Matrix Metalloproteinase-2 and -9 Differentially Regulate Smooth Muscle Cell Migration and Cell-Mediated Collagen Organization

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Objectives—Smooth muscle cells (SMCs) produce both matrix metalloproteinase (MMP)-2 and MMP-9, enzymes with similar in vitro matrix degrading abilities. We compared the specific contributions of these enzymes to SMC-matrix interactions in vitro and in vivo.

Methods and Results—Using genetic models of deficiency, we investigated MMP-2 and MMP-9 roles in SMC migration in vivo in the formation of intimal hyperplasia and in vitro. In addition, we investigated potential effects of MMP-2 and MMP-9 genetic deficiency on compaction and assembly of collagen by SMCs.

Conclusions—MMP-2 and MMP-9 genetic deficiency decreased by 81% and 65%, respectively (P < 0.01), SMC invasion in vitro and decreased formation of intimal hyperplasia in vivo (P < 0.01). However, we found that MMP-9, but not MMP-2, was necessary for organization of collagen by SMCs. Likewise, we found that MMP-9 deficiency resulted in a 50% reduction of SMC attachment to gelatin (P < 0.01), indicating that SMCs may use MMP-9 as a bridge between the cell surface and matrix. Furthermore, we found that the hyaluronan receptor, CD44, assists in attachment and utilization of MMP-9 in SMCs. Understanding the specific roles of these MMPs, generally thought to be similar, could improve the design of therapeutic interventions aimed at controlling vascular remodeling. (Arterioscler Thromb Vasc Biol. 2004;24:54-60.)

Key Words: matrix metalloproteinase ▶ smooth muscle cells ▶ vascular remodeling ▶ migration ▶ collagen organization

Vascular smooth muscle cells (SMCs) comprise the major cellular component of the normal arterial wall. Their main functions are maintaining proper vascular tone and producing the extracellular matrix (ECM) scaffold of the vessel wall. SMCs are also major drivers of vascular pathologies, including the formation of intimal lesions occurring naturally or induced by vascular interventions.1 Migration, proliferation, and subsequent production of ECM, including fibrillar collagens,2 leads to formation of SMC-rich intimal and neointimal lesions, which occlude the arterial lumen. In addition, SMC-rich lesions may enhance the inward or constrictive geometrical remodeling of arteries.2

See page 10

The movement of SMCs through the matrix necessitates degradation of various components, including the basement membrane and elastic lamina, likely with the aid of a family of enzymes called matrix metalloproteinases (MMPs). SMCs produce pro-MMP-2 basally in normal blood vessels and in culture and other MMPs, including MMP-9, after cytokine stimulation in vitro, in human atherosclerotic lesions,3,4 and after balloon injury.5 MMP inhibition either through adenoviral delivery of tissue inhibitor of metalloproteinase-16 or by a synthetic general MMP inhibitor7 was shown to decrease SMC migration and subsequent intimal hyperplasia in the rat vascular injury model. MMP-2 and -9 are largely thought to have similar functions based on sharing substrate affinity in vitro,8 namely short collagens, degradation products of interstitial collagen, and elastin. To begin to elucidate potential differences in their roles in vivo in relation to vascular remodeling, we comparatively investigated the functional effect of MMP-2 or MMP-9 genetic deficiency on SMC interaction with matrix in vivo and in vitro using genetically deficient MMP-2 or MMP-9 (KO) mice.

Methods

Mouse Strains

The Institutional Animal Care and Use Committee of Emory University approved all the animal protocols used in this study. Mice with genetic deletions (KO) of either MMP-29 (a generous gift from Dr Itohara, RIKEN Brain Institute, Wako, Saitama, Japan), MMP-910 (a generous gift from Drs Shipley and Senior, Washington University, St Louis, Mo), or CD4411 (Jackson Laboratories, Bar Harbor, Maine) were all bred into the same C57BL6/J background. C57BL6/J wild-type (WT) mice (Jackson Laboratories) were used.
for controls. Mice that express LacZ gene driven by the MMP-9 promoter GelB/LacZ12 were a generous gift from Dr E. Fini (University of Miami School of Medicine, Miami, FL).

