Matrix Metalloproteinase-9 Overexpression Enhances Vascular Smooth Muscle Cell Migration and Alters Remodeling in the Injured Rat Carotid Artery


Abstract—Matrix metalloproteinase-9 (MMP-9) has been implicated in the pathogenesis of atherosclerosis as well as intimal hyperplasia after vascular injury. We used Fischer rat smooth muscle cells (SMCs) overexpressing MMP-9 to determine the role of MMP-9 in migration and proliferation as well as in vessel remodeling after balloon denudation. Fischer rat SMCs were stably transfected with a cDNA for rat MMP-9 under the control of a tetracycline-regulatable promoter. In this system, MMP-9 was overexpressed in the absence, but not in the presence, of tetracycline. In vitro SMC migration was determined using a collagen invasion assay as well as a Boyden chamber assay. In vivo migration was determined by measuring the invasion into the medial and intimal layers of transduced SMCs seeded on the outside of the artery. Transduced SMCs were also seeded on the luminal surface, and the effect of local MMP-9 overexpression on vascular structure was measured morphometrically at intervals up to 28 days. MMP-9 overexpression enhanced SMC migration in both the collagen invasion assay and Boyden chamber in vitro, increased SMC migration into an arterial matrix in vivo, and altered vessel remodeling by increasing the vessel circumference, thinning the vessel wall and decreasing intimal matrix content. These results demonstrate that MMP-9 enhances vascular SMC migration in vitro and in vivo and alters postinjury vascular remodeling. (Circ Res. 1999;85:1179-1185.)

Key Words: matrix metalloproteinase-9 • migration • rat • smooth muscle cell • remodeling

Remodeling of the vessel wall in response to injury involves smooth muscle cell (SMC) proliferation and migration,1,2 apoptosis,3 and changes in the extracellular matrix in part mediated by matrix metalloproteinases (MMPs).4 Arterial injury induces MMP expression, and MMP-9 specifically is transiently upregulated in the first 24 hours.5,6 MMP-9 degrades the vessel wall collagens (types III, IV, and V), elastin, entactin, and vitronectin.7 Active synthesis is correlated with unstable angina and plaque disruption in the coronary and carotid arteries8–10 and with the development of abdominal aortic aneurysms.11 Like MMP-2, MMP-9 has been shown to be required for SMC migration.12,13 To test the hypothesis that MMP-9 plays a role in SMC physiology and vascular remodeling, rat aortic SMCs were transfected with the cDNA for rat MMP-9 under the control of a tetracycline-regulated promoter (TnA). This approach permitted us to examine the effects of MMP-9 on vascular SMC migration through matrix components in vitro and in vivo and of local metalloproteinase overexpression on postinjury vascular remodeling.

Materials and Methods

Transfection
Fischer 344 SMCs were cotransfected with pSV2NEO plus a plasmid containing tTA protein controlled by the Tet operator.14 Using transient transfection with a tetracycline-dependent β-galactosidase construct, a clone showing low activity with tetracycline and high activity without tetracycline was selected for stable transfection. We used a hygromycin-resistance plasmid and Tet-regulated expression vector with both MMP-9 (from Dr P. Basset) and a myc-tagged β-galactosidase reporter gene under control of TnA-dependent promoters.

Cell Culture
Cells were maintained in DMEM with 10% calf serum and 1 mg/mL tetracycline. To compare cells expressing or not expressing MMP-9, cells were washed twice with PBS and maintained 48 hours in 10% serum with or without tetracycline.

SMC Proliferation
SMC(tTA-MMP-9) cells were plated at 5000 cells/cm² in 10% serum with or without tetracycline. Cells were counted daily in quadruplicate (n=3).

