Increased Expression of Membrane-Type Matrix Metalloproteinase and Preferential Localization of Matrix Metalloproteinase-2 to the Neointima of Balloon-Injured Rat Carotid Arteries

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Background—Remodeling of the injured vascular wall is dependent on the action of several extracellular proteases. Previous studies have shown that expression of matrix metalloproteinases (MMP-2 and MMP-9) is upregulated after vascular injury and that MMP-2 is required for the migration of cultured vascular smooth muscle cells across complex extracellular matrix barriers. The present study examined changes in the expression of membrane-type metalloproteinase (MT–MMP-1), a putative regulator of MMP-2, in the tissue localization of MMP-2, and in the expression of activated and latent forms of MMP-2 and the tissue inhibitor of metalloproteinases, TIMP-2, in rat carotid arteries subjected to balloon catheter injury.

Methods and Results—MT–MMP-1 mRNA levels increased sixfold after 3 days of injury, coinciding with an increase in MMP-2 activation assessed by gelatin zymography. Western blotting and gelatin zymography showed an increase in MMP-2 protein levels beginning 5 to 7 days after injury; immunocytochemistry and Western blotting showed that the increase occurred preferentially in the developing neointima.

Conclusions—These results show that increased expression of MT–MMP-1 and activation of MMP-2 occurs early after injury to the rat carotid artery and that at later times MMP-2 is preferentially localized to the developing neointima. (Circulation. 1998;97:82-90.)

Key Words: restenosis ■ neointima ■ extracellular matrix ■ metalloproteinases ■ carotid arteries

Restenosis remains the “Achilles heel” of percutaneous transluminal angioplasty, occurring in 25% to 50% of patients within 6 months of the procedure.¹–⁴ Numerous attempts to modify the fibroproliferative response to arterial injury, either through pharmacological interventions or mechanical devices, have met with very limited success.⁵–⁹ This failure reflects, in part, the complexity of the pathophysiological process of neointima formation after balloon injury and the difficulty in identifying the appropriate cellular or molecular target(s) for therapeutic intervention.¹⁰–¹² An understanding of the events involved in ECM remodeling, which is required for VSMC migration and, possibly, proliferation,¹³ may provide additional targets for modifying restenosis.

As is the case for atherosclerosis, the lesion formed after percutaneous transluminal coronary angioplasty is partly a result of aberrant smooth muscle accumulation in the intima. This is a consequence of VSMC proliferation and migration stimulated by platelet activation, thrombin generation, and the release of various growth factors and cytokines.¹⁴–¹⁸ Although there is no ideal experimental animal model for restenosis, balloon catheter injury to the rat common carotid artery is the most thoroughly investigated. The first response to vascular injury in the rat is a dramatic increase in the proliferation of VSMCs in the media, which occurs 1 to 3 days after injury.¹⁹,²⁰ The second phase of lesion development, beginning at day 3, involves the migration of proliferating and nonproliferating VSMCs through the internal elastic lamina into the intima,¹⁶,¹⁹ where VSMCs are normally not found in the rat. During the third phase of lesion development, VSMCs proliferate within the intima, with cell numbers reaching a maximum at ≈14 days after injury.¹⁶,¹⁹,²¹

The turnover and remodeling of the ECM have been shown to be important events in a number of physiological and pathological processes, such as blastocyst implantation, wound healing, and tumor invasion.²²–²⁴ VSMCs in vivo are surrounded by and embedded in extracellular matrices, which exert biochemical and mechanical barriers to VSMC movement. ECM degradation and remodeling requires the action of extracellular proteinases,²⁵–²⁷ among which the matrix metalloproteinases have been shown to play an essential role.²⁸–²⁹

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This enzyme family consists of a number of structurally related proteinases capable of degrading a wide array of ECM proteins, including interstitial collagen, basement membrane collagen, denatured collagen (gelatin), laminin, various proteoglycans, and elastin. The MMPs are secreted in latent form and are activated in situ by limited proteolysis. All active MMPs are inhibited by a naturally occurring class of low molecular weight proteins known as the TIMPs.

