. This approaches relies on counting a large number of sections to gain a representative selection of the whole artery.

The 60% reduction in migration seen *in vivo* is greater than that observed *in vitro.* One would anticipate that a higher local concentration of TIMP1 was achieved around transfected smooth muscle cells of the arterial wall than in the conditioned media of cells in culture. In addition to possible higher concentrations of TIMP1 it may also be secreted in more important sites at the perimeter of the cell. Several groups have highlighted the importance of focalizing proteolysis, via the MMPs, to the leading edges of a cell, often via integrin interactions (Ward *et al.* 1994; Brooks *et al.* 1996; Basbaum and Werb, 1996). It has been shown that Gelatinase A is localized to the surface of invasive cells via the αvß3 integrin which can also act as a cell surface receptor for adenoviruses (Brooks *et al.* 1996). It is therefore possible that adenoviral gene delivery may localize transgene

expression to potentially invasive cells because the virus can use the integrins expressed on these cells to gain entry. In theory, at least, gene transfer strategies may have functional advantages over pharmacological MMP inhibitors because they would be better able to harness these normal cellular processes.

In vivo detection of cell proliferation was carried out using a Ki 67 antibody which reacts with cells at all stages of the cell cycle but not with cells in G_0 or early G_1 (Brown and Gatter, 1990). The most commonly used immunohistochemical methods of assessing cellular proliferation utilize antibodies to bromodeoxyuridine (BrdU), Ki 67, and proliferating cell nuclear antigen (PCNA) (Yu *et al.* 1992). BrdU is a thymidine analogue which is incorporated into the cells during S phase and, therefore, demonstrates active DNA synthesis. BrdU is administered *in vivo* , usually over a 24 hour period, when proliferation is to be assessed and then detected in tissue sections using monoclonal anti-BrdU antibodies. PCNA is a nuclear antigen which is essential for DNA synthesis, acting as an auxiliary protein for DNA polymerase δ (Bravo *et al.* 1987). PCNA immunostaining may, however, overestimate rates of proliferation. Ki 67 is a non-histone protein which is expressed at all stages of the cell cycle but not with cells in G_0 or early G_1. It has been shown in a number of studies to correlate with flow cytometry, thymidine labeling, and bromodeoxyuridine labeling, reviewed by (Yu *et al.* 1992). A disadvantage of Ki 67 based techniques has been the sensitivity of the antigen to fixation but a subset of anti-Ki 67 antibodies can be used in paraffin embedded tissue (Leers *et al.* 1997).

In vivo detection of cell proliferation using Ki 67 antibodies showed no difference between Av1LacZ4 infected arteries and Av1.TIMP1 arteries. There was, however, a decrease in cell number in the media and neointima of the Av1.TIMP1 infected arteries four days after vascular injury. The total number of cells will not be influenced by migration differences between the two groups because both neointima and media were counted. The *in vitro* results and the *in vivo* proliferation indexes measured using Ki 67 in this

study (and using BrdU labeling in the study of Forough *et al* using retroviral gene transfer (Forough *et al.* 1996b)) do not suggest a reduction in DNA synthesis. There appears to be a selective loss of cells in the Av1.TIMP1 treated group which may possibly be due to necrosis or apoptosis.

The inhibition of the matrix metalloproteinase enzymes could result in the early onset of matrix accumulation after vascular injury and therefore be associated with adverse remodeling of the vessel wall. No negative remodeling could, however, be identified when the area subtended by the external elastic laminae in the Av1.TIMP1 and Av1.LacZ4 arteries were compared. A modification of the method described by Coats *et al* to investigate the collagen content of the vessels 14 days after gene transfer was used (Coats, Jr. *et al.* 1997).

Collagen content is most frequently measured by analysis of hydroxyproline content (Laurent, 1997). This method can however prove unreliable when applied to small samples, such as the rat carotid artery. A number of authors have investigated methods which allow estimation of collagen content using digital image analysis of histochemical sections (Whittaker *et al.* 1994; Coats, Jr. *et al.* 1997; Karim *et al.* 1995; Pickering and Boughner, 1990). Picrosirius red is a histochemical stain for collagen which is more specific than Masson's trichrome (Whittaker *et al.* 1994). Digital subtraction image analysis has been employed to optimize the brightness contrast between collagen and surrounding structures. Two approaches have been used, the first relies on the birefringence of PSR stained collagen under polarized light (Pickering and Boughner, 1990), and the second on the chemical elimination of cytoplasmic staining using phosphomolybdic acid (Dolber and Spach, 1987). Background yellow staining using picrosirius red is of particular importance in the myocardium where collagen fibrils may be obscured by myoplasmic staining. This is successfully eliminated in arterial specimens by phosphomolybdic acid treatment (Dolber and Spach, 1987). Quantitative comparison of polarized light image analysis and hydroxyproline has only been applied to rabbit arteries and showed only moderate correlation ($r=0.65$) (Karim *et al.*

1995) when compared with myocardial specimens (r=0.98) (Pickering and Boughner, 1990). This may reflect the small size of the arteries and thickness of sections (5μm) which is not suited to analysis with circularly polarized light which highlights fiber orientation. The specimens analyzed in this thesis were examined visually under circularly polarized light with dark ground microscopy but the light levels produced poor images which were beyond the limit of detection of the Lucia image analysis system. Analysis of integral density of the picrosirius red staining was therefore employed (Kratky *et al.* 1996).

The picrosirius red stained sections are translucent apart from the red collagen staining which varies in density according to the amount of collagen present (see Figure 56). Integral density of the medial and neointimal layers of picrosirius stained sections thus reflects the total amount of collagen present in that area of a section. This unexpectedly showed a higher total collagen staining in the Av1.LacZ4 treated group than in the Av1.TIMP1 group. When these results were normalized to the area measured, the density of picrosirius red staining per mm^2 was the same in both groups. This suggests that while the absolute amount of collagen is greater in the Av1.LacZ4 group than the Av1.TIMP1 group there is no difference in the collagen content per unit area. The lower collagen content in the Av1.TIMP1 group reflects the reduced area of neointima present. These results are similar to those found using a nonspecific pharmacological MMP inhibitor (GM6001) in the rabbit iliac double injury model where radiolabeled hydroxyproline was assayed (Strauss *et al.* 1996). In this study a nonsignificant 25% reduction in neointimal area was accompanied by a significant 33% reduction in collagen content in the inhibitor treated group compared with placebo. Both collagen synthesis and degradation were reduced by MMP inhibition.

In another study using the rat model of vascular injury the same MMP inhibitor (GM6001) reduced *in vivo* smooth muscle cell migration by 97% four days after injury (Bendeck *et al.* 1996). The final lesion was not altered by this pharmacological inhibitor when neointimal areas were

compared at 14 days and this has been explained by a phase of 'catch up' growth which was measured between ten and fourteen days after injury. One interpretation of the results from pharmacological inhibitors of the MMPs in the vascular system, was that inhibiting smooth muscle cell migration in this way was not sufficient to inhibit lesion growth.

The results presented in this thesis show that inhibition of migration of smooth muscle cells *in vivo* by TIMP1 gene transfer reduces neointimal hyperplasia. This supports earlier analysis of the rat model that suggested that 50% of the neointimal cells are derived through proliferation and 50% from migration, and may explain the incomplete inhibition of neointimal hyperplasia by inhibition of either proliferation or migration alone (Clowes and Schwartz, 1985). The difference between pharmacological inhibitors and the studies of gene transfer may be due to the specificity or level of inhibition of the MMPs. In contrast to the studies discussed above, one report using a broad spectrum MMP inhibitor, Batimastat, in the rat carotid model showed a reduction in neointimal hyperplasia at 14 days after injury (Zempo *et al.* 1996). This may be because Batimastat is a less specific inhibitor of the matrix metalloproteinases than CT1746 or the other inhibitors which have failed to effect a change in lesion size (Bendeck *et al.* 1996; Strauss *et al.* 1996). Batimastat also inhibits other metalloproteinases, such as the ADAMs, whose role as a TNFα converting enzyme has been investigated (Black *et al.* 1997). There is a marked discrepancy between the concentrations at which all these compounds inhibit MMPs *in vitro* and the much larger doses required to see biological effects *in vivo* leading to speculation that they were not acting solely by MMP dependent mechanisms. Thus the studies of pharmacological inhibitors have failed to prove the specific role of MMPs in vascular injury *in vivo.*

TIMP1 gene transfer was chosen in this thesis because while TIMP1 inhibits all the MMPs it has a particular specificity for Gelatinase B which is upregulated soon after vascular injury. It is also possible that the important difference in the gene transfer approach to vascular injury is the reduction

in cell number in the arterial wall after TIMP1 gene transfer. This effect was seen *in vivo* but not *in vitro* suggesting that it is partially mediated by interaction of the transgene or vector with other components of the arterial wall itself. If this is due to a specific biological effect of TIMP1 on either necrotic or programmed cell death it may influence the application of this viral vector not only in the response to vascular injury but also in tumour metastasis.

In summary, it has been shown that direct *in vivo* gene transfer of TIMP1 using an adenoviral vector inhibits neointimal development by 30%, and that a similar reduction in smooth muscle cell migration, demonstrated both *in vivo* and *in vitro,* may be the underlying mechanism. A reduction in cell number in the arterial wall followed TIMP1 gene transfer *in vivo* which parallels that seen *in vitro* by other groups (Forough *et al.* 1996b). The mechanism of this novel effect will add to our understanding of the role of the TIMPs in the vasculature. In this study the use of TIMP gene transfer provides specific evidence of the role of the MMPs in vascular smooth muscle cell migration and the response to vascular injury. To date, the majority of gene transfer studies of the response to arterial balloon injury have focused on reducing proliferation of smooth muscle cells, but these results suggest that migration of these cells has an equally important role. Ultimately, the unwanted aspects of the response to vascular injury may be best targeted by inhibition of both migration and proliferation of smooth muscle cells. The matrix metalloproteinases and their inhibitors are a major regulatory component in these responses.

CHAPTER 9 CONCLUSIONS

ORIGINAL FINDINGS AND CONCLUSIONS

Pharmacological inhibition of the MMPs.

At the time this work commenced no data were published on the effect of MMP inhibitors *in vivo* in the vascular system. The studies described in Chapter 3. Pharmacological Inhibition Of The MMPs , identified the limitations of the compound examined. The poor solubility and the lack of correlation between *in vitro* and *in vivo* effects appears common to all pharmacological inhibitors and makes them a poor tool for the study of the biology of the MMPs in the vasculature. As shown in this thesis, and by others, they do not inhibit neointimal formation after vascular injury, despite an initial effect on migration of VSMCs, (Bendeck *et al.* 1996; Strauss *et al.* 1996). This has ruled out their therapeutic application in the vasculature after systemic administration.

After the studies of CT1746 were complete, one report of a reduction in neointimal hyperplasia in the rat model was published using a broad spectrum pharmacological MMP inhibitor, Batimastat (Zempo *et al.* 1996). Perhaps the most interesting aspect of this paper is the contrast with the previous results using several different inhibitors. Clearly Batimastat has some unique action which either prevents the catch up phenomenon seen with the other inhibitors or has an unrelated effect on lesion development. Inhibition of the disintegrin and metalloproteinase family (ADAMs), which can prevent of activation of TNFα may be one explanation (Black *et al.* 1997). This raises new questions about cross talk between the ADAMs and MMPs which can only be addressed using highly specific inhibitors of each. Gene transfer of native inhibitors, such as the TIMPs, may help to answer these questions.

Adenoviral Gene Transfer To The Arterial Wall.

During this project I developed techniques for reproducible and quantifiable propagation and purification of adenoviral vectors and studied a number of herpes virus vectors. These studies show that viral gene transfer is an accessible tool for vascular biologists.

The results of these marker gene transfer studies highlight many of the difficulties associated with viral gene transfer. Efficient *in vitro* expression of the transgene was achieved with relatively low titres of adenovirus giving expression in 100% of cells. Little toxicity was seen. The versatile adenoviral vector system allowed the same techniques to be applied rapidly to a number of cell types. In contrast current herpes virus vectors showed inefficient gene transfer to smooth muscle cells and significant toxicity.

In comparison, the *in vivo* results for both types of viral vector showed relatively low levels of expression. Herpes viruses proved too inefficient to be suitable for further use *in vivo* in the vasculature. Using adenovirus the expression observed was more efficient but was lost completely when titres were increased over a relatively narrow range. Others have shown that this is due to the combination of host immune responses and tissue injury, and can result in neointimal hyperplasia, the exact opposite of the desired therapeutic effect (Newman *et al.* 1995).

Paralleling the experimental findings in these studies the field of gene transfer has passed from a period of optimism, through skepticism, into a more constructive acceptance of the difficulties associated with therapeutic application. The work in this thesis shows that *in vivo* gene delivery requires *in vivo* titration of the optimal amount virus used. Reproducibility of surgical techniques and control of coagulation had to be formally assessed in order to ensure a reproducible result and measure the biological effect of a transgene. These aspects of gene delivery would be much more difficult to control in a human population with greater biological variability of the subjects. This diversity would effect not only size of the target artery, and the structure and content of the atherosclerotic plaque, but also pre-existing levels of adenoviral antibodies.

This study does, however, show that the unwanted response to vascular injury can be reduced using adenoviral gene transfer of TIMP1 *in vivo*. This proves that low levels of transfer of marker genes is not always predictive of the biological response to a 'therapeutic' transgene. The use of a transgene that encodes a secreted product or has a bystander effect substantially improves the prospects of altering a biological end point *in vivo* (Chang *et al.* 1995a; Simari *et al.* 1996; Guzman *et al.* 1994). Comparison of photomicrographs of arteries infected with Av1.TIMP1 (Figure 52) and Av1.LacZ4 (Figure 27) illustrate the transmural distribution of a diffusable gene product compared to a nuclear localized protein. The TIMP1 will thus have a biological effect on many cells within the vessel wall which were not successfully transfected.

Inhibition Of Neointimal Hyperplasia By Adenoviral Gene Transfer Of TIMP1.

A novel gene transfer vector, Av1.TIMP1, was designed and characterized during these studies. The vector was isolated by screening a number of potential recombinant viruses, purified and grown in quantities required for *in vivo* studies. The vector was able to consistently express human TIMP1 in rat vascular smooth muscle cells in quantities proportional to the amount of virus used to infect cells in culture. The protein produced by infection with Av1.TIMP1 was detectable with a highly specific anti human TIMP1 monoclonal antibody and was shown to retain its biological function using reverse zymography. *In vitro* studies of the functional effects of the Av1.TIMP1 virus showed a minimal increase in proliferation at high titre compared to cells infected with a marker virus, Av1.LacZ4. No change in proliferation was seen when Av1.TIMP1 infected cells were compared to control uninfected cells. Invasion of smooth muscle cells into a matrigel barrier was inhibited by 27% by infection with Av1.TIMP1 supporting the underlying hypothesis of this thesis. Migration through uncoated

membranes was also seen to be substantially reduced by TIMP1 although some of this reduction may be due to effects of viral infection as a smaller decrease in migration was seen in marker virus infected cells.

For *In vivo* studies the TIMP1 adenoviral vector was delivered to the rat carotid artery following balloon injury. Four days later immunoreactive protein was identified and migration of smooth muscle cells reduced by 60%. Neointimal area 14 days after injury showed a 30% reduction in the animals receiving the Av1.TIMP1 virus compared with controls. Further studies showed that there was no difference in proliferation of smooth muscle cells in Av1.TIMP1 infected arteries compared with controls two days after injury, confirming that the change in final lesion size is not mediated by proliferation. An unexpected loss of cells was observed in the Av1.TIMP1 arteries four days after injury compared with control. The striking reduction in cell number may have been due to necrosis or programmed cell death but was not accompanied by an inflammatory cell infiltrate. Analysis of the collagen content of arteries using picrosirius red staining excluded the possibility of any adverse accumulation of matrix in the final lesion in the Av1.TIMP1 infected group. These findings support the hypothesis that arterial balloon injury involves MMP-dependent smooth muscle cell migration, which can be attenuated *in vivo* by the transmural expression of TIMP-1 using adenoviral gene transfer.

A large number of studies have successfully reduced neointimal hyperplasia by preventing proliferation of smooth muscle cells (Chang *et al.* 1995b; Yang *et al.* 1996; Chang *et al.* 1995c; Bennett *et al.* 1994; Abe *et al.* 1994; Morishita *et al.* 1994; Simons *et al.* 1994; Chang *et al.* 1995a; Simari *et al.* 1996; Guzman *et al.* 1994; Simari *et al.* 1996; Morishita *et al.* 1995). However smooth muscle cell proliferation may sometimes be advantageous in the vasculature, for example, in stabilizing a vulnerable atherosclerotic plaque. The work in this thesis shows that neointimal formation can be reduced significantly without altering proliferation, raising the possibility of an exciting alternative target for therapy. Using the established method of detection of migration in this model, the number of neointimal cells four days after injury is clearly reduced. Substantial

changes are clearly occurring in the arterial wall between two and four days after injury. The considerable loss of cells in the arterial media may have caused an apparent reduction in estimated migration to the neointima due to an overall paucity of cells in the vessel wall. Because these two possible events were analysed at the same time point in these studies it was not possible to distinguish their relative contributions. *In vitro* results showed no visible effect on cell survival but did demonstrate reduced migration. Four days after injury is the only time point at which *in vivo* migration can be quantified in this model, because after four days intimal cell number reflects both proliferation and migration. Detection of *in vivo* inhibition of MMPs might support the role of migration but unfortunately this technique has only proved applicable to larger animals such as rabbits. Gene transfer of TIMP1 reduces neointimal formation; possible contributory mechanisms are the reduction in medial cell number four days after injury or reduction in neointimal cell count at the same time point.

Adenoviral vectors have a significant advantage over retroviral vectors in facilitating the study of migration of smooth muscle cells after vascular injury. Unless used in very high titre retroviral vectors are too inefficient to transfer genes directly to native smooth muscle cells (Zhu *et al* 1997). Adenoviral vectors, however, allow direct genetic modification of native smooth muscle cells of the arterial wall whose migration can then be observed. It is likely, however, that transfer of multiple genes in combination may be necessary to produce further reductions in lesion size after vascular injury. An adenoviral vector which expresses genes that can inhibit both migration and proliferation may be the next step.

These studies have highlighted the differences between some pharmacological means of inhibition of the MMPs and gene transfer of TIMP1. The ability of TIMP1 to inhibit formation of a neointima may be in part due to its wider distribution in the vessel wall than can be achieved with the pharmacological inhibitors. The *in vitro* and *in vivo* results suggest

support the contribution of the inhibition of smooth muscle cells migration to this result. There may however be additional properties of TIMPs, possibly independent of their ability to prevent matrix breakdown by the MMPs, which have prevented the 'catch up' phenomenon seen in the majority of pharmacological studies (Bendeck *et al.* 1996; Strauss *et al.* 1996; Kenagy *et al.* 1996). The loss of cells in the TIMP1 treated arteries was only observed *in vivo* and may have prevented or compensated for any catch up smooth muscle cell growth. This novel effect may represent either necrosis or apoptosis. It is known that in some circumstances extracellular matrix contact can mediate cell survival and the replacement of specific components or imbalance in the matrix composition of the arterial wall might mediate the cell loss observed (Khwaja *et al.* 1997; Weaver *et al.* 1996). TIMP3 has recently been shown to mediate cell death by stabilizing TNFα receptors but it is not known if this is a property of other members of the TIMP family (Smith *et al* 1997). The ability of the matrix disintegrins (ADAMs) to regulate TNFα activation gives a biological precedent for matrix mediated control of cell survival (Black *et al.* 1997) The TIMPs may potentially influence not only the structure of the matrix but also modulate important cytokines as well interact with MMPs and integrins which can influence cell motility forming a matrix-regulated signaling system.