SMC Migration

In Vitro
Two methods were used. First, microchemotaxis chambers with 10-µm-pore filters coated with Matrigel were used with platelet-derived growth factor (PDGF)-BB as a chemotactant.15 For conditioned-medium experiments, normal SMCs were suspended in serum-free medium conditioned 48 hours by SMC(tTA-MMP-9) cells in the presence or absence of tetracycline. Second, 10% F-127 pluronic gel containing PDGF-BB (20 ng/mL; 2 mL/cm²) was overlaid by collagen (1 mL).
Vitrogen). SMC(TA-MMP-9) cells, suspended in 10% calf serum (Tet+ or Tet−) with 5 mmol/L hydroxyurea, were plated on the collagen at 1.0×10^4 cells/cm^2. The MMP inhibitor, BB-94 (1 μmol/L), was used with DMSO controls in some experiments (in the top chamber for chemotaxis). The number of cells migrating into the collagen after 48 hours was determined by phase contrast microscopy.

**In Vivo**

The left carotid artery was stripped of adventitia and flushed with saline. A steel spatula chilled in liquid nitrogen was applied for 2 seconds. The artery thawed for 30 seconds. Freezing/thawing was confirmed visually, and this procedure was repeated 3 times. Medium without or with transduced cells (2.5×10^5 cells/mL; 0.5 mL) was flooded over the vessel after flow was restored. After 15 minutes, the wound was closed.

Rats received tetracycline (1 mg/mL, in 2.5% sucrose; 100 mg/kg per day) or vehicle alone in their drinking water. BB-94 or vehicle was given daily (30 mg/kg per day IP). After 7 days animals were perfusion fixed. The number of intimal and medial cells in cross sections from the center of the injured area was determined to avoid SMCs migrating from uninjured regions.

Animal care and procedures were conducted at the University of Washington Medical Center in accordance with state and federal laws and under protocols approved by the University of Washington Animal Care and Use Committee. Animal care complied with the Principles of Laboratory Animal Care as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (US Department of Health and Human Services, NIH Publication No. 80-23, revised 1985).

**Luminal SMC Seeding**

SMC seeding was performed as described. Rats were treated with tetracycline or vehicle as above beginning 2 days before surgery and were euthanized after 7, 14, or 28 days. One group received tetracycline or vehicle as above beginning 2 days before surgery and were euthanized after 7, 14, or 28 days. One group received tetracycline (Tet-) or vehicle alone in their drinking water. BB-94 or vehicle was given daily (30 mg/kg per day IP). After 7 days animals were perfusion fixed. The number of intimal and medial cells in cross sections from the center of the injured area was determined to avoid SMCs migrating from uninjured regions.

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**Zymography and Western Blotting**

Arteries were extracted in 50 mmol/L Tris, 0.2% Triton X-100, 10 mmol/L CaCl_2, and 2.0 mol/L guanidine hydrochloride, pH 7.5. Equal amounts of protein (10 to 20 μg) or of conditioned medium from cells grown under serum-free conditions for 48 hours were loaded, and gelatin zymography or reverse zymography was performed. Western blotting was performed using 5 μg/mL anti-MMP-9.

**Statistics**

Results are expressed as mean±SEM. The Wilcoxon signed-rank and Mann-Whitney tests were used for in vitro and in vivo experiments, respectively. Differences of P<0.05 were considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**In Vitro**

**Metalloproteinase Expression and Cell Proliferation**

Conditioned medium collected from SMC(TA-MMP-9) cells maintained with tetracycline showed a faint gelatinolytic band at ~116 kDa, which was dramatically increased when tetracycline was removed (Figure 1A). This band was identified as MMP-9 by an antibody specific for rat MMP-9 (Figure 1B). Treatment of the conditioned medium with p-aminophenylmercuric acid increased the amount of the ~80-kDa band, present in small amounts in Tet− cells, consistent with this band being activated MMP-9. A 71-kDa band (MMP-2) was present in all SMCs grown in culture. Overnight incubation of the gelatin zymogram with tetracycline before staining to evaluate whether it had any direct effect on metalloproteinase activity demonstrated no significant effect of tetracycline on MMP activity at doses up to 50 μg/mL.

SMC growth in a 5-day period was not affected by MMP-9 overexpression (final cell densities, Tet+, 2.5±0.7×10^4 cells/mm^2; Tet−, 1.9±0.5×10^3 cells/mm^2; P=NS). In addition, there were no differences in cell morphology (data not shown).