Previous studies have shown that MMP expression and/or activation is upregulated in human atherosclerotic lesions and in rat arteries after balloon catheter injury. In addition, peptide-based inhibitors of MMP activity have been shown to block VSMC migration both in vivo and in vitro. Our previous in vitro studies had demonstrated that activation of MMP-2 (72 kD type IV collagenase/gelatinase A) is a critical step in the migration of VSMCs through a reconstituted basement membrane similar in composition to that surrounding VSMCs in vivo. Unlike many other MMPs, the closely related MMP-9, the activation of MMP-2 is not mediated by serine proteases such as plasmin but instead through its association with a cell surface activator. A family of cell surface–associated proteins with the potential to activate MMP-2 have recently been identified. These activators are membrane-spanning metalloproteinases, with the prototype being MT–MMP-1 (or MMP-14). The present study was undertaken to characterize the changes in the in vivo expression of MMP-2 and MT–MMP-1 after vascular injury.

Methods

Carotid Injury

Six-month-old male Wistar rats were used for all studies. Injury of the left common carotid artery was performed in the standard fashion. Briefly, animals were anesthetized with sodium pentobarbital (40 mg/kg body wt), ketamine (2 mg/kg body wt) and xylazine (8 mg/kg body wt), given intraperitoneally. A 2F Fogerty balloon catheter (Baxter) was inserted through an incision made in the external carotid artery and advanced along the length of the common carotid artery to the aortic arch. The balloon was then inflated and passed three times along the length of the carotid artery and advanced along the length of the common carotid artery to the aortic arch. The balloon was then inflated and passed three times along the length of the carotid artery. The balloon catheter was removed and the external carotid artery was permanently ligated. Animals were killed at varying time intervals, and completeness of surgery and the second in which the vessels were extracted from sham-operated animals (anesthetized and operated but no catheter inserted).

Neointimal Tissue Isolation

Fourteen days after injury, the carotid artery was isolated and the adventitia stripped away from the vessel. The vessel was then incised longitudinally and the neointima was dissected away from the media with the use of fine forceps and a dissecting microscope. The medial and neointimal cell layers were then snap-frozen in liquid nitrogen.

RNA Isolation and Analysis

RNA was isolated from vessels that had been snap-frozen in liquid nitrogen by use of the guanidium isothiocynate procedure except that 20 μg of nuclease-free glycogen (Molecular Biology Grade, Boehringer Mannheim) was added before ethanol precipitation to act as a nonspecific carrier. For RNA used in Fig 1, an additional proteinase K digestion step was included to remove possible contaminating protein. Each sample represents a pooling of RNA from three arteries. Northern blotting with 4 μg of total RNA was performed as previously described. Each sample represents a pooling of RNA from three arteries. Northern blotting with 4 μg of total RNA was performed as previously described. Each sample represents a pooling of RNA from three arteries. Northern blotting with 4 μg of total RNA was performed as previously described. Each sample represents a pooling of RNA from three arteries. Northern blotting with 4 μg of total RNA was performed as previously described. Each sample represents a pooling of RNA from three arteries.

A cDNA probe for rat MMP-2 has been described previously. The rat TIMP-2 cDNA was obtained by screening a rat aortic VSMC cDNA library (AZAP II, Stratagene Cloning Systems, La Jolla, Calif) with a partial human TIMP-2 cDNA. The authenticity of these probes was verified by dideoxy sequencing. 18S rRNA was detected with an oligonucleotide probe as described previously.

A rat MT–MMP-1 (MMP-14) cDNA probe for Northern blotting was obtained by screening a rat VSMC cDNA library with an 823 bp probe for human MMP-14 that was generated by reverse transcription–polymerase chain reaction (RT-PCR) with total RNA from concanavalin A–treated (100 μg/mL) HT1080 cells and oligonucleotide primers whose sequences were based on the published sequence for human MT–MMP-1. The sense primer (5’-CCAAGATCTGT GACGGAAACTTG-3’) corresponds to amino acids 317 to 324 and is located in a region of the protein that is conserved among many other MMPs. The antisense primer (5’-CCTTGTCACAGGACCTGTCG-3’).
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GAAC-3′) corresponds to the terminal 4 amino acids of the protein as well as some of the 3′ untranslated region and is unique for MT-MMPs. Screening of the rat cDNA library with this probe resulted in the isolation of an approximately 2300 bp rat cDNA for MT-MMP (pBS–MMP–14/9) encoding the entire rat MT-MMP-1 protein. The DNA sequence of pBS–MMP–14/9 was identical to that of rat skin MT-MMP-1.1,2

