Deficiency of Gelatinase A Suppresses Smooth Muscle Cell Invasion and Development of Experimental Intimal Hyperplasia

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Background—Although it has been demonstrated that matrix metalloproteinases (MMPs) play an important role in the arterial remodeling in atherosclerosis and restenosis, it is not clear which MMP is involved in which process. To define the role of MMP-2 in arterial remodeling, we evaluated the influence of the targeted deletion of the MMP-2 gene on vascular remodeling after flow cessation in the murine carotid arteries.

Methods and Results—The left common carotid arteries of wild-type and MMP-2–deficient mice were ligated just proximal to their bifurcations, and the animals were then processed for morphological and biochemical studies at specific time points. MMP-2 activity and mRNA levels increased in ligated carotid arteries of wild-type mice on the basis of observation by gelatin zymography and quantitative real-time RT-PCR. There was significantly less intimal hyperplasia in MMP-2–deficient mice at 2 and 4 weeks after ligation than there in wild-type mice. Arterial explants from the aorta of MMP-2–deficient mice showed that smooth muscle cell (SMC) migration was inhibited in comparison with wild-type mice. The chemoattractant-directed invasion through a reconstituted basement membrane barrier was significantly reduced in cultured SMCs derived from MMP-2–deficient mice, although no difference was observed in SMC migration across the filter or in proliferative response between the control and MMP-2–deficient mice.

Conclusions—in a mouse carotid artery blood flow cessation model, MMP-2 contributes to intimal hyperplasia mainly through the SMC migration from the media into the intima by degrading and breaching the extracellular matrix proteins surrounding each cell and the internal elastic lamina. (Circulation. 2003;108:1375–1381.)

Key Words: metalloproteinases ■ remodeling ■ blood flow ■ carotid arteries

Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent proteinases that degrade collagen and other matrix proteins, are involved in the accelerated breakdown of the extracellular matrix associated with vascular remodeling during the development of atherosclerosis and vascular injury–induced neointimal formation.1–2 Various kinds of MMPs were shown to be upregulated and activated in the atherosclerotic lesions as well as in injury-induced vascular walls in human and animal models.3–7 Recent efforts focused on finding ways to control the action of MMPs through the use of nonselective synthetic inhibitors, or intrinsic inhibitors. The use of various synthetic nonselective inhibitors of MMPs and tissue inhibitors of MMPs (TIMPs), which are intrinsic MMP inhibitors, has been shown to reduce injury-induced intimal hyperplasia as well as constrictive arterial remodeling,6–11 suggesting that one or some of these MMPs play a key role in vascular remodeling. An important but elusive goal has been to determine which of these enzymes is critical in vascular remodeling. However, because of the current absence of selective MMP inhibitors, experiments have provided little insight into the role of individual MMPs.

MMP-2, also called gelatinase A, a major MMP derived from vascular smooth muscle cells (SMCs), degrades various extracellular matrix proteins and such barriers as the basement membrane.1,2 Several lines of evidence indicate that MMP-2 is upregulated and activated in human atherosclerotic lesions as well as in various animal models of neointimal formation, including the rat balloon injury model and electric burning or flow cessation of murine arteries.3–7 We and others have also demonstrated that MMP-2 activation is a critical step in the migration of SMCs through a collagen or a...
reconstituted basement membrane barrier in vitro. However, whether MMP-2 has a specific biological role in vascular remodeling remained unknown. Studies using animal models have led to important insights into the cellular and molecular events underlying vascular remodeling. In this study, we evaluated the influence of the targeted deletion of the MMP-2 gene on vascular remodeling after flow cessation in the murine carotid arteries.

Methods

Animals and the Flow Cessation Model
All animal studies were conducted in accordance with the Animal Care and Use Committee guidelines of the Nagoya University School of Medicine. To induce a null mutation into the MMP-2 gene, the promoter and first exon region was replaced with the pgk-neo gene by gene targeting as previously described. The mutant mouse has been shown to be slower growth rate than as compared with that of wild-type mouse. Male vessels. However, the mutant mouse has been shown to be slower

Histology and Morphometry
Mice were euthanized and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline under physiological pressure. Fixed ligated arteries were embedded and frozen in OCT media. For the morphometric analysis, serial sections (5 μm thick) at 2 mm proximal to the ligated site were stained with hematoxylin and eosin. Perimeters of the lumen, the internal elastic lamina (IEL), and the external elastic lamina (EEL) were obtained by tracing the contours on digitized images. The intimal and medial areas were calculated by subtracting the area defined by the lumen from the area defined by the IEL and subtracting the area defined by the EEL from the area defined by the EEL. The neointimal area was determined by subtracting the lumen area from the area defined by the EEL.

For immunohistochemical analysis, sections were preincubated with 5% serum and then incubated with antibodies against human MMP-2 (Fuji Chemical Co), MMP-9 (Chemicon), SM α-actin, proliferating cell nuclear antigen (PCNA, Sigma-Aldrich), or CD45 (Santa Cruz Inc) followed by the biotinylated second antibody, avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP Kit, Vector Laboratories), and visualized with Vector Red (Vector Laboratories). Counterstaining was performed with hematoxylin.

Gelatin Zymography and mRNA Quantification
For gelatin zymography, each aliquot of the conditioned medium or 20 μg protein extracts of carotid arteries were mixed with SDS sample buffer without reducing agent and loaded onto a 10% SDS-polyacrylamide gel containing 1 mg/mL gelatin, as described in detail previously. Digestion bands were quantified by an image analyzer system (NIH image 1.62) and compared with a human MMP-2 standard (Oncogene Research Products).

Changes in the mRNA levels of MMPs and TIMPs in ligated carotid arteries were quantified by real-time reverse transcription and polymerase chain reaction (RT-PCR). The total RNA was extracted from carotid arteries, and then reverse-transcribed. The synthesized cDNA was quantified by using TaqMan quantitative PCR analysis of each gene with the ABI PRISM 7700 Detection System according to the manufacturer’s protocol. Specifically, primer and probe sequences used for mouse MMP-2 were (forward) 5’-CCCCCCATGAAGCCTTGTITACCC (reverse) 5’-TTGTAGGAAGGTGCGCTTGGAA (probe) 5’-ATATGCTATGGAA-CACCGGCT/GA, for mouse MMP-9 were (forward) 5’-AGACCAAG-GTGACACCCTGGTTC (reverse) 5’-GGCACGCGTTGAAATGATCTAGAG (probe) 5’-GCCAGCGATGCGCCATGAC/GC for mouse MMP-3 (forward) 5’-CATGGGAGACTGTTGCGCTTTTGAT (reverse) 5’-CTGAGGGATGCATCATTAG (probe) 5’-GGGCGTGATGGAAGACCTTCTT (reverse) 5’-GGGCGTATGCCAAGAAGTATCGACT, (probe) 5’-GGGCGTATGCCAAGAAGTATCGACT, (probe) 5’-TGAGGACCCCAAGAATGCTTAGGC, for mouse TIMP-1 (forward) 5’-GCCCTACCA C AC C CCAGTCATGGA, (reverse) 5’-GCCCCGTTAGGAAAGACTCTT, (probe) 5’-TTGAGGAGGGCGAAGATGGGCGG, for mouse TIMP-2 (forward) 5’-TCCGGTATCCCTGTCTACA, (reverse) 5’-TGCCGATGCGCTTCTTCT (probe) 5’-TTCCCGGGGTAAGTGGCCTGGCCTGGGA. Each RNA quantity was normalized to its respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA quantity.

Preparation of Explants and Migration Assay
Preparation of aortic explants was performed as previously described. Briefly, thoracic aortas of male mice (age, 10 weeks) were removed and opened out, and the endothelium was removed by gentle abrasion. After periadventitial fat was removed, the aortas were cut into 1×1×1 mm explants, using an McIlwain tissue chopper. The explants were individually plated with the lumen side down into collagen type I-coated 96-well plates and cultured in 50 μL of Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% BSA, transferrin, insulin, platelet-derived growth factor BB (PDGF-BB, 10 ng/mL, PeproTech), and fibroblast growth factor 2 (FGF-2, 10 ng/mL, Roche Diagnostics). Explants were examined daily for evidence of any cell migration onto the plastic culture surface and were counted as positive if ≥1 cell was observed as described previously. To characterize the cell migrating from explants, cells were fixed and stained for SM α-actin.

Migration, Invasion, and Proliferation Assays
SMCs were isolated from the aortas of wild-type and MMP-2 KO mice by the explants method and were cultured with DMEM supplemented with 10% FBS and antibiotics. For the experiments, SMCs in a subconfluent state at the 3rd to 5th passages were used.

Migration and invasion assays were performed with Transwell (Costar) 24-well tissue culture plates composed of a polycarbonate membrane containing 5-μm pores. For migration assay, inner chamber membrane was coated with 50 μL of Matrigel solution (50 μg/mL, Becton Dickinson) at 4°C overnight to avoid polymerization and then rinsed with DMEM. For invasion assay, 20 μL of the Matrigel solution (0.7 mg/mL) was added to a membrane and polymerized at 37°C for 6 hours. SMCs were then seeded on the inner chamber of the Transwell at a concentration of 5×10⁵ cells/100 μL. The inner chamber was placed into the outer chamber, which contained recombinant human PDGF-BB (10 ng/mL), and incubated for 6 hours (migration) or 14 hours (invasion) at 37°C. The cells that migrated onto the outer side of the membrane were fixed and stained. The number of migrated cells was counted in the 6 to 8 randomly chosen fields of the duplicated chambers at a magnification of ×200 for each sample.

For the proliferation assay, SMCs were plated on collagen-coated 96-well plates (5×10⁵ cells/well) and then incubated with DMEM containing 0.3% BSA and PDGF-BB (10 ng/mL) for 3 days. Relative cell numbers per well were estimated with the use of the Cell Titer 96AQ Assay kit (Promega). Values are expressed as mean±SEM. Significant differences were analyzed by using a Student’s t test or ANOVA followed by a Dunnett multiple-comparison post hoc test. A value of P<0.05 is considered to be statistically significant.
Results

Neointimal Formation in the Carotid Artery Ligation Model

Significant intimal lesion formation was observed in wild-type mice at day 28 after ligation and to a lesser extent at day 14 after ligation (Figure 1). In contrast, much less intimal lesion formation was observed in MMP-2 KO mice at days 14 and 28 after ligation, compared with wild-type mice. Intimal lesion contained mainly SMα-actin-positive cells. Quantitative measurements revealed that significant lower levels of intimal areas in MMP-2 KO mice were detected at days 14 and 28 compared with those of wild-type but that no difference in media thickness was observed between wild-type and KO (Figure 2). Therefore, the ratio of intimal to medial area was higher in MMP-2 KO mice. The total cell numbers in the intima at day 28 were significantly higher in wild-type mice, whereas PCNA index was not different. There was no significant difference in the average circumference of EEL between the two genotypes at days 14 and 28 after ligation.

Figure 1. Representative light micrographs of ligated carotid arteries from wild-type and MMP-2 KO mice. MMP-2 staining was positive for MMP-2 in intimal and medial lesions, although staining was much less pronounced in media than in intima. No staining was observed in adventitia in wild-type mice as well as in the whole arterial wall in MMP-2 KO mice. Intimal lesion was positive for SMα-actin in wild-type and MMP-2 KO mice (inset).

Figure 2. Morphometry of ligated arteries of wild-type and MMP-2 KO mice 2 and 4 weeks after ligation. A, Intimal and medial areas of ligated carotid arteries. B, Ratio of intimal area to medial wall area. C, Total nuclei were counted in intima and media from cross sections. D, PCNA labeling index. PCNA labeling index was defined as percentage of total cells within a given area positive for PCNA staining. E, External elastic lamina circumference of ligated carotid arteries. Data represent mean±SEM (n=7 except for MMP-2 KO at baseline, n=6). *P<0.05, **P<0.01 vs wild-type mice.
Time Course of MMP Expression and Localization
A dramatic increase in MMP-9 (92 kDa) was observed at days 1 and 3 after ligation compared with the level before the ligation, and the MMP-9 level was then decreased at days 7, 14, and 28 in wild-type mice (Figure 3A). In contrast, there was no change in the levels of MMP-2 up to day 3, but the increase in both the pro-MMP-2 and active-MMP-2 levels was observed from day 7 through day 28 in wild-type mice (Figure 3, A and C). As expected, no MMP-2 activity was detected in MMP-2 KO mice, and a similar time course of MMP-9 level was observed (Figure 3B). However, quantitative analysis demonstrated an increase of gelatinolytic activity by MMP-9 in MMP-2 KO mice (Figure 3D). MMP-2 and MMP-9 mRNA expression increased in wild-type mice at days 3 and 14 after ligation compared with the levels before ligation (Figure 4). Higher levels of MMP-9 mRNA in ligated arteries were observed in MMP-2 KO mice, although there was no significant difference in MMP-3 mRNA between the two genotypes. In contrast, mRNA expression of TIMP-1 and TIMP-2 were decreased in MMP-2 KO mice compared with wild-type not only at the baseline but also during follow-up periods after ligation.

Immunohistochemical analysis revealed that strong staining for MMP-2 was observed in the neointimal region and that weak staining was detected in the medial region (Figure 1). No staining was observed in the adventitia in wild-type mice as well as in any regions of the vessel wall in MMP-2 KO mice. CD45 staining of cross sections taken on day 3 after ligation produced positive-stained cells in the adventitia in both wild-type (Figure 5) and MMP-2 KO mice (data not shown). Abundant MMP-9 expression was noted in CD45-positive leukocytes localized at adventitia at day 3 in wild-type mice (Figure 5). The number of MMP-9–positive cells decreased in adventitia at day 7 after ligation. MMP-9 staining pattern was not different between the two genotypes. In contrast, no increase in MMP-2 staining was observed at day 3, but strong staining was detected in medial region in wild-type at day 7 after ligation (Figure 5).
SMC Migration and Invasion

Cell migration from the arterial explants of wild-type mice was observed at day 2, and migrating cells were detected in 98% of the explants by day 5 (Figure 6, A and B). These migrating cells stained positