Neointima Formation after Acute Vascular Injury

Role of Counteradhesive Extracellular Matrix Proteins

Mark W. Majesky, PhD

Restenosis currently limits the long-term beneficial effects of balloon coronary angioplasty. Two important cellular events in the development of clinically significant luminal narrowing after angioplasty are 1) increased production of extracellular matrix proteins and 2) acquisition of a motile phenotype by vascular smooth muscle cells. In this paper, smooth muscle cell responses that produce a fibrocellular neointima after acute vascular injury are reviewed. Particular emphasis is placed on specialized extracellular matrix proteins implicated in cell movement and tissue repair. Tenascin and thrombospondin are large, modular extracellular matrix glycoproteins; they possess both adhesive and counteradhesive domains and are expressed at high levels during smooth muscle cell migration and neointima formation after balloon injury to rat carotid artery. The ability of both tenascin and thrombospondin to down-regulate the assembly and activity of focal adhesions (points of cell–extracellular matrix adhesive interactions) may be important in the conversion of stationary, quiescent smooth muscle cells to cells that are able to move and divide within the strongly adhesive vessel wall. Moreover, tenascin is present in the extracellular matrix as a large 6-armed oligomer (a hexabrachion) that contains both cell-binding and matrix protein-binding domains in each of the hexabrachion arms. The large size and multidomain structure of tenascin and thrombospondin suggest that these proteins may be particularly well suited to form a nascent provisional matrix at sites of 1) neointima formation after acute vascular injury, 2) new growth and expansion within primary atherosclerotic plaques, and 3) intimal repair and luminal narrowing in restenosis after angioplasty. ***(Texas Heart Institute Journal 1994;21:78-85)***

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Restenosis currently limits the long-term beneficial effects of balloon coronary angioplasty.[1] Although satisfactory initial increases in blood flow can be achieved with angioplasty balloons or atherectomy devices, long-term failure rates of 20% to 40% occur within 6 to 12 months due to restenosis.[2] The clinical problem can be factored into 2 components: 1) thrombosis and blood coagulation and 2) fibrocellular intimal thickening with luminal narrowing. New fibrinolytic and antithrombotic agents promise greater clinical control over complications due to blood coagulation; however, little progress has been made toward controlling fibrocellular intimal thickening and luminal narrowing, which remain major clinical problems.[1,2]

Animal models have been used to identify the cellular responses involved in repair of acute vascular injury. Perhaps the most thoroughly studied model is the response of the rat common carotid artery to a balloon catheter injury.[3] As with any animal model of a complex human disease process, the rat carotid model has advantages and limitations. It continues to be very useful for exploring the mechanisms whereby normally stationary and quiescent smooth muscle cells (SMCs) become motile and proliferative within a damaged artery and go on to form a neointima at the luminal surface. The early events (hours to days) after angioplasty injury in human arteries are not readily accessible for experimental study. The major limitations of the model are that 1) there is no preexisting intimal thickening, as is found in human diseased vessels, and 2) SMCs are almost completely responsible for the response to injury in the rat, whereas a mixed population of macrophages, T-cells, plaque microvascular endothelial cells, and SMCs typically interact in complex ways after angioplasty injury to a preexisting advanced human atherosclerotic plaque.

This paper reviews the current understanding of how a neointima forms in the rat carotid artery after balloon angioplasty. Special emphasis is placed on the roles

of the "counteradhesive" extracellular matrix (ECM) glycoproteins tenascin[4] and thrombospondin[5] in those events. It is important to note that neointima formation observed after rat carotid injury is a different process than the acute wound repair generally found in other tissues. Arterial SMCs not only repair the damage to the media, but they go on to construct a new layer of vessel wall in what was previously fluid-filled space. In doing so, these cells reexpress many phenotypic features displayed by fetal SMCs during vessel wall formation in the embryo,[6] such as the production and organization of an extensive ECM upon which formation of the neointima rests. The nature of that matrix and the factors derived from both within and outside the neointima that regulate its formation are discussed.

Cellular Events in Rat Carotid Wound Repair

The SMC responses to rat carotid balloon injury occur in 3 distinct stages, each characterized by different cellular events controlled by different factors.[3] During the first 48 hours (stage 1), normally stationary and quiescent SMCs enter the cell cycle and divide.[7] Between 10% and 30% of surviving SMCs synchronously enter S phase beginning around 24 hours after wounding.[8] Upon completion of the 1st round of SMC replication, the number of SMCs in the injured media is nearly restored to normal. By 48 to 72 hours after wounding, most SMCs in the injured artery have returned to the quiescent state. However, an important subpopulation of SMC escapes growth inhibition, continues to replicate, and migrates to the luminal surface.[9,10] The cells in this subpopulation serve as progenitors for most of the neointimal SMCs that are found at later times. A key question to be answered is whether this SMC subpopulation is a distinct subset specialized for arterial wound repair or whether it is derived by a stochastic process within damaged arteries.[11]

During days 2 to 5 after injury (stage 2), SMCs migrate toward the lumen, colonize the intimal surface, and begin to form a new layer of arterial wall, the neointima. Movement of SMCs to the luminal surface is presumed to follow a gradient of chemoattractants in the wall,[12] to depend upon production of matrix degrading proteases,[3,9] and to require the synthesis of new ECM proteins.[11,13] During the next few weeks (stage 3), the number of SMCs in the neointima continues to increase.[7] At the same time, neointimal SMCs synthesize and assemble abundant quantities of ECM, including fibronectin,[13,14] collagen,[13,15] tenascin,[16] thrombospondin,[17] elastin,[11,18] and proteoglycans.[15] These steps are essential for constructing a new tissue layer in the fluid-filled space. Between 1 and 3 months after injury, SMC DNA synthesis within the neointima is greatly reduced. Despite little or no change in the number of SMCs, the neointima more than doubles in size because of continued production of ECM at high levels.[7,19] Since the external diameter of the rat carotid artery does not change during repair of balloon injury, expansion of the neointima results in a narrowed vascular lumen.

Growth Factors Produced by SMCs after Acute Vascular Injury

Because SMC replication after balloon injury has been found to be largely independent of platelet products,[20] attention has shifted in recent years to identification of growth factors synthesized or released by SMCs themselves. Regenerating SMCs have been shown to produce platelet-derived growth factor-A (PDGF-A), particularly those SMCs along the luminal surface of the developing neointima[21,22] (Fig. 1A). The distribution of PDGF-A transcripts within the neointima is intriguing because this growth factor co-localizes with SMCs that are also actively

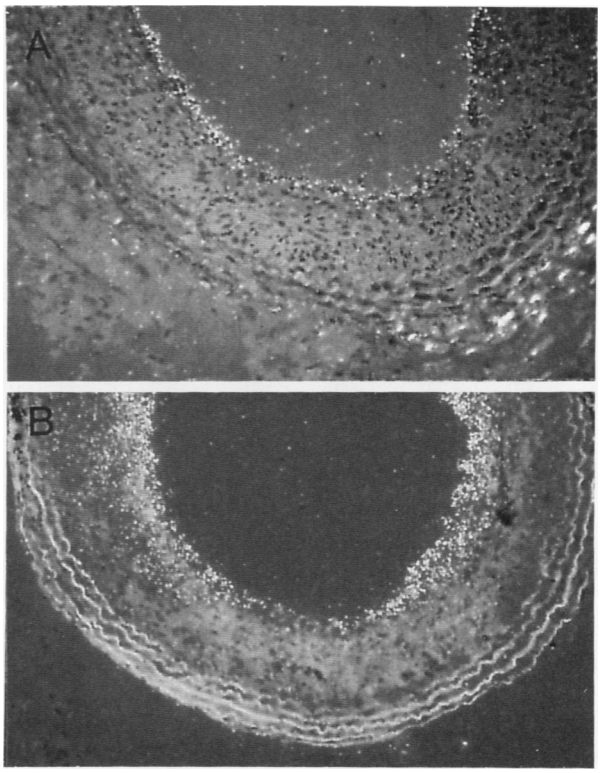

Fig. 1 *Localization of platelet-derived growth factor-A (PDGF-A) and PDGF β-receptor mRNAs in rat carotid neointima by in situ hybridization. Serial sections of injured left carotid artery were obtained 2 weeks after balloon injury. Sections were hybridized as described.[21]* ***A****) PDGF-A transcripts are localized to smooth muscle cells (SMCs) that line the luminal surface.* ***B****) PDGF β-receptor mRNA is also concentrated in SMCs at or near the luminal surface and, in addition, is found in SMCs located deeper within the neointima. Micrographs were made using polarized light epiluminescence and bright field illumination. (orig. x100)*

proliferating. However, this co-localization must be interpreted with caution since expression of the PDGF α-receptor gene is greatly reduced in neointimal SMCs.[21] It is not clear whether cells expressing PDGF-A (or their nearest neighbors) can respond to the PDGF-AA that is produced. Neointimal SMCs do, however, contain abundant PDGF β-receptor mRNA (Fig. 1B), raising the possibility that the PDGF-BB released from platelets or made by neointimal SMCs themselves stimulates chronic SMC replication at the luminal surface. However, Ferns and colleagues[12] reported that infusion of antibodies capable of neutralizing PDGF-BB had little or no effect on SMC replication. More likely, the increased expression of PDGF β-receptor is important for directed SMC migration into the intima, since infusion of PDGF-BB itself greatly stimulated SMC migration in vivo,[23] and neutralizing antibodies to PDGF significantly reduced SMC movement into the intima.[12] The stimuli that maintain luminal SMC replication late after injury have yet to be clearly identified (see below).

Smooth muscle cells actively engaged in arterial wound repair also produce transforming growth factor-β_1 (TGF-β_1) and TGF-β_3 (Fig. 2). We found that TGF-β_1 mRNA levels were increased within 6 to 8 hours after injury and remained elevated for at least 2 weeks.[13] Transcripts for TGF-β_3 were also elevated at 6 hours after wounding, but then returned to basal levels by 24 to 48 hours after injury. Immunolocalization studies showed that nearly all the neointimal SMCs stain strongly for TGF-β_1 in an intracellular pattern consistent with cellular synthesis of the growth factor rather than accumulation of TGF-β_1 released from platelets.[13] A similar staining pattern for TGF-β_1 was reported for human restenotic lesions.[24] Infusion of purified, recombinant TGF-β_1 into rats with a preexisting neointima produced a modest increase in intimal, but not medial, SMC DNA synthesis.[13] It is likely that the major role of TGF-β_1 produced by neointimal SMCs is to stimulate the abundant synthesis and organization of ECM that enables construction of this new tissue layer of arterial wall.

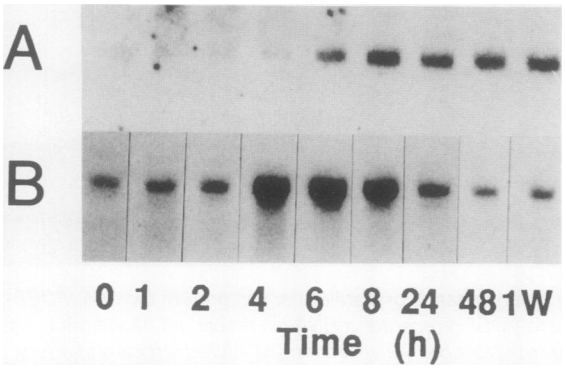

Fig. 2 *Effects of carotid injury on transforming growth factor-β_1 (TGF-β_1) and TGF-β_3 mRNA levels. Total cellular RNA was isolated at the times indicated after left carotid injury, subjected to electrophoresis (12 μg/lane), and hybridized with a ^{32}P-labeled human TGF-β_1 (**A**) or murine TGF-β_3 (**B**) cDNA probe.*