**Western Blotting**

Individual carotid arteries or isolated medial and neointimal cell layers were minced with a razor blade and extracted in 250 μL of a solution containing 50 mmol/L Tris-HCl, pH 7.6, 0.2 mol/L NaCl, 5 mmol/L CaCl₂, and 0.02% Brij-35 (wt/vol), using a tissue homogenizer. Samples were centrifuged at 10 000 rpm for 10 minutes and the supernatants were used immediately or stored at −70°C. Protein concentration was measured using bovine serum albumin as a standard and the BCA reagent (Pierce Chemical). Extracts containing equal amounts of protein (10 μg) were denatured by boiling for 5 minutes in 50 mmol/L Tris-HCl, pH 6.5, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 μmol/L dithiothreitol (final concentrations) and separated on 10% SDS-polyacrylamide gels at 120 V. The protein bands were then transferred to nitrocellulose membranes by a semidry technique (Bio-Rad Laboratory, Hercules, Calif) at 10 V/cm for 3 hours. The membrane was rinsed and blocked with 5% nonfat dry milk for 1 hour and then incubated overnight at 4°C in 0.05% Tween 20–phosphate-buffered saline with a rabbit polyclonal antibody to MMP-2 (Ab 45) at a concentration of 1 μg/mL. Immune complexes were detected with a horseradish peroxidase–conjugated anti-rabbit antibody and a chemiluminescent detection method (ECL, Amersham Corp, Arlington Heights, Ill). The results of immunoblotting were quantified with an Ultrascan XL–enhanced laser densitometer (Pharmacia LKB Biotechnology Inc).

**Immunohistochemistry**

Isolated carotid arteries were fixed for 3 to 4 hours in 10% formalin and transferred to 70% ethanol. The sections, embedded with the use of low-temperature paraffin, were cut 5 μm thick and placed on silanated slides. After deparaffinization and hydration, the endogenous peroxidase was blocked by immersion in 2 mL 30% H₂O₂ and 98 mL of methanol for 30 minutes at 21°C. After adding serum blocking solution (Zymed Labs), MMP-2 antibody (Ab 45) was applied and incubated overnight at 4°C. Subsequent washing, the addition of biotinylated secondary antibody, and color development were carried out as described by the manufacturer (Zymed Labs). Control slides were treated with nonimmune serum instead of primary antibody.

**Gelatin Zymography and Quantitation**

Arterial extracts prepared as described for Western blotting were denatured in 50 mmol/L Tris-HCl, pH 6.5, 2% SDS, 0.1% bromophenol blue, and 10% glycerol (final concentrations) and then subjected to electrophoresis in 10% SDS–polyacrylamide gels containing 0.1% (wt/vol) gelatin (Novex Chemical). All gels were run under nonreducing conditions. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 30 minutes at 21°C and then incubated at 37°C for 18 hours in 50 mmol/L Tris-HCl, pH 7.5, 0.2 mol/L NaCl, 5 mmol/L CaCl₂, and 0.02% Brij-35. Gels were stained with Coomassie blue R-250. Both latent and active forms of metalloproteinase produce clear areas of lysis in the gel. Immunoprecipitation of extracts was performed before zymography as described previously.36 rMMP-2 was used for standardization and was generously provided by Dr Rafi Fridman (Wayne State University, Detroit, Mich). rMMP-2 was converted from its latent to active form with APPA as previously described.36 Quantitation of lysis was performed by scanning the gels on a Molecular Devices Personal Densitometer and analyzing the image with Image Quant 3.3 software. Standard curves were generated with nonactivated or APPA-activated recombinant human MMP-2 and the plots of picograms of enzyme against gel band area (OD×nm) were found to be linear over the range of 1 to 100 pg for both nonactivated (latent) and activated MMP-2. Arterial extracts were then diluted until their gel band areas were within the linear range of the assay.