In summary, this thesis studied the *in vitro* and *in vivo* effects of inhibition of the matrix metalloproteinase enzymes on smooth muscle cells. The pharmacological inhibitor studied (CT1746) proved ineffective in altering the response to vascular injury *in vivo*. Adenoviral and Herpes vectors encoding marker genes were studied *in vivo* and *in vitro* to determine the optimal type of vector and conditions for successful gene transfer to the balloon injured rat carotid artery. A novel adenoviral vector encoding human TIMP1 was developed and its ability to express biologically active TIMP1 in a dose dependent fashion demonstrated in smooth muscle cells. This vector was used to demonstrate that *in vivo* arterial gene transfer of

TIMP1 attenuates lesion development after vascular injury, with a marked reduction in the number of smooth muscle cells migrating to the neointima and in the medial cell number.

FUTURE WORK

In all research the results that are most exciting are the ones that raise more questions than they answer. A number of specific questions which have arisen as a result of the work in this thesis are outlined below as well as a personal view of some of the possible future directions for the field of gene transfer to the arterial wall.

These studies have highlighted some possible additional properties of TIMP1. The mechanism underlying the early loss of cells from the media of arteries after transfer of TIMP1 remains unexplored. Is this a necrotic or apoptotic effect of TIMP1? In either case the effect appears only to be present *in vivo* and therefore may be mediated by cell cell or cell matrix interactions. This could be dissected by *in vitro* experiments on different matrices and by co-culture. Possible mediators might be TNFα (either by a change in activation by MMPs or by a change in the level of receptors) or Tenascin C which is a matrix component known to be associated with cell survival. The effects of TIMP1 on migration are likely to involve more than the ability to digest matrix, changes in adhesion and the loss of pro-migratory matrix components such as Laminin V may be important.

These studies have looked only at TIMP1 but it is likely that the other TIMPs also have an important role in the vasculature. Further experiments might study the ability of adenoviral vectors encoding TIMPs 2-4 in the same *in vitro* and *in vivo* models. The changes in baseline expression of these TIMPs and the MMPs in response to gene transfer would give interesting insights into the ability of the vessel wall to adapt. Longer term studies of the lesion in the arterial wall after TIMP gene transfer would also

be desirable to discover if the formation of a lesion has been avoided or only postponed.

Ultimately it is likely that transfer of multiple genes in combination may be necessary to produce greater reductions in lesion size after vascular injury. It is now possible using internal ribosome entry sites to have expression of two genes in series in a single vector. An adenoviral vector which expresses genes that can inhibit both migration and proliferation may be the next step.

This thesis has demonstrated some of the difficulties of viral gene transfer as well as its ability to achieve a modification of the vessel wall response to injury. The majority of these difficulties relate to the humoural and cellular immune responses that arise even when a replication deficient vector is used. Second and third generation adenoviruses promise to reduce this effect but remain unproven as yet. New vectors employing lentiviruses, adeno-associated viruses and more heavily deleted adenoviruses continue to be developed in addition to the viral conjugate and liposomal vectors. To date no trials of viral gene transfer vectors have been approved for human use in the vasculature.

In addition to developing new vectors and broader targets for vascular gene transfer it is necessary to apply it to large animal models which allow studies that closely reflect the clinical situation. The TIMP1 vector used in this study will be studied in the porcine coronary model of vascular injury which allows investigation of gene transfer to both normal and pre-injured arteries using delivery devices developed for human clinical use. The potential for rapid application of the results to clinical practice exists.

Adenoviral gene transfer is established as a powerful tool to investigate the pathophysiology of the vasculature. The current adenoviral vectors may not be ready for clinical use but human vascular gene therapy has already become a reality with three clinical trials of plasmid gene transfer to the vessel wall which have begun recruiting patients in Europe and the USA. This raises exciting challenges for those working in this emerging field where only a short distance separates the benchtop from bedside.

REFERENCES

Anonymous (1992) Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? Results of the MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. Multicenter European Research Trial with Cilazapril after Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MERCATOR) Study Group [comment]. *Circulation* **86**, 100-110.

Anonymous (1997) Human gene marker/therapy clinical protocols. *Hum. Gene Ther.* **7**, 2287-2313.

Abe, J., Zhou, W., Taguchi, J., Takuwa, N., Miki, K., Okazaki, H., Kurokawa, K., Kumada, M. and Takuwa, Y. (1994) Suppression of neointimal smooth-muscle cell accumulation in-vivo by antisense cdc2 and cdk2 oligonucleotides in rat carotid-artery. *Biochemical And Biophysical Research Communications* **198**, 16-24.

Ali, M., Lemoine, N.R. and Ring, C.J.A. (1994) The use of DNA viruses as vectors for gene therapy. *Gene Ther.* 367-384.

Amalfitano, A., Begy, C.R. and Chamberlain, J.S. (1996) Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3352-3356.

An, Z., Wang, X., Willmott, N., Chander, S.K., Tickle, S., Docherty, A.J., Mountain, A., Millican, A.T., Morphy, R., Porter, J.R., Epemolu, R.O., Kubota, T., Moossa, A.R. and Hoffman, R.M. (1997) Conversion of highly malignant colon cancer from an aggressive to a controlled disease by oral

administration of a metalloproteinase inhibitor. *Clin. Exp. Metastasis* **15**, 184-195.

Andersen, H.R., Maeng, M., Thorwest, M. and Falk, E. (1996) Remodeling rather than neointimal formation explains luminal narrowing after deep vessel wall injury: insights from a porcine coronary (re)stenosis model. *Circulation* **93**, 1716-1724.

Anderson, I.C., Shipp, M.A., Docherty, A.J. and Teicher, B.A. (1996) Combination therapy including a Gelatinase inhibitor and cytotoxic agent reduces local invasion and metastasis of murine Lewis lung carcinoma. *Cancer Res.* **56**, 715-718.

Asahara, T., Bauters, C., Pastore, C., Kearney, M., Rossow, S., Bunting, S., Ferrara, N., Symes, J.F. and Isner, J.M. (1995) Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery [see comments]. *Circulation* **91**, 2793-2801.

Au, Y.P., Kenagy, R.D., Clowes, M.M. and Clowes, A.W. (1993) Mechanisms of inhibition by heparin of vascular smooth muscle cell proliferation and migration. *Haemostasis* **23** Suppl 1, 177-182.

Baker, A.H., Wilkinson, G.W.G., Hembry, R.M., Murphy, G. and Newby, A.C. (1996) Development of recombinant adenoviruses that drive high level expression of the human metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and -2 genes: Characterization of their infection into rabbit smooth muscle cells and human MCF-7 adenocarcinoma cells. *Matrix Biology* **15**, 383-395.

Basbaum, C.B. and Werb, Z. (1996) Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. *Current Opinions in Cell Biology* **8**, 731-738.

Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C. and Chambon, P. (1990) A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* **348**, 699-704.

Bendeck, M.P., Zempo, N., Clowes, A.W., Galardy, R.E. and Reidy, M.A. (1994) Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circ. Res.* **75**, 539-545.

Bendeck, M.P., Irvin, C. and Reidy, M.A. (1996) Inhibition of matrix metalloproteinase activity inhibits smooth muscle cell migration but not neointimal thickening after arterial injury. *Circ. Res.* **78**, 38-43.

Bennett, M.R., Anglin, S., McEwan, J.R., Jagoe, R., Newby, A.C. and Evan, G.I. (1994) Inhibition of vascular smooth muscle cell proliferation *in vitro* and *in vivo* by c-myc antisense oligodeoxynucleotides. *J. Clin. Invest.* **93**, 820-828.

Bennett, M.R. and Schwartz, S.M. (1995) Antisense therapy for angioplasty restenosis. Some critical considerations. *Circulation* **92**, 1981-1993.

Bertaux, B., Hornebeck, W., Eisen, A.Z. and Dubertret, L. (1991) Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases. *J. Invest. Dermatol.* **97**, 679-685.

Birkedal-Hansen, H., Moore, W., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., De Carlo, A. and Engler, J.A. (1993) Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 197-250.

Black, R.A., Durie, F.H., Ottenevans, C., Miller, R., Slack, J.L., Lynch, D.H., Castner, B., Mohler, K.M., Gerhart, M., Johnson, R.S., Itoh, Y., Okada, Y. and Nagase, H. (1996) Relaxed specificity of matrix metalloproteinases (MMPs) and imp insensitivity of tumour-necrosis-factor-alpha (TNF-alpha) production suggest the major TNF-alpha converting-enzyme is not an MMP. *Biochemical And Biophysical Research Communications* **225**, 400-405.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. and Cerretti, D.P. (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor- alpha from cells. *Nature* **385**, 729-733.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Bravo, R., Frank, R., Blundell, P.A. and Macdonaldbravo, H. (1987) Cyclin PCNA is the auxiliary protein of DNA polymerase-delta. *Nature* **326**, 515-517.

Brett, A.J., Haddara, W., Prevec, L. and Graham, F.L. (1994) An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8802-8806.

Brinckerhoff, C.E. (1991) Joint destruction in arthritis - metalloproteinases in the spotlight. *Arthritis And Rheumatism* **34**, 1073-1075.

Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T.L., Aimes, R.T., Stetler Stevenson, W.G., Quigley, J.P. and Cheresh, D.A. (1996) Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* **85**, 683-693.

Brown, D.C. and Gatter, K.C. (1990) Monoclonal antibody Ki-67: its use in histopathology. *Histopathology* **17**, 489-503.

Brown, P.D. (1994) Clinical-trials of a low-molecular-weight matrix metalloproteinase inhibitor in cancer. *Annals Of The New York Academy Of Sciences* **732**, 217-221.

Cao, J., Rehemtulla, A., Bahou, W. and Zucker, S. (1996) Membrane type matrix metalloproteinase 1 activates pro-Gelatinase A without furin cleavage of the N-terminal domain. *J. Biol. Chem.* **271**, 30174-30180.

Carmeliet, P., Moons, L., Stassen, J.M., DeMol, M., Bouche, A., vandenOord, J.J., Kockx, M. and Collen, D. (1997) Vascular wound healing and neointima formation induced by perivascular electric injury in mice. *American Journal Of Pathology* **150**, 761-776.

Carmichael, D.F., Stricklin, G.P. and Stuart, J.M. (1989) Systemic administration of TIMP in the treatment of collagen-induced arthritis in mice. *Agents Actions* **27**, 378-379.