h = hours; W = week

In normal arteries, SMCs contain basic fibroblast growth factor (bFGF) in an intracellular location that is isolated from interaction with bFGF receptors.[25] The extensive cell injury and cell death that occurs after balloon injury is sufficient to account for release of bFGF into the wall and activation of bFGF receptors at the surface of surviving SMCs.[26] The bFGF released into the damaged artery is important for the initiation of SMC replication. Lindner and Reidy[26] showed that infusion of neutralizing antibodies to bFGF reduced the frequency of ^{3}H-thymidine-labeled SMC nuclei by about 50%.[26] Although these and related studies clearly establish the importance of bFGF in the *initiation* of rat carotid SMC replication after acute vascular injury, the role that bFGF may play later in the sequence of neointima formation is unclear. When neutralizing antibodies to bFGF were given during the active phase of neointimal SMC proliferation, they had little or no effect on DNA synthesis.[27]

α-Thrombin, a multifunctional wound repair protease, is known to be present and enzymatically active within injured arteries soon after the injury itself.[28] α-Thrombin has been shown to stimulate SMC replication in vitro[29] and to increase PDGF-A gene expression in cultured SMCs with a time course very similar to that found after carotid injury in vivo.[21,30] Indeed, we found that infusion of the thrombin antagonist PPACK prevented increases in PDGF-A mRNA levels normally seen 6 hours after balloon injury to baboon brachial arteries.[30] In addition, the selective α-thrombin antagonist hirudin inhibited neointimal thickening in rabbit femoral arteries after balloon injury.[31] The persistent generation of active α-thrombin in regenerating arteries over a period of days[28] suggests that α-thrombin could play vital roles not only in the early events after balloon injury[30] but also in the continued SMC replication, PDGF-A expression, and ECM production (see below) occurring at the luminal surface of the developing neointima.

Angiotensin II has also been implicated in the initiation of SMC replication in the rat carotid artery.[32,33] Prescott and colleagues[32] showed that DuP753, a selective antagonist of the AT_1 subtype of angiotensin II receptor, produced a 53% inhibition of SMC proliferation during the first 48 hours after balloon injury to rat carotid artery. This degree of inhibition is comparable to that reported for antibodies to bFGF.[26] In contrast, the angiotensin-converting enzyme (ACE)

inhibitor benazeprilat had no effect on initiation of SMC replication after injury, but greatly reduced the extent of neointimal thickening due to inhibition of SMC migration.[32] At least part of the effect of ACE inhibitors on neointimal thickening may be due to increases in local bradykinin levels at the luminal surface.[34]

Although the growth factors described above were initially studied because of their potential role in promoting SMC replication in injured arteries, each of these growth factors also regulates ECM production by vascular SMCs. A recent report by O'Brien and co-authors[35] showed that SMC replication in human restenotic lesions is not substantially greater than that in normal arteries. These results emphasize the potential importance of ECM production in the development of increased intimal mass and luminal narrowing in human arterial restenosis. Two ECM proteins whose expression is highly responsive to growth factors known to be present at sites of acute vascular injury are tenascin[36,37] and thrombospondin.[5,38]

Molecular Mechanisms for SMC Movement, Structural Remodeling, and Repair

To move forward from a description of cellular responses to injury to the molecular mechanisms for SMC movement, structural remodeling, and repair, we need to ask, "What proteins do SMCs use to adhere to the ECM and how are they regulated?" Cell attachment to the ECM involves recognition of adhesive matrix proteins by focal adhesions, which are specialized complexes of cytoskeletal and membrane-associated proteins (for review, see Burridge and co-authors[39]). Binding of an adhesive ligand (for example, fibronectin) to the extracellular domain of an integrin receptor leads to the assembly of cytoplasmic and cytoskeletal proteins, including talin, vinculin, paxillin, and α-actinin, to form insertion points for cytoskeletal stress fibers, thus connecting the cytoskeleton with the ECM. Focal adhesions are dynamic structures that can exhibit rapid remodeling within minutes.[40] The assembly and disassembly of focal adhesions is associated with cell movement and changes in cell–ECM interactions.[39,41]

Within the blood vessel wall, SMCs are typically surrounded by a strongly adhesive ECM. Under normal conditions, SMCs make stable contacts with the matrix and remain stationary and quiescent. Strong attachments to the ECM are necessary for transmission of contractile force to structural elements of the vessel wall so that changes in peripheral resistance and blood flow distribution can be produced by SMC contraction. After vascular injury, however, SMCs must acquire a motile phenotype to repair the wound. Movement of SMCs within the strongly adhesive vessel wall requires expression of specialized matrix proteins with counteradhesive properties, together with secreted, matrix-degrading proteases[9] and new cell surface matrix receptors. Therefore, the strong attachment activity of focal contacts must be modulated to permit cells to make weaker and more transient connections with the matrix as they migrate or remodel the existing structural elements of the vessel wall.[41]

The Counteradhesive Matrix Protein, Tenascin. Tenascin, also called cytotactin, is a large ECM glycoprotein with adhesion-modifying activity whose expression is tightly linked to cell surface events that regulate cell movement, selective adhesion, differentiation, and development (for reviews, see references 4 and 36). Tenascin is composed of 6 polypeptide subunits of 200 to 280 kDa each (variation in size due to alternative splicing) that are disulfide-linked at their amino termini to form an oligomeric structure called a hexabrachion[4] (Fig. 3). Each tenascin subunit has a modular structure comprising distinct functional domains. The amino terminus forms a triple-stranded coiled coil and is responsible for interchain disulfide bonding. Next are found 14.5 cysteine-rich, epidermal growth factor–like repeats that possess antiadhesive activity[42] followed by a variable number of fibronectin type III domains resulting from alternative splicing of tenascin pre-mRNA. Tenascin has been reported to have both adhesive and antiadhesive properties.[42,43] This functional diversity may depend, in part, upon the pattern of fibronectin type III repeats that are included or excluded by alternative splicing.[44] At the C-terminus is a domain homologous to the β and γ chains of fibrinogen with putative Ca^{2+}-binding and cell-adhesion activities.[43]

When plated onto a substrate of tenascin, many different types of cells either do not attach at all[45] or they attach weakly but remain rounded and do not spread out.[36] In contrast, cells plated onto fibronectin