**Results**

Total RNA was isolated from carotid arteries at various times after balloon catheter injury and analyzed by Northern blotting for the expression of MT–MMP–1 (MMP–14), MMP–2, and the tissue inhibitor of metalloproteinase activity TIMP–2. Fig 1a shows a representative Northern blot of total RNA isolated from control and balloon-injured vessels and hybridized with a cDNA probe for rat MT–MMP–1. A graph of the results from 4 different Northern blots representing 12 different injured vessels is shown in Fig 1b. MT–MMP–1 mRNA levels were relatively low in uninjured vessels but increased significantly in balloon–injured vessels as early as 1 day after injury. By 3 days after injury, the relative mRNA levels had increased sixfold over uninjured vessels. MT–MMP–1 mRNA levels then returned to control values by 7 days after injury.

Figs 2A, 2B, and 2C show a Northern blot from one series of pooled RNA samples for MMP–2, TIMP–2, and 18S rRNA, respectively. Fig 2D is a graphical representation of the data from 4 sets of pooled samples (representing 12 vessels in all). Although MMP–2 mRNA levels appear to rise over the
course of injury, 18S rRNA also rose. This may be due to changes in the amount of 18S rRNA relative to total RNA occurring after injury as the cellular composition of the vessel changes but is more likely a consequence of contaminating proteins in the RNA preparation resulting in altered spectrophotometric determination of total RNA. This explanation is consistent with the fact that when RNA is treated with proteinase K, which was done in later preparations of total RNA, a uniform hybridization profile for 18S rRNA is seen (Fig 1). When the MMP-2 mRNA signal is normalized to 18S rRNA, there was no significant change in MMP-2 mRNA observed over the 28 days after balloon injury, although there was a trend toward reduced expression early (1 day) after injury. Two TIMP-2 mRNA species were observed in the cell extracts (Fig 2B)—one at 3500 bp and another at 1000 bp. No significant change was observed in the level of the 1000 bp TIMP-2 mRNA after day 1 of injury, but there was a significant reduction in the 3500 bp mRNA levels at days 5, 7, and 14 after injury. Total TIMP-2 mRNA (1000 bp + 3500 bp), however, did not significantly change over the course of injury.

Western blot analysis performed on equal amounts of total protein from different vessel extracts with an affinity-purified antibody to MMP-2 showed that in contrast to the mRNA data, MMP-2 protein levels in the vessels increased significantly from 5 days up to 1 month after injury (Fig 3A). When these data were combined with that of three other independent Western blotting analyses, MMP-2 protein levels were found to increase fourfold after injury and to remain elevated even at 28 days after injury (Fig 3B). Immunohistochemistry was performed on cross sections of the vessels to localize the specific area of the vessel wall where increased MMP-2 expression was occurring (Fig 4, A through H). In uninjured vessels, endothelial cells stained strongly and VSMCs weakly for MMP-2 (Fig 4A). Control preparations with nonimmune serum used in place of the primary antibody showed virtually no background or nonspecific staining (Fig 5A). VSMCs within the media continued to stain with anti-MMP-2 at early time points after injury, and there appeared to be no grossly visible differences in staining intensity during these times (Fig 4, B and C). Between 5 and 14 days after injury, overall staining of the vessel increased, with most of the increased staining confined to the neointima (Fig 4, D through G). Staining of the vessel then decreased to control levels by 2 months after injury (Fig 4H). Fig 5B shows a lower-power view of a stained injured vessel at 14 days after injury to demonstrate that staining is confined to the neointima and media and is not in the adventitia.

To corroborate the observation of increased expression of MMP-2 in the neointima compared with the medial cell layer and to quantify the difference, separate extracts of these layers from 14 days after injury vessels were prepared and analyzed by Western blotting (Fig 6A). Quantification of the result indicated that the neointima expressed approximately 4 times the amount of MMP-2 than did the media (Fig 6B). MMPs are secreted as inactive zymogen that must be activated by cleavage of an N-terminal "pro" segment before they are capable of enzymatic activity. As a possible physiologic activator of MMP-2, the increase in MT-MMP-1 expression after balloon injury (Fig 1) could lead to MMP-2 activation. To directly measure MMP-2 activation, changes in the mobility of MMP-2 as seen by gelatin zymography were assessed in the vessel extracts. Fig 7A shows a representative zymogram of tissue extracts from uninjured and injured vessels. Two bands migrating in the 68 to 72 kD molecular mass range were seen. On the basis of control experiments described in Fig 7B, it was possible to assign the upper and lower bands of latency as the latent and activated forms of MMP-2 (labeled L and A), respectively. Also shown in Fig 7A is the transient presence of a band migrating below the 96 kD molecular weight marker, which may represent 92 kD type IV collagenase (MMP-9). This band appears within 1 hour of injury but disappears between 3 and 5 days after injury.