**Cell Culture**

SMCs were isolated from MMP-9 KO, MMP-2 KO, CD44 KO, and WT mice and expanded in culture using the explant method previously described in detail.13 Cells cultured in DMEM (Mediatech, a mouse anti-

**SMC Migration Assays In Vitro**

Three-dimensional cell migration was determined using the modified Boyden chamber assay with a gelatin-coated membrane. The lower chambers contained either no chemoattractant control, 10% FBS, or 50 nmol/L platelet-derived growth factor (PDGF)-BB in DMEM. After 6 hours, cells were scraped from the upper surface, the membrane was fixed with formalin (Fisher Scientific), migrated cell nuclei were stained with Hoechst 33258 (Sigma), and the membrane was analyzed using a fluorescence microscope (Axioscope, Zeiss) to count cell numbers (n=8 independent fields for each condition). To examine 2D migration, we used the scratch-wound migration assay, as previously described.13 Cells were stained for actin using rhodamine-conjugated phalloidin and nuclei with Hoechst (Sigma) 6, 24, or 48 hours after the wounding. Migrated cell number was determined as the number of nuclei in front of the wound edge (n=3 independent microscope fields for each condition per experiment, 3 independent experiments per time point).

**Animal Model of Intimal Hyperplasia**

To initiate formation of intimal hyperplasia as a model for in vivo SMC migration, we used the carotid ligation model and performed morphological measurements as described in detail previously.14

**Assays for Collagen Gel Compaction and Collagen Assembly by SMCs**

Collagen gel compaction was determined by seeding collagen with MMP-2 KO, MMP-9 KO, CD44 KO, or WT SMC.13 Cell-dependent gel compaction was measured 48 hours after seeding in culture using tritiated water (Dupont NEN) exclusion as a quantitative measure of compaction, normalized by counts obtained from cell-free collagen gels (n=4 independent gels for each condition). Collagen assembly was measured using a novel assay with FITC-labeled (Fluos labeling kit, Roche) rat-tail collagen (Becton Dickenson), which we previously described in detail.13 Briefly, 10 to 100 µg/ml FITC-labeled collagen (subgelling concentrations) in serum-free DMEM was added to quiesced, confluent SMC seeded in 96-well tissue culture plates. After washing collagen monomers unbound after 24 hours, SMC monolayers and associated assembled collagen were fixed. Nuclei were counterstained with Hoechst. Fluorescence was measured using a Cytofluor 3000 (Applied Biosystems) with green fluorescence (λex, 480 nm; λem, 530 nm) as a measure for collagen assembly and blue fluorescence (λex, 360 nm; λem, 460 nm) for cell density. Wells with no cultured cells were used to control for collagen self-assembly. The possibility to restore collagen contraction and assembly by providing MMP-9 was tested by adding purified mouse MMP-910 in PBS. Blocking experiments were performed using the rat anti-mouse CD44 blocking antibody purified from the KM201 hybridoma cell line (ATCC).

**SMC Attachment Assays**

Cell attachment assays to rat-tail collagen (0.1 to 10 µg/well), gelatin (1 to 100 µg/well, Sigma), or purified MMP-9 were performed by seeding SMCs in 96-well tissue culture plates for 6 hours. After washing, attached cells were stained with 1% crystal violet (Sigma) and solubilized (5% acetic acid). Cell attachment was calculated as optical density (OD) measured at 590 nm using a microplate reader (Bio-Rad) of sample minus OD of wells blocked with BSA (Sigma), normalized by cell attached to FBS. The effect of the gelatinase selective cyclic peptide inhibitor H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH16 (Bachem) on WT SMC attachment to gelatin (50 µg/well) was tested at concentrations ranging from 0.1 to 10 µmol/L. Potential rescue of MMP-9 KO SMC attachment to gelatin (50 µg/well) was determined by adding purified MMP-9. To demonstrate the role of CD44, rat anti-mouse CD44 blocking antibody was added to WT SMC presented with gelatin (50 µg/well) for attachment (n=6 independent wells for each condition).

**Statistical Analysis**

Values are given as mean±SEM. All comparisons were done using Student’s t test for comparisons between groups. P<0.05 was considered significant, and P<0.01 was considered highly significant.