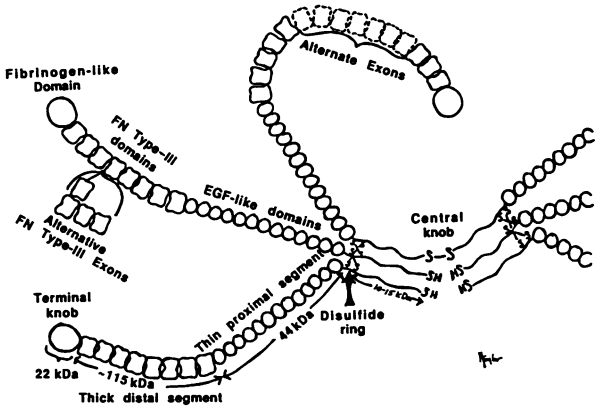

Fig. 3 *Structure of tenascin*

EGF = epidermal growth factor; FN = fibronectin

attach and spread fully. In a detailed analysis of the adhesion properties of tenascin versus fibronectin, Lotz and coworkers[46] reported that fibroblasts or glial cells plated at 4 °C would attach to either fibronectin or tenascin as the sole substrate protein. When the same cells were warmed to 37 °C, the strength of adhesion to fibronectin increased by an order of magnitude, whereas the adhesive strength to tenascin was unchanged or actually *reduced*. When the substrate was a mixture of fibronectin and tenascin, the presence of tenascin reduced the adhesion strengthening of cells adhering to fibronectin in the substrate mixture. The "strengthening" response of cells adhering to fibronectin is most likely due to integrin-mediated assembly of focal adhesions, an energy-requiring process. Two mechanisms have been proposed to explain the counteradhesive effects of tenascin: 1) a steric blocking effect on fibronectin–integrin binding by the large tenascin molecule in its native form (Mr >1,000 kDa),[43,45] and 2) an active, receptor-mediated signaling pathway leading to disassembly of focal adhesions.[42,44] Both mechanisms have received experimental support and both may have physiologic relevance. The recent finding that small recombinant domains of tenascin retain counteradhesive activity in a concentration-dependent and saturable manner[42] gives strong support to the hypothesis that as yet unidentified cell surface receptors mediate the "antiadhesive" effects of tenascin by an active signaling mechanism.

The Counteradhesive Protein, Thrombospondin. Similarly, endothelial cells that were plated onto a substrate of thrombospondin attached but failed to spread out or to form focal adhesions.[47] Moreover, addition of thrombospondin to endothelial cells that had already formed stable focal adhesions on a fibronectin substrate stimulated focal adhesion disassembly through interactions that involved the heparin-binding domain of thrombospondin.[47] Thus tenascin and thrombospondin can be viewed as adhesion modifiers that act to regulate focal adhesion assembly and disassembly in cells attached to a fibronectin-rich ECM.

Rapid Increase of Tenascin Gene Expression after Arterial Injury. We examined the expression of tenascin during repair of balloon injury in the rat carotid model. Tenascin mRNA was undetectable in normal, uninjured rat carotid arteries. Similarly, Mackie and associates[37] found only barely detectable amounts of tenascin protein in the media of elastic arteries by immunostaining. By 6 hours after injury, large increases in tenascin mRNA levels were observed in injured left carotid arteries (Fig. 4). Tenascin gene expression remained elevated for 2 weeks, by which time a neointima had formed that was equal in thickness to the original media.

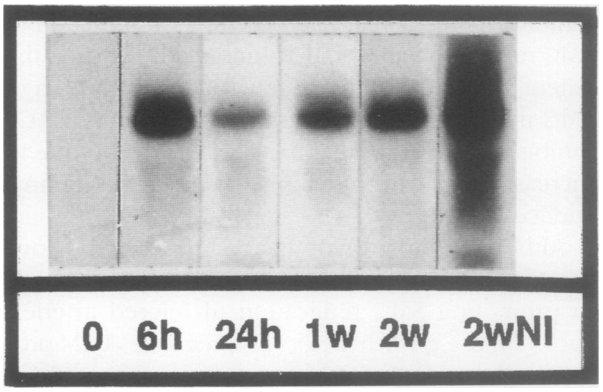

Fig. 4 *Tenascin mRNA levels in balloon-injured rat carotid artery. Total cellular RNA was isolated at the times indicated after left carotid injury, subjected to electrophoresis (10 μg/lane), and hybridized with a ^{32}P-labeled murine tenascin cDNA probe.*

h = hours; NI = neointimal surface; w = weeks

The distribution of tenascin protein in injured rat carotids was examined by immunostaining with a previously characterized polyclonal antibody raised against purified rat tenascin.[37] We observed large increases in immunoreactive protein in the developing neointima and in the adventitia, with much smaller increases in the media itself. The increased staining in the neointima seen 2 weeks after injury was similar to that reported by Hedin's group in 1991.[16] The intense staining for tenascin in the newly forming neointima, together with the absence of tenascin in platelets or in plasma,[48] strongly suggests that neointimal SMCs themselves are the source of tenascin synthesis. We used en face, in situ hybridization analysis to show that within small focal clusters of SMCs that are among the first to appear at the luminal surface after injury, nearly all of the SMCs exhibited strong hybridization to tenascin antisense riboprobes (Fig. 5). Moreover, in the regenerating endothelium, cells at the leading edge of the regenerating sheet were uniformly positive for tenascin expression, whereas endothelial cells at points further back in the monolayer were negative (Fig. 5).

