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Invited Review

Phenotypic modulation of smooth muscle cells during formation of neointimal thickenings following vascular injury

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Summary. Smooth muscle cells build up the media of mammalian arteries and constitute one of the principal cell types in atherosclerotic and restenotic lesions. Accordingly, they show a high degree of plasticity and are able to shift from a differentiated, contractile phenotype to a less differentiated, synthetic phenotype, and then back again. This modulation occurs as a response to vascular injury and includes a prominent structural reorganization with loss of myofilaments and formation of an extensive endoplasmic reticulum and a large Golgi complex. At the same time, the expression of cytoskeletal proteins and other gene products is altered. As a result, the cells lose their contractility and become able to migrate from the media to the intima, proliferate, and secrete extracellular matrix components, thereby contributing to the formation of intimal thickenings. The mechanisms behind this change in morphology and function of the smooth muscle cells are still incompletely understood. A crucial role has been ascribed to basement membrane proteins such as laminin and collagen type IV and adhesive proteins such as fibronectin. A significant role is also played by mitogenic proteins such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). An improved knowledge of the regulation of smooth muscle differentiated properties represents an important part in the search for new methods of prevention and treatment of vascular disease.

Key words: Smooth muscle cells, Differentiated properties, Basement membrane, Fibronectin, Intimal thickening

Introduction

Smooth muscle cells (SMCs) build up the media of the arterial wall and play an important role in the pathogenesis of atherosclerosis and restenosis after angioplasty (Ross and Fuster, 1996; Schwartz and Reidy, 1996). In the early stages of these processes, SMCs migrate from the media to the intima, where they later proliferate and secrete extracellular matrix components, thereby contributing to the mass of the developing intimal lesions. This change in function of the differentiated and quiescent cells normally found in the media is accompanied by an extensive structural reorganization and is referred to as a transition from a contractile to a synthetic phenotype. Noticeably, it is a reversible process and after the formation of an intimal plaque, the SMCs are able to regain a contractile state. The morphological and functional properties of the cells will therefore differ markedly in different stages of the disease process. As a consequence, analysis of biopsy material taken at a certain point will not necessarily reflect the state of the cells at the time the lesions are formed.

During the last 10-15 years, considerable interest has been paid to the control of vascular SMC differentiation. The object has been to widen our knowledge of the embryonic development of the vasculature as well as the involvement of SMCs in vascular disease. Particular attention has been given to the role of polypeptide growth factors in the regulation of SMC migration and proliferation (Bobik and Campbell, 1993; Libby and Ross, 1996). Recently, the extracellular matrix has also been brought into focus as a possible source of signalling molecules influencing the phenotypic state of the SMCs (Assoian and Marcantonio, 1996; Ruoslahti and Engvall, 1997). Both in vitro and in vivo models have been used extensively in all parts of this work. In the present article, a short summary of the literature regarding the modification of the SMCs following vascular injury is presented. For details concerning the study of SMCs in culture, the reader is referred to recent reviews (Owens, 1995; Thyberg, 1996).

Development of the arterial wall

The phenotypic modulation of SMCs in the early stages of vascular disease is in many ways a reversal of

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the differentiation process seen during development of the vascular system. Hence, a brief account of vasculogenesis will be given as background to the coming discussion. In the embryo, the heart and the main blood vessels are first formed by mesodermal cells (angioblasts) that differentiate into endothelial cells (Fishman, 1996; Beck and D'Amore, 1997; Risau, 1997). As soon as a closed circulatory system is established, new vessels arise by budding from preexisting vessels. This latter process is called angiogenesis and is important in the formation of new blood vessels also later in life, e.g. during wound healing, tumor growth, and other diseases. Among the many mitogens that act on endothelial cells, vascular endothelial growth factor (VEGF) has been identified as a key regulator of vasculogenesis and angiogenesis (Ferrara and Davis-Smyth, 1997). Accordingly, both VEGF (Breier et al., 1992) and VEGF receptors (Millauer et al., 1993) are expressed in association with developing blood vessels and mice deficient in VEGF (Carmeliet et al., 1996a; Ferrara et al., 1996) or VEGF receptors (Fong et al., 1995; Shalaby et al., 1995) fail to form normal blood vessels and die in utero. In like manner, the endothelial receptor tyrosine kinases Tie-1 and Tie-2 have been found essential in vasculogenesis (Dumont et al., 1994; Sato et al., 1995; Maisonpierre et al., 1997).

Once the primary vascular network is established, local mesodermal cells gather around the endothelial tubes and differentiate into SMCs, supposedly under influence of factors released by the endothelial cells (Hungerford et al., 1996). The monoclonal antibody used to define these early cells of the smooth muscle lineage recognizes a 100 kD protein identified as smooth muscle α-actinin (Hungerford et al., 1997). Recently, it has also been suggested that embryonic endothelial cells may transdifferentiate into SMCs and take part in formation of the vessel media (De Ruiter et al., 1997). Among the molecular markers associated with developing vascular SMCs, many are of cytoskeletal origin (Glukhova and Koteliansky, 1995; Owens, 1995; Katoh and Periasamy, 1996). One of the first to appear is smooth muscle α-actin. However, nonmuscle ß-actin is initially the principal actin isoform and smooth muscle α-actin becomes the predominant isoform at a later stage (Kocher et al., 1985; Owens and Thompson, 1986; Glukhova et al., 1990a). Other cytoskeletal proteins that show successively increasing expression levels during maturation of the vessel wall include smooth muscle myosin heavy chains (Glukhova et al., 1990a; Miano et al., 1994), heavy caldesmon, calponin, and SM22 (Frid et al., 1992; Duband et al., 1993; Miano and Olson, 1996).

Extracellular matrix components and their receptors, the integrins, evidently play a crucial role in control of blood vessel formation (Luscinskas and Lawler, 1994; Glukhova and Koteliansky, 1995; Farhadian et al., 1996). The adhesive protein fibronectin is produced early during vasculogenesis, whereas laminin and other basement membrane components appear in maturing vessels (Risau and Lemmon, 1988). In accord with this observation, serious defects in vascular development occur in mouse embryos lacking fibronectin (George et al., 1993) or fibronectin receptors (Yang et al., 1993). The production of fibronectin and laminin variants by the SMCs is also changed in a developmentally regulated pattern. Expression of fibronectin containing extra domain A (ED-A) and B (ED-B) is high in the fetus and then decreases after birth, whereas fibronectin lacking these domains continues to be expressed at a high level (Glukhova et al., 1990b; Peters and Hynes, 1996; Peters et al., 1996). There is further a shift in synthesis of laminin from ß1-chain containing isoforms in the fetus to ß2-chain containing isoforms in the adult (Glukhova et al., 1993). At the same time, the integrin $\alpha_1\beta_1$ is the main laminin receptor in fetal aorta, whereas both $\alpha_1\beta_1$ and $\alpha_3\beta_1$ appear in the adult (Belkin et al., 1990; Duband et al., 1992; Glukhova et al., 1993).