**Results**

**Genetic Deficiency in MMP-2 or MMP-9 Reduces SMC Ability to Migrate In Vitro**

Using a modified Boyden chamber assay, we found that either MMP-2 or MMP-9 genetic deficiency resulted in significant impairment of SMC migration through a gelatin-coated membrane when either FBS or PDGF-BB was used as chemoattractant (PDGF-BB, 59±18 for MMP-2 KO and 108±26 for MMP-9 KO; WT, 312±112; P<0.05 for either KO versus WT). Interestingly, this remained significant even in the presence of exogenous MMPs existent in the FBS used as chemoattractant, likely explaining increased overall invasion levels with FBS. We then investigated in vitro migration of SMCs induced by wounding of a SMC monolayer. Activation of MMP-9 transcription was demonstrated only in cells at the wound edge after 6 hours using SMCs isolated from the GelB/LacZ mice12 (Figure 1). Translation into MMP-9 protein was verified after 24 hours by positive MMP-9 immunostaining in the SMC migrating from the wound edge. We found a decrease in both SMC number (Figure 1) and average distance migrated (data not shown) for both MMP-2 KO and MMP-9 KO compared with WT SMCs at 6, 24, and 48 hours after wounding. Addition of a gelatinase-selective cyclic peptide inhibitor16 reduced migration of WT SMC to levels comparable to MMP-2 KO or MMP-9 KO SMCs (online Figure 1, available at http://atvb.ahajournals.org). Interestingly, addition of a general MMP inhibitor to WT SMC resulted in only a modest 16% additional inhibition compared with MMP-2 KO or MMP-9 KO SMC, suggesting the gelatinases as the major MMP contributors to SMC migration.

**MMP-2 or MMP-9 Deficiency Reduces the Extent of Intimal Hyperplasia In Vivo**

MMP-2 KO and MMP-9 KO mice had significantly reduced carotid artery intimal hyperplasia compared with WT mice. At 14 days after intervention, we determined that MMP-2 KO and MMP-9 KO had significantly fewer intimal SMCs (Figure 2). This difference was maintained at 28 days after ligation (108±26 for MMP-2 KO and 59±18 for MMP-9 KO versus 312±112 for WT, P<0.05 for either KO versus WT). The reduction in intimal thickness found in MMP-2 KO or MMP-9 KO carotid arteries (21.6±5.6 µm for MMP-2 KO and 10.0±1.2 µm for MMP-9 KO versus 63.6±16.1 for WT, P<0.05 for either KO versus WT) likely contributed to reduced lumen loss compared with WT. Measurements of the
external elastic lamina perimeters indicated little change in vessel size ($P=\text{NS}$ for time course or MMP-2 or MMP-9 versus WT), suggesting minimal geometric remodeling at the times examined. Of note, biochemical analyses indicated that there was no compensatory increase in the levels of latent or activated MMP-9 produced in the MMP-2 KO or of MMP-2 in the MMP-9 KO (data not shown).

**MMP-9 But Not MMP-2 Assists With In Vitro Organization of Collagen Type I by SMCs**

To investigate the potential role of MMP-2 and MMP-9 in SMC interaction with collagen, we performed in vitro collagen gel compaction assays. We found that MMP-9 KO but not MMP-2 KO SMCs had an impaired ability to compact collagen gels (Figure 3, $P<0.05$ for MMP-9 KO versus WT, NS for MMP-2 versus WT). To gain mechanistic insight into this process, we used a novel assay that allows quantification of cell-mediated supramolecular assembly of collagen monomers.\(^5\) After adding subgelling concentration of collagen monomers to cultured MMP-2 KO, MMP-9 KO, and WT SMC, levels of fluorescence detectable after 24 hours were significantly different (8.68±0.42 arbitrary units [AU] for MMP-2 KO, 5.15±0.42 AU for MMP-9 KO versus 8.76±0.64 AU for WT, $P<0.01$ for MMP-9 KO versus WT, NS for MMP-2 KO versus WT), indicating that MMP-9 but not MMP-2 genetic deficiency impaired SMC ability to assemble collagen.
SMC ability to attach to type I collagen or the nonfibrillar collagen analog gelatin was tested as a necessary event for organization of a collagenous matrix. Neither MMP-9 nor MMP-2 deficiency altered SMC attachment to fibrillar collagen (not shown). On the other hand, MMP-9 deficiency resulted in an impaired capacity of SMCs to attach to gelatin (Figure 3). At 50 μg gelatin per well, WT SMC attached at 52%, MMP-2 KO at 47%, and MMP-9 KO at 22% of a substrate (FBS) control (P<0.01 for MMP-9 KO versus WT, NS for MMP-2 KO versus WT). Addition of a gelatinase-selective cyclic peptide inhibitor resulted in a dose-dependent decrease in WT SMC attachment to gelatin (online Figure I, WT+10 μmol/L inhibitor attached at 26.2±4.3% versus 51.6±1.0% of control, P<0.01). Taken together, these results indicate that MMP-9 is necessary for proper SMC organization of collagen likely through SMC-mediated attachment and assembly of nonfibrillar collagens.