**SMC Migration**

In the Boyden chamber using a filter coated with Matrigel, overexpression of MMP-9 did not alter migration (data not shown). Because this could result from low levels of MMP-9 produced during the 5-hour experiment, we studied migration of nontransfected Fischer rat aortic SMCs incubated in the presence of 48-hour conditioned medium harvested from Tet− cells overexpressing MMP-9. These cells demonstrated a significant increase in migration to PDGF-BB over cells exposed to conditioned medium from Tet+ cells overexpressing MMP-9. This increase was completely blocked by BB-94 but not by DMSO (data not shown). In addition, the Tet− SMCs overexpressing MMP-9 demonstrated a ~2-fold increase in migration through 3-dimensional collagen gel over 48 hours compared with Tet+ SMCs. The MMP inhibitor BB-94 also abolished these differences (Figure 2).

**In Vivo**

**Metalloproteinase Expression**

Ten days after internal cell seeding, rat carotid arteries were harvested and processed for zymography. The vessels of animals treated with tetracycline (Tet+) demonstrated primarily 62-kDa and 57-kDa bands, which we believe represent active MMP-2 as shown by Sang et al. and barely visible bands at 116, 71, and 66 kDa. Vessels of animals not treated with tetracycline (Tet−) showed large increases in bands at 116, 80, 71, and 66 kDa. We believe that the 116-kDa band is the proform of MMP-9 and the 80-kDa band is the active MMP-9 as observed by O’Connell et al. (Figure 3). The 71-kDa and 66-kDa bands are consistent with proMMP-2. However, because these bands increased after inducing MMP-9, it is possible that these are MMP-9
control vessels. We were unable to detect TIMP-3. It has been no differences between Tetlated. TIMP-2 was identified in all tissue samples, but there were internal cell seeding to determine whether TIMPs were upregu-

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Tissue Inhibitor of Metalloproteinase (TIMP) Activity in Tissues

We performed reverse zymography on carotid extracts after internal cell seeding to determine whether TIMPs were upregulated. TIMP-2 was identified in all tissue samples, but there were no differences between Tet+, Tet−, and uninjured contralateral control vessels. We were unable to detect TIMP-3. It has been shown previously that there is no significant TIMP-1 expression at 7 days after balloon injury in the rat carotid model.26 We were unable to identify any significant TIMP-1 expression in seeded vessels at 10 days (data not shown). The minimal TIMP-1 expression detected was the same in Tet+ and Tet− groups and was comparable with the uninjured contralateral carotid.

Morphometry Analysis

MMP-9 overexpression by SMCs seeded on the luminal surface produced significant changes in vessel morphology at 14 days (Figure 4). MMP-9 overexpression caused an increase in luminal area and vessel dilation as determined by an increase in internal elastic lamina (IEL) length at 14 days which persisted at 28 days. Some animals were treated with tetracycline for the first 14 days after seeding and without tetracycline for the subsequent 14 days to produce delayed MMP-9 upregulation (Tet+/Tet−). These vessels were dilated and exhibited increased luminal area at 28 days (Figures 5A and 5B). Positive remodeling, defined as an increase in luminal area without an increase in wall thickness and expressed as luminal index (luminal diameter/cross sectional wall thickness), occurred in the Tet− group compared with the Tet+ group at 14 days (Figure 5C). Intimal area was less in the Tet− group at 7 days but was equal to the Tet+ group at 14 and 28 days (Figure 5D). Nuclear density in the MMP-9–seeded intimas was greater in the Tet− group at 7, 14, and 28 days (Figure 6) than that of the Tet+ group. The volume fraction of extracellular matrix in the intima as measured by electron microscopy was decreased in the MMP-9–overexpressing Tet− group at 14 days (55.0±3.4% [Tet−] versus 68.1±1.7% [Tet+], P<0.05, n=4).