Elastin, collagen, and proteoglycans are quantitatively the main extracellular matrix components of the vessel wall (Wight, 1996). In large arteries, an inner and outer elastic lamina separate the media from the intima and adventitia, respectively. Moreover, elastic lamellae divide the media into distinct cell layers. During development of the cardiovascular system, tropoelastin gene expression starts early (Holzenberger et al., 1993). In the rat, it peaks in the late fetal and early postnatal periods and ceases 2-3 months after birth (Belknap et al., 1996). Collagen type I and III are the main collagen types in blood vessels and are organized in fibrillar bundles around the SMCs in the media. They are both essential during vasculogenesis and mice lacking collagen type I die in utero due to rupture of blood vessels (Löhler et al., 1984). In the absence of collagen type III, collagen type I fibrillogenesis is disturbed and most of the animals die shortly after birth as a result of blood vessel defects (Liu et al., 1997). The major types of proteoglycans found in the interstitial matrix of the vessel wall include the large chondroitin sulfate proteoglycan versican, the small leucine-rich dermatan sulfate proteoglycans decorin and biglycan, and the keratan sulfate proteoglycan lumican. In the basement membranes underlying the endothelial cells in the intima and surrounding the SMCs in the media, the heparan sulfate proteoglycan perlecan is also found (Wight, 1996). During vasculogenesis, perlecan gene expression starts at the time when SMC replication begins to decline and thereafter remains high (Weiser et al., 1996). Otherwise, only little is known about proteoglycan production in the fetal vasculature.

Although the mechanisms of action still are incompletely understood, platelet-derived growth factor (PDGF - a dimer of A and/or B chains) and PDGF receptors (the α subunit binds the A as well as the B chain of PDGF and the ß subunit only the B-chain) have been found necessary for normal cardiovascular development. Mice deficient for the PDGF B-chain die perinatally and show dilation of large arteries and diffuse

hemorrhages. However, the number of SMCs in the arterial media is not clearly reduced and the primary role of PDGF B is apparently not to stimulate cell multiplication (Levéen et al., 1994). On the other hand, no major defects in large blood vessels are observed in PDGF ß-receptor mutant mice, conceivably due to compensation by the α-receptor (Soriano, 1994). In agreement with this notion, the number of SMCs in the arterial media of mice lacking PDGF α-receptors was found to be reduced (Schatteman et al., 1995). Similar effects were seen in embryos treated with antibodies against PDGF-AA (Schatteman et al., 1996). Conspicuous disturbances in formation of the vessel media were also detected following inactivation of the gene for tissue factor, the primary cellular initiator of blood coagulation (Carmeliet et al., 1996b).

In parallel with the aforementioned changes in gene expression, the morphology of the SMCs is distinctly modified. Quantitative electron microscopic analyses indicate that the cells in the media of the developing rat aorta have a prominent endoplasmic reticulum and Golgi complex during most of the fetal and early postnatal period (Gerrity and Cliff, 1975; Nakamura, 1988). Autoradiographic studies further reveal that these cells replicate (Berry et al., 1972) and secrete extracellular matrix components (Ross and Klebanoff, 1971; Gerrity et al., 1975). As a result, the vessel grows in size, with a marked increase in cell mass as well as collagen and elastin content (Looker and Berry, 1972). However, myofilaments gradually fill up larger and larger parts of the cytoplasm. At the same time, the endoplasmic reticulum and the Golgi complex become smaller. In the rat, this shift of the medial SMCs from a synthetic phenotype to a more specialized contractile phenotype is completed at an age of 2-3 months, i.e. when the aorta reaches its final size (Gerrity and Cliff, 1975).

Atherogenesis and restenosis after angioplasty

Atherogenesis is generally considered as an inflammatory-fibroproliferative response to vascular injury (Ross and Fuster, 1996). It is a complex process involving endothelial cells and SMCs of the vessel wall itself (Di Corleto and Gimbrone, 1996; Owens, 1996), extracellular matrix components (Wight, 1996), lipoproteins (Chisolm and Penn, 1996), platelets, monocytes/macrophages and lymphocytes derived from the blood (Hansson and Libby, 1996; Raines et al., 1996), and a large collection of cytokines, growth factors and other biologically active molecules (Libby and Ross, 1996). Since the lesions of atherosclerosis develop slowly over a number of years, it is difficult to follow their evolution directly in man. According to recent classifications, at least eight types of lesions can be distinguished on a morphological basis (Stary et al., 1994; Stary, 1996). Among them, three major groups will be mentioned here: *the fatty streak* (type I-II), a small local accumulation of lipid-laden macrophages (foam cells) and a few SMCs in the intima; *the fibrous plaque* (type IV-V), a larger accumulation of intra- and extracellular lipid (lipid core) surrounded by a cap of SMCs and a dense extracellular matrix; and, *the complicated plaque* (type VI), characterized by the occurrence of erosions or fissures and thrombotic deposits or hemorrhage. Clinical symptoms mainly appear in the third group and it is also in this group that surgical intervention by angioplasty, atherectomy, or bypass grafting is utilized. Even though the short-term effects of these procedures are usually beneficial, restenosis due to vascular remodelling and neointimal hyperplasia remains an important and so far unresolved, long-term complication (Landau et al., 1994; Anderson and King, 1996; Bauters et al., 1996).

Because of the limited availability of biopsy material and the consequent difficulties to follow the formation of atherosclerotic and restenotic lesions in man, numerous animal models have evolved (Armstrong and Heistad, 1990; Jackson, 1994). Recently, the mouse has found increasing usage in this field (Breslow et al., 1996), and the apolipoprotein E-deficient mouse has been established as a model in which atherosclerotic lesions resembling those observed in humans develop within a few months (Plump et al., 1992; Nakashima et al., 1994). Even if the rat resists most attempts to induce a true atherosclerotic state, it has been extensively used to explore the role of the SMCs in neointimal hyperplasia after vascular injury (Schwartz and Reidy, 1996). Hence, the present discussion will to a large extent relate to this model. In order of appearance, the following points will be treated: transition of medial SMCs from a contractile to a synthetic phenotype; migration of SMCs from the media to the intima; proliferation of SMCs in the intima; secretion of extracellular matrix components in the intima; and redifferentiation of SMCs in the intima. Finally, a brief recapitulation of the literature dealing with prevention of intimal thickening and restenosis will be given.

Modulation from a contractile to a synthetic phenotype

Structural reorganization

According to the response-to-injury hypothesis, atherosclerotic lesions emanate as a reaction to damage of the arterial wall (Ross and Fuster, 1996). The principal causes of the damage are believed to be either chemical (increased low-density lipoprotein levels, smoking) or mechanical (hypertension, shearing stress). In the balloon injury model introduced by Clowes et al. (1983), a balloon catheter is inserted into an artery, inflated with air or liquid, and passed through the vessel a few times. As a result of this procedure, the endothelium is denuded and one of the main barriers regulating passage of macromolecules from the blood into the vessel wall is lost (Clowes et al., 1978; Penn et al., 1994, 1997). In addition, the inflated catheter exerts a direct pressure on the underlying media. It seems likely

that the loss of the permeability barrier and the strain on the media are both important to bring about the neointimal lesions, although the former factor appears to be the most significant. Thus, lesions also form in the absence of medial injury (Fingerle et al., 1990). Moreover, the application of a hydrogel barrier in order to prevent contact between the arterial wall and the blood was found to strongly reduce intimal thickening after balloon injury (West and Hubbell, 1996). In any case, several days normally pass after the acute injury before SMCs appear in the intima and start to produce a lesion protruding into the vessel lumen. The signalling machinery behind this process is therefore complex to resolve.