Carter, A.J., Laird, J.R., Farb, A., Kufs, W., Wortham, D.C. and Virmani, R. (1994) Morphologic characteristics of lesion formation and time course of smooth muscle cell proliferation in a porcine proliferative restenosis model. *J. Am. Coll. Cardiol.* **24**, 1398-1405.

Chang, M.W., Barr, E., Lu, M.M., Barton, K. and Leiden, J.M. (1995a) Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *J. Clin. Invest.* **96**, 2260-2268.

Chang, M.W., Barr, E., Seltzer, J., Jiang, Y.Q., Nabel, G.J., Nabel, E.G., Parmacek, M.S. and Leiden, J.M. (1995b) Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* **267**, 518-522.

Chang, M.W., Ohno, T., Gordon, D., Lu, M.M., Nabel, G.J., Nabel, E.G. and Leiden, J.M. (1995c) Adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene inhibits vascular smooth muscle cell proliferation and neointima formation following balloon angioplasty of the rat carotid artery. *Mol. Med.* **1**, 172-181.

Chirivi, R.G.S., Garofalo, A., Crimmin, M.J., Bawden, L.J., Stoppacciaro, A., Brown, P.D. and Giavazzi, R. (1994) Inhibition of the metastatic spread and growth of b16-bl6 murine melanoma by a synthetic matrix metalloproteinase inhibitor. *International Journal Of Cancer* **58**, 460-464.

Clark, I.M., Rowan, A.D., Edwards, D.R., Bech Hansen, T., Mann, D.A., Bahr, M.J. and Cawston, T.E. (1997) Transcriptional activity of the human tissue inhibitor of metalloproteinases 1 (TIMP-1) gene in fibroblasts involves elements in the promoter, exon 1 and intron 1. *Biochem. J.* **324**, 611-617.

Clesham, G.J., Browne, H., Efstathiou, S. and Weissberg, P.L. (1996) Enhancer stimulation unmasks latent gene transfer after adenovirus-

mediated gene delivery into human vascular smooth muscle cells. *Circ. Res.* **79**, 1188-1195.

Clowes, A.W., Reidy, M.A. and Clowes, M.M. (1983) Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab. Invest.* **49**, 327-333.

Clowes, A.W., Clowes, M.M., Fingerle, J. and Reidy, M.A. (1989) Kinetics of cellular proliferation after arterial injury. V. Role of acute distension in the induction of smooth muscle proliferation. *Lab. Invest.* **60**, 360-364.

Clowes, A.W., Clowes, M.M., Au, Y.P., Reidy, M.A. and Belin, D. (1990) Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circ. Res.* **67**, 61-67.

Clowes, A.W., Clowes, M.M., Vergel, S., Muller, R.K., Powell, J.S., Hefti, F. and Baumgartner, H.R. (1991) Heparin and cilazapril together inhibit injury-induced intimal hyperplasia. *Hypertension* **18**, 1165-1169.

Clowes, A.W., Clowes, M.M., Kirkman, T.R., Jackson, C.L., Au, Y.P. and Kenagy, R. (1992) Heparin inhibits the expression of tissue-type plasminogen activator by smooth muscle cells in injured rat carotid artery. *Circ. Res.* **70**, 1128-1136.

Clowes, A.W. and Clowes, M.M. (1985) Kinetics of cellular proliferation after arterial injury. II. Inhibition of smooth muscle growth by heparin. *Lab. Invest.* **52**, 611-616.

Clowes, A.W. and Clowes, M.M. (1986) Kinetics of cellular proliferation after arterial injury. IV. Heparin inhibits rat smooth muscle mitogenesis and migration. *Circ. Res.* **58**, 839-845.

Clowes, A.W. and Schwartz, S.M. (1985) Significance of quiescent smooth muscle migration in the injured rat carotid artery. *Circ. Res.* **56**, 139-145.

Coats, W.D., Jr., Whittaker, P., Cheung, D.T., Currier, J.W., Han, B. and Faxon, D.P. (1997) Collagen content is significantly lower in restenotic versus nonrestenotic vessels after balloon angioplasty in the atherosclerotic rabbit model. *Circulation* **95**, 1293-1300.

Coffin, R.S., Howard, M.K., Cumming, D.V., Dollery, C.M., McEwan, J., Yellon, D.M., Marber, M.S., MacLean, A.R., Brown, S.M. and Latchman, D.S. (1996) Gene delivery to the heart *in vivo* and to cardiac myocytes and vascular smooth muscle cells *in vitro* using herpes virus vectors. *Gene Ther.* **3**, 560-566.

Coffin, R.S. and Latchman, D.L. (1996) Herpes simplex virus based vectors. In: Latchman, D.S. (Ed.) *Genetic manipulation of the nervous system.* pp. 99-111. London: Academic Press]

Corcoran, M.L. and Stetler Stevenson, W.G. (1995) Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a cAMP-dependent mechanism. *J. Biol. Chem.* **270**, 13453-13459.

Couffinhal, T., Kearney, M., Sullivan, A., Silver, M., Tsurumi, Y. and Isner, J.M. (1997) Histochemical staining following LacZ gene transfer underestimates transfection efficiency. *Human Gene Therapy* **8**, 929-934.

Curran, T. and Franza, B.R. (1988) Fos and Jun - the AP-1 connection. *Cell* **55**, 395-397.

Datta, S.K., Tumilowicz, J.J. and Trentin, J.J. (1993) Lysis of human arterial smooth muscle cells infected with herpesviridae by peripheral blood mononuclear cells: implications for atherosclerosis. *Viral. Immunol.* **6**, 153-160.

Debbas, M. and White, E. (1993) Wild type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* **7**, 546-554.

Denhardt, D.T., Feng, B., Edwards, D.R., Cocuzzi, E.T. and Malyankar, U.M. (1993) Tissue inhibitor of metalloproteinases (TIMP, aka EPA): structure, control of expression and biological functions. *Pharmacol. Ther.* **59**, 329-341.

Docherty, A.J., Lyons, A., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J., Murphy, G. and Reynolds, J.J. (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. *Nature* **318**, 66-69.

Dolber, P.C. and Spach, M.S. (1987) Picrosirius red staining of cardiac muscle following phosphomolybdic acid treatment. *Stain Technology* **62**, 23-26.

Dollery, C.M., McEwan, J.R. and Henney, A.M. (1995) Matrix metalloproteinases and cardiovascular disease. *Circ. Res.* **77**, 863-868.

Eccles, S.A., Box, G.M., Court, W.J., Bone, E.A., Thomas, W. and Brown, P.D. (1996) Control of lymphatic and hematogenous metastasis of a rat mammary- carcinoma by the matrix metalloproteinase inhibitor batimastat (bb- 94). *Cancer Research* **56**, 2815-2822.

Editorial (1987) Restenosis following angioplasty. *Lancet* 1440-1441.

Engelhardt, J.F., Ye, X., Doranz, B. and Wilson, J.M. (1994) Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6196-6200.

Fabunmi, R.P., Baker, A.H., Murray, E.J., Booth, R.F. and Newby, A.C. (1996) Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa Gelatinases and tissue inhibitors or metalloproteinases-1, -2, and -3 in rabbit aortic smooth muscle cells. *Biochem. J.* **315**, 335-342.

Faxon, D.P., Sanborn, T.A., Weber, V.J., Haudenschild, C., Gottsman, S.B., McGovern, W.A. and Ryan, T.J. (1984) Restenosis following transluminal angioplasty in experimental atherosclerosis. *Arteriosclerosis* **4**, 189-195.

Feldman, L.J., Steg, P.G., Zheng, L.P., Chen, D., Kearney, M., McGarr, S.E., Barry, J.J., Dedieu, J.F., Perricaudet, M. and Isner, J.M. (1995) Low-efficiency of percutaneous adenovirus-mediated arterial gene transfer in the atherosclerotic rabbit [see comments]. *J. Clin. Invest.* **95**, 2662-2671.

Feldman, L.J., Pastore, C.J., Aubailly, N., Kearney, M., Chen, D., Perricaudet, M., Steg, P.G. and Isner, J.M. (1997) Improved efficiency of arterial gene transfer by use of poloxamer 407 as a vehicle for adenoviral vectors. *Gene Ther.* **4**, 189-198.

Flotte, T.R. and Ferkol, T.W. (1997) Genetic therapy - past, present, and future. *Pediatric Clinics Of North America* **44**, 153

Flugelman, M.Y., Jaklitsch, M.T., Newman, K.D., Casscells, W., Bratthauer, G.L. and Dichek, D.A. (1992) Low level *in vivo* gene transfer into the arterial wall through a perforated balloon catheter [see comments]. *Circulation* **85**, 1110-1117.

Forough, R., Hasenstab, D., Koyama, N., Lea, H., Clowes, M. and Clowes, A.W. (1996a) Generating antibodies against secreted proteins using vascular smooth muscle cells transduced with replication-defective retrovirus. *Biotechniques* **20**, 694-701.

Forough, R., Koyama, N., Hasenstab, D., Lea, H., Clowes, M., Nikkari, S.T. and Clowes, A.W. (1996b) Overexpression of tissue inhibitor of matrix metalloproteinase-1 inhibits vascular smooth muscle cell functions *in vitro* and *in vivo*. *Circ. Res.* **79**, 812-820.

Freestone, T., Turner, R.J., Coady, A., Higman, D.J., Greenhalgh, R.M. and Powell, J.T. (1995) Inflammation and matrix metalloproteinases in the enlarging abdominal aortic-aneurysm. *Arteriosclerosis Thrombosis And Vascular Biology* **15**, 1145-1151.

French, B.A., Mazur, W., Ali, N.M., Geske, R.S., Finnigan, J.P., Rodgers, G.P., Roberts, R. and Raizner, A.E. (1994) Percutaneous transluminal *in vivo* gene transfer by recombinant adenovirus in normal porcine coronary arteries, atherosclerotic arteries, and two models of coronary restenosis. *Circulation* **90**, 2402-2413.