We also seeded a control series of rats using SMCs transfected with the identical tetracycline promoter system and the reporter gene luciferase. There were no significant differences between the Tet+ and Tet− groups in vessel circumference at 7 days (2.2±0.1 mm [Tet+] versus 1.9±0.1 mm [Tet−], P=NS, n=4) or at 14 days (2.3±0.1 mm [Tet+] versus 2.1±0.1 mm [Tet−], P=NS, n=4 to 5). Luminal area also showed no significant differences at 7 days (0.25±0.03 mm2 [Tet+] versus 0.20±0.01 mm2 [Tet−], P=NS) or 14 days (0.12±0.03 mm2 [Tet+] versus 0.14±0.01 mm2 [Tet−], P=NS). The intimal areas were not significantly different at 7 days (0.14±0.01 mm2 [Tet+] versus 0.10±0.02 mm2 [Tet−], P=NS), although the intimal area of the Tet+ luciferase-seeded group was larger at 14 days (0.30±0.03 mm2 [Tet+] versus 0.21±0.01 mm2 [Tet−], P<0.05). There was no difference in nuclear density in the control luciferase-seeded intimas at 14 days (6571±473 cells/mm2 [Tet+] versus 6673±280 cells/mm2 [Tet−], P=NS).

SMC Proliferation

We measured the neointimal BrdU labeling index in the luminally seeded vessels at 7 days after seeding. There was no difference in labeling index between Tet+ (17.7±1.4%) and Tet− (20.0±1.7%, P=NS, n=6) seeded animals at 7 days.

SMC Migration

Extraluminal SMC Seeding of Rat Carotid Artery

We decellularized the carotid artery by snap-freezing and seeded SMCs(TA-MMP-9) cells on the adventitial surface and stained for α-actin (Figure 7A). At 7 days, there were significantly more SMCs (α-actin positive) in the media and intima in the Tet− group than the Tet+ group. Carotid arteries frozen but treated with medium alone showed no cells in either the media or intima in any of the segments (n=5) (Figure 7B). Treatment of the animals with the metalloproteinase inhibitor BB-94 abolished the enhanced migration seen with MMP-9 overexpression (Figure 7C).

Discussion

Increased metalloproteinase activity may contribute to the arterial pathology seen in aneurysmal degeneration,11,27 atherosclerotic
plaque formation, and disruption,\textsuperscript{28,29} as well as intimal hyperplasia after angioplasty and surgical reconstruction.\textsuperscript{30,31} Vascular SMCs predominate in the vessel wall and play a central role in its response to injury.\textsuperscript{3} Although normal SMCs do not produce MMP-9 under quiescent conditions, they are transiently stimulated to do so under conditions of injury. We hypothesized that an increase in MMP-9 activity might alter the local proteolytic balance, enhance SMC migration and matrix degradation, and contribute to pathological changes in a diseased vessel. We attempted to test this hypothesis by using SMCs transfected with a tetracycline-regulatable gene encoding rat MMP-9 and seeded into the luminal surface of the denuded rat carotid artery to form a neointima.

Figure 4. Photomicrographs of hematoxylin/eosin–stained carotid arteries (×63) taken from rats that were balloon injured, seeded with transduced SMCs, and treated with tetracycline (Tet+) or vehicle alone (Tet−) for 14 days. Bar=0.2 mm.

Figure 5. Effects of MMP-9 overexpression on morphology of internally seeded, balloon-injured rat carotid arteries quantified by digital morphometry. A, Vessel circumference measured by IEL length. Open bars, Tet−; closed bars, Tet+; cross-hatched bars, Tet+ 14 days followed by Tet− 14 days. *Tet− vs Tet+, \( P<0.05 \); #Tet+/Tet− vs Tet+, \( P<0.05 \). B, Luminal area. *Tet− vs Tet+, \( P<0.05 \); #Tet+/Tet− vs Tet+, \( P<0.05 \). C, Luminal index (luminal diameter/cross-sectional wall thickness) as an indicator of positive remodeling. *Tet− vs Tet+, \( P<0.05 \). D, Intimal area. *Tet− vs Tet+, \( P<0.05 \). Data are mean±SEM.
Effects of MMP-9 on Cell Proliferation and Migration