Fig 7C shows a graphical representation of the changes in the relative content of activated to latent MMP-2 in the vessels after injury. Although uninjured vessels contained both bands, there was a large and significant increase in the ratio of activated to latent MMP-2 between 1 and 5 days after injury. The ratio remained elevated up to 14 days after injury and then declined to uninjured levels between 21 and 28 days after injury.

Discussion

The remodeling of the vascular wall in response to injury is critically dependent on the action of extracellular proteases, such as the MMPs, and is accompanied by the migration and proliferation of VSMCs. A number of laboratories have shown that expression of the type IV collagenases/gelatinases (MMP-9 and MMP-2) are upregulated after vascular inju-
The present results extend these observations by providing quantitative analyses of the changes in expression of activated and latent forms of MMP-2 and localizing its increased expression to the developing neointima. In addition, we show that TIMP-2 mRNA levels decrease during the development of the neointima and that the expression of MT-MMP-1, a membrane-spanning metalloproteinase and putative regulator of MMP-2, is markedly increased early after injury and preceded the changes in MMP-2 activation.

MMP activity can be increased through a number of different mechanisms, including an increase in MMP protein levels, increases in the relative amount of the protein that has been processed by limited proteolytic digestion to its activated form, and reduction in specific inhibitors of activity and activation, such as the endogenous TIMPs. We show that MMP-2 protein levels increase after injury (Fig 3) and that the increase occurred without an apparent concomitant increase in MMP-2 mRNA levels (Fig 2). The increase in MMP-2 protein levels was primarily confined to the intima, which was evident from immunocytochemistry of the vessels and Western blotting analyses of the separated neointimal and medial cell layers. The data in Fig 3 on MMP-2 protein levels and in Fig 7A on zymogram activity show a slight reduction in expression between 14 and 28 days after injury, which on the surface seems contradictory to the intense staining seen in the neointima at 21 days. However, these measurements were made on extracts of the entire vessel (media + neointima), and localized changes in MMP-2 protein expression in the neointima are likely to be obscured by the lack of such changes in the media and some of the neointima. The difference in MMP-2 protein expression in the medial and neointimal cell layers after injury may reflect differences in the translational efficiency or protein stability of MMP-2 expression by medial and neointimal VSMCs reflect the active growth state of the neointima, or a response to hemodynamic stress at the interface of the lumen and the neointima.

Because TIMP-2 forms a unique complex with MMP-2, is more effective than TIMP-1 in inhibiting MMP-2 activity, and inhibits not only MMP-2 activity but also its activation (ie, the proteolytic activation of the latent enzyme), TIMP-2 mRNA levels before and after injury were measured by Northern blotting. As is shown in Fig 2 and has been described by others, two mRNA transcripts were detected. Both of these transcripts have been shown to encode functional
TIMP-2 proteins. Vessel injury caused a redistribution of the relative amounts of the two mRNA species, increasing the relative expression of the 1000 kb species after injury (Fig 2). This was due primarily to a reduction in expression of the 3500 kb species. As a consequence, overall TIMP-2 mRNA levels remained essentially unchanged. We also show that there is an overall increase in the relative amount of activated MMP-2. Activation of MMP-2 is not mediated by serine proteases, such as plasmin, as is the case for other MMPs. Instead, protease inhibitor studies suggest that MMP-2 is activated by metalloproteinases themselves and requires interaction with cell surface protein(s). This unique requirement for MMP-2 activation may ensure that its profound proteolytic ability is localized to either migrating cells or those involved in ECM turnover. Recent studies have identified one possible cell surface activator of MMP-2, which has now been cloned and designated as membrane type-MMP (MT-MMP-1). MT-MMP-1 contains a single transmembrane domain that positions its catalytic domain on the exterior surface of a cell. MMP-2 binds MT-MMP and becomes activated possibly through its limited proteolysis by MT-MMP-1. In cell culture, MT-MMP-1 expression can be stimulated by the lectin concanavalin A, suggesting that clustering of cell surface molecules can lead to its increased expression. Consistent with this mechanism is the observation that in dermal fibroblasts, MMP-2 activation is blocked by a peptide that inhibits the binding of the integrin αβ to collagen. Whether this activation occurs through MT-MMP-1 expression is not known. It is nonetheless intriguing that while α integrin levels are undetectable in the VSMCs of uninjured vessels, they are readily detected in cultured VSMCs, which express high levels of MT-MMP-1 (M.T. Crow and C. Bilato, unpublished observations, NIA-NIH, 1997). If α integrin levels were to increase in vessels after injury, a possible connection between ECM-integrin interactions and MMP-2 activation could exist.