The structural reorganization of the SMCs following balloon injury of animal arteries has been studied by a combination of electron microscopic, cytochemical, and chemical methods. Within one week after the operation, many of the SMCs in the innermost part of the media show a decrease in the number of actin filaments and a concurrent increase in size of the endoplasmic reticulum and the Golgi complex. Some of these cells move through fine openings in the inner elastic lamina and spread over the exposed luminal surface of the vessel. Subsequently, they start to replicate and in another week a multilayered neointima consisting of synthetic SMCs surrounded by a newly deposited extracellular matrix is established (Grünwald et al., 1987; Manderson et al., 1989; Thyberg et al., 1995). At the same time, the cytoskeletal profile of the cells is modified with shifts from smooth muscle α-actin to nonmuscle ß-actin (Gabbiani et al., 1984), from smooth muscle myosin to nonmuscle myosin (Benzonana et al., 1988), and from desmin to vimentin (Gabbiani et al., 1982). Part of the SMCs in atherosclerotic and restenotic lesions likewise show an altered morphology with a predominance of secretory organelles rather than actin filaments (Ross et al., 1984; Mosse et al., 1985; Nakamura and Ohtsubo, 1992; Stary et al., 1994; Chen et al., 1997a). It is also well recognized that the cytoskeletal composition is changed during atherogenesis with shifts from smooth muscle to nonmuscle isoforms of actin, myosin, and other associated proteins (Kocher and Gabbiani, 1986; Glukhova et al., 1988; Aikawa et al., 1993; Sartore et al., 1997).

In a recent study on rat carotid arteries, about two thirds of the SMCs in the inner layer of the media were found to have adopted a synthetic phenotype one week after balloon injury, whereas the cells in the outer layers of the media for the main part retained a contractile phenotype as judged by electron microscopic analysis and staining for smooth muscle α-actin. It was further evident that all the SMCs appearing in the intima were initially in a synthetic phenotype (Thyberg et al., 1997a). Together with the results referred to above, these observations suggest that the SMCs of the intimal lesions originate from medial SMCs and that the latter have to convert into a synthetic phenotype before they are able to populate the intima (Fig. 1). In a similar manner, the SMCs of atherosclerotic and restenotic lesions probably derive from phenotypically modified SMCs in the media. The fact that both contractile and synthetic SMCs are usually found in such lesions may be explained by the ability of the cells to redifferentiate after an earlier change in phenotype (see below). Lately, it has also been demonstrated that adventitial myofibroblasts may contribute to neointimal formation in injured arteries (Shi et al., 1996a,b; Wilcox and Scott, 1996). However, in this case a deeper damage with medial disruption exposing the adventitia to the lumen is required. Finally, it has been proposed that intimal SMCs may arise from a small population of undifferentiated stem cells, but good evidence for the existence of such cells in the vessel wall is still lacking.

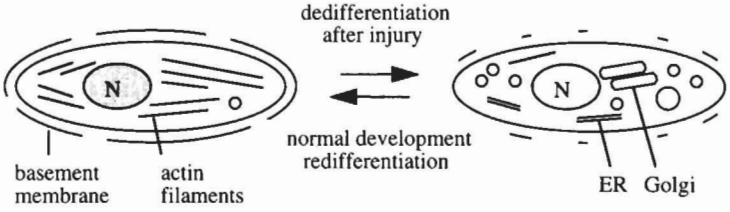
The mechanism behind the change in smooth muscle phenotype following vascular injury is still poorly understood. Previous in vitro studies have revealed that the transition from a contractile to a synthetic phenotype is not dependent on serum mitogens. Thus, freshly isolated SMCs go through this process when seeded on a substrate of plasma fibronectin in a defined medium, conceivably by signalling via integrin receptors (Thyberg, 1996). In agreement with this notion, it was reported that the early SMC activation after balloon catheterization is not prohibited in animals made deficient in platelets, a major source of serum growth factors (Fingerle et al., 1989). Moreover, it was recently observed that vascular injury is associated with internalization of caveolae from the surface of the SMCs (Thyberg et al., 1997b). These fine invaginations of the plasma membrane are enriched in signalling molecules (Couet et al., 1997) and it seems likely that they are implicated in the control of cell differentiation. However, their exact role in smooth muscle phenotypic modulation remains to be elucidated. The loss of the endothelial barrier will also lead to an increased transfer of lipoproteins into the arterial wall, but it is not known how this influences the phenotypic state of the SMCs (Olsson et al., 1995; Nielsen, 1996). Despite the absence of the endothelium, no immigration of monocytes and lymphocytes into the arterial wall is noted, and the latter cell types are apparently not required in the activation of the SMCs and the formation of a neointima. Likewise, lymphocytes were found to play a minor role in the formation of atherosclerotic plaques in the apolipoprotein E-deficient mouse (Dansky et al., 1997).

The search for genes involved in the control of smooth muscle differentiation and phenotypic modulation is still in its beginning (Liau and Han, 1995; McQuinn and Schwartz, 1995; Owens, 1995, 1996). Using differential screening of a fetal aortic cDNA library, H19 was identified as a developmentally regulated gene expressed in SMCs of fetal but not adult vessels (Han and Liau, 1992). Although its function remains elusive, the H19 gene was shown to be reexpressed in the neointima of injured rat arteries (Kim et al., 1994) and in human atherosclerotic plaques (Han et al., 1996). Using a similar approach, tropoelastin and

procollagen type I were recognized as genes with a markedly stronger expression in newborn than in adult rat aorta and a noticeable reactivation in the neointima of injured arteries (Majesky et al., 1992). Comparing freshly isolated and late-passage rat aortic cells, a number of gene markers distinguishing differentiated and proliferating SMCs were described (Shanahan et al., 1993). Most of these genes encode cytoskeletal or extracellular matrix proteins and are probably a result rather than a cause of the change in differentiated properties of the SMCs. More interesting as potential regulatory elements are the homeobox genes, several of which have been found to be expressed in vascular SMCs in a phenotype-specific pattern (Patel et al., 1992; Gorski et al., 1993; Miano et al., 1996). Another gene preferentially expressed in arterial SMCs and reported to be down-regulated by vascular injury is APEG-1 (Hsieh et al., 1996). After injury of the rat aorta, the fos, jun and myc proto-oncogenes are rapidly induced (Miano et al., 1993a,b). In vitro, the fos and jun genes were similarly found to be activated already during the isolation of the SMCs (Hultgårdh-Nilsson et al., 1997). As in the case with the other genes referred to above, the precise function of these immediate-early genes in the phenotypic modulation is not known.

Role of extracellular matrix components

In the last several years, considerable interest has been focussed on the role of the extracellular matrix in vascular biology (Assoian and Marcantonio, 1996; Ruoslahti and Engvall, 1997; Shattil and Ginsberg, 1997). A concept that has attracted the attention of our laboratory is that adhesive proteins produced locally in the arterial wall or derived from the blood take part in determining the smooth muscle phenotype (Thyberg, 1996). As mentioned above, the SMCs of developing arteries migrate and proliferate in a fibronectin-rich matrix and later become surrounded by a basement membrane as the vessels mature (Risau and Lemmon, 1988). In vitro studies have further demonstrated that a substrate of fibronectin promotes the transition of adult



Cytoskeletal proteins	smooth muscle α-actin smooth muscle myosin	nonmuscle β-actin nonmuscle myosin
Predominant organelles	actin filaments	endoplasmic reticulum Golgi complex
Caveolae	abundant	few
Basement membrane	present	largely lacking
Contractility	high	low
Migratory activity	low	high
Secretory activity	insignificant	extracellular matrix components, matrix metalloproteinases, growth factors
Proliferative activity	insignificant	able to divide and produce growth factors

Fig. 1. Schematic overview of the phenotypic modulation of SMCs following vascular injury.

rat aortic SMCs from a contractile to a synthetic phenotype, whereas substrates of the basement membrane proteins laminin and collagen type IV retain the cells in a contractile phenotype (Hedin et al., 1988). In vivo, a potential scenario could be that vessel injury activates proteolytic enzymes degrading the basement membrane enclosing the SMCs, exposing the plasmalemma to fibronectin and other molecules penetrating into the intima and the inner parts of the media after removal of the endothelium, thus generating signals altering the phenotype of the SMCs. As a result, an earlier genetic program is turned on and like the SMCs of embryonic vessels, the modified SMCs start to migrate, proliferate and secrete extracellular matrix components. Eventually, the endothelium regenerates and the SMCs cease to divide, deposit a new basement membrane around themselves, and resume a contractile phenotype (Figs. 1, 2).

Recent immunoelectron microscopic findings in the injured rat carotid artery confirm the above model (Thyberg et al., 1997a). One week after the operation, a large part of the SMCs in the inner layer of the media had lost most of the intracellular staining for smooth muscle α-actin as well as the pericellular reactivity for laminin. In addition, a network of fibronectin had been laid down on the exposed subendothelial tissue and served as a substrate for movement of SMCs into the intima. After two weeks, the SMCs were still mainly in a synthetic state and had grown in number and formed a multilayered neointima. Except for fine strands of fibronectin, collagen fibrils and elastic fibers were now found extracellularly. After five weeks, the intimal SMCs had regained a basement membrane positive for laminin and only small amounts of fibronectin were detected in the pericellular matrix. Moreover, most of the cells had readopted a contractile phenotype with a cytoplasm rich in filaments of smooth muscle α-actin (Thyberg et al., 1997a). These observations illustrate the ability of the SMCs to shift between a contractile and a synthetic phenotype.

In support of the model presented above, it has also been demonstrated that vascular SMCs have integrin receptors for fibronectin and laminin/collagen type IV both in vivo and in vitro (Belkin et al., 1990; Clyman et

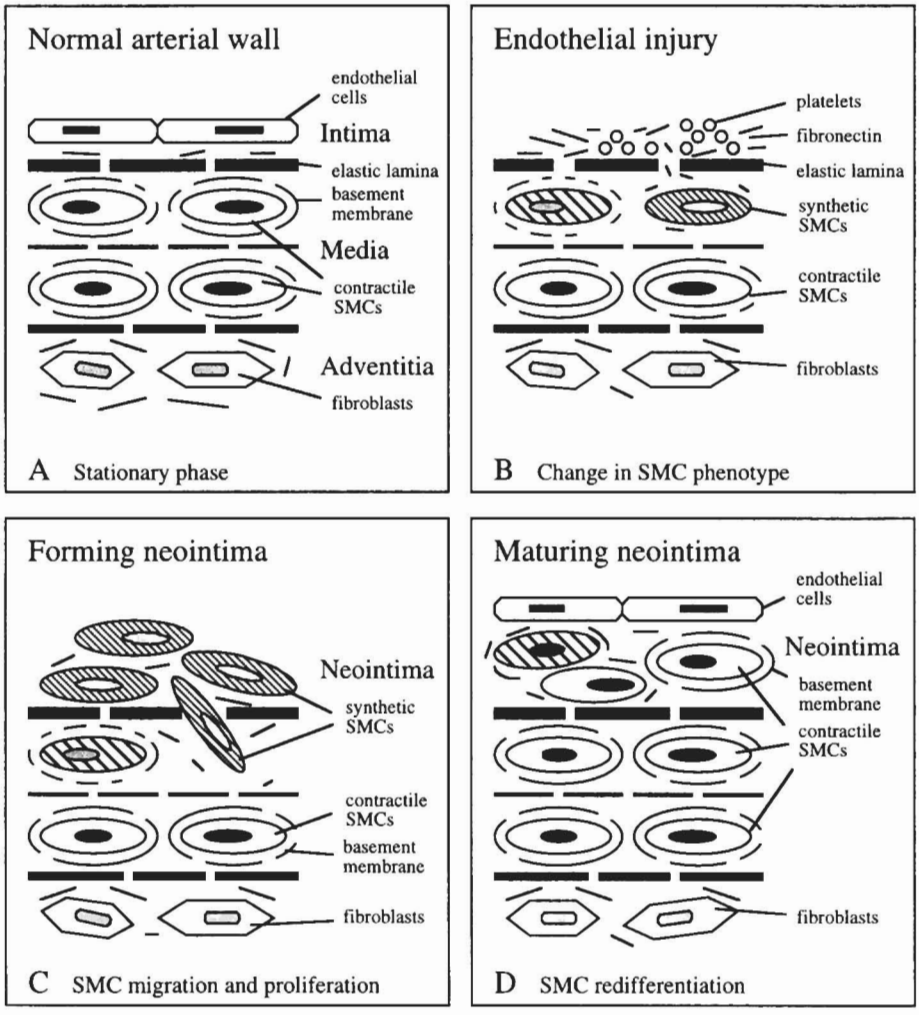


Fig. 2. Schematic model of the formation of neointimal thickenings following vascular injury.

al., 1990; Glukhova et al., 1993). Not long ago, it was further noticed that freshly isolated SMCs seeded on a substrate of fibronectin show a progressive increase in phosphotyrosine content of focal adhesions and a concomitant increase in tyrosine phosphorylation of a few proteins, including focal adhesion kinase (FAK). Treatment with the tyrosine kinase inhibitor genistein blocked assembly of focal adhesions, tyrosine phosphorylation of FAK, and transition of the SMCs into a synthetic phenotype as judged morphologically. On the other hand, cells seeded on laminin did not form focal adhesions and the levels of protein tyrosine phosphorylation remained low (Hedin et al., 1997). In this context, it is also interesting to mention that FAK is expressed at high levels in the media of embryonic vessels, where the SMCs are still in a synthetic phenotype (Polte et al., 1994). Even if it needs to be clarified why SMCs behave differently when interacting with fibronectin and laminin/collagen type IV, these results are in good agreement with the idea that the extracellular matrix not only serves as a passive mechanical support but also generates signals that influence cell behavior (Juliano and Haskill, 1993; Meredith et al., 1996). Examples of the involvement of extracellular matrix components in SMC functions such as migration and proliferation are given below.