MMP-9 inhibitors have been shown to decrease proliferation in rabbit but not baboon aortic explants. MMP inhibitors decreased the first round of proliferation after balloon injury. We found that MMP-9–overexpressing cells in culture demonstrated no difference in cell growth from cells not expressing MMP-9, nor was there a difference in the BrdU labeling index in vivo in luminally seeded cells in animals treated with tetracycline or vehicle alone. We found that SMCs that overexpress MMP-9 invaded a thick collagen matrix more readily than cells not expressing MMP-9. In addition, the conditioned medium of MMP-9–overexpressing cells significantly increased the migration of normal cultured SMCs through a basement membrane–coated filter. The increase in migration was inhibited by the metalloproteinase inhibitor BB-94 in all cases. BB-94 also appeared to decrease migration in the Tet+ group, although this difference was not statistically significant. We believe that this inhibition is most likely a result of inhibition of constitutively expressed MMP-2 as reported by Pauly et al. We did not observe an increase in migration of MMP-9–overexpressing cells through a basement membrane–coated filter, although we did observe a similar increase in migration when conditioned medium from these cells was added to the migration assay (128% of control). We believe that this effect is due to the larger amount of MMP-9 that is present in medium that has been conditioned for 48 hours than that expressed by cells in the 5-hour migration assay.

To study the effects of MMP-9 on SMC migration in vivo, we stripped the adventitia, killed all endogenous artery wall cells by snap-freezing, and seeded SMCs into the perivascular space. MMP-9 overexpression by Tet−SMCs enhanced migration toward the lumen of the vessel compared with Tet+ controls, and this enhancement was abolished by systemic administration of BB-94. Our combined in vitro and in vivo findings suggest that the upregulation of MMP-9 in the rabbit carotid artery after balloon injury may serve to enhance migration of SMCs from the media to the intima in the “second wave” of postinjury intimal hyperplasia and may function in both an autocrine and paracrine manner. Because frozen carotid artery segments that were not seeded with SMCs demonstrate no cellularity in either the media or intima at 7 days, it is unlikely that SMCs came from an adjacent, uninjured arterial segment. In a separate study, the intima and media formed by external seeding of SMCs, which were prelabeled in vitro with BrdU, contained 100% BrdU-positive cells (Hasenstab D, Lea H, Hart CE, Lok S, Clowes AW, unpublished data, 1999). We conclude that the medial and intimal cells are adventitiously seeded SMCs that have transmigrated. These in vivo data confirm and extend previous in vitro observations that MMP-9 enhances migration of SMCs through arterial explants and of monocytes through a reconstituted basement membrane. The mechanism for increased migration as a result of MMP-9 overexpression is unknown. Vitrogen is largely type I collagen (95 to 98%), although it does contain some type III collagen (2% to 5%). Type I collagen in its fibrillar form is not a known substrate for MMP-9, and it is unlikely that increased degradation of type I collagen is the mechanism. One potential explanation is that MMP-9 cleaves type III collagen to reveal a promigratory sequence. Such a process has been shown for MMP-2, which cleaves the γ-2 subunit of laminin-5, not a previously known substrate for MMP-9. Collagen and elastin fragments have been shown to enhance chemotaxis for neutrophils, monocytes, and fibroblasts. In this regard, MMP-9 has also been shown to bind to fibrin in vitro and may direct SMC migration after injury through the release of chemotactic peptides and localize MMP-9 activity in an unstable atherosclerotic plaque. In addition, MMP-9 may degrade matrix components synthesized by the SMCs producing promigratory signals. Although MMP-9 appears to be overexpressed primarily in its proform, we believe that it may be exerting its effects through local activation by, for example, MMP-2 that is present in active form.

In addition to causing matrix degradation, MMP-9 might alter cell-matrix binding characteristics that enhance migration in an activation- and proteolysis-independent manner. MMP-9 has been shown to bind type I collagen without degrading it and

![Figure 6. Effects of MMP-9 overexpression (Tet−, closed bars) on nuclear cell density after balloon injury and luminal seeding at 7, 14, and 28 days. Open bars, Tet+; closed bars, Tet−; cross-hatched bars, Tet+ 14 days followed by Tet− 14 days. *Tet− vs Tet+, P<0.05; #Tet+ 14 days/Tet−14 days vs Tet+, P<0.05. Data are mean±SEM.](image)