α-Thrombin-Stimulated Tenascin Synthesis by Cultured SMCs. Although the conversion of stationary SMCs to cells capable of active migration is thought to be an important step in vascular wound repair, the factors that stimulate the production of counteradhesive matrix proteins by SMCs within injured arteries are not well defined. We tested the ability of the multifunctional wound repair protease α-thrombin, present at nearly all sites of acute vascular injury in vivo, to stimulate the production of tenascin and thrombospondin by rat aortic SMCs in vitro. α-Thrombin reproducibly increased the synthesis and secretion of tenascin (4- to 16-fold) and thrombospondin (2- to 4-fold) in 5 different isolates of rat

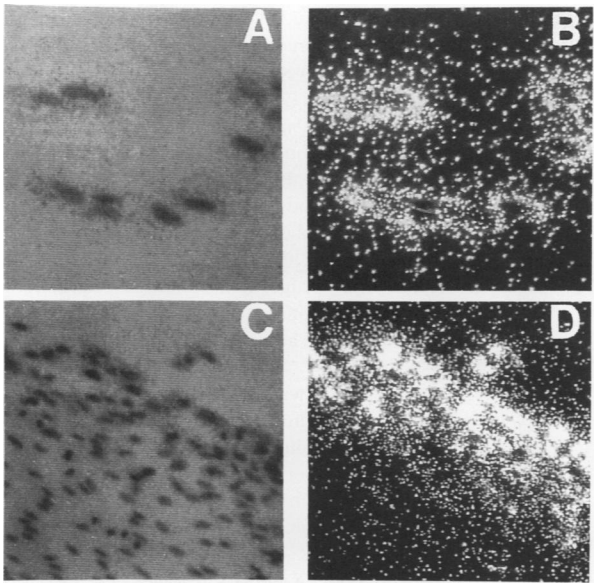

Fig. 5 *Distribution of tenascin mRNA by en face in situ hybridization. Balloon-injured rat carotid arteries were excised 4 days after wounding. The cells present at the luminal surface were examined by an en face Haütchen technique. Cells were hybridized with 35S-riboprobes transcribed from a rat tenascin cDNA clone.* ***A, B****) Neointimal SMCs. Note that essentially all SMCs at the luminal surface are strongly positive for tenascin gene expression.* ***C, D****) Regenerating endothelial cells. Note that the leading edge is strongly positive, but the more distal monolayer is uniformly negative. (orig. x400)*

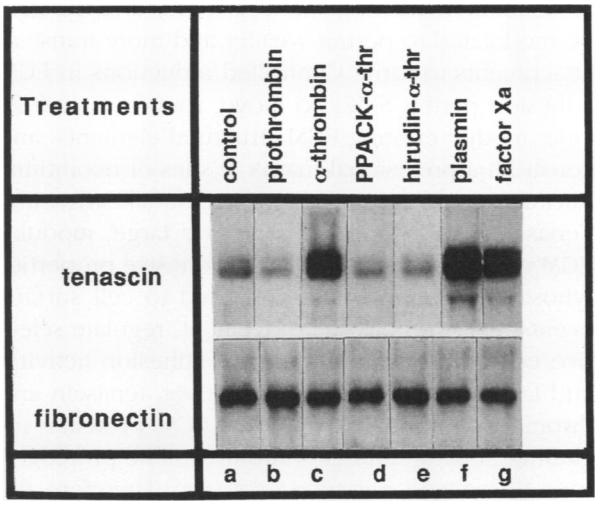

Fig. 6 *Effects of α-thrombin on tenascin mRNA levels in rat aortic SMCs in culture. Rat aortic smooth muscle cells were grown to confluence, growth-arrested for 48 hours, and exposed to the agents indicated for 6 hours. Total cellular RNA was isolated, subjected to electrophoresis (10 μg/lane), blotted, and hybridized with a ^{32}P-labeled murine tenascin cDNA probe. Blot was exposed to film for 18 hours. Prothrombin, α-thrombin, and α-thrombin derivatives were tested at 1 U/mL or equivalent concentration. Plasmin and factor Xa were tested at 0.1 U/mL.*