Galis, Z.S., Muszynski, M., Sukhova, G.K., Simon Morrissey, E., Unemori, E.N., Lark, M.W., Amento, E. and Libby, P. (1994a) Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ. Res.* **75**, 181-189.

Galis, Z.S., Sukhova, G.K., Lark, M.W. and Libby, P. (1994b) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J. Clin. Invest.* **94**, 2493-2503.

Galis, Z.S., Muszynski, M., Sukhova, G.K., Simon Morrissey, E. and Libby, P. (1995a) Enhanced expression of vascular matrix metalloproteinases induced *in vitro* by cytokines and in regions of human atherosclerotic lesions. *Ann. N. Y. Acad. Sci.* **748**, 501-507.

Galis, Z.S., Sukhova, G.K. and Libby, P. (1995b) Microscopic localization of active proteases by *in situ* zymography: detection of matrix metalloproteinase activity in vascular tissue. *FASEB J.* **9**, 974-980.

Gearing, A.J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A.H., Drummond, A.H., Galloway, W.A., Gilbert, R., Gordon, J.L. and *et al* (1994) Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* **370**, 555-557.

Geary, R.L., Koyama, N., Wang, T.W., Vergel, S. and Clowes, A.W. (1995) Failure of heparin to inhibit intimal hyperplasia in injured baboon arteries. The role of heparin-sensitive and -insensitive pathways in the stimulation of smooth muscle cell migration and proliferation. *Circulation* **91**, 2972-2981.

Geary, R.L., Williams, J.K., Golden, D., Brown, D.G., Benjamin, M.E. and Adams, M.R. (1996) Time course of cellular proliferation, intimal hyperplasia, and remodeling following angioplasty in monkeys with established atherosclerosis. A nonhuman primate model of restenosis. *Arterioscler. Thromb. Vasc. Biol* **16**, 34-43.

Giannelli, G., FalkMarzillier, J., Schiraldi, O., Stetler Stevenson, W.G. and Quaranta, V. (1997) Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* **277**, 225-228.

Glagov, S. (1994) Intimal hyperplasia, vascular remodelling, and the restenosis problem. *Circulation* **89**, 2888-2891.

Goldberg, G.I., Strongin, A., Collier, I.E., Genrich, L.T. and Marmer, B.L. (1992) Interaction of 92-kDa type IV Collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial Collagenase, and activation of the proenzyme with Stromelysin. *J. Biol. Chem.* **267**, 4583-4591.

Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**, 59-74.

Graham, F.L. and Prevec, L. (1991) Manipulation of adenoviral vectors. In: Murray, E.J. (Ed.) *Gene transfer and expression vectors.* pp. 109-127. Clifton, NJ: Humana]

Green, M., Pina, M. and Kimes, R.C. (1967) Biochemical studies of adenovirus multiplication. XII. Plaquing efficiencies of purified human adenoviruses. *Virology* **31**, 562-565.

Greene, J., Wang, M., Liu, Y.E., Raymond, L.A., Rosen, C. and Shi, Y.E. (1996) Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J. Biol. Chem.* **271**, 30375-30380.

Greenwald, R.A. (1987) Tetracyclines inhibit human synovial Collagenase *in vivo* and *in vitro*. *J Rheumatol* **14**, 28-32.

Greenwald, R.A., Moak, S.A., Ramamurthy, N.S. and Golub, L.M. (1992) Tetracyclines suppress matrix metalloproteinase activity in. *Journal Of Rheumatology* **19**, 927-938.

Greenwald, R.A. (1994) Treatment of destructive arthritic disorders with MMP inhibitors - potential role of tetracyclines. *Annals Of The New York Academy Of Sciences* **732**, 181-198.

Guzman, R.J., Lemarchand, P., Crystal, R.G., Epstein, S.E. and Finkel, T. (1993) Efficient and selective adenovirus-mediated gene transfer into vascular neointima. *Circulation* **88**, 2838-2848.

Guzman, R.J., Hirschowitz, E.A., Brody, S.L., Crystal, R.G., Epstein, S.E. and Finkel, T. (1994) *In vivo* suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10732-10736.

Halpert, I., Sires, U.I., Roby, J.D., Potterperigo, S., Wight, T.N., Shapiro, S.D., Welgus, H.G., Wickline, S.A. and Parks, W.C. (1996) Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. *Proceedings Of The National Academy Of Sciences Of The United* **93**, 9748-9753.

Hansson, G.K., Jonasson, L., Seiffert, P.S. and Stemme, S. (1989) Immune Mechanisms in Atherosclerosis. *Atherosclerosis* 567-578.

Hasenstab, D., Forough, R. and Clowes, A.W. (1997) Plasminogen activator inhibitor type 1 and tissue inhibitor of metalloproteinases-2 increase after arterial injury in rats. *Circ. Res.* **80**, 490-496.

Hawkes, S.P., Shubayev, V., Kishani, N., Leco, K.J., Beaudry, P.P., Tretiak, M., Urbanski, S.J., Kossakowska, A.E. and Edwards, D.R. (1996) Quantitative reverse zymography for analysis of TIMPs in cell culture and

tissue extracts. *Inhibitors of metalloproteinases in Development and Disease* I-29(Abstract)

Hayakawa, T., Yamashita, K., Tanzawa, K., Uchijima, E. and Iwata, K. (1992) Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett.* **298**, 29-32.

Hayakawa, T., Yamashita, K., Ohuchi, E. and Shinagawa, A. (1994) Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J. Cell Sci.* **107**, 2373-2379.

Henney, A.M., Wakeley, P.R., Davies, M.J., Foster, K., Hembry, R., Murphy, G. and Humphries, S.E. (1991) Localization of Stromelysin gene expression in atherosclerotic plaques by *in situ* hybridization. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8154-8158.

Herron, G.S., Unemori, E., Wong, M., Rapp, J.H., Hibbs, M.H. and Stoney, R.J. (1991) Connective tissue proteinases and inhibitors in abdominal aortic aneurysms. Involvement of the vasa vasorum in the pathogenesis of aortic aneurysms. *Arterioscler. Thromb.* **11**, 1667-1677.

Huard, J., Goins, W.F. and Glorioso, J.C. (1995) Herpes simplex type 1 vector mediated gene transfer to muscle. *Gene Ther.* **2**, 385-392.

Isner, J.M., Walsh, K., Rosenfield, K., Schainfeld, R., Asahara, T., Hogan, K. and Pieczek, A. (1996a) Arterial gene therapy for restenosis. *Hum. Gene Ther.* **7**, 989-1011.

Isner, J.M., Walsh, K., Symes, J., Pieczek, A., Takeshita, S., Lowry, J., Rosenfield, K., Weir, L., Brogi, E. and Jurayj, D. (1996b) Arterial gene

transfer for therapeutic angiogenesis in patients with peripheral artery disease. *Hum. Gene Ther.* **7**, 959-988.

Jackson, C.L., Raines, E.W., Ross, R. and Reidy, M.A. (1993) Role of endogenous platelet-derived growth factor in arterial smooth muscle cell migration after balloon catheter injury. *Arterioscler. Thromb.* **13**, 1218-1226.

James, T.W., Wagner, R., White, L.A., Zwolak, R.M. and Brinckerhoff, C.E. (1993) Induction of Collagenase and Stromelysin gene expression by mechanical injury in a vascular smooth muscle-derived cell line. *J. Cell Physiol.* **157**, 426-437.

Kaner, R.J., Medina, J., Nicholson, A.C., Ursea, R., Schwartz, S.M. and Hajjar, D.P. (1993) Developmentally regulated herpesvirus plaque formation in arterial smooth muscle cells. *Circ. Res.* **73**, 10-14.

Karakiulakis, G., Missirlis, E. and Maragoudakis, M.E. (1990) Basement membrane collagen-degrading activity from a malignant tumour is inhibited by anthracycline antibiotics. *Biochim. Biophys. Acta* **1035**, 218-222.

Karim, M.A., Miller, D.D., Farrar, M.A., Eleftheriades, E., Reddy, B.H., Breland, C.M. and Samarel, A.M. (1995) Histomorphometric and biochemical correlates of arterial procollagen gene-expression during vascular repair after experimental angioplasty. *Circulation* **91**, 2049-2057.

Kenagy, R.D., Nikkari, S.T., Welgus, H.G. and Clowes, A.W. (1994) Heparin inhibits the induction of three matrix metalloproteinases (Stromelysin, 92-kD Gelatinase, and Collagenase) in primate arterial smooth muscle cells. *J. Clin. Invest.* **93**, 1987-1993.

Kenagy, R.D., Vergel, S., Mattsson, E., Bendeck, M., Reidy, M.A. and Clowes, A.W. (1996) The role of plasminogen, plasminogen activators, and matrix metalloproteinases in primate arterial smooth muscle cell migration. *Arterioscler. Thromb. Vasc. Biol.* **16**, 1373-1382.

Khwaja, A., Rodriguez Viciana, P., Wennstrom, S., Warne, P.H. and Downward, J. (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**, 2783-2793.

Kirschenlohr, H.L., Metcalfe, J.C., Weissberg, P.L. and Grainger, D.J. (1995) Proliferation of human aortic vascular smooth muscle cells in culture is modulated by active TGF beta. *Cardiovasc. Res.* **29**, 848-855.

Kleiner, D.E. and Stetler Stevenson, W.G. (1994) Quantitative zymography: detection of picogram quantities of Gelatinases. *Anal. Biochem.* **218**, 325-329.

Knox, J.B., Sukhova, G.K., Whittemore, A.D. and Libby, P. (1997) Evidence for altered balance between matrix metalloproteinases and their inhibitors in human aortic diseases. *Circulation* **95**, 205-212.

Kratky, R.G., Ivey, J. and Roach, M.R. (1996) Collagen quantitation by video-microdensitometry in rabbit atherosclerosis. *Matrix Biology* **15**, 141-144.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680-685.

Lafont, A., Guzman, L.A., Whitlow, P.L., Goormastic, M., Cornhill, J.F. and Chisolm, G.M. (1995) Restenosis after experimental angioplasty.

Intimal, medial, and adventitial changes associated with constrictive remodeling. *Circ. Res.* **76**, 996-1002.