![Figure 7. Effect of MMP-9 on migration of transduced SMCs from the adventitial side into the media and intima of the snap-frozen carotid artery. A, Representative cross section of a carotid artery that has been frozen, externally seeded with transduced cells for 7 days in the absence of tetracycline, and stained with antibody to smooth muscle α-actin. L indicates lumen; EEL, external elastic lamina. B, Representative cross section of a hematoxylin/eosin–stained carotid artery showing lack of medial and intimal cells at 7 days after freezing and treating with cell-free medium. Bar=50 μm. C, Inhibition of SMC migration into the media and the intima by BB-94. *Tet− vs Tet+, P<0.05. Data are mean±SEM.](image)
might, through a receptor such as CD44, which binds MMP-9, increase SMC binding to matrix. Overexpression of proMMP-9 might also alter the formation of basement membranes after injury by the binding of proMMP-9 to free (not triple-helical) α2(IV) collagen. MMP-9 might also affect SMC migration by interacting with integrins as illustrated by the association of MMP-2 with αβ3 and MMP-9 with β1 integrins (B. Levkau et al, unpublished data, 1999).

MMP-9 Overexpression and Vessel Remodeling

Multiple cellular processes are integrated in vascular remodeling and require changes in cell proliferation, cell migration, extracellular matrix deposition and degradation, and cell death. For example, coronary arteries compensate for large atherosclerotic plaques and maintain lumen size by dilating (compensatory vascular remodeling). Our observations suggest that MMP-9 plays an active role in the remodeling process, causing increased vessel circumference, lumen size, and cell nuclear density with decreased extracellular matrix content. We believe that the alterations in remodeling are due to regulated MMP-9 overexpression, because differences are most striking at late time points after injury when endogenous MMP-9 expression has largely disappeared.

The finding that MMP-9 induces vascular dilation is novel. MMPs have been implicated in vessel dilation in response to increased flow, but the specific MMP responsible for the change was not identified. It is particularly striking that arterial vasodilation and remodeling occur at 28 days when MMP-9 overexpression is delayed for the first 14 days, given that initial SMC proliferation and injury-induced MMP expression have largely returned to baseline.

MMP-9 overexpression may induce vessel dilation through several mechanisms. First, MMP-9 may degrade matrix that directly alters the mechanical properties of the vessel. Increased arterial collagen content has been shown to decrease distensibility in a rat model of hypoxic pulmonary hypertension. Matrix degradation by MMP-9 overexpression may similarly increase the distensibility of the vessel. For example, MMP-9 degrades elastin, the major elastic component of the vessel responsible for vessel recoil. Although there was no obvious difference in collagen composition or evidence of elastin breakdown by Movat’s staining, subtle changes in matrix composition may have occurred and altered the mechanical properties of the vessel. Second, it is well known that the endothelium plays a major role in regulating the vascular tone and acute medial and adventitial enlargement through NO. It is possible that endothelial migration and regrowth are stimulated by MMP-9 given the role of MMP-1 in angiogenesis. However, our seeded vessels demonstrated minimal reendothelialization at the injured region as assessed by Evan’s blue staining (data not shown), and there was no endothelium present in the areas through which sections were cut. Therefore, it is unlikely that the endothelium mediates the observed changes in morphology.

Finally, the observation that vascular SMC αβ3 integrin mediates arteriolar vasodilation in response to RGD peptides and elastase-generated collagen fragments in an ex vivo model of arteriolar function suggests another possible mechanism for MMP-9 remodeling. Cleavage of matrix substrates by MMP-9 may produce RGD fragments that induce vasodilation through integrin-mediated signaling.

Our observations support the hypothesis that increased MMP-9 proteolytic activity seen after vascular injury increases SMC migration and alters vascular remodeling. Increased sub-strate cleavage and extracellular matrix loss likely lead to modified cell-matrix and cell-cell interactions. Degradation products may in turn provide signals for SMC migration, contractile responses, and formation and disruption of the atherosclerotic plaque. In addition to contributing to plaque growth (increased SMC migration) and instability (increased proteolytic activity), our observations suggest a novel and even beneficial role for MMP-9. It produces adaptive remodeling that may serve to provide room for a growing plaque by inducing vessel dilation and increasing the luminal area. Stenosis or plaque rupture might occur only after this adaptive process has been maximized and overwhelmed.

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References


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