Another possible connection between MMP-2 activation and integrin expression is the recent study showing that the integrin complex αβ, which is upregulated in response to injury and linked to the activation of intracellular signaling pathways necessary for VSMC migration, is a cell-surface binding site for MMP-2. Binding to αβ, leads to activation of MMP-2, presumably by causing conformational alterations in the protein that make it susceptible to autocatalysis. Both MT-MMP-1 and αβ represent not only novel ways by
which MMP-2 can become activated but also a mechanism to localize the proteolytic activity of MMP-2 to the migrating VSMCs, and both may play significant roles in regulating the biological activity of MMP-2. A dual mechanism for activation and localization may explain why in our data, MMP-2 activation remains elevated after MT–MMP-1 expression returns to baseline levels. When MT–MMP-1 mRNA levels return to baseline soon after the onset of injury, a \( \alpha \beta \) integrin levels may remain elevated, supporting increased activation of MMP-2.

Finally, we also saw a significant reduction in the mRNA levels for the endogenous MMP inhibitor TIMP-2, which could translate into increased activity and activation of MMP-2. TIMP-2 may be particularly relevant to the MMP-associated events in vessel injury, since it is 2 times and 10 times more effective in inhibiting MMP-2 and MMP-9 activity, respectively.38 The expression and/or activation of both of these MMPs is a consistent finding after balloon catheter injury in a number of species, including the rat,15,18 pig,19 and rabbit.41 In addition, TIMP-2 has been shown to inhibit surface activation of MMP-2.39,40

The functional significance of MMP expression and activation after injury to the vessel has been partially addressed with the use of synthetic peptide inhibitors of MMPs. Using the balloon-injured rat carotid artery model, it has been shown that a peptide MMP inhibitor effectively blocked the early development of the neointima, presumably by interfering with the early migration of vascular cells.22 At 14 days after injury, however, no difference in neointima thickening between untreated and peptide-treated vessels could be observed, as the result of the persistent proliferation of those cells in the intima.34 It is unclear whether these observations mean that MMP inhibitor therapy for the treatment of restenosis is questionable or merely reflect the inappropriateness of the rat balloon injury model for human restenotic disease. A recent
study in a double-injury rabbit model did show prolonged efficacy of the same MMP peptide inhibitor on the development of the second neointima, which in contrast to the first neointima contains a higher percentage of ECM. This type of lesion is reminiscent of human restenotic lesions that contain large amounts of ECM material and usually form on an existing intimal cell layer. The effectiveness of MMP inhibition in this situation may be the consequence of unexpected effects on inhibiting collagen accumulation in the neointima. Although rat neointimal lesions are mostly cellular, it is of interest that we see preferential localization of MMP-2 expression to the developing neointima of the rat that persists beyond the period in which cell recruitment to the neointima is thought to occur. MMP-2 may therefore be involved in the control of collagen accumulation in the developing neointima and MMP inhibitors may have better therapeutic potential in situations in which vascular lesions are predominantly ECM rich.

In summary, we show that MMP-2 protein levels are significantly increased in the developing neointima after balloon injury of the rat carotid artery. The relative amount of activated MMP-2 also increases after injury, with the increase coinciding with VSMC migration and preceded by increased expression of a potential cell surface MMP-2 activator, MT-MMP. Expression of the 3500 kb mRNA species for TIMP-2 decreases during injury, whereas the 1000 kb mRNA species remains unchanged. These results identify a number of control points through which MMP-2 activation and activity is regulated after balloon injury to the rat carotid artery. The elevated expression of MMP-2 protein in the neointima during a period in which contribution of VSMC migration to neointimal development is thought to be minimal suggests that MMP-2 may play an important role in extracellular matrix remodeling unrelated to promoting cell movement.

References


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