Migration of smooth muscle cells into the intima

Electron microscopic and immunocytochemical studies have shown that it is only phenotypically modified SMCs (i.e. cells in a synthetic phenotype) that migrate from the media to the intima after endothelial damage (Thyberg et al., 1995, 1997a). This is in good agreement with in vitro studies indicating that smooth muscle α-actin (an actin isoform that is downregulated in synthetic SMCs) retards cellular motility (Rønnov-Jessen and Petersen, 1996). In order to penetrate the inner elastic lamina, the SMCs pass through fine preexisting holes in this highly cross-linked protein membrane. However, to do so they must liberate themselves both from the basement membrane (probably done already during the change in phenotype) and the collagenous matrix filling the pericellular space. In recent years, large interest has been paid to the function of matrix metalloproteinases (MMPs) in this process. These enzymes make up a family of zinc-dependent endopeptidases with a crucial role in normal as well as pathological tissue remodelling, the expression of which is tightly controlled by cytokines and growth factors (Birkedal-Hansen, 1995; Dollery et al., 1995).

In culture, SMCs stimulated by mechanical injury or exposure to cytokines and growth factors produce both MMPs (secreted and membrane-bound) and inhibitors (TIMPs) regulating their activity (James et al., 1993; Galis et al., 1994a; Kennedy et al., 1997; Pickering et al., 1997a; Shofuda et al., 1997). The ability to do so is phenotype-dependent and mainly associated with cells in a synthetic state (Pauly et al., 1994; Sasaguri et al., 1994). Thus, newly isolated rat SMCs seeded on substrates of fibronectin or basement membrane proteins (laminin/collagen type IV) were found to express stromelysin (MMP-3) mRNA at a high level in close connection with the shift from a contractile to a synthetic phenotype, whereas type IV collagenase (MMP-2) mRNA showed a more constant level (Hultgårdh-Nilsson et al., 1997). Moreover, in vitro studies have demonstrated that other types of proteases, secreted by the SMCs or derived from the blood, may take part in the degradation of the extracellular matrix and the activation of latent forms of the MMPs (Clowes et al., 1990; Kenagy et al., 1996; Lee et al., 1996; Galis et al., 1997).

An upregulation of MMPs and other proteases has likewise been observed following balloon catheter injury (Bendeck et al., 1994; Southgate et al., 1996; Reidy et al., 1996; Webb et al., 1997). Furthermore, treatment of the animals with MMP inhibitors was found to repress migration of SMCs from the media to the intima after endothelial denudation (Bendeck et al., 1996a; Zempo et al., 1996). In a similar manner, local overexpression of TIMP-1 using transfected cells implanted onto injured rat carotid arteries was reported to inhibit neointima formation (Forough et al., 1996). Taken together, these findings suggest a key role of MMPs in the movement of SMCs into the intima after endothelial damage. An increased expression of MMPs has also been noted in human atherosclerotic and restenotic lesions (Henney et al., 1991; Galis et al., 1994b, 1995; Nikkari et al., 1995, 1996; Knox et al., 1997). In this case, MMPs are produced by SMCs as well as macrophages and are believed to be involved not only in cellular migration but also in destabilization of the tissue, thereby leading to an increased risk of plaque rupture.

Although the SMCs need to degrade part of the surrounding extracellular matrix to be able to migrate, they also have to interact with this scaffold to advance from the media to the intima. The macromolecules used for this purpose include collagen type I and III, inherent components of the vascular extracellular matrix, and fibronectin, a plasma protein deposited in the intima and the inner parts of the media (close to the fenestrae in the inner elastic lamina) following destruction of the endothelial barrier (Thyberg et al., 1997a). In vitro and in vivo studies have indicated that the interaction of the SMCs with these fibril-forming proteins is for the main part mediated via the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrins (Skinner et al., 1994; Hoshiga et al., 1995; Gotwals et al., 1996; Bilato et al., 1997), and that migration occurs at an intermediate adhesion strength (Di Milla et al., 1993; Koyama et al., 1996). It has further been found that RGD-containing peptides (i.e. peptides containing the cell-attachment sequence of fibronectin and other adhesive proteins) limit neointima formation in catheter-injured arteries, at least partly due to an inhibition of SMC migration (Choi et al., 1994; Matsuno et al., 1994).

To induce a directed migration of SMCs from the

media to the intima, a chemotactic gradient is also required. Among the factors that may serve this function, PDGF and basic fibroblast growth factor (bFGF) have attracted special attention (Fingerle et al., 1989; Ferns et al., 1991; Jawien et al., 1992; Jackson et al., 1993; Jackson and Reidy, 1993; Bornfeldt, 1996; Myllärniemi et al., 1997). PDGF is released from degranulating platelets adhering to the subendothelial matrix after balloon injury. In addition, PDGF as well as bFGF may be discharged from damaged endothelial cells (Barrett et

