The mechanisms of coronary restenosis: insights from experimental models

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Summary. Since its introduction into clinical practice, more than 20 years ago, percutaneous transluminal coronary angioplasty (PTCA) has proven to be an effective, minimally invasive alternative to coronary artery bypass grafting (CABG). During this time there have been great improvements in the design of balloon catheters, operative procedures and adjuvant drug therapy, and this has resulted in low rates of primary failure and short-term complications. However, the potential benefits of angioplasty are diminished by the high rate of recurrent disease. Up to 40% of patients undergoing angioplasty develop clinically significant restenosis within a year of the procedure. Although the deployment of endovascular stents at the time of angioplasty improves the short-term outcome, 'in-stent' stenosis remains an enduring problem. In order to gain an insight into the mechanisms of restenosis, several experimental models of angioplasty have been developed. These have been used together with the tools provided by recent advances in molecular biology and catheter design to investigate restenosis in detail. It is now possible to deliver highly specific molecular antagonists, such as antisense gene sequences, to the site of injury. The knowledge provided by these studies may ultimately lead to novel forms of intervention. The present review is a synopsis of our current understanding of the pathological mechanisms of restenosis.

Keywords: balloon angioplasty, smooth muscle cells, endothelium, proliferation, migration, rat, rabbit, pig

The first clinical application of coronary angioplasty using a balloon catheter was performed by Gruentzig (1978), however, it was originally used experimentally several years earlier (Baumgartner 1963). Since its introduction,

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balloon angioplasty has been shown to be safe and effective. In comparisons with CABG, PTCA appears to be an 'acceptable alternative' with similar outcomes (CABRI 1995; Pocock *et al.* 1995, 1996; BARI 1996) After a 6.5-years follow up of the RITA cohort (Henderson *et al.* 1999), report that patients undergoing PTCA and CABG have similar outcomes with regard to the primary clinical end-points of death or non-fatal MI. However patients undergoing PTCA had a significantly

higher requirement for repeat procedures, and suffered a higher prevalence of angina. PTCA is currently used in more than 500 000 patients per annum, world-wide. Acute closure, and primary failure rates are low, however, restenosis remains a major long-term complication (Meier 1989).

Experimental models of restenosis

Restenosis is a narrowing at the site of balloon dilatation (Waller *et al.* 1991). It develops rapidly following the procedure and may be accompanied by recurrent symptoms (Glazier *et al.* 1989). The precise mechanisms for restenosis probably vary between patients, but is likely to be a combination of artery wall remodeling and intimal hyperplasia (Austin *et al.* 1985). Several animal models of restenosis have been developed and these have led to an improvement in our understanding of the cellular mechanisms involved.

Cellular events following angioplasty

Detailed analysis of the cell kinetics following balloon catheter injury has been available since the late 1970s and early 1980s. This knowledge has relied almost entirely on the use of experimental animal models.

Platelet activation

Very rapidly after balloon injury, circulating platelets and leucocytes cover the de-endothelialized surface of the blood vessel (Steele *et al.* 1985; Ferns *et al.* 1991c; Groves *et al.* 1995). The platelets have a flattened appearance, and are clearly involved in the polar release of their contents into the injured artery wall (Winocour *et al.* 1989). Platelet aggregation is stimulated by the release of subendothelial elements, such as collagen. The process is self-sustaining due to the release of platelet associated pro-aggregatory substances, such as ADP and thromboxane A2. However, within 24 h of balloon injury, the arterial surface becomes significantly less thrombogenic (Kinlough-Rathbone *et al.* 1983), and the turnover of platelets diminishes (Winocour *et al.* 1989).
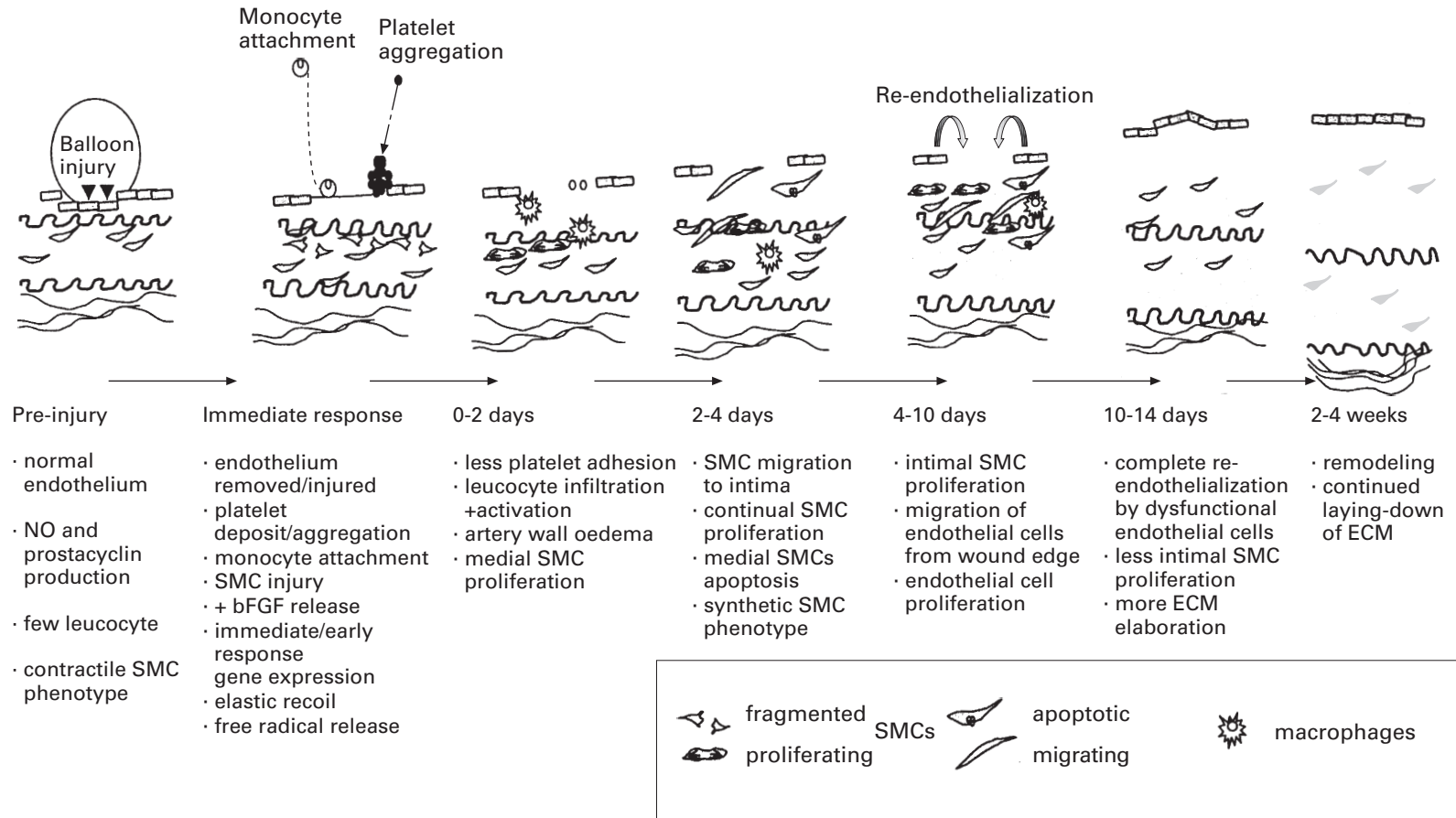
Studies using antiplatelet treatment (aspirin plus dipyridamole) have indicated the importance of platelets to restenosis in the atherosclerotic rabbit (Faxon *et al.* 1984), although this has not be confirmed by more recent studies (Sun *et al.* 1993). However, recent clinical trials using glycoprotein IIb/IIIa receptor blockers have reinforced the importance of platelets in restenosis (Lincoff *et al.* 1999).

Leucocyte infiltration

Inflammatory cells are involved in the acute response to arterial injury. In the subtle injury associated with the placement of a silastic 'collar' around the rabbit carotid artery, neutrophils were found to be an important early, though transient, participant (Booth *et al.* 1989). However, following balloon injury, neutrophils do not form a major constituent of the cellular infiltrate, particularly in the rat (Jonasson *et al.* 1988; Verheyen *et al.* 1988; Ferns *et al.* 1991c). In this model, monocyte-derived macrophages comprise approximately 1% of the neo-intimal cell population (Ferns *et al.* 1991c). In the cholesterol-fed rabbit these macrophages are transformed into macrophage-derived foam cells, and constitute a substantial part of the neo-intima (Ferns *et al.* 1992b). The importance of leucocytes to the response to injury has recently been demonstrated by blocking their entry into the artery wall using monoclonal antibodies to cell adhesion molecules (Liao *et al.* 1997). Mac-1 blockade reduces experimental neointimal thickening, suggesting that leucocyte recruitment and infiltration of injured arteries may be a useful target for preventing intimal hyperplasia (Rogers *et al.* 1998), and activated circulating monocytes expressing CD11b/CD18 are a good marker of restenosis risk in patients undergoing angioplasty. The role of T lymphocytes in neo-intimal development is unclear, and their importance may vary with the animal model used. Jonasson *et al.* (1988) have reported that T cells constitute 0.3% of the neo-intimal cell population. Their importance to neo-intimal thickening has been assessed in several ways, but the results of these experiments is inconsistent. The use of cyclosporin A at high doses inhibited neo-intimal thickening in the rat (Jonasson *et al.* 1988), however, this was not found to be the case in the rabbit when cyclosporin A was used within the therapeutic range (Ferns *et al.* 1990), nor did the absence of functional T cells affect the cell kinetics of smooth muscle cell proliferation (Ferns *et al.* 1991c). However Hansson (1994) has reported that T cell depletion is associated with enhanced neo-intimal thickening in the rat.

Smooth muscle kinetics

The characteristic histological feature of restenosis in man and experimental animal models is smooth muscle cell accumulation within the neointima (Austin *et al.* 1985; Nobuyoshi *et al.* 1989; Waller *et al.* 1991). Balloon injury is initially associated with medial smooth muscle cell death (Clowes *et al.* 1983). There follows several phases of cellular activity (Figure 1). These include:

Monocyte
attachment

Platelet
aggregation

Re-endothelialization

Balloon
injury

Pre-injury	Immediate response	0–2 days	2–4 days	4–10 days	10–14 days	2–4 weeks
· normal endothelium	· endothelium removed/injured	· less platelet adhesion	· SMC migration to intima	· intimal SMC proliferation	· complete re-endothelialization by dysfunctional endothelial cells	· remodeling
· NO and prostacyclin production	· platelet deposit/aggregation	· leucocyte infiltration +activation	· continual SMC proliferation	· migration of endothelial cells from wound edge	· less intimal SMC proliferation	· continued laying-down of ECM
· few leucocyte	· monocyte attachment	· artery wall oedema	· medial SMCs apoptosis	· endothelial cell proliferation	· more ECM elaboration	
· contractile SMC phenotype	· SMC injury	· medial SMC proliferation	· synthetic SMC phenotype			
	· + bFGF release					
	· immediate/early response gene expression					
	· elastic recoil					
	· free radical release					

fragmented SMCs   apoptotic   macrophages

proliferating   migrating

Figure 1. Cellular changes and time course after balloon angioplasty.

- the co-ordinated proliferation of medial smooth muscle cells, driven by growth factors released from platelets and cell lysis;
- migration of a subpopulation of medial smooth muscle cells into the intima in response to the release of chemo-attractants such as platelet-derived growth factor (PDGF) (Ferns *et al.* 1991c; Rutherford *et al.* 1997);
- proliferation of intimal smooth muscle cells;
- elaboration of extracellular matrix molecules, such as hyaluronan and chondroitin sulphate by smooth muscle cells of a synthetic phenotype;
- vascular remodeling, including the removal of excess extracellular matrix by matrix metalloproteases (MMPs).

It is a combination of these events that ultimately lead to luminal narrowing, and an understanding of the factors that orchestrate each stage in this process may provide new targets for therapeutic intervention. It is apparent that medial smooth muscle cells are heterogeneous with respect to their responsiveness to chemo-attractants and mitogens released following balloon injury (Clowes *et al.* 1983; Haudenschild & Grunwald 1985). Smooth muscle cell (SMC) proliferation and phenotypic modulation are important aspects of the arterial response to injury in experimental models of restenosis.

Changes in smooth muscle cell phenotype

SMC undergo profound changes following balloon injury. These changes include: a decrement in myofilament density (Manderson *et al.* 1989); altered desmin and vimentin positivity (Kocher *et al.* 1984); an increase in β–actin expression (Kocher *et al.* 1984; Barja *et al.* 1986); and changes in extracellular matrix elaboration (Snow *et al.* 1990). These changes appear to be partially reversible (Grunwald *et al.* 1987). Smooth muscle cells are also induced to express MHC class II molecules following injury (Jonasson *et al.* 1988). It has been hypothesized that this is due to the local release of γ–interferon by activated T lymphocytes within the neo-intima. It is unclear to what extent this may be important in restenosis, although, the cells expressing MHC class II molecules were nondividing cells.

In vivo and *in vitro* observations strongly suggest that marked differences exist in the growth, and matrix-producing capabilities of phenotypically distinct SMC subpopulations within the artery wall (Frid *et al.* 1997).

SMC migration from the tunica media to the intima in the ballooned rat carotid artery occurs intensively between the second and fifth days after balloon injury. About 80% of the migrating cells are reported to be in the

G1 and S phases of the cell cycle. Positive staining for PDGF-B, elastase III B, MMP-I, and MMP-9 of these cells was detected on the fifth day after injury (Yoshida *et al.* 1997).

Smooth muscle cell apoptosis

Acute apoptotic cell death after vascular injury is a highly regulated process governed by cellular redox state and the relative expression of antiapoptotic genes. Apoptotic cell death is a feature of human and experimental angio-plasty. (Perlman *et al.* 1997) found that up to 70% of medial cells appeared apoptotic within 30 min of injury using the TUNEL assay technique. This was associated with a marked down-regulation of bcl-X expression, particularly in the cells nearest the lumen. Fas ligand induces apoptosis in Fas-bearing cells and (Sata *et al.* 1998) showed that Fas-ligand gene transfer to the balloon injured rat carotid artery inhibited intimal hyperplasia.

Modifying the vascular redox state by the administration of antioxidants, markedly attenuated both stress-activated protein kinase activation and the induction of apoptosis (Pollman *et al.* 1999). It has been suggested that the induction of apoptosis in vascular smooth muscle cells is modulated by cell phenotype. In contrast to medial smooth muscle cells, neointimal cells were found to be relatively resistant to apoptotic death induced by balloon injury (Kollum *et al.* 1997). This resistance to balloon injury-induced death was associated with an upregulation of the antiapoptotic mediator bcl-xL in neointimal smooth muscle cells.

Extra cellular matrix elaboration

The elaboration of extracellular matrix molecules by intimal smooth muscle cells contributes substantially to intimal thickening, hence intimal cross-sectional area continues to increase for some time after cell proliferation has ceased (reviewed by Wight 1995). The size of the neointima reaches a plateau between 1 and 4 months and gradually diminishes in size through the removal of extracellular matrix.

Re-endothelialization

The major stimuli for intimal and medial smooth muscle cell proliferation have not been identified, however, factors released by endothelial cells appear to be critical in modulating this effect. Re-endothelialized segments of artery are often associated with less neo-intimal thickening. However this is partially dependent on the extent of the original injury (Walker *et al.* 1983; Reidy & Silver 1985). Deep and wide injuries may be associated

with incomplete re-endothelialization and substantial neo-intimal thickening, whereas superficial, narrow lesions may heal without intimal hyperplasia (Reidy & Silver 1985). Arteries that have sustained extensive endothelial damage may not become fully re-endothelialized but instead become lined by phenotypically modified smooth muscle cells (Stemerman *et al*. 1977; Reidy 1985). Rapid endothelial cell recoverage of Fogarty balloon-injured arteries in the rabbit may not limit intimal hyperplasia in the centre of the lesion. It is possible that the inability of regenerated endothelium to inhibit intimal hyperplasia at this site is due to its initially dedifferentiated and possibly dysfunctional phenotype (Doornekamp *et al*. 1997).

Tanaka *et al*. (1993) found that balloon injury was associated with prolonged expression of activation markers on the regenerating endothelial cells (ICAM-1 and VCAM-1), SMCs (ICAM-1 and class II MHC antigens) and leucocytes. Cell adhesion molecules were upregulated for approximately one week following balloon injury on surviving endothelium and circulating leucocytes. If the selectins were blocked, lumen size remained larger, and intimal size was reduced (Barron *et al*. 1997). However (Guzman *et al*. 1995) reported that blocking leucocyte adhesion with a monoclonal antibody to CD18/CD11 did not significantly affect lumen diameter, or percentage stenosis in the rabbit. By contrast (Rogers *et al*. 1998) found that a monoclonal antibody to Mac-1 (CD11b/CD18) immediately before injury and for 14 days thereafter significantly reduced leucocyte infiltration and intimal hyperplasia.

La Veau *et al*. (1990) reported that vascular wall reactivity responses were lost after balloon angioplasty, and that the duration of impaired responses was related to the extent of injury (Weidinger *et al*. 1990). Bosmans *et al*. (1996) investigated the changes in expression of nitric oxide synthase following balloon injury. This was studied using immunocytochemistry and functionally using isolated arterial segments in organ culture. They reported that endothelium dependent responses of the injured arteries remained impaired for 10 weeks after injury, although re-endothelialization was complete by 2 weeks. Balloon injury was associated with an early reduction in the release of prostacyclin and thromboxane, although this was not sustained (Mattsson *et al*. 1992, 1993b). The early vasospasm often associated with angioplasty did not appear to be due to endothelin (Mattsson *et al*. 1993a).

Putative mediators of restenosis

Several growth factors and cytokines probably orchestrate the complex sequence of events described above.

They are likely to be derived from a number of sources including platelets, mononuclear cells, endothelial cells and smooth muscle cells. Platelets in particular are a rich repository of growth factors, such as PDGF, IGF, TGF and EGF (Assoian *et al*. 1984) and have been shown to be important in driving the early cellular events (Fingerle *et al*. 1990). Activated monocytes are also a rich source of growth factors and cytokines (Nathan 1987). Although they are not present in great abundance in normo-cholesterolaemic animals (Ferns *et al*. 1991a), they are found in substantial numbers in diseased human arteries and in some hyperlipidaemic animal models (Rosenfeld & Ross 1990; Ferns *et al*. 1993a). Mononuclear cells remain within the injured artery wall for a considerable time, and hence may contribute to the sustained expansion of the neo-intima. Smooth muscle cells can also be stimulated to elaborate growth factors, including PDGF and IGF that have the potential to act in a paracrine, or autocrine manner. Smooth muscle cell necrosis may also lead to the release of intracellular growth factors such as bFGF. Although the endothelium also has the potential to release growth factors, including PDGF, IGF and bFGF, it appears to exert an overall inhibitory effect on neo-intimal thickening. This may be related to its release of nitric oxide. Other endothelium-derived factors promote endothelial cell re-growth, enhancing re-endothelialization and thereby indirectly inhibiting neo-intimal thickening. Activated T cells are a source of γ−interferon, a putative inhibitor of SMC proliferation Hansson *et al*. 1988).