SMC = smooth muscle cell; α-thr = α-thrombin

aortic SMCs (passage 1 through 12).* Maximal increases in tenascin mRNA levels were found 6 to 8 hours after α-thrombin addition and were completely blocked by preincubation with hirudin, a highly specific thrombin antagonist (Fig. 6). A synthetic peptide mimicking the first 10 amino acids of the tethered ligand sequence of the activated thrombin receptor (SFLLRNPNDK)[49] was a full agonist for tenascin gene expression in rat aortic SMCs. In contrast, peptides inactive as thrombin receptor agonists produced no increases in tenascin mRNA levels. These data suggest that α-thrombin produced at sites of acute vascular injury in vivo may, by itself or in combination with other growth factors, activate tenascin and thrombospondin gene expression in SMCs during arterial wound repair.

Relation of Rat Carotid Repair to Human Restenosis

It is uncertain at this time which of the cellular events described above for repair of balloon injury to rat carotid artery may be most relevant to human restenosis. Recent studies suggest that SMC proliferation, prominent in the rat carotid model, may not be of central importance in human restenosis.[35] This emphasizes the need to know more about other mechanisms that may produce luminal narrowing in damaged human arteries. One such mechanism is the production and organization of a new ECM at sites of intimal injury that could lead to plaque expansion without increases in SMC replication. A similar process occurs in the rat carotid artery late (>1 month) after balloon injury wherein an approximate doubling of intimal thickness is found despite little or no increase in the number of SMCs.[7-19] Large multidomain ECM proteins like tenascin and thrombospondin could play several roles in human restenosis, including 1) selectively reducing adhesion of SMCs to ECM, thereby allowing SMCs to move into areas of active repair of angioplasty injury, 2) binding to and organizing cells and matrix proteins into a nascent tissue network, and 3) expanding intimal plaque volume by virtue of the large size of these proteins (particularly tenascin) and multivalent binding sites for even larger ECM molecules, specifically the proteoglycans.

Summary

Smooth muscle cells in the vessel wall are surrounded by and embedded within a strongly adhesive extracellular matrix (ECM). Structural remodeling and wound repair events require that strong

*Pindur J, Dong XR, Majesky MW. Unpublished observation; August 1993.

attachment interactions between SMCs and the ECM be modulated to permit weaker and more transient attachments to form. Controlled reductions in ECM adhesion permit SMCs to move, round up and divide, modify existing ECM structural elements, and construct a provisional matrix at sites of neointimal thickening, plaque expansion, or luminal narrowing. Tenascin and thrombospondin are large, modular ECM glycoproteins with counteradhesive properties whose expression is tightly linked to cell surface events that promote cell movement, regulate selective cell adhesion, reduce focal adhesion activity, and facilitate tissue repair. Moreover, tenascin and thrombospondin bind to other ECM proteins, including heparan and chondroitin sulfate proteoglycans, fibronectin, and type 1 collagen. Therefore, the large size and multimeric structure of tenascin and thrombospondin may be particularly well suited to organize nascent ECM proteins into a functional tissue network. Synthesis of tenascin, a specialized matrix protein and adhesion modulator, is greatly increased during arterial wound repair. It is reasonable to consider the possibility that inhibition of tenascin expression, either alone or in combination with other proteins involved in SMC movement and wall remodeling, could be an effective strategy to limit restenosis after balloon angioplasty.

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