Hyaluronic Acid Receptor CD44 Contributes to SMC Utilization of MMP-9 for Collagen Assembly

Our results suggested that SMCs bind MMP-9 and use it for assembly of fibrillar collagen. Based on a previous suggestion from the literature, we investigated the potential contribution of the hyaluronan cell-surface receptor CD44. First, we verified that MMP-9 and CD44 colocalize in situ in the remodeled carotid artery using double immunocytochemistry (Figure 4). Positive double staining was found mainly perivascularly in areas of highest collagen and hyaluronic acid accumulation, supporting participation of MMP-9 and CD44 in the distribution and organization of these matrix components. Use of specific antibodies and Picrosirius red (not shown) suggested that MMP-9 deficiency was associated with decreased accumulation and organization of fibrillar collagen in the remodeled artery, additionally supporting the cooperation between MMP-9 and CD44 in vivo.

We thus tested the suggested MMP-9/CD44 cooperation using in vitro experiments examining SMC-matrix interactions. We found that similar to MMP-9 KO SMC, CD44 KO SMCs had impaired capacity to compact collagen gels (Figure 4). To specifically question the role of MMP-9, we added back-purified mouse MMP-9. This rescued the compaction of collagen by MMP-9 KO SMC but not by CD44 KO SMC, supporting the need for both molecules. To demonstrate the

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**Figure 2.** MMP-2 or MMP-9 genetic deficiency impairs neointimal hyperplasia of mouse carotid artery. Top, Representative fluorescence micrographs of remodeling mouse carotid arteries (28 days; blue, nuclei; green, elastic laminae; scale bar=100 μm). Note the difference in neointima thickness (arrows) in the 3 different mouse strains. Insets, Intima of uninjured carotid arteries does not contain SMCs. Left, Quantification intimal cell numbers support impairment of SMC migration attributable to MMP-2 or MMP-9 deficiency. Middle, MMP-2 or MMP-9 deficiency also reduces average intimal thickness. Right, Reduction of neointimal hyperplasia results in reduced lumen loss, indicated by lumen area. *P<0.05 vs WT.
involvement of CD44, we used an anti-CD44 blocking antibody, which reduced compaction of collagen gels by WT SMC to levels similar to MMP-9 KO and CD44 KO SMC. CD44-deficiency similarly impaired SMC capacity to assemble fibrillar collagen from exogenous monomers (Figure 4), which could not be rescued by addition of purified MMP-9, as in the case of MMP-9 KO SMC. The impairment seemed to be attributable to decreased CD44 KO SMC adhesion to gelatin compared with the WT SMC and similar to the MMP-9 KO SMC. The anti-CD44 blocking antibody decreased the WT SMC ability to attach to gelatin.

Next, we found that both WT and MMP-9 KO SMC, but not CD44 KO SMC, attached to MMP-9–coated cell culture plates (Figure 4). Attachment of WT and MMP-9 KO SMCs to MMP-9 could be blocked using the anti-CD44 antibody, indicating that the cell-surface receptor CD44 is instrumental for the use of MMP-9 by SMCs to attach to and organize fibrillar collagen.