The biochemical sequelae of balloon injury

Growth factors and cytokines

The effects of growth factors are likely to be synergistic or at least additive. In their review of clinical atherosclerosis Fuster *et al*. (1993) drew attention to the complexity of growth factor/cytokine interactions in the development of arterial lesions. Most animal models of restenosis are a gross simplification of this complexity. They are usually single injury models, in arteries with no pre-existing stenotic lesion. They are often in normo-cholesterolaemic animals in whom the restenotic lesion is almost entirely composed of vascular smooth muscle cells. And they are usually models in which other risk factors (e.g. diabetes and hypertension) are not present.

The studies of Ross & Glomset (1976) identified platelet-derived growth factor (PDGF) as a potent stimulator of the VSMC proliferation. However several other growth factors, cytokines and factors involved in the coagulation and fibrinolytic cascades have

subsequently been shown to be VSMC mitogens and chemo attractants.

Growth factors are rapidly released from platelets and dying cells after balloon injury. Ligand binding to cell surface receptors results in transient activation and signal transduction. The expression of early response genes is induced within minutes of balloon injury. Among these are c-fos, c-jun, which are upregulated within 30 min, closely followed by c-myc, the expression of which peaks by 2 h (Bauters *et al.* 1992). Activation of the immediate-early genes results in G_o/G_1 traverse and entry into the cell cycle. This is co-ordinated by a number of cyclin-dependent kinases, and is associated with the inactivation of several tumour suppressor genes (reviewed by Muller 1997).

Increased polyamine synthesis appears to play an important role in the response to balloon injury, with a transient increase in the expression of ornithine decarboxylase, preceding medial smooth muscle cell proliferation (Thyberg & Fredholm 1987; Majesky *et al.* 1990; Nishida *et al.* 1990).

Balloon injury to rat carotid arteries results in temporally related changes in the expression of PDGF receptors (Cercek *et al.* 1990; Majesky *et al.* 1990) and their state of tyrosine phosphorylation. Furthermore, tyrosine phosphorylation of PDGF beta receptors is associated with PI-3 kinase activation (Panek *et al.* 1997).

The profound changes in growth factor and growth factor receptor expression may partially reflect feedback autoregulation, but it is also likely that cross-modulation occurs. For example at low concentrations TGFβ is known to induce PDGF and PDGF receptor expression by smooth muscle cells (Battegay *et al.* 1990). VSMC exposed simultaneously to both TGF-β1 and PDGF exhibited diminished migration (50%) when compared to cells treated only with PDGF. TGF-β1 can partially reverse the stimulatory effect of PDGF on VSMC migration *in vitro* possibly by modifying the actin cytoskeleton and the distribution of the $\alpha_V\beta_3/\beta_5$ integrins (Engel & Ryan 1997).

The importance of PDGF as a SMC chemoattractant has been demonstrated in several animal models, including the rat, rabbit and nonhuman primates, by using specific inhibitors of PDGF. In these models, the BB-isoform of PDGF has emerged as being particularly important in restenosis. Although PDGF is a potent mitogen for mesenchymal cells, a neutralizing antibody was found to inhibit intimal cell accumulation without having a significant effect on SMC proliferation (measured as thymidine uptake) in the balloon rat carotid (Ferns *et al.* 1991b). This was more recently confirmed using a PDGF B-chain specific antibody (Rutherford *et al.* 1997).

Administering PDGF-BB to the injured rat carotid was also found to stimulate SMC migration rather than proliferation (Jawien *et al.* 1992). Rat neo-intimal SMC also express PDGF-BB but are heterogeneous in this respect (Lindner *et al.* 1995).

The interaction between the growth factors has been investigated by (Rutherford *et al.* 1997) who have demonstrated that PDGF-BB and bFGF are major determinants of the initial response to balloon injury in the rat. Basic FGF is widely expressed in cells of the artery wall and it is upregulated following balloon injury (Lawrence *et al.* 1995). It appears to have a major role in early medial SMC proliferation, endothelial cell regrowth and possibly SMC migration (Lindner & Reidy 1991; Lindner *et al.* 1992; Jackson & Reidy 1993; Lawrence *et al.* 1995; Rutherford *et al.* 1997) and extracellular matrix elaboration.

Vascular remodeling has emerged as a major component of luminal narrowing (Pasterkamp *et al.* 1997). Vascular imaging has shown that intimal thickening is not the sole determinant of lumen size. The overall cross-sectional area of an artery may increase, due to the effects of haemodynamic forces, partially compensating for the encroachment of the neo-intima. This process is probably mediated by growth factors and matrix metalloproteinases (MMPs) acting on adventitial cell and extracellular matrix proteins. However, there has been some debate and disagreement about the relative importance of remodeling in the rabbit model (Gertz *et al.* 1994; Kakuta *et al.* 1994; Post *et al.* 1994, 1995; Lafont *et al.* 1995).

Transforming growth factor-beta-1 (TGFβ1) plays a central role in tissue repair owing to its effects on cell growth and extracellular matrix synthesis. TGFβ1 stimulates adventitial fibroblast differentiation into myofibroblasts after injury, a process that may affect arterial remodelling (Shi *et al.* 1996). TGFβ also causes an upregulation of lysyl oxidase, an enzyme involved in collagen and elastin cross-linking, and which may, therefore contribute to arterial remodeling after vascular injury (Shanley *et al.* 1997).

The local inflammatory response elicited by balloon injury is associated with leucocyte activation and the release of inflammatory cytokines such as IL-1 and TNFα. IL-1 is a pleotropic cytokine which has been shown to stimulate SMC proliferation via an autocrine loop (Raines *et al.* 1989). IL-1 is also a T cell chemoattractant, and stimulates cell adhesion molecule expression on endothelial and VSMC (Wang *et al.* 1994). Replication of arterial SMCs after balloon injury in rabbits occurs in regions of TNF-α but not IL-1α expression and correlates inversely with the presence of bFGF. Therefore, SMC-derived TNF-α serves as a marker of a

modulated SMC phenotype after acute vascular injury and may contribute to local cellular activation and proliferation of SMCs at sites of arterial injury (Tanaka *et al.* 1996). IL-1β and TNF-α separately induce and synergistically increase P-selectin-dependent leucocyte rolling and firm adhesion (Thorlacius *et al.* 1997), enhancing leucocyte entry into the artery wall.

IL-1 also has potential to stimulate IGF-1 production by various cells (Glazebrook *et al.* 1998). IGF-1 is a SMC mitogen that exerts its effects via specific cell surface receptors (reviewed by Ferns *et al.* 1991a). IGF-1 expression is upregulated by balloon injury (Bornfeldt *et al.* 1990) as is its receptor. However the role of IGF-1 in restenosis is unclear. Motani *et al.* (1995) have reported that IGF-1 enhances platelet aggregation, a process that may promote the delivery of platelet associated factors to the injured artery wall. Although the mitogenic effects of IGF-1 could be inhibited *in vitro* by IGF-BP1, the administration of IGF-BP1 did not significantly affect the intimal response to injury in the rat carotid (Motani *et al.* 1995). These data suggest that IGF-1 is probably not a major contributor to intimal thickening in the rat model.

Transcription factors

The binding of cytokines and growth factors to their respective receptors stimulate mitogen-activated protein (MAP) kinases. MAP kinase are involved in nuclear signalling. Phosphatases down-regulate MAP kinase activity. Lai *et al.* (1996) found that MAP kinase phosphatase-1 (MKP-1) expression fell after balloon injury, whereas MAP kinase expression rose. In vitro, over-expression of MKP-1 was associated with growth arrest. Hu *et al.* (1997) have presented data demonstrating the importance of MAP kinases in mediating the SMC proliferative response. Extracellular signal-regulated kinases-2 (ERK-2) and Jun N-terminal protein kinase (JNK) were rapidly upregulated. Kinase activation is followed by increased c-fos and c-jun expression and activator protein-1 (AP-1) DNA binding activity. Balloon injury rapidly causes the activation of the MAP kinases, including extracellular signal-regulated kinases (ERK), stress activated protein kinases (SAPK) and c-Jun N-terminal protein kinases (JNK) in the rat carotid artery (Hu *et al.* 1997).

Farnesylation appears to be an important process in activating ras-proteins. Chemla *et al.* (1997) inhibited the expression of the enzyme responsible for catalysing this reaction using antisense oligonucleotides and also inhibited intimal hyperplasia. Farnesylation is also affected by the HMGCoA reductase inhibitor, lovastatin, which was found to inhibit luminal narrowing in a double-injury model

of the hypercholesterolaemic rabbits (Gellman *et al.* 1991).

NF-kB and its inhibitory proteins (IK-B) form an auto-regulatory system that has a potential role in vascular disease. Immediately after balloon catheter injury, the levels of IKBα, IKBβ and p105 were found to be dramatically reduced whereas the expression of the NFKB regulated genes, vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic protein (MCP)-1, were increased in VSMCs. In chronically denuded vessels, SMCs on the luminal surface continued to express high levels of VCAM-1 and MCP-1, which may account for the continuing presence of macrophages. These findings provide a link between the activation of NFKB and intimal lesion formation (Landry *et al.* 1997).

The myocyte enhancer binding factor-2 (MEF2) family of transcription factors are known to regulate the differentiation of skeletal and cardiac muscle. In the rat model of restenosis, the expression of MEF2A,B and D mRNAs were found to be upregulated in the neointima, with the highest levels in the layer of cells nearest to the lumen. These data suggest that the MEF2s are associated with the activated smooth muscle phenotype (Firulli *et al.* 1996). It was also shown that after balloon injury in the rat there was an increased immuno-reactivity of ETS-1 transcription factor that activates expression of matrix-degrading proteinases such as collagenase and stromelysin (Hultgardh-Nilsson *et al.* 1996)

The coagulation cascade

Plasmin is a fibrinolytic enzyme derived from plasminogen by proteolytic activation. Balloon injury is associated with altered interactions between plasminogen and the artery wall (Hatton *et al.* 1988), and changes in plasminogen activator expression (Clowes *et al.* 1990). In uninjured vessels the expression of both urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) is low. Following injury uPA expression rises to an initial peak within 24 h. Subsequently mRNA levels for both tPA and uPA rise to a maximum at approximately 7 days after injury, hence uPA is expressed during the early mitogenic phase, whilst tPA is present during the phase of medial smooth muscle cell migration (Reidy *et al.* 1996).

von Willebrand factor

von Willebrand factor (vWF) is a well-characterized multimeric glycoprotein present in platelets and plasma and synthesized by vascular endothelial cells and megakaryocytes.

The deposition of vWF in the media of the injured precedes vascular smooth muscle proliferation and endothelial regrowth (Giddings *et al.* 1997). However its absence in the von Willebrand disease pig was not associated with a significant effect on lesion size or SMC proliferation.

Renin-angiotensin system

The renin-angiotensin paracrine/autocrine networks are responsible for the local generation and receptor binding of angiotensin II within the vessel wall (Nakamura *et al.* 1988). There is also evidence that angiotensin II may modulate smooth muscle growth *in vitro* and *in vivo* (Dzau *et al.* 1989). Enalaprilat, an ACE inhibitor reduced intimal hyperplasia (Law *et al.* 1994). Neo-intimal thickening was shown to be enhanced by aldosterone and inhibited by spironolactone, an aldosterone antagonist (Van Belle *et al.* 1995).

Renin mRNA is upregulated in the balloon-injured rat carotid artery and this precedes neointima formation (Iwai *et al.* 1997). The renin-angiotensin system is in a heightened state of activation in the aortae of spontaneously hypertensive Sprague-Dawley rats SHR both before and after balloon injury compared with normotensive rats (Jandeleit-Dahm *et al.* 1997).

Huckle *et al.* (1996) have demonstrated that that balloon injury enhances the expression of angiotensin II receptor subtypes following balloon injury. PAI-1 regulates fibrinolysis and extracellular matrix turnover. It has been suggested that PAI-1 activity is modulated by angiotensin II. Hamdan *et al.* (1996) used the ACE inhibitor Captopril to investigate this possible interaction. After balloon injury levels of PAI-1 mRNA were increased by 3 h, falling to baseline at 2 days and rising again between 4 and 7 days. Captopril inhibited the induction of PAI-1 at the 7 day time point, but not the early phase of induction. Cilazapril, another ACE inhibitor reduced the expression of bFGF in medial SMCs after injury (Iwata *et al.* 1998). Powell *et al.* (1991) have found that the benefits of Cilazapril treatment were greater when treatment was started before angioplasty, and inhibition was enhanced by combining treatment with heparin. Miyauchi *et al.* (1998) used Cilazapril in the lathryritic rat model. In this animal, connective tissue is abnormal and the internal elastic lamina is fractured following balloon injury. The ACE inhibitor was administered for 1 week before injury, and for 3 weeks afterwards. The authors found that Cilazapril inhibited intimal hyperplasia in animals receiving a mild injury, but not when the injury was sufficiently severe to rupture the internal elastic lamina. ATII receptor type 1 blockade with a selective non peptide antagonist, TCV-116, inhibited the induction of immediate/early genes, such as c-fos, c-jun. Egr-1, ornithine decarboxylase and fibronectin (Kim & Iwao 1997). There was a corresponding reduction in intimal hyperplasia, and the content of extracellular matrix in the neo-intima.

Reactive oxygen and nitrogen species

The benefits of antioxidant treatment in clinical trials, and experimental animal models of restenosis, support the notion that free radicals play an important role in restenosis (Ferns *et al.* 1992a, b; Konneh *et al.* 1995; Tardif *et al.* 1997). The local release of free radicals can be detected directly using 'spin-traps' combined with mass spectrometry (McCormick *et al.* 1998). These free radical species lead to the formation of lipid peroxides (Blann *et al.* 1993) and isoprostanes, products of arachidonic acid oxidation (Reilly *et al.* 1997; Witztum & Berliner 1998). Golino *et al.* (1996) have also demonstrated that free radicals may enhance tissue factor expression by endothelial cells, potentially causing activation of the extrinsic coagulation pathway.

Endothelial cells in areas adjacent to the site of injury are likely to be dysfunctional. Short-term exposure of endothelial cells to low oxygen tension results in the elaboration of predominantly vasoconstricting effectors, while longer-term and more severe hypoxic exposure generates factors that can induce smooth muscle proliferation and remodeling (Faller 1999).

Peroxynitrite (derived by reaction of superoxide with nitric oxide) and transition metal ions (perhaps released by injury to the vessel wall) may contribute to lipid peroxidation in atherosclerotic lesions (Darley-Usmar & Halliwell 1996; Van *et al.* 1997).

Nitric oxide (NO) may be involved in the response to balloon injury in several ways. NO, which is normally derived from a healthy endothelium, inhibits platelet and leucocyte adhesion and platelet aggregation (Gonzalez-Fernandez *et al.* 1998) and may thereby limit thrombosis and inflammation. NO also stimulates the differentiation of SMCs and inhibits their proliferation and migration (Sarkar & Webb 1998). Groves *et al.* (1993) have reported that the NO donor SIN-1 (3-morpholino-sydnonimine) inhibits platelet deposition on the balloon-injured artery, and work from the same group subsequently reported that the orally active nitric oxide donor, molsidomine, inhibited intimal and medial SMC proliferation in arteries where the injury was moderate but not when rupture of the internal elastic lamina was present (Groves *et al.* 1995). More recently, Provost *et al.* (1997) have reported that SIN-1 inhibited platelet and neutrophil

adhesion, and attenuated mural thrombosis and post angioplasty vasoconstriction. There is functional evidence suggesting that endothelial denudation stimulates inducible nitric oxide synthase (iNOS) activity in the vascular wall (Gonzalez-Fernandez *et al.* 1998). In vitro studies have shown that iNOS expression in smooth muscle cells is reduced by endothelial cells. It was shown that platelets prevent iNOS protein expression early after endothelial balloon damage, an effect that can be avoided with GP IIb/IIIa blocking agents (Gonzalez-Fernandez *et al.* 1998). Adenovirus-mediated transfer of the gene encoding NO synthase (NOS) in balloon-injured arteries may restore NO production and inhibit neointima formation. Human endothelial constitutive NOS cDNA (AdCMVceNOS) gene transfer to balloon-injured rat carotid arteries restored vascular NO production and reduced neointima formation, at least in part because of an antiproliferative effect on medial SMCs (Janssens *et al.* 1998).

L-arginine, a precursor of NO, inhibits intimal hyperplasia after experimental balloon angioplasty in the hypercholesterolemic rabbit whereas the NOS inhibitor L-NAME enhances lesion formation (Le Tourneau *et al.* 1999). However, McNamara *et al.* (1993) and Greenlees *et al.* (1997) found that although L-arginine reduced intimal thickening in the cholesterol-fed rabbit it appeared to adversely affect endothelial function at 4 weeks after injury. This may be related to the effects of lipids on nitric oxide synthase activity. Local, intramural delivery of L-arginine enhanced NO generation and reduced intimal hyperplasia (Schwarzacher *et al.* 1997).

Endothelin

Endothelin-1 (ET-1) is a SMC mitogen and its circulating levels are elevated after PTCA. ET-1 may be derived from dysfunctional endothelial cells and possibly from activated macrophages. Exogenous ET-1 administration promotes neointimal formation, suggesting that ET-1 is involved in the pathogenesis of balloon-induced lesion formation in the rat (Douglas *et al.* 1994).

Endothelin-converting enzyme-1 (ECE-1) is responsible for the conversion of pro-ET-1 to biologically active ET-1 and is expressed in neointimal smooth muscle cells in rat balloon-injured arteries, and in both smooth muscle cells and macrophages in human coronary atherosclerotic lesions. Blockade of ECE-1 was effective in reducing neointima formation after balloon injury. Thus, ECE-1 activity may contribute to the process of injury-induced neointimal formation and atherosclerosis through the autocrine/paracrine effects of endothelin-1 (Minamino *et al.* 1997).

Using quantitative radioligand binding (Viswanathan *et al.* 1997) investigated the expression of endothelin receptor subtypes ET(A) and ET(B) in the rat carotid artery after balloon-catheter injury. These studies and the use of selective ET receptor antagonists suggest that the ET(B) receptor may have a significant role in injury-induced vascular smooth muscle proliferation and neointima formation. Similarly, Azuma *et al.* (1995) have examined the effects of balloon injury on the expression of subtypes of the endothelin-1 receptor, in the rabbit and showed that the relative expression was greater for ET(B) than ET(A) receptor, with ET(B) being mainly expressed within the neo-intima, and ET(A) in the tunica media.

Lipoprotein interactions

Arterial injury is associated with a marked increase in permeability for plasma constituents. Lipoprotein influx increases dramatically and this results in a 10-fold increase in cholesterol ester accumulation within 5 days (Schwenke & Zilversmit 1987). This is probably also related to changes in the synthesis of lipoprotein-binding proteoglycan by smooth muscle cells (Alavi *et al.* 1989), alterations in smooth muscle cell phenotype (Parlavecchia *et al.* 1989), and vessel wall cholesterol esterase activity (Hajjar *et al.* 1981).

In the rabbit, high levels of β–VLDL are associated with a significantly larger neo-intima following injury (Walker & Bowyer 1984; Ferns *et al.* 1993a). The entry of β–VLDL into the artery wall has the potential to enhance leucocyte recruitment, due to monocyte chemotactic peptide-1 release (Berliner *et al.* 1997), and to prolong the duration of endothelial dysfunction (Stemerman 1981). Lp(a) is another lipoprotein that may influence the development of the neo-intima by its inhibition of tPA activation of plasminogen (Miles *et al.* 1989). Although it is not present in the plasma of most mammals apart from primates, several apo(a) transgenic animal models have been developed that have allowed an investigation of the role of Lp(a) in restenosis (Luoma 1997). The accumulation of Lp(a) is significantly greater in injured vessels than in normal vessels. Moreover, the accumulation of Lp(a) was relatively greater than for LDL (Nielsen *et al.* 1996). The degradation rate of Lp(a) is markedly increased in atherosclerotic lesions of rabbits, supporting the notion that Lp(a) may contribute to foam cell formation (Nielsen *et al.* 1998).

Extra cellular matrix molecules and their reciprocal effects on cell function

The extracellular matrix is now recognized as a

biologically active constituent of the artery wall. It consists of structural, adhesive, and counteradhesive fibrous proteins embedded in a hydrated ground substance of glycosaminoglycans. Resident arterial cells are able to detect small differences in the specific composition and distribution of matrix components. Following injury there is a perturbation of matrix formation and degradation, that can lead to remodeling. Modifying cell–matrix interactions may provide a novel therapeutic approach in the prevention of unfavourable remodeling that leads to restenosis (Coats & Faxon 1997). After balloon injury, SMC migration is dependent on chemotactic factors and the effects of matrix metalloproteinases that free SMCs from their ECM 'cage'. *In vitro* bFGF and PDGF-BB but not PDGF-AA increased the migration of SMC from primate aortic explants. This process was found to be dependent on endogenous MMP2 and MMP9 activity (Kenagy *et al.* 1997).

Following balloon angioplasty there is a significant increase in collagen, elastin, and proteoglycan synthesis, up to 4–10 times above control. This increase in synthesis is accompanied by a significant increase in collagen and elastin content (by approximately 35%) that coincides with the temporal increase in cross-sectional area (Strauss *et al.* 1994). Analysis of cultured SMCs from the ballooned rat carotid artery showed that these cells express higher levels of type VIII collagen in response to serum and growth factors, notably platelet-derived growth factor (PDGF)-BB. VSMCs adhere significantly less to type VIII collagen than to type I collagen substrata and showed greater PDGF-BB-stimulated migration (by 2.2-fold) on type VIII collagen than on type I collagen (Sibinga *et al.* 1997). Degradation of newly synthesized collagen is an important mechanism regulating collagen accumulation and the matrix metalloproteinases MMPs have an integral role in collagen turnover after balloon angioplasty (Strauss *et al.* 1996).

The accumulation of proteoglycans (PGs), such as decorin, versican and hyaluronan, is regulated by growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β. PG deposition is localized to areas of intense growth factor immunostaining. Notably, decorin and TGF-β_1 are distributed similarly, predominantly in the macrophage-rich core, whereas biglycan is prominent in the smooth muscle cell matrix adjoining TGF-β1-positive macrophages. Versican and hyaluronan are enriched in the extracellular matrix adjacent to both PDGF-and TGF-β1-positive cells. PG accumulation varies with lesion severity, structural characteristics, and in relation to PDGF and TGF-β expression (Evanko *et al.* 1998). PDGF was associated with a reduction in perlecan expression whilst only the

PDGF-AA isoform was associated with increased syndecan-1 mRNA expression. In vitro the migratory response and PG synthesis induced by PDGF-AA was accompanied by changes in the morphology of SMCs. PDGF-AA dramatically induced the spreading of SMCs, whereas heparin lyase treatment of PDGF-AA-stimulated cultures diminished cell spreading. PDGF-AA selectively modifies heparan sulphate PG accumulation on SMCs and thereby potentially influences the interactions of SMCs with heparin-binding ECM proteins. These interactions, in turn, generate signals that may suppress SMC migration (Koyama *et al.* 1998). Purified intimal-medial proteoglycan (PG) extracts from rat aortae were analysed for protein and glycosaminoglycan (GAG) content and GAG distribution pattern. The neointima of injured aortas synthesized sulphated PG at a significantly higher concentration than the intima of normal aortae. Size exclusion chromatography revealed that neointima synthesized higher molecular weight PG, and in a higher proportion, than PG from normal aortae. PG accumulating in neointima of injured aortas showed a significantly altered GAG distribution pattern (Ismail *et al.* 1997).

Matrix metalloproteinases

The role of basement membrane-degrading matrix metalloproteinases (MMPs) in enabling vascular smooth muscle cell migration after vascular injury has been demonstrated in several animal models. Vascular SMCs constitutively elaborate the zymogen form of gelatinase A (MMP-2). MMP-2 forms complexes with its inhibitor, the tissue inhibitor of metalloproteinases (TIMP)-2 at sites of vascular injury. Thrombin may activate MMP-2 *in situ* and thereby facilitate cell migration and proliferation. In the case of complicated atherosclerotic plaques, episodes of intraplaque haemorrhage or plaque disruption may promote plaque instability by increasing local matrix-degrading activity (Galis *et al.* 1997). Upregulation of metalloproteinase MMP-2 and MMP-9 accompanies neointima formation in rat, pig and cholesterol-fed rabbits models of angioplasty (Bendeck *et al.* 1996; Jenkins *et al.* 1998). Similar findings have been reported in organ cultures of human saphenous veins (George *et al.* 1997). *In situ* hybridization detected increased expression of TIMP-4 as early as 24 h after injury and a marked induction in neointimal cells 7 days after injury. The temporal relationship between the upregulation of TIMP-4, its accumulation, and the onset of collagen deposition suggest an important role for TIMP-4 in the proteolytic balance of the vasculature controlling both smooth muscle migration and collagen accumulation in the injured arterial wall (Dollery *et al.*

1999). Secretion of TIMP-1 and TIMP-2 is greatly increased during neointima formation in human saphenous veins *in vitro*. TIMP-1 is readily released, whereas TIMP-2 remains partially attached and TIMP-3 exclusively attached to the extracellular matrix. Regulation of TIMP expression is therefore an important determinant of net MMP activity during neointima formation, restricting this to the pericellular environment (Kranzhofer *et al.* 1999). Expression of TIMP-3 mRNAs is increased by PDGF and TGF-β, particularly when used in combination. Divergent regulation of gelatinase and TIMP expression implies that either net synthesis or net degradation of basement membrane can be mediated by appropriate combinations of growth factors and cytokines (Fabunmi *et al.* 1996). TIMP-2 overexpression reduces neointimal thickening, primarily by inhibiting MMP activity and hence smooth muscle cell migration (George *et al.* 1998). TIMP-1 is upregulated in the rabbit aorta in response to a balloon-catheter induced de-endothelialization at the mRNA and protein levels (Wang *et al.* 1996a).

Bone associated genes

Osteopontin is an arginine-glycine-asparatate (RGD) containing adhesive glycoprotein. It is constitutively expressed in rat arteries and is upregulated after balloon injury (Giachelli *et al.* 1995) with a peak at approximately 1–3 days (Wang *et al.* 1996b). Osteopontin is chemotactic for VSMCs and may therefore be involved in vascular remodeling. Osteopontin's expression is regulated by PDGF-AB and −BB but not PDGF-AA (Wang *et al.* 1996b). Shanahan *et al.* (1997) have identified another bone-associated gene, osteoglycin, that is upregulated following balloon injury, using differential cDNA library screening. In vitro osteoglycin is downregulated by a number of growth factors, including bFGF, PDGF, ATII and TGFβ. In uninjured arteries, cells in the media and adventitia expressed osteoglycin, whereas after balloon injury, neointimal cells also expressed osteoglycin.

Animal models

Many of the earlier studies of the mechanisms of restenosis were undertaken in the rat and rabbit carotid artery (Clowes *et al.* 1983; Reidy *et al.* 1983; Richardson *et al.* 1984). Because the calibre of these arteries is smaller than a human coronary, it is not possible to use an angioplasty catheter, and many of these studies used a Fogarty embolectomy catheter. In order to produce complete de-endothelialization, a partially inflated catheter is repeatedly drawn through the artery bed. Hence the forces to which the artery wall is subjected are different in magnitude and nature to those experienced in the clinical setting. A further problem is that coronary angioplasty is used to dilate arteries with pre-existing disease, whereas most of the published data using experimental animal models have been performed in normal arteries. The compliance of human arteries containing advanced atherosclerotic lesions, often with regions of calcification, is poor, and PTCA is consequently accompanied by considerable tissue damage, including intimal tears and splitting of the tunica media (Waller 1989; Brady & Warren 1991). The degree of injury produced by angioplasty has been shown to affect the biological response that ensues, and hence data from animal models must be interpreted with caution. Despite the use of different experimental models, different forms of vascular injury, different methods of analysis and different definitions of arterial remodelling, most animal studies have demonstrated the importance of remodeling in the maintenance of vascular patency in both atherogenesis and in restenosis following angioplasty particularly in the nonhuman primate. Balloon catheterization as usually performed clinically can cause a much deeper injury than simple endothelial denudation. However these models have been useful for an understanding human restenosis, and for testing therapies aimed at preventing restenosis after balloon angioplasty or other coronary interventional procedures.

The rat model

Although the rat is not the ideal animal model for human disease, the ballooned rat carotid model remains the best characterized to date. The rat common carotid artery is easily accessible via the external carotid artery and the common carotid is a relatively long unbranched arterial segment, that permits the harvesting of a reasonable amount of tissue for histological and biochemical analysis. However the rat carotid has a relatively simple structure. The intima consists of a monolayer of endothelial cells apposed to the basement membrane, or internal elastic lamina. For this reason it is not an ideal model of the human coronary artery, that has a well developed intima. The model has proved useful in the study of the kinetics of smooth muscle cell response to injury. Another major difference between the rat carotid model and clinical angioplasty is the extent of injury. The gentle passage of the embolectomy catheter in the rat model does not usually create extensive tears or lacerations in the tunica media whereas this is a consistent finding in man. Indolfi *et al.* (1995) have found that SMC proliferation after balloon injury is proportional to the extent of injury.

Several antisense oligonucleotide strategies have been used to inhibit intimal hyperplasia, targeting different processes. Both Abe *et al.* (1994) and Morishita *et al.* (1994) used this approach to inhibit the cell cycle related molecules cdc2 and cdk2. They reported a 47% reduction in intimal cell accumulation when inhibited singly, with a near complete inhibition when cdc2 and cdk2 antisense oligonucleotides were used in combination. Bennett *et al.* (1997) found that antisense oligonucleotides to c-myc, administered adventitially, suppressed medial SMC replication 2 days after injury, and intimal thickening at 14 days. Luminal area was increased at this time point. Most of the effect of the oligonucleotide was due to suppression of cell migration. Antisense c-myb oligonucleotides were found to have a similar effect (Villa *et al.* 1995). Various adenovirus vector constructs have also been used to elucidate the mechanisms of restenosis. Chang *et al.* (1995b) transfected injured arteries with a adenoviral vector encoding nonphosphorylatable retinoblastoma gene product. This reduced SMC proliferation. Overexpression of the cyclin/cyclin-dependent kinase inhibitor, p21, similarly inhibited SMC proliferation and neointima formation (Chang *et al.* 1995a). p21 acts by inhibiting cyclin dependent kinases required for the initiation of the S phase of the cell cycle and inhibition is associated with inhibition of the phosphorylation of Rb. Gax is a homeobox gene that is expressed by VSMCs and is down regulated by mitogen stimulation. It is also down regulated following balloon injury (Weir *et al.* 1995). When microinjected into VSMCs gax protein inhibits cell cycle entry into S-phase. This inhibitory effect is associated with an upregulation of p21 and reduced cdk2 activity. Local delivery of gax using an adenoviral vector, inhibited neointima formation and luminal narrowing (Smith *et al.* 1997). The proto-oncogene c-Harvey-ras is thought to be a key transducer in growth factor signalling. Ueno *et al.* (1997) inhibited c-Harvey-ras *in vivo*, transfecting a dominant negative gene construct into the injured rat carotid artery, and inhibited intimal hyperplasia in this way. The local delivery of an adenoviral vector expressing hirudin, an inhibitor of thrombin, was found to reduce neointima formation, supporting the notion that local thrombin activity has a role in its pathogenesis (Rade *et al.* 1996).

Using differential display Autieri *et al.* (1995, 1996) have reported a number of novel genes upregulated following balloon injury in the rat. These include protein kinase C regulatory protein 14−3-3 gamma, BART-1 and allograft inflammatory factor-1. The expression of AIF-1 was increased at 1 and 3 days, declining by 7 days. *In vitro* AIF-1 is expressed by human SMCs stimulated by serum and cytokines. 14−3-3 may play a role in cell proliferation by activating protein kinase RAF-1.

Healthy endothelial cells elaborate nitric oxide due to a constitutively expressed nitric oxide synthase (ecNOS). Douglas *et al.* (1994) have examined time-dependent changes in vascular reactivity after balloon injury in the rat carotid. Angioplasty was associated with an increased sensitivity to ET-1 induced contractility, which they suggest is due to the loss of ET-1 induced release of nitric oxide. Chen *et al.* (1998) expressed ecNOS aberrantly in VSMCs that were subsequently seeded onto injured arteries. These cells produced NO locally, inhibited neointima formation, and caused vessel dilatation. Nitric oxide donors are known to inhibit SMC proliferation *in vitro*. Seki *et al.* (1995) found that a novel NO donor (FK409) suppressed intimal hyperplasia by 48%, whereas isosorbide mononitrate (ISMN) was ineffective. FK409 inhibited intimal and medial SMC proliferation, and had a significant effect on intimal hyperplasia even when administered on the day of injury and for 4 days subsequently.

The development of an intimal proliferative lesion after balloon catheter de-endothelialization has been studied in congenitally athymic nude rats lacking T lymphocytes. There did not appear to be any significant effect on SMC kinetics in the nude animals (Ferns *et al.* 1991c). Hancock *et al.* (1994) investigated the cell types and cytokine expression associated with intimal hyperplasia after balloon injury in the Sprague-Dawley rat. Large numbers of CD4+/ED1 + cells but no T cells were identified by immunocytochemistry. IL-1β, TNFα and IL-8 were widely expressed, together with PDGF and TGFβ. The authors administered an anti CD4 monoclonal antibody to the rats, and this was associated with a reduction in intimal hyperplasia, and reduced monocyte accumulation.

The importance of PDGF in the rat model of restenosis has become evident over recent years. Following balloon injury there are well characterized temporal changes in the expression and phosphorylation state of PDGF receptor subunits ($\alpha-$ and $\beta-$subunits) (Majesky *et al.* 1990; Panek *et al.* 1997). Abe *et al.* (1996) have found that injury is associated with tyrosyl phosphorylation of PDGF receptor subunits ($\alpha$and β) but not EGF receptor, nor insulin receptor substrate-1 (IRS-1). An angiotensin II receptor antagonist was found to inhibit tyrosyl phosphorylation of PDGF receptor subunits and also intimal hyperplasia.

Platelet-derived growth factor accounts for approximately 50% of the mitogenic activity of a rat platelet releasate. PDGF-BB and PDGF-AB were both potent chemotactic agents for the nude rat carotid smooth muscle cells with a peak response at approximately 10 ng/ml. In contrast, PDGF-AA, transforming growth factor beta, and basic fibroblast growth factor were only weak chemoattractants for these cells (Ferns *et al.*

1991c). In the balloon injury rat model PDGF appears to be a major chemoattractant (Ferns *et al.* 1991b; Jawien *et al.* 1992). Lindner *et al.* (1995) found that a subpopulation of SMCs, comprising approximately 10% of the total express PDGF-B chain. More recently, Rutherford *et al.* (1997) have reported that the PDGF-BB isoform and bFGF act synergistically and account for the majority of the chemotactic effect in this model. In contrast, Bjornsson *et al.* (1991) found that administration of acidic FGF intravenously inhibited intimal thickening and promoted endothelial regrowth. The receptors for FGF are upregulated following balloon injury, and this fact has been used to target toxins (such as saporin, using a bFGF conjugate) to proliferating intimal cells (Casscells *et al.* 1992; Farb *et al.* 1997). Monocytes adhere preferentially to the migrating cells at the edge of the regenerating endothelium and Wempe *et al.* (1997) have reported that this is probably due to an induction of monocyte chemotactic peptide-1 (MCP-1) stimulated by bFGF.

The stimuli for intimal hyperplasia are probably derived from the blood and the injured artery wall. West & Hubbell (1996) attempted to clarify the relative importance of factors from these two sources by using a hydrogel barrier that blocked thrombus formation after balloon injury. The authors measured the intimal response to injury, endogenous bFGF mobilization within the artery wall, thrombosis and medial cell re-population. They reported that factors other than PDGF-BB and thrombin contributed to intimal hyperplasia, and that a luminally derived factor was necessary for bFGF mobilization. Platelet adhesion and aggregation occur rapidly after arterial injury, causing the release of several platelet-associated factors. Zahger *et al.* (1995) used a platelet GP 1b receptor antagonist to inhibit platelet adhesion at the site of injury, a recombinant form of the GP 1b binding domain of von Willebrand factor (vWF) which inhibits platelet binding to vWF. It was found to reduce platelet adhesion by more than 80% and reduced intimal thickening and luminal stenosis, without significantly affecting SMC proliferation.

TGFβ mRNA was found to be increased 5-fold by 24 h after injury, with significant increases being apparent as early as 6 h, and remaining elevated for up to 2 weeks (Majesky *et al.* 1991). Increased levels of TGFβ immunoreactivity were associated with increased expression of fibronectin, and collagen, and infusion of TGFβ into injured arteries stimulated SMC DNA synthesis (Majesky *et al.* 1991). More recently, Ward *et al.* (1997) investigated the expression of several other TGFβ−related genes following balloon injury. TGFβ−1 and TGFβ−3 were both induced within 48 h. There were also elevations in TGFβ-type-1 (ALK-2 and ALK−5) and -2 receptors. The

expression of two integrins (α_v and β_3), normally responsive to TGFβ were also upregulated. These changes could be inhibited substantially by the tyrosine kinase inhibitor, genistein. A TGFβ antagonist, Tranilast, also attenuated these effects *in vivo* (Ward *et al.* 1998).

The role of IGF-I in intimal hyperplasia is unclear (Ferns *et al.* 1991a; Motani *et al.* 1995). Yumi *et al.* (1997) used a specific inhibitor of growth hormone and the IGF-1 axis, octreotide to probe this issue. Octreotide inhibited IGF-I mRNA expression following injury, but had no effect on IGF-BP4, which was upregulated following balloon injury. Treatment with octreotide inhibited neo-intimal thickening by up to 90%.

Endothelin-related proteins are rapidly modulated following balloon injury (Wang *et al.* 1996c; Viswanathan *et al.* 1997). Endothelin converting enzyme (ECE-1) expression preceded that of prepro-ET-1 and -3. Expression of the ET(A) and ET(B) receptors were markedly upregulated within 3 days of injury. Douglas & Ohlstein (1993) reported that ET-1 stimulates rat SMC proliferation *in vitro*, and enhanced intimal hyperplasia when administered *in vivo*. They also reported that an ET receptor blocker inhibited intimal thickening in the rat. Douglas *et al.* (1994) compared the effects of an ET(A) receptor antagonist *vs.* a dual ET(A)/(B) antagonist. They found that the dual antagonist was effective in inhibiting intimal hyperplasia, but the specific ET(A) antagonist was ineffective. These data suggest that the ET(B) receptor subtype is involved in the development of intimal hyperplasia. The possible role of endothelin in intimal hyperplasia has been strengthened by the demonstration that SB 217242, and TAK-044, both mixed ET(A)/(B) receptor antagonist (Tsujino *et al.* 1995; Chandra *et al.* 1998) reduced the intima/media ratio by more than 40%. Endothelin converting enzyme (ECE-1) is a key neutral metalloprotease involved in the processing of endothelin-1, and is normally present in endothelial cells. Minamino *et al.* (1997) examined the expression and localization of ECE-1 following carotid injury. ECE-1 mRNA increased between 2 and 5 days after injury. In the absence of endothelium, ECE-1 was detected in the neointimal smooth muscle cells up to 14 days after injury. The authors concluded that ECE-1 may contribute to neointimal thickening through an autocrine, or paracrine effect on ET-1.

The importance of vascular remodeling has received less attention in the rat model. However, Jenkins *et al.* (1998) have recently investigated the expression of matrix metalloproteinase following balloon injury. Previous studies demonstrated that MMP-2 and MMP-9 were upregulated in this model, and that MMP-2 is involved in SMC migration. Jenkins *et al.* (1998) examined the effects on

membrane-type MMP-1 that may be involved in the regulation of MMP-2. It may do so by affecting the tissue localization of MMP-2, its activation state, and the expression of an inhibitor, tissue inhibitor of metalloproteinase (TIMP-2). They reported that MT-MMP-1 steady-state mRNA levels were increased 6-fold within 3 days of injury, and that there was a temporal relationship with increased MMP-2 activity, measured by zymography. Collagen type $\alpha 1$ VIII and $\alpha 2$ VIII mRNA are both upregulated after balloon injury (Sibinga *et al.* 1997). The pattern of expression indicates that collagen type VIII is expressed by activated, dedifferentiated SMCs of the intima and media. In vitro expression could be stimulated by PDGF-BB, and SMCs were less adherent to collagen type VIII than to type I collagen. SMCs were also more responsive to chemotactic agents on a matrix of collagen type VIII vs. collagen type I. Proteoglycan accumulation varies with lesion severity and with local growth factor elaboration (Evanko *et al.* 1998).

Heat shock proteins (HSPs) are a family of highly conserved proteins, expressed during periods of physiological stress, that are protective during re-exposure to stress. Neschis *et al.* (1998) induced HSPs in Sprague-Dawley rats by whole body hyperthermia and subsequently these animals were subject to balloon injury. Hyperthermia treatment was associated with a significant reduction in intimal hyperplasia and medial cell proliferation.

The response to balloon injury is sexually dimorphic due to the protective effects of oestrogens. Oparil *et al.* (1997) found that exogenous oestrogen was ineffective in inhibiting intimal hyperplasia in male rats, although serum 17β–oestradiol concentrations reached physiological female levels. Progestin (medroxyprogesterone acetate) blocks the effects of oestrogen, and enhanced the response to balloon injury in female rats, but the response in male rats was unaffected. The oestrogen, oestradiol, enhanced the rate of functional recovery of the endothelium after balloon injury (Krasinski *et al.* 1997).

The rabbit model

The rabbit model of balloon injury was one of the first to be used (Groves *et al.* 1979). Rabbits are very responsive to atherogenic diets, developing hyperlipidaemia and early lesions soon after initiation. Indeed, Tashiro *et al.* (1998) found that small amounts of saturated fatty acid (such as lard) promoted the formation of intimal thickening without affecting serum lipid levels. There are also inbred rabbit strains with genetic abnormalities of lipid metabolism, equivalent to human conditions such as Familial Hypercholesterolaemia (the Watanabe Heritable hyperlipidaemic rabbit; Takagi *et al.* 1997) and Familial combined hyperlipidaemia (Seddon *et al.* 1987). For this reason it is possible to investigate the combined effects of mechanical injury and dyslipidaemia (Faxon *et al.* 1982; Alavi *et al.* 1983). Moreover it has recently been possible to establish transgenic rabbit models. For example a human apolipoprotein A1 transgenic rabbit line has been created (Duverger *et al.* 1996) which may be useful to explore the role of high density lipoprotein in the response to balloon injury.

More *et al.* (1994) and Stadius *et al.* (1994) have presented the detailed cell kinetics in the rabbit after balloon injury. Re-endothelialization was complete by day 14 and could be enhanced by administration of vascular permeability factor (Callow *et al.* 1994). Cellular proliferation reached a maximum by day 7 for the media and day 14 for the intima, before returning to baseline by one month. Urokinase type plasminogen activator (uPA) increased within 2 h of injury and remained elevated at 24 h but fell below baseline by 7 days, whilst tissue type plasminogen activator (tPA) fell after injury, rising to baseline by 7 days (More *et al.* 1995). Stadius *et al.* (1996) also examined the effect of double injury in the rabbit.

The importance of PDGF in the rabbit model of restenosis is also clear. PDGF B-chain is expressed in the balloon-injured artery, with PDGF-B chin mRNA peaking by 7 days, and remain elevated for 21 days, whereas PDGF A-chain is only modestly increased. PDGF receptor alpha and beta subunits are also upregulated (Uchida *et al.* 1996). Trapidil, a PDGF antagonist, inhibited intimal hyperplasia, and increased luminal area in the atherosclerotic rabbit (Liu *et al.* 1990).

Using immuno-cytochemical analysis, Lawrence *et al.* (1995) found that following balloon injury bFGF positive cells were found in the media and in recently migrated cells of the neo-intima for up to 14 days after injury.

The collagen content of ballooned rabbit artery was found to increase over the first four weeks after injury, however, the ratio of collagen subtypes was unchanged (Coats *et al.* 1996). Coats *et al.* (1997) subsequently reported an inverse relationship between collagen content and gelatinase activity. Strauss *et al.* (1996) investigated the turnover of collagen and its regulation by MMP in a double injury model. Peak collagen synthesis and degradation occurred at one week, as did MMP-2 activity. The expression of matrix metalloproteinase-2 is increased in the neo-intima of the rabbit (Wang *et al.* 1998) and its inhibition results in a reduction in VSMC proliferation and migration. MMP-2 requires activation for full activity. This appears to be due to membrane type MMP-1 (MT-MMP-1) (Wang & Keiser 1998).

In vitro, heparan sulphate PGs (HSPG) maintained VSMCs in a quiescent state by inhibiting phenotypic change and DNA synthesis. Aorta-derived HSPG reduced neo-intimal thickening by 35% when applied peri-adventially (Bingley *et al.* 1998). Hyaluronic acid, a major constituent of aortic GAG also reduced intimal thickening and monocyte influx in the cholesterol-fed rabbit (Ferns *et al.* 1995), as did low molecular weight heparin (Currier *et al.* 1991) which may be partly related to its effect on SMC proliferation (Oberhoff *et al.* 1998).

Endotoxin-stimulatable tissue-factor expression was increased in circulating mononuclear cells following balloon angioplasty, and this was blunted by treating animals with L-arginine (Corseaux *et al.* 1998). Brown *et al.* (1996) and Asada *et al.* (1998) have investigated the effects of inhibiting fibrin formation using a tissue factor pathway inhibitor administered locally immediately after injury. Jang *et al.* (1995) investigated the contribution of the coagulation cascade on restenosis in the hyper-cholesterolaemic rabbit using inhibitors for different levels of the coagulation pathway. Factor VIIa binding to tissue factor was blocked with active-site inactivated factor VIIa (DEGR-VIIa). Recombinant tissue factor pathway inhibitor (TFPI), binds to factor Xa and inhibits tissue factor-VIIa complex formation, and recombinant tick anticoagulant peptide (TAP) and hirudin block factor Xa and thrombin, respectively. DEGR-VIIa and TFPI inhibited neo-intimal hyperplasia indicating the importance of the extrinsic coagulation pathway, especially factor VII and tissue factor in the arterial response to injury. Thrombin inhibition, by hirudin, suppressed arterial thrombin activity (Barry *et al.* 1996), and restenosis (Barry *et al.* 1997). Hirudin probably operates partly by reducing tissue factor expression. Gertz *et al.* (1998) have reported that hirudin inhibits tissue factor expression in the injured rabbit femoral artery and porcine coronary artery. Ragosta *et al.* (1996) found that hirudin did not inhibit cell proliferation. Recombinant tissue plasminogen activator inhibits intimal hyperplasia in the hypercholesterlaemic rabbit (Kanamasa *et al.* 1996).

The efficiency of adeno-virus transfection is very low (approximately 0.2% of intimal and medial cells) in atherosclerotic rabbit arteries (Feldman *et al.* 1996). However a vector construct containing the growth arrest homeobox gene (Gax) reduced intimal thickening (Maillard & Walsh 1996; Maillard *et al.* 1997).

The pig model

The anatomical and physiological similarities between human and porcine cardiovascular systems have led to the use of the pig to model restenosis (Steele *et al.* 1985). It is possible to use the same balloon catheters and similar inflation pressure as used clinically, and the morphology of the lesion that follows balloon injury is similar to that seen in human postangioplasty restenosis (Schwartz *et al.* 1990; Karas *et al.* 1992).

Groves *et al.* (1995) have provided detailed kinetic data of VSMC proliferation in the pig and examined the possible variations with respect to degree of injury (medial dilatation *vs.* medial tearing). Although medial enlargement was similar for both degrees of injury, they found that the total thickening of the injured artery was greater when a deep tear breached the media. The effects of balloon injury on SMC phenotype previously shown in the rat have also been demonstrated in the pig (De Leon *et al.* 1997). Non-muscle myosin heavy chain-B isoform is expressed by proliferating VSMCs, and its expression following balloon injury in the pig is associated with cell proliferation in the adventitia and intima. However, nonmuscle myosin heavy chain-B isoform expression was not limited to proliferating cells.

von Willebrand factor (vWF), is involved in platelet–vessel wall interactions. Giddings *et al.* (1997) have investigated the sequential changes in tissue distribution of vWF following balloon injury, and report that it is localized to the cells of the tunica media for up to 7 days, and may be involved in medial smooth muscle cell proliferation. There are some significant differences between pig and human platelet function, for example their responsiveness to agonists such as adrenaline and arachidonic acid (reviewed by Softeland *et al.* 1992).

Following balloon injury, arteries sometimes undergo vasoconstriction. Lam *et al.* (1987) found that the arterial spasm is related to the degree of platelet deposition, and could be inhibited by aspirin. Although neutrophils are not a major cellular component of the ballooned artery wall in most animal models, neutropenia induced by treatment with cyclophosphamide was associated with reduced platelet aggregation and deposition, and reduced vasoconstrictor response (Merhi *et al.* 1994). Lam *et al.* (1992) have reported that cod liver oil at a dose of 1 ml/kg/day reduced platelet deposition after deep carotid injury by balloon injury. This was also associated with reduced vasoconstriction. However, using porcine arteries *ex vivo*, Fischell *et al.* (1989) reported that the vasoconstriction may also be endothelium-dependent, particularly in situations where residual endothelial cells remain on the partially denuded artery. Vasoconstriction appeared to be mediated by cyclo-oxygenase-derived products, and could be blocked by indomethacin. The pig model has also been used to show the importance of arterial remodeling in late luminal narrowing; (Post *et al.* 1994;

Andersen *et al*. 1996). Lamawansa *et al*. (1997) also present evidence that the sympathetic innervation may influence arterial remodeling and wall thickening.

Studies in the pig model have shown that endothelin stimulates SMC proliferation via the ET(A) receptor. Burke *et al*. (1997) have reported that an orally active ET(A) receptor antagonist, A127722.5, inhibited intimal thickening and luminal narrowing. It also reduced the collagen content of the tunica media. These data support the role of endothelin in restenosis, although the relative importance of ET(A) and ET(B) receptors may differ between the animal models.

The pig has also been used to test a number of putative intervention strategies which have provided a clearer insight into the mechanisms of restenosis. For example Banai *et al*. (1998) have reported that a specific blocker of PDGF-receptor tyrosine kinase (tyrphostin AG1295) when delivered locally to the site of balloon injury, significantly reduced intimal thickening, and luminal narrowing. Selective $\alpha_V\beta_3$ integrin blockade, using a cyclic Arginine-Glycine-Asparagine (RGD) pepidometic antagonist, inhibited neointimal hyperplasia in the pig coronary artery (Srivatsa *et al*. 1997). Antithrombin III is the major physiological inhibitor of thrombin. Combined with heparin, ATIII significantly inhibited the reduction in minimal luminal diameter at 4 weeks after angioplasty (Ali *et al*. 1996). There was a modest reduction in neo-intimal proliferation associated with treatment with the ACE inhibitor, trandolapril (Huber *et al*. 1993). Three Ang II receptor antagonists, including AT1 selective (L-158 809), balanced AT1/AT2 (L-163 082), and AT2 selective (L-164 282) agents, were evaluated for their ability to inhibit vascular intimal thickening in a porcine coronary artery model of vascular injury. It was shown that chronic blockade of Ang II receptors by either site-selective or balanced AT1/AT2 antagonists is insufficient to inhibit intimal hyperplasia after experimental coronary vascular injury in the pig. Unlike in the rat carotid artery, Ang II is not a major mediator of intimal thickening in the pig coronary artery (Huckle *et al*. 1996).

The calibre of the pig coronary artery has allowed the local delivery of antisense oligonucleotides. Using this model, Gunn *et al*. (1997) have demonstrated that an antisense oligonucleotides to c-myb, a early gene expressed after vascular injury, reduced c-myb mRNA and protein, and subsequently led to a significant reduction in the intimal response to injury. However this study was confounded by nonspecific effects of the oligonucleotides.

Shi *et al*. (1997) investigated the cellular origin of type I collagen and elastin after balloon injury. By the second day after injury, type I pro-collagen was expressed by cells of the adventitia. This was followed by enhanced neo-intimal expression of pro-collagen and elastin.

The non-human primate model

The arterial response to angioplasty in atherosclerotic monkeys appears to closely parallel that seen in humans. Plaque fracture, delayed recoil, intimal hyperplasia, and remodeling each appear to be important in determining late lumen calibre. In monkeys that were fed an atherogenic diet and subsequently underwent experimental angioplasty, the pattern of matrix and integrin expression within the injured artery wall is in many ways analogous to that of healing wounds (Geary *et al*. 1996). The provisional extracellular matrix laid down after injury is invaded by leucocytes and alpha-actin-positive smooth muscle cells. These SMCs express hyaluronan and are associated with versican proteoglycans expression. Versican is expressed throughout the neointima as it enlarges, but expression later diminishes. Procollagen-I expression initially increases in the adventitia and then subsequently within the developing neointima. Procollagen-I expression persists within the adventitia and in the neointima in SMCs nearest the lumen. Elastin staining is prominent within the mature neointima but is not evident at earlier time points. Integrin expression also increases within the injured artery wall. $\alpha_V\beta_3$ staining characteristic of the fibrin[ogen] receptor, increases in the injured media and is then apparent throughout the newly formed neointima. $\alpha_2\beta_1$ integrin expression (collagen receptor) increased in the neointima in SMCs nearest the lumen (Geary *et al*. 1998).

The glycoprotein (GP) IIb/IIIa found on platelets cross-links fibrinogen and vWF, thereby mediating platelet aggregation. Guth *et al*. (1997) studied the effects of blocking GP IIb/IIIa, using a new nonpeptidic molecule, BIBU52. The inhibition of platelet aggregation by BIBU52 was found to be species specific. BIBU52 inhibited aggregation in platelet rich plasma from rhesus and marmoset but was ineffective in rat plasma.

Recently, Giese *et al*. (1999) selectively blocked PDGFα and β receptor subunits using monoclonal antibodies and demonstrated that PDGF probably promotes neointima formation via PDGF receptor β subunit.

Lipoprotein(a) (Lp(a)) has been proposed as a risk factor for both restenosis and coronary heart disease. Recently, Ryan *et al*. (1997) identified Lp(a) in the arterial wall during the early phases of neointimal growth in cynomolgus monkeys. Ryan *et al*. (1998) also investigated the relationship between circulating Lp(a) levels and the extent of early neointima formation. A four-fold increase in circulating plasma Lp(a) levels did not result

in a statistically significant enhancement in neointima formation at 14 days after balloon injury.

The mouse model

Because of its small size, it has not been possible to develop an authentic mouse model of angioplasty. Models of arterial injury have been developed which are associated with endothelial injury and intimal thickening (Lindner *et al.* 1993; Kumar & Lindner 1997). Carmeliet *et al.* (1997b) have described a mouse model of restenosis induced by perivascular femoral artery injury using an electric current. Endothelial injury in the mouse femoral artery was also induced by photochemical reaction between localized irradiation by green light and intravenous injection of rose bengal (Miyazawa *et al.* 1998). Although none of these models is ideal for studying the complete process of restenosis, it is possible that they will provide reliable information when combined with the use of transgenic strains such as those described by Liao *et al.* (1996) and Leppanen *et al.* (1998). The use of transgenic mouse models has demonstrated that leucocyte-endothelium adhesion receptors play a significant part in promoting monocyte recruitment and consequently lesion growth (Dong & Wagner 1998). Mechanical and electric vascular injury of plasminogen activator inhibitor-1 (PAI-1) deficient mice showed that an inhibitory role by PAI-1 was played in neointima formation and that the adenoviral gene transfer reduced neointimal formation (Carmeliet *et al.* 1997a).

Conclusions

Animal models of intimal hyperplasia have provided a profound insight into the cellular and biochemical mechanisms of restenosis following angioplasty. They have been less useful in predicting the efficacy of therapeutic interventions. Nevertheless, these models have provided a test-bed for novel strategies including the new molecular approaches. It is clear that interspecies differences, and the relative simplicity of the animal models have yielded some misleading results that have not been translated into clinical benefit. Nonetheless, they have collectively provided a number of novel therapeutic targets, and as their sophistication increases their reliability as models of human diseases will improve pari passu.

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