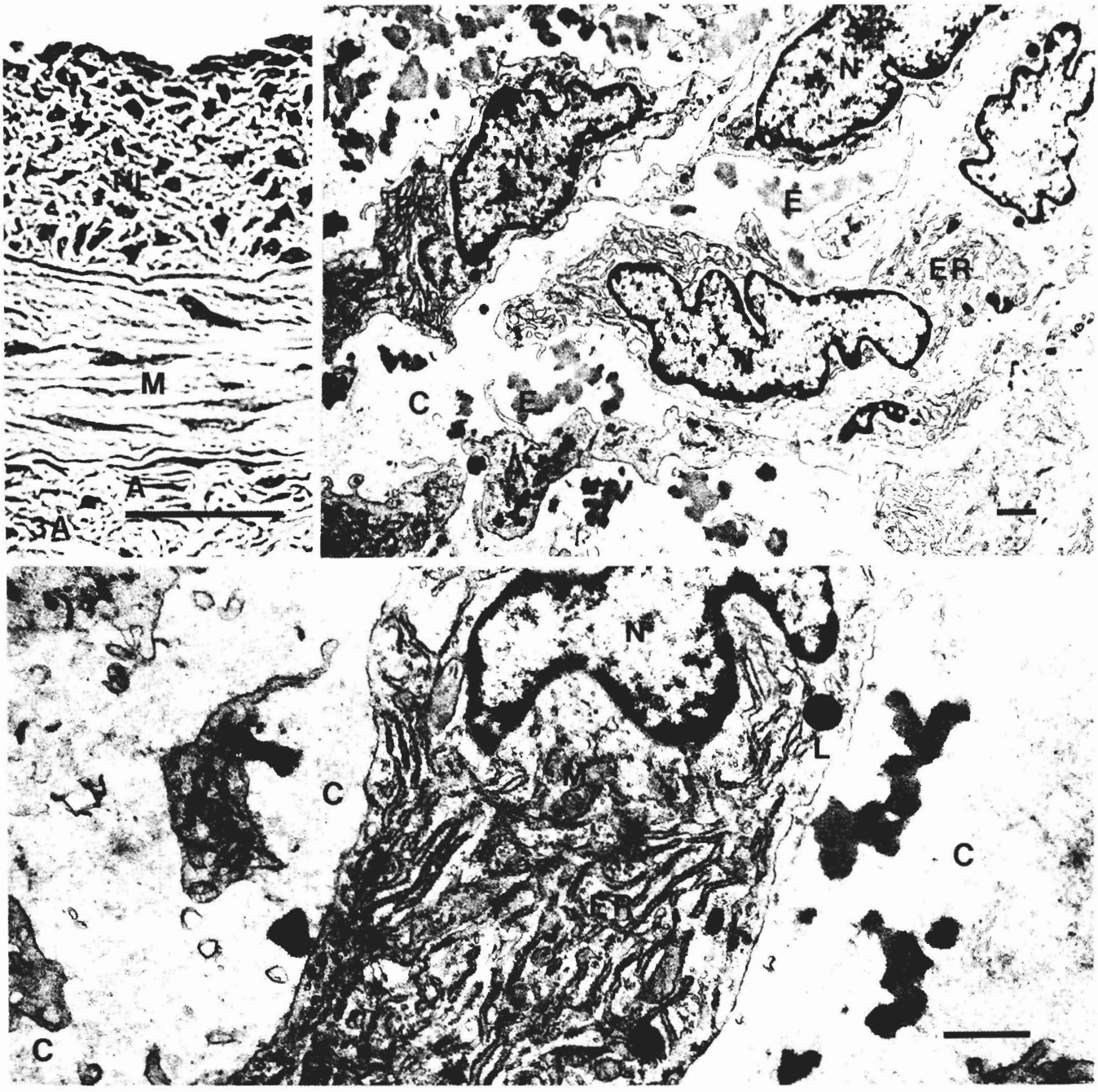


Fig. 3. Light **(A)** and electron **(B, C)** micrographs of neointimal thickenings in the rat carotid artery two weeks after balloon injury. In the light micrograph, NI marks the neointima, M the media, and A the adventitia. In the electron micrographs, synthetic SMC with an extensive endoplasmic reticulum (ER) are shown. They are surrounded by a dense extracellular matrix with collagen fibrils (C) and elastic fibers (E) as major structural components. L: lysosomes; M: mitochondria; N: nucleus. Bars: A 50 μm; B, C, 1 μm.

al., 1984; Speir et al., 1991) or mobilized from storage sites in the extracellular matrix (Vlodavsky et al., 1991; Raines and Ross, 1992; Raines et al., 1992; Taipale and Keski-Oja, 1997). The mode of action of the mitogens may in this case be to stimulate MMP production (Birkedal-Hansen, 1995; Dollery et al., 1995; Kennedy et al., 1997; Pickering et al., 1997a), integrin expression (Janat et al., 1992; Seki et al., 1996; Pickering et al., 1997b), and rearrangement of the actin cytoskeleton (Claesson-Welsh, 1996; Bikfalvi et al., 1997). Although few details are known, cytokines (Libby and Ross, 1996) and eicosanoids (Jeremy et al., 1996) are other factors that could be involved in the control of SMC migration after vascular injury.

Intimal proliferation of smooth muscle cells

The number of SMCs that migrate from the media to the intima in the course of atherogenesis and restenosis is limited and the impressive size often reached by the lesions is likely to be dependent on cell proliferation in the intima. Accordingly, SMC replication has been well documented in animal models such as the balloon-injured rat carotid artery (Clowes et al., 1983, 1989; Miano et al., 1993a,b; Indolfi et al., 1995; Wei et al., 1997). For about a week following the appearance of the first SMCs in the intima, many mitoses are noted here and a neointima with densely packed cells and a thickness equivalent to or even larger than the media is formed (Fig. 3A). During this period, the SMCs are all in a synthetic phenotype as distinguished morphologically (Fig. 3B). Later, only few dividing cells are detected, but some additional growth in thickness of the neointima takes place by accumulation of extracellular matrix components (Fig. 4A). However, within a few weeks essentially all of the neointimal SMCs regain a contractile phenotype as judged by the decrease in size of the secretory apparatus and the reappearance of actin filaments (Fig. 4B - Thyberg et al., 1995, 1997a).

The investigations referred to above indicate that the phase of rapid SMC proliferation in the intima after endothelial damage is restricted in time also when lesions of large dimensions are formed. This could explain the difficulties to find evidence for active cell proliferation in atherosclerotic and restenotic lesions (O'Brien et al., 1993; Rekhter and Gordon, 1995; Geary et al., 1996; Schwartz and Reidy, 1996). Although species differences probably exist, it may therefore

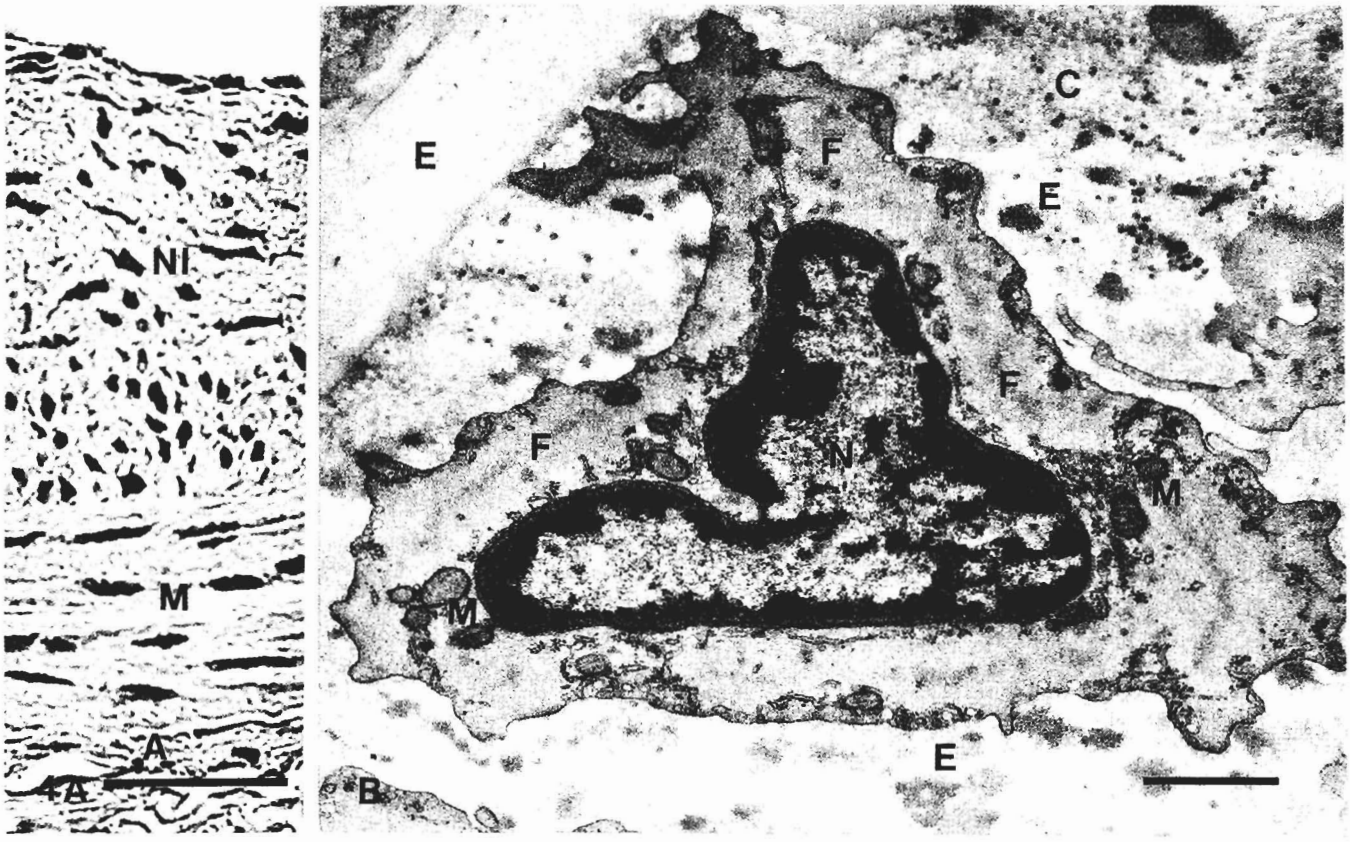


Fig. 4. Light **(A)** and electron **(B)** micrographs of neointimal thickenings in the rat carotid artery five weeks after balloon injury. In the light micrograph, NI marks the neointima, M the media, and A the adventitia. In the electron micrograph, a contractile SMC with a cytoplasm dominated by actin filaments (F) is shown. C: collagen fibrils (cross-sectioned); E: elastic fibers; M: mitochondria; N: nucleus. Bars: A, 50 μm; B, 1 μm.

be too early to exclude the importance of SMC multiplication in the formation of neointimal plaques in humans. Moreover, a variety of polypeptide mitogens and other molecules that stimulate growth of animal as well as human SMCs have been identified (Bobik and Campbell, 1993; Libby and Ross, 1996). Testing of growth-promoting activity is usually done in vitro. Nevertheless, it has been demonstrated that systemic or local perivascular administration of PDGF (Jawien et al., 1992) and bFGF (Cuevas et al., 1991; Lindner et al., 1991) stimulates SMC replication in injured arteries. In addition, treatment of balloon catheter injured animals with antibodies against PDGF (Ferns et al., 1991; Jackson et al., 1993; Rutherford et al., 1997) and bFGF (Lindner and Reidy, 1991; Jackson and Reidy, 1993; Rutherford et al., 1997) or a PDGF receptor tyrosine kinase inhibitor (Myllärniemi et al., 1997) was found to inhibit neointimal hyperplasia, apparently by neutralization of endogenous growth factors.

One problem in interpreting experiments of this type is that molecules such as PDGF and bFGF act both as mitogens and chemoattractants (see above). Hence, it may be hard to discriminate between these effects. In the case of PDGF and bFGF mobilized from the subendothelial matrix or released from degranulating platelets and damaged endothelial cells, the first effect is presumably to stimulate migration of SMCs into the intima. The subsequent proliferation of SMCs at this site may be driven either by factors from the sources just mentioned or by factors produced by the cells in the neointima and acting in an autocrine or paracrine manner. In support of the latter possibility, both PDGF and PDGF receptors were shown to be expressed by the cells in the neointima following balloon injury (Majesky et al., 1990; Okazaki et al., 1992; Lindner et al., 1995; Uchida et al., 1996; Panek et al., 1997). Moreover, the PDGF receptors are activated and display a peak in tyrosine phosphorylation one to two weeks after the operation (Abe et al., 1997). In a similar manner, both bFGF and bFGF receptors are found in the forming neointima (Olson et al., 1992; Lindner and Reidy, 1993). Albeit at varying levels, PDGF (Barrett and Benditt, 1988; Wilcox et al., 1988; Rekhter and Gordon, 1994a; Murry et al., 1996) and bFGF (Brogi et al., 1993; Hughes et al., 1993) are also expressed in human atherosclerotic plaques. Furthermore, production of PDGF in the arterial wall after in vivo gene transfer was shown to induce neointimal thickening by stimulation of SMC migration and proliferation (Nabel et al., 1993; Pompili et al., 1995).

Transforming growth factor-ß1 (TGF-ß1) is another factor that has been found important in the reaction of the vessel wall to endothelial damage. Thus, both medial and neointimal SMCs in injured arteries were demonstrated to express TGF-ß1 (Majesky et al., 1991; Wolf et al., 1994). In addition, treatment of balloon catheter-injured animals with TGF-ß1 was found to enhance neointimal growth by stimulation of SMC proliferation and extracellular matrix production (Majesky et al., 1991; Kanzaki et al., 1995), whereas treatment with antibodies against TGF-ß1 had the opposite effect (Wolf et al., 1994). Other examples of factors that have been found to be expressed in damaged arteries and could be significant in promoting SMC proliferation include insulin-like growth factor-1 (Khorsandi et al., 1992; Häyry et al., 1995), heparin-binding epidermal growth factor-like growth factor (Nakata et al., 1996), interleukin-1 (Moyer et al., 1991), and tumor necrosis factor-α (Tanaka et al., 1996).

Secretion of extracellular matrix components

Secretion of extracellular macromolecules is an integral part in the phenotypic modulation of the SMCs following vascular injury and plays a crucial role both in the early migratory and proliferative phase of neointimal growth and the later phase distinguished by deposition of a collagen- and elastin-rich matrix around the cells (Wight, 1996). One molecule that lately has attracted much attention as a stimulus of SMC migration is osteopontin, an RGD-containing and strongly acidic glycoprotein (Giachelli et al., 1995). It is expressed by SMCs in association with the phenotypic modulation in primary culture and deposited extracellularly in a fibrillar pattern (Hultgårdh-Nilsson et al., 1997). It interacts with $\alpha_v ß_3$and other integrins and promotes adhesion, spreading, and migration of cultured SMCs (Liaw et al., 1994, 1995; Yue et al., 1994). In vivo, osteopontin is produced at raised levels in the neointima of balloon injured rat arteries (Giachelli et al., 1993; De Blois et al., 1996; Wang et al., 1996) and treatment of the animals with antibodies against osteopontin was found to inhibit the growth in size of the neointima (Liaw et al., 1997). In human atherosclerotic and restenotic lesions, osteopontin is expressed not only by SMCs but also by endothelial cells and macrophages, and increased levels of osteopontin appear in plasma after angioplasty (Ikeda et al., 1993; O'Brien et al., 1994; Panda et al., 1997).

Other molecules that are synthesized by SMCs after vascular injury and have been proposed to support their migration are tenascin, a large hexameric glycoprotein (Hedin et al., 1991; Hahn et al., 1995), thrombospondin, a trimeric glycoprotein also released by degranulating platelets (Miano et al., 1993b), and collagen type VIII, a network-forming collagen (Bendeck et al., 1996b; Sibinga et al., 1997). Hyaluronan (Riessen et al., 1996), a glycosaminoglycan component of many extracellular matrices, and CD44 (Jain et al., 1996), a cell surface proteoglycan and the principal receptor for hyaluronan, likewise show high levels of expression in injured arteries and have been implicated in SMC migration and proliferation. Two members of the syndecan family of transmembrane heparan sulfate proteoglycans, syndecan-1 and syndecan-4 (ryudocan), also demonstrate an early rise in expression following endothelial denudation and may act by binding mitogens such as bFGF to the surface of the SMCs (Nikkari et al., 1994;

Cizmeci-Smith et al., 1997). As a part of the vessel wall response to damage, the phenotypically modified SMCs of the media and the developing neointima further manufacture fetal forms of fibronectin containing ED-A and ED-B (Glukhova et al., 1989; Bauters et al., 1995; Dubin et al., 1995). Directly after the loss of the endothelial barrier, plasma fibronectin is also deposited in the intima and the inner portions of the media (Thyberg et al., 1997a). As mentioned previously, this layer of fibronectin may help to bring about a change in phenotype of the SMCs inside the openings in the internal elastic lamina and to create a chemotactic gradient along which these cells can migrate into the intima (Fig. 2). In a similar manner, and especially following deeper vascular injury, fibrin thrombi may also form and promote neointima growth (Bosmans et al., 1997).

During the early stages of intimal thickening the extracellular matrix is scanty, but following the peak in SMC proliferation collagen fibrils and elastic fibers take up a gradually increasing part of the tissue as judged morphologically (Thyberg et al., 1995). Accordingly, mRNA for procollagen type I and tropoelastin were found to be expressed at high levels in the neointima two weeks after balloon injury of the rat carotid artery (Majesky et al., 1992; Nikkari et al., 1994). In a rabbit model of restenosis, a significant increase in collagen, elastin and proteoglycan synthesis was likewise noted one to two weeks after injury (Strauss et al., 1994). Moreover, both in rats and rabbits it has been demonstrated that maximal tropoelastin expression in neointimal SMCs occurs after cessation of DNA synthesis, confirming that the peaks in cell replication and extracellular matrix secretion are separated in time (Belknap et al., 1996; Aoyagi et al., 1997). Similar conclusions have been reached in studies on collagen secretion in human atherosclerotic plaques (Rekhter and Gordon, 1994b; Gordon and Rekhter, 1997). Nonetheless, it may to a large extent be the same substances that are responsible for stimulation of cell proliferation and extracellular matrix production. Thus, growth factors like PDGF, bFGF, and TGF- ß1 have been found to affect both of these processes (Bobik and Campbell, 1993). As discussed above, such factors are produced and sequestered locally in the injured vessel and may act here over an extended period of time (Vlodavsky et al., 1991; Medalion et al., 1997; Taipale and Keski-Oja, 1997).

Redifferentiation of smooth muscle cells and endothelial regeneration

Following endothelial denudation, the initial change in phenotype in the media, the migration into the intima, and the stages of cellular proliferation and secretion of extracellular matrix components, the SMCs of the newly formed and thickened intima eventually readopt a contractile phenotype. This is observed structurally as an increase in the fractional volume of myofilaments and and a concurrent decrease in the fractional volume of secretory organelles (Grünwald et al., 1987; Manderson et al., 1989; Thyberg et al., 1995). At the same time, the expression of smooth muscle α-actin (Kocher et al., 1991; Thyberg et al., 1997a) and smooth muscle myosin (Aikawa et al., 1997) is augmented. However, it is not known if the redifferentiated intimal SMCs are fully equivalent to the cells in the normal arterial media. In any case, it is evident that the SMCs have a remarkable ability to shift phenotype in a reversible manner.

At least in part, the redifferentiation of the neointimal SMCs may be related to the regeneration of the endothelial cell layer and the restoration of a permeability barrier between the blood and the vessel intima. Thus, a comparison of balloon-injured rat carotid and femoral arteries has revealed that the more rapid reformation of the endothelium in the latter vessel is accompanied by a faster resumption of a contractile phenotype of the SMCs and a less prominent thickening of the intima (Thyberg et al., unpublished observations). SMCs of injured as well as atherosclerotic arteries have been found to produce endothelial cell mitogens such as bFGF (Olson et al., 1992; Brogi et al., 1993; Hughes et al., 1993; Lindner and Reidy, 1993) and VEGF (Couffinhal et al., 1997; Ruef et al., 1997; Tsurumi et al., 1997) and this could be an important stimulus of endothelial regeneration. However, in the case of VEGF partly contradictory observations have been made. Thus, no clear effect on endothelial cell replication was noted after intra-arterial infusion and it was suggested that this factor may not be a mitogen for large-vessel endothelium in vivo (Lindner and Reidy, 1996).

Pharmacological treatment

In view of its central role in the development of atherosclerotic and restenotic lesions, the vascular SMC has found broad interest as a target for pharmacological therapy (Jackson and Schwartz, 1992; Bobik and Campbell, 1993; Bauters et al., 1996). Two principal approaches for drug design can be envisioned, to interfere either with the transition of the cells from a contractile to a synthetic phenotype or the resulting ability to migrate, proliferate, and secrete extracellular matrix components. However, these steps in the modification of the SMCs are closely interconnected and usually not kept apart in the evaluation of drug effects. In the first case, the treatment will mainly be preventive in character. In the second case, it may also serve to halt the progress of the disease process. As examples of substances that have been reported to inhibt neointimal thickening after vascular injury in laboratory animals, the following can be mentioned: heparin (Clowes and Clowes, 1985; Pukac et al., 1991; Lindner et al., 1992), matrix metalloproteinase inhibitors (Bendeck et al., 1996a; Zempo et al., 1996), tyrosine kinase inhibitors (Fukumoto et al., 1996; Golomb et al., 1996; Myllärniemi et al., 1997), growth factor antibodies (Rutherford et al., 1997), angiotensin converting enzyme

inhibitors and angiotensin receptor antagonists (Powell et al., 1989; Kauffman et al., 1991; Prescott et al., 1991), mevalonate and cholesterol synthesis inhibitors (Gellman et al., 1991), calcium channel blockers (Schmitt et al., 1995), and microtubule inhibitors (Axel et al., 1997). In spite of the positive effects in experimental models, only limited success has so far been obtained in trials to reduce the incidence of restenosis in humans (Bauters et al., 1996).

In recent years, increasing attention has also been paid to the application of gene therapy for vascular disease (Finkel and Epstein, 1995; Feldman et al., 1996; Gibbons and Dzau, 1996; Nabel and Nabel, 1996). This approach may be of particular interest in the prevention of restenosis after angioplasty, a process that is initiated by tissue damage in connection with a surgical procedure and develops over a limited period of time. In this case, antisense oligonucleotides or recombinant genes are transferred to the vasculature in an attempt to suppress the function of specific genes. So far, the main object of this work has been to inhibit SMC proliferation. The probes that have been used for this purpose include antisense oligonucleotides targeting the PDGF ß-receptor (Sirois et al., 1997), the proto-oncogenes c-myc (Biro et al., 1993) and c-myb (Simons et al., 1992), cyclin G1 and the cyclin-dependent kinases cdc2 and cdk2 (Abe et al., 1994; Zhu et al., 1997), proliferating cell nuclear antigen (Speir and Epstein, 1992), and the transcription factors E2F (Morishita et al., 1995) and NF-kB (Autieri et al., 1995). Other examples of constructs that have been utilized are recombinant genes encoding cytotoxic fusion proteins binding to growth factor receptors (Pickering et al., 1993; Farb et al., 1997), the cyclin-dependent kinase inhibitor p27 (Chen et al., 1997b), a constitutively active form of the retinoblastoma protein (Chang et al., 1995), enzymes making the cells sensitive to ganciclovir (Ohno et al., 1994) or 5-fluorocytosine (Harrell et al., 1997), and ß-interferon (Stephan et al., 1997). The animal studies described above provide a promising basis for the use of gene transfer in the treatment of human vascular disease. However, several details need to be refined before this can become a clinical routine.

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