**Discussion**

MMPs, specifically gelatinases MMP-2 and MMP-9, have been implicated in SMC migration, contributing to the intimal thickening characteristic of vascular lesions in vivo. Overexpression of MMP-9 in SMCs in an injured rat carotid artery led to an increase in SMC migration, expansive remodeling, and a decrease in ECM. Investigations using viral transduction of tissue inhibitors of metalloproteinases (TIMPs) showed that expression of TIMP-1, a natural inhibitor of MMP-9, decreased SMC migration in vitro and formation of neointimal hyperplasia in vivo after balloon injury of rat carotid arteries. Overexpression of TIMP-2, a specific inhibitor of MMP-2, inhibited in vitro SMC invasion through basement membrane proteins and also reduced neointima formation in rat balloon-injured carotid arteries. TIMP-1, -2, and -3 were shown to alter SMC behavior in vitro, all 3 inhibiting cell migration, but also proved to have MMP-independent effects. The inhibitor effects of various treatments, such as dexamethasone, doxycycline, and green teas catechins, on SMC migration in vitro and intimal hyperplasia in vivo were also attributed to general MMP inhibition. Other very recent observations suggest that MMPs may be involved with processing of cell-cell adhesion molecules, which would release cells, but the potential specific contribution of MMP-2 and MMP-9 have yet to be defined.

Interestingly, a previous report suggested that MMP-3 but not MMP-9 deficiency decreased in vitro SMC migration in response to a scratch wound. However, the addition of FBS to the culture medium during the assay likely provided the MMP-2 and MMP-9 necessary for SMC migration, as also suggested by our results using FBS. We recently showed that MMP-9 genetic deficiency resulted in less intimal thickening and lumen loss in a mouse model of carotid artery injury with a concurrent increase in adventitial collagen.

In this study, we found that both MMP-2 and MMP-9 facilitate SMC migration and intimal thickening, contributing to arterial lumen loss in vivo. Furthermore, we show that in vitro both MMP-2 and MMP-9 genetic deficiencies result in a significant decrease in SMC migration in response to wounding and impair invasion through gelatin to levels comparable to those produced by nonspecific chemical inhibition of all other MMPs. However, neither MMP-2 nor MMP-9 alone completely reduced SMC migration, suggesting that these 2 MMPs have separate contributions and neither could compensate for the lack of the other.

Interestingly, our data support a new role for MMP-9, but not for MMP-2, in SMC attachment to the matrix, which is distinct from the known role in degradation of matrix. This may facilitate cell attachment to degraded collagen and traction during migration. We believe that such properties may serve to guide cell migration, maybe for the purpose of repopulation and healing of areas of high tissue remodeling. Furthermore, our detailed investigation based on specific inhibition of MMP-2 or MMP-9 indicates that MMP-9 but not MMP-2 genetic deficiency led to an impaired ability for
SMCs to compact collagen gels and to assembly collagen fibers from exogenous collagen monomers added in culture, activities essential in the process of tissue contraction during wound healing. Several recent pieces of information based mostly on circumstantial associations or use of nonspecific inhibitors\(^26,27\) implicate MMPs in cell-mediated collagen gel compaction. We suggest that MMP-9 may be very important for wound healing. In relation to remodeling of arteries, the same processes, ie, collagen gel compaction, fibrillar collagen assembly, and accumulation, are thought to be major contributors to the constrictive (inward or negative) remodeling associated with arterial stenosis.

Our results suggest that MMP-9 participates in SMC compaction of collagen gels and fibrillar collagen assembly by potentially acting as a bridge between SMC and collagen monomers. We suggest that this capacity is mediated through the cell-surface CD44, also known as the hyaluronic acid receptor, previously shown to colocalize with MMP-9 to promote cell-mediated collagen IV degradation and tumor cell invasiveness,\(^17\) and now detected by us in situ within the arterial wall in the areas of highest collagen accumulation. These assumptions are based on matching experiments revealing the impaired capacity of MMP-9 KO or CD44-deficient SMC or of WT SMC in the presence of CD44-blocking antibodies to perform these matrix-related SMC functions, rescued by addition of purified MMP-9 to compact collagen or to fibrillar collagen assembly by MMP-9 KO SMC but not by CD44 KO SMC.

We thus found that whereas MMP-2 and MMP-9 may have similar matrix-degrading abilities, these MMPs have distinct contributions to SMC interaction with matrix. Such differences will likely lead to different regulation of the complex process of vascular remodeling, involving cell migration, degradation, and reorganization of complex matrices. A better understanding of such newly emerging functional differences in vivo is essential for the fine tuning of cell-matrix interactions for therapeutic or tissue engineering purposes.

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