Landau, C., Lange, R.A. and Hillis, L.D. (1994) Percutaneous transluminal coronary angioplasty. *N. Engl. J. Med.* **330**, 981-993.

Laurent, G.J. (1997) Rates of collagen-synthesis in lung skin and muscle obtained *in vivo* by a simplified method using [3H] labeled proline. *Biochem. J.* **206**, 535-544.

Leco, K.J., Khokha, R., Pavloff, N., Hawkes, S.P. and Edwards, D.R. (1994) Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem.* **269**, 9352-9360.

Lee, E., Vaughan, D.E., Parikh, S.H., Grodzinsky, A.J., Libby, P., Lark, M.W. and Lee, R.T. (1996) Regulation of matrix metalloproteinases and plasminogen activator inhibitor-1 synthesis by plasminogen in cultured human vascular smooth muscle cells. *Circ. Res.* **78**, 44-49.

Lee, S.W., Trapnell, B.C., Rade, J.J., Virmani, R. and Dichek, D.A. (1993) *In vivo* adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries. *Circ. Res.* **73**, 797-807.

Leers, M.P., Theunissen, P.H., Ramaekers, F.C. and Schutte, B. (1997) Multi-parameter flow cytometric analysis with detection of the Ki67-Ag in paraffin embedded mammary carcinomas. *Cytometry* **27**, 283-289.

Lemarchand, P., Jones, M., Yamada, I. and Crystal, R.G. (1993) *In vivo* gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. *Circ. Res.* **72**, 1132-1138.

Li, J.J., Ueno, H., Tomita, H., Yamamoto, H., Kanegae, Y., Saito, I. and Takeshita, A. (1995) Adenovirus-mediated arterial gene transfer does not require prior injury for submaximal gene expression. *Gene Ther.* **2**, 351-354.

Libby, P., Schwartz, D., Brogi, E., Tanaka, H. and Clinton, S.K. (1992) A cascade model for restenosis. A special case of atherosclerosis progression. *Circulation* **86**, III47-52.

Lim, C.S., Chapman, G.D., Gammon, R.S., Muhlestein, J.B., Bauman, R.P., Stack, R.S. and Swain, J.L. (1991) Direct *in vivo* gene transfer into the coronary and peripheral vasculatures of the intact dog [see comments]. *Circulation* **83**, 2007-2011.

Lindner, V., Fingerle, J. and Reidy, M.A. (1993) Mouse model of arterial injury. *Circ. Res.* **73**, 792-796.

Lochmuller, H., Jani, A., Huard, J., Prescott, S., Simoneau, M., Massie, B., Karpati, G. and Acsadi, G. (1994) Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum. Gene Ther.* **5**, 1485-1491.

Matrisian, L.M. (1990) Metalloproteinases and their inhibitors in matrix remodeling. *Trends. Genet.* **6**, 121-125.

McGeehan, G.M., Becherer, J.D., Bast, R.C., Jr., Boyer, C.M., Champion, B., Connolly, K.M., Conway, J.G., Furdon, P., Karp, S., Kidao, S. and *et al* (1994) Regulation of tumour necrosis factor-alpha processing by a metalloproteinase inhibitor. *Nature* **370**, 558-561.

McMillan, W.D., Patterson, B.K., Keen, R.R. and Pearce, W.H. (1995) *In situ* localization and quantification of seventy-two-kilodalton type IV Collagenase in aneurysmal, occlusive, and normal aorta. *J. Vasc. Surg.* **22**, 295-305.

Melchiori, A., Albini, A., Ray, J.M. and Stetler Stevenson, W.G. (1992) Inhibition of tumour-cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment. *Cancer Research* **52**, 2353-2356.

Mesri, E.A., Federoff, H.J. and Brownlee, M. (1995) Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice. *Circ. Res.* **76**, 161-167.

Mignatti, P. and Rifkin, D.B. (1993) Biology and biochemistry of proteinases in tumour invasion. *Physiological Reviews* **73**, 161-195.

Mintz, G.S., Pichard, A.D., Kent, K.M., Satler, L.F., Popma, J.J. and Leon, M.B. (1994) Intravascular ultrasound comparison of restenotic and *de novo* coronary artery narrowings. *Am. J. Cardiol.* **74**, 1278-1280.

Mintz, G.S., Popma, J.J., Pichard, A.D., Kent, K.M., Satler, L.F., Wong, C., Hong, M.K., Kovach, J.A. and Leon, M.B. (1996) Arterial remodeling after coronary angioplasty: a serial intravascular ultrasound study. *Circulation* **94**, 35-43.

Mittereder, N., March, K.L. and Trapnell, B.C. (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* **70**, 7498-7509.

Moncada, S. (1997) Nitric oxide in the vasculature: physiology and pathophysiology. *Annals Of The New York Academy Of Sciences* **811**, 60-69.

More, R.S., Underwood, M.J., Brack, M.J., de Bono, D.P. and Gershlick, A.H. (1995) Changes in vessel wall plasminogen activator activity and smooth muscle cell proliferation and activation after arterial injury. *Cardiovasc. Res.* **29**, 22-26.

Morishita, R., Gibbons, G.H., Ellison, K.E., Nakajima, M., Vonderleyen, H., Zhang, L.N., Kaneda, Y., Ogihara, T. and Dzau, V.J. (1994) Intimal hyperplasia after vascular injury is inhibited by antisense cdk-2 kinase oligonucleotides. *Journal Of Clinical Investigation* **93**, 1458-1464.

Morishita, R., Gibbons, G.H., Horiuchi, M., Ellison, K.E., Nakama, M., Zhang, L., Kaneda, Y., Ogihara, T. and Dzau, V.J. (1995) A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5855-5859.

Muller, D.W., Ellis, S.G. and Topol, E.J. (1992) Experimental models of coronary artery restenosis [published erratum appears in J Am Coll Cardiol 1992 Jun;19(7):1678]. *J. Am. Coll. Cardiol.* **19**, 418-432.

Murphy, G. and Reynolds, J.J. (1993) Extracellular Matrix Degradation. In: Anonymous *Connective Tissue and Its Heritable Disorders.* pp. 287-316. Wiley-Liss, Inc.]

Nabel, E.G., Plautz, G. and Nabel, G.J. (1990) Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall. *Science* **249**, 1285-1288.

Nagase, H., Enghild, J.J., Suzuki, K. and Salvesen, G. (1990) Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (Stromelysin) by proteinases and (4-aminophenyl) mercuric acetate. *Biochemistry* **29**, 5783-5789.

Newman, K.D., Dunn, P.F., Owens, J.W., Schulick, A.H., Virmani, R., Sukhova, G., Libby, P. and Dichek, D.A. (1995) Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. *J. Clin. Invest.* **96**, 2955-2965.

Nikkari, S.T., Jarvelainen, H.T., Wight, T.N., Ferguson, M. and Clowes, A.W. (1994) Smooth muscle cell expression of extracellular matrix genes after arterial injury. *Am. J. Pathol.* **144**, 1348-1356.

Nikkari, S.T., O'Brien, K.D., Ferguson, M., Hatsukami, T., Welgus, H.G., Alpers, C.E. and Clowes, A.W. (1995) Interstitial Collagenase (MMP-1) expression in human carotid atherosclerosis. *Circulation* **92**, 1393-1398.

Ohno, T., Gordon, D., San, H., Pompili, V.J., Imperiale, M.J., Nabel, G.J. and Nabel, E.G. (1994) Gene therapy for vascular smooth muscle cell proliferation after arterial injury [see comments]. *Science* **265**, 781-784.

Pauly, R.R., Passaniti, A., Bilato, C., Monticone, R., Cheng, L., Papadopoulos, N., Gluzband, Y.A., Smith, L., Weinstein, C., Lakatta, E.G. and *et al* (1994) Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV Collagenase activity and is inhibited by cellular differentiation. *Circ. Res.* **75**, 41-54.

Pepine, C.J., Hirshfeld, J.W., MacDonald, R.G., Henderson, M.A., Bass, T.A., Goldberg, S., Savage, M.P., Vetrovec, G., Cowley, M., Taussig, A.S.

and *et al* (1990) A controlled trial of corticosteroids to prevent restenosis after coronary angioplasty. M-HEART Group. *Circulation* **81**, 1753-1761.

Pickering, J.G., Ford, C.M., Tang, B. and Chow, L.H. (1997) Coordinated effects of fibroblast growth factor-2 on expression of fibrillar collagens, matrix metalloproteinases, and tissue inhibitors of matrix metalloproteinases by human vascular smooth muscle cells - evidence for repressed collagen production and activated degradative capacity. *Arteriosclerosis Thrombosis And Vascular Biology* **17**, 475-482.

Pickering, J.G. and Boughner, D.R. (1990) Fibrosis in the transplanted heart and its relation to donor ischemic time - assessment with polarized-light microscopy and digital image- analysis. *Circulation* **81**, 949-958.

Post, M.J., Borst, C. and Kuntz, R.E. (1994) The relative importance of arterial remodeling compared with intimal hyperplasia in lumen renarrowing after balloon angioplasty. A study in the normal rabbit and the hypercholesterolemic Yucatan micropig [see comments]. *Circulation* **89**, 2816-2821.

Post, M.J., deSmet, B.J.G.L., VanderHelm, Y., Borst, C. and Kuntz, R.E. (1997) Arterial remodeling after balloon angioplasty or stenting in an atherosclerotic experimental model. *Circulation* **96**, 996-1003.

Powell, J.S., Clozel, J.P., Muller, R.K., Kuhn, H., Hefti, F., Hosang, M. and Baumgartner, H.R. (1989) Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* **245**, 186-188.

Puente, X.S., Pendas, A.M., Llano, E., Velasco, G. and Lopez Otin, C. (1996) Molecular cloning of a novel membrane-type matrix

metalloproteinase from a human breast carcinoma. *Cancer Res.* **56**, 944-949.

Quax, P.H.A., Lamfers, M.L.M., Grimbergen, J.M., Teeling, J., Hoeben, R.C., Amerongen, G.P.V. and Vanhinsbergh, V.W.M. (1996) Effects of infection with recombinant adenovirus on human vascular endothelial and smooth-muscle cells. *Fibrinolysis* **10**, 71-74.

Rade, J.J., Schulick, A.H., Virmani, R. and Dichek, D.A. (1996) Local adenoviral-mediated expression of recombinant hirudin reduces neointima formation after arterial injury. *Nat. Med.* **2**, 293-298.

Rajagopalan, S., Meng, X.P., Ramasamy, S., Harrison, D.G. and Galis, Z.S. (1996) Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases *in vitro* - implications for atherosclerotic plaque stability.-. Journal Of Clinical Investigation 98, 2572-2579.

Raza-Ahmad, A., Klassen, G.A., Murphy, D.A., Sullivan, J.A., Kinely, C.E., Landymore, R.W. and Wood, J.R. (1995) Evidence of type 2 herpes simplex infection in human coronary arteries at the time of coronary bypass surgery. *Can. J. Cardiol.* **11**, 1025-1029.

Rennick, R., Campbell, J.H. and Campbell, G.R. (1988) Vascular smooth muscle cell phenotype and growth behavior can be influenced by macrophages *in vitro*. *Atherosclerosis* **71**, 35-43.

Rhoads, J.L., Birx, D.L., Wright, D.C., Brundage, J.F., Brandt, B.L., Redfield, R.R. and Burke, D.S. (1991) Safety and immunogenicity of multiple conventional immunizations administered during early HIV infection. *J Acquir Immune Defic Syndr* **4**, 724-731.

Rubenstein, R.C., McVeigh, U., Flotte, T.R., Guggino, W.B. and Zeitlin, P.L. (1997) CFTR gene transduction in neonatal rabbits using an adeno-associated virus (AAV) vector. *Gene Therapy* **4**, 384-392.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Detection and analysis of proteins expressed from cloned genes. In: Sambrook, J., Fritsch, E.F. and Maniatis, T. (Eds.) *Molecular cloning. A laboratory manual.* 2nd Edn. Cold Spring Harbour Laboratory Press]

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. and Seiki, M. (1994) A matrix metalloproteinase expressed on the surface of invasive tumour cells [see comments]. *Nature* **370**, 61-65.

Schulick, A.H., Newman, K.D., Virmani, R. and Dichek, D.A. (1995) *In vivo* gene transfer into injured carotid arteries. Optimization and evaluation of acute toxicity. *Circulation* **91**, 2407-2414.

Schwartz, R.S., Chu, A., Edwards, W.D., Srivatsa, S.S., Simari, R.D., Isner, J.M. and Holmes, D.R., Jr. (1996) A proliferation analysis of arterial neointimal hyperplasia: lessons for antiproliferative restenosis therapies. *Int. J. Cardiol.* **53**, 71-80.

Shofuda, K., Yasumitsu, H., Nishihashi, A., Miki, K. and Miyazaki, K. (1997) Expression of three membrane-type matrix metalloproteinases (MT-MMPs) in rat vascular smooth muscle cells and characterization of MT3-MMPs with and without transmembrane domain. *J. Biol. Chem.* **272**, 9749-9754.

Simari, R.D., San, H., Rekhter, M., Ohno, T., Gordon, D., Nabel, G.J. and Nabel, E.G. (1996) Regulation of cellular proliferation and intimal formation following balloon injury in atherosclerotic rabbit arteries. *J. Clin. Invest.* **98**, 225-235.

Simons, M., Edelman, E.R. and Rosenberg, R.D. (1994) Antisense proliferating cell nuclear antigen oligonucleotides inhibit intimal hyperplasia in a rat carotid-artery injury model. *Journal Of Clinical Investigation* **93**, 2351-2356.

Southgate, K.M., Davies, M., Booth, R.F. and Newby, A.C. (1992) Involvement of extracellular-matrix-degrading metalloproteinases in rabbit aortic smooth-muscle cell proliferation. *Biochem. J.* **288**, 93-99.

Southgate, K.M., Fisher, M., Banning, A.P., Thurston, V.J., Baker, A.H., Fabunmi, R.P., Groves, P.H., Davies, M. and Newby, A.C. (1996) Upregulation of basement membrane-degrading metalloproteinase secretion after balloon injury of pig carotid arteries. *Circ. Res.* **79**, 1177-1187.

Sperti, G., van Leeuwen, R.T., Quax, P.H., Maseri, A. and Kluft, C. (1992) Cultured rat aortic vascular smooth muscle cells digest naturally produced extracellular matrix. Involvement of plasminogen-dependent and plasminogen-independent pathways. *Circ. Res.* **71**, 385-392.

Stetler Stevenson, W.G., Hewitt, R. and Corcoran, M. (1996) Matrix metalloproteinases and tumour invasion: from correlation and causality to the clinic. *Semin. Cancer Biol.* **7**, 147-154.

Smith, M.R., Kung, H., Durum, S.K., Colburn, N.H., and Sun, Y. (1997) TIMP-3 induces cell death by stabilizing TNF- alpha receptors on the surface of human colon carcinoma cells. *Cytokine*. **9**(10):770-780.

Strauss, B.H., Robinson, R., Batchelor, W.B., Chisholm, R.J., Ravi, G., Natarajan, M.K., Logan, R.A., Mehta, S.R., Levy, D.E., Ezrin, A.M. and Keeley, F.W. (1996) *In vivo* collagen turnover following experimental

balloon angioplasty injury and the role of matrix metalloproteinases. *Circ. Res.* **79**, 541-550.

Takino, T., Sato, H., Shinagawa, A. and Seiki, M. (1995) Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family. *J. Biol. Chem.* **270**, 23013-23020.

Talbot, D.C. and Brown, P.D. (1996) Experimental and clinical studies on the use of matrix metalloproteinase inhibitors for the treatment of cancer. *European Journal Of Cancer* **32A**, 2528-2533.

Tyagi, S.C., Kumar, S. and Katwa, L. (1997) Differential regulation of extracellular matrix metalloproteinase and tissue inhibitor by heparin and cholesterol in fibroblast cells. *Journal Of Molecular And Cellular Cardiology* **29**, 391-404.

Urbanski, S.J., Edwards, D.R., Maitland, A., Leco, K.J., Watson, A. and Kossakowska, A.E. (1992) Expression of metalloproteinases and their inhibitors in primary pulmonary carcinomas. *British Journal Of Cancer* **66**, 1188-1194.

Vincenti, M.P., Clark, I.M. and Brinckerhoff, C.E. (1994) Using inhibitors of metalloproteinases to treat arthritis -easier said than done. *Arthritis And Rheumatism* **37**, 1115-1126.

Vine, N. and Powell, J.T. (1991) Metalloproteinases in degenerative aortic disease. *Clin. Sci. Colch.* **81**, 233-239.

von der Leyden, H.E., Gibbons, G.H. and Morishita, R. (1995) Gene therapy inhibiting neointimal vascular lesion: *in vivo* transfer of endothelial

cell nitric oxide synthase gene. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 1137-1141.

Wagner, J.A. and Gardner, P. (1997) Toward cystic fibrosis gene therapy. *Annual Review Of Medicine* **48**, 203-216.

Waller, B.F., Orr, C.M., Pinkerton, C.A., VanTassel, J.W. and Pinto, R.P. (1990) Morphologic observations late after coronary balloon angioplasty: mechanisms of acute injury and relationship to restenosis. *Radiology* **174**, 961-967.

Walther, S.E. and Denhardt, D.T. (1996) Directed mutagenesis reveals that two histidines in tissue inhibitor of metalloproteinase-1 are each essential for the suppression of cell migration, invasion, and tumourigenicity. *Cell Growth Differ.* **7**, 1579-1588.

Wang, H., Moore, S. and Alavi, M.Z. (1996) Synthesis of tissue inhibitor of metalloproteinase-1 (TIMP-1) in rabbit aortic neointima after selective de-endothelialization. *Atherosclerosis* **126**, 95-104.

Wang, X., Fu, X., Brown, P.D., Crimmin, M.J. and Hoffman, R.M. (1994) Matrix metalloproteinase inhibitor bb-94 (batimastat) inhibits human colon-tumour growth and spread in a patient-like orthotopic model in nude-mice. *Cancer Research* **54**, 4726-4728.

Ward, R.V., Atkinson, S.J., Reynolds, J.J. and Murphy, G. (1994) Cell surface-mediated activation of proGelatinase A: demonstration of the involvement of the C-terminal domain of proGelatinase A in cell surface binding and activation of proGelatinase A by primary fibroblasts. *Biochem. J.* **304**, 263-269.

Watson, S.A., Morris, T.M., Robinson, G., Crimmin, M.J., Brown, P.D. and Hardcastle, J.D. (1995) Inhibition of organ invasion by the matrix metalloproteinase inhibitor batimastat (bb-94) in 2 human colon-carcinoma metastasis models. *Cancer Research* **55**, 3629-3633.

Watson, S.A., Morris, T.M., Parsons, S.L., Steele, R.J.C. and Brown, P.D. (1996) Therapeutic effect of the matrix metalloproteinase inhibitor, batimastat, in a human colorectal-cancer ascites model. *British Journal Of Cancer* **74**, 1354-1358.

Weaver, V.M., Fischer, A.H., Peterson, O.W. and Bissell, M.J. (1996) The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumourigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. *Biochem. Cell Biol.* **74**, 833-851.

Webb, K.E., Henney, A.M., Anglin, S., Humphries, S.E. and McEwan, J. (1997) The expression of matrix metalloproteinases and their inhibitor TIMP-1 in the rat carotid artery carotid following balloon injury. *Arterioscler. Thromb. Vasc. Biol.* **17**, 1837-1844.

Whittaker, P., Kloner, R.A., Boughner, D.R. and Pickering, J.G. (1994) Quantitative assessment of myocardial collagen with picrosirius red staining and circularly-polarized light. *Basic Research In Cardiology* **89**, 397-410.

Will, H. and Hinzmann, B. (1995) cDNA sequence and messenger-RNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. *European Journal Of Biochemistry* **231**, 602-608.

Willard, J.E., Landau, C., Glamann, D.B., Burns, D., Jessen, M.E., Pirwitz, M.J., Gerard, R.D. and Meidell, R.S. (1994) Genetic modification of the vessel wall. Comparison of surgical and catheter-based techniques for delivery of recombinant adenovirus. *Circulation* **89**, 2190-2197.

Wilson, J.M. (1996) Adenoviruses as gene-delivery vehicles. *N. Engl. J. Med.* **334**, 1185-1187.

Yanagi, H., Sasaguri, Y., Sugama, K., Morimatsu, M. and Nagase, H. (1991) Production of tissue Collagenase (matrix metalloproteinase 1) by human aortic smooth muscle cells in response to platelet-derived growth factor. *Atherosclerosis* **91**, 207-216.

Yang, Z.Y., Simari, R.D., Perkins, N.D., San, H., Gordon, D., Nabel, G.J. and Nabel, E.G. (1996) Role of the p21 cyclin-dependent kinase inhibitor in limiting intimal cell proliferation in response to arterial injury. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7905-7910.

Yei, S.P., Mittereder, N., Wert, S., Whitsett, J.A., Wilmott, R.W. and Trapnell, B.C. (1994) In-vivo evaluation of the safety of adenovirus-mediated transfer of the human cystic-fibrosis transmembrane conductance regulator cDNA to the lung. *Human Gene Therapy* **5**, 731-744.

Yu, C.C., Woods, A.L. and Levison, D.A. (1992) The assessment of cellular proliferation by immunohistochemistry: a review of currently available methods and their applications. *Histochem. J.* **24**, 121-131.

Zempo, N., Kenagy, R.D., Au, Y.P., Bendeck, M., Clowes, M.M., Reidy, M.A. and Clowes, A.W. (1994) Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. *J. Vasc. Surg.* **20**, 209-217.

Zempo, N., Koyama, N., Kenagy, R.D., Lea, H.J. and Clowes, A.W. (1996) Regulation of vascular smooth muscle cell migration and proliferation *in vitro* and in injured rat arteries by a synthetic matrix metalloproteinase inhibitor Regulation of vascular smooth-muscle cell-migration and proliferation in-vitro and in injured rat arteries by a synthetic matrix metalloproteinase inhibitor. *Arteriosclerosis Thrombosis And Vascular Biology* **16**, 28-33.

Zhang, W.W., Koch, P.E. and Roth, J.A. (1995) Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *Biotechniques* **18**, 444-447.

Zoldhelyi, P., McNatt, J., Xu, X.M., Loose Mitchell, D., Meidell, R.S., Clubb, F.J., Jr., Buja, L.M., Willerson, J.T. and Wu, K.K. (1996) Prevention of arterial thrombosis by adenovirus-mediated transfer of cyclooxygenase gene. *Circulation* **93**, 10-17.

APPENDICES

APPENDIX 1 HISTOLOGICAL STAINING PROTOCOLS

Haematoxylin and Eosin

1) Sections were rehydrated by immersion in Xylene solutions for three minutes twice, they were then placed in the following alcohols for 3 minutes: 100% ethanol, 100% ethanol, 70% ethanol and finally tap water.
2) Sections were stained for 15 minutes in Cole's Haematoxylin made as follows: 1.5g Haematoxylin was heated in 250 ml dH_2O until dissolved. 700ml 10% saturated aqueous potassium alum and 50ml 1% iodine in 95% alcohol were added and the solution briefly boiled before rapid cooling. The solution was filtered prior to use.
3) The sections were briefly washed in a tray of running tap water.
4) They were then differentiated in acid alcohol (1%HCl in 70% ethanol) for 15 seconds.
5) Sections were washed for 5 minutes in dH_2O.
6) Sections were then stained with 1% Eosin (in tap water) for five minutes.
7) Sections were briefly washed in water.
8) The slides were then rinsed in 70% ethanol and dehydrated for 3 minutes in the following series of solutions: 70% ethanol, 90% ethanol, 100% ethanol, Xylene, Xylene.
9) The slides were mounted in DPX (BDH)
10) This results in blue nuclei, red muscle, and pink collagen.

Verhoeff's-van Gieson

1) Sections were rehydrated by immersion in Xylene solutions for three minutes twice, they were then placed in the following alcohols for 3 minutes: 100% ethanol, 100% ethanol, 70% ethanol and finally tap water.
2) Sections were placed in 0.5% potassium permanganate for five minutes and then rinsed in dH_2O.
3) Sections were then placed for three minutes in 1% oxalic acid and rinsed in dH_2O and then 95% ethanol.
4) Sections were stained in Miller's stain (BDH) for two hours.
5) They were rinsed in 95% ethanol for ten seconds and then in dH_2O.
6) The sections were stained in van Gieson's stain (BDH) for four minutes.
7) The slides were then rinsed in 70% ethanol and dehydrated for 3 minutes in the following series of solutions: 70% ethanol, 90% ethanol, 100% ethanol, Xylene, Xylene.
8) The slides were mounted in DPX (BDH).
9) This results in black staining of elastic fibres.

Nuclear Fast Red

1) Sections were rehydrated by immersion in Xylene solutions for three minutes twice, they were then placed in the following alcohols for 3 minutes: 100% ethanol, 100% ethanol, 70% ethanol and finally tap water.
2) Sections were placed in 0.1% Nuclear fast red (Sigma) in 5% aluminium sulphate for 5 minutes.
3) Sections were then washed in dH_2O.
4) The slides were then rinsed in 70% ethanol and dehydrated for 3 minutes in the following series of solutions: 70% ethanol, 90% ethanol, 100% ethanol, Xylene, Xylene.
5) The slides were mounted in DPX (BDH).

Modified Nuclear Fast Red Protocol For X-Gal Stained Blocks.

1) All tissue incubated in X-Gal prior to fixation was embedded in paraffin using short cycles designed for biopsy tissues to minimize exposure to organic solvents, in particular xylene, which will wash out the blue pigment product of the X-Gal reaction.
2) Sections were rehydrated by immersion in Xylene solutions for 20 seconds twice, they were then placed in the following alcohols for 20 seconds: 100% ethanol, 100% ethanol, 70% ethanol and finally tap water.
3) Sections were placed in 0.1% Nuclear fast red (Sigma) in 5% aluminium sulphate for 5 minutes.
4) Sections were then washed in dH_2O.
5) Slides were then mounted in aquapolymount, an aqueous mountant in which the blue pigment product of the X-Gal reaction is not soluble.
6) Where possible sections were photographed or the images stored on a computer image analysis system shortly after staining.

APPENDIX 2 PUBLICATIONS ARISING FROM THE RESEARCH

The following papers and reviews have been /are to be published in peer reviewed journals:

Dollery, C.M., McEwan, J.R., and Henney, A.M. Matrix metalloproteinases and cardiovascular disease. *Circ.Res.* 77(5):863-868, 1995.

Coffin, R.S., Howard, M.K., Cumming, D.V., Dollery, C.M., McEwan, J., Yellon, D.M., Marber, M.S., MacLean, A.R., Brown, S.M., and Latchman, D.S. Gene delivery to the heart *in vivo* and to cardiac myocytes and vascular smooth muscle cells *in vitro* using herpes virus vectors. *Gene Ther.* 3(7):560-566, 1996.

Dollery, C.M, Humphries, S.E., McClelland A,
Latchman DS, McEwan, J.R. Expression of Tissue Inhibitor of Matrix Metalloproteinases 1 using an adenoviral vector inhibits smooth muscle cell migration and reduces neointimal hyperplasia in the rat model of vascular balloon injury. *J Clin Invest* 1998. (submitted, under second review)

Dollery, C.M, McEwan, J.R., Wang, M. Liu Y.E., Shi, Y.E. TIMP 4 Protein Is Regulated By Vascular Injury In Rats. *Circ.Res* 1998 (submitted, under second review)

Book Chapters

C Dollery, M Marber. Gene therapy. In Difficult Concepts in Cardiology Volume III, Ed: Jackson G. Martin Dunitz 1997.

Abstracts

C M Dollery, S E Humphries, A McClelland, DS Latchman, J R McEwan. *In vivo* gene transfer after vascular injury- Expression of TIMP1 reduces neointima formation. British Cardiac Society May 1998 (submitted).

Clare M Dollery, Jean R. McEwan, Mingsheng Wang, Yiliang E. Liu, Yuenian E. Shi. TIMP 4 protein is regulated by vascular injury in the rat model of angioplasty restenosis. British Cardiac Society May 1998 (submitted).

Dollery, C.M., McClelland, A., Latchman, D.S., Henney, A.M., Humphries, S.E., and McEwan, J.R. Adenoviral gene transfer of human TIMP-1 inhibits smooth muscle cell migration and neointima formation in injured rat carotid arteries. *Circulation* 96:2693, 1997.

Dollery CM, McClelland A, Rollence M *et al. In vivo* and *In vitro* adenoviral vascular gene transfer of tissue inhibitor of metalloproteinases- 1. *Eur Heart J*, 18 Suppl: 1298,1997.

Dollery CM, McClelland A, Rollence M *et al. In vivo* and *In vitro* arterial gene transfer of TIMP1 using an adenoviral vector. *JMCC* 29: 5; S89, 1997.

Packwood L, Andrews W, Dollery CM, Yiu P, McClelland A, Humphries SE, McEwan JR. Adenoviral gene transfer of the marker gene ß Galactosidase, to a pre-injured pig coronary artery. *JMCC* 29: 5; Sa71, 1997.

Dollery CM, McClelland A, Rollence M *et al. In vivo* arterial gene transfer of TIMP-1 using an adenoviral vector. *Heart* 77 (Suppl 1) P76, 1997.

Dollery CM, McClelland A, Rollence M *et al. In vivo* and *In vitro* arterial gene transfer of TIMP1 using an adenoviral vector. *Clinical Science* 1997 May: Suppl

Dollery CM, McClelland A, Rollence M *et al.* Overexpression of TIMP-1 in vascular smooth muscle cells by adenoviral gene transfer. TIMPs 96. 1996.

Dollery CM, Webb, KA, McClelland A

Overexpression of TIMP-1 in vascular smooth muscle cells by adenoviral gene transfer. J Vasc Res. 33 Suppl 1: P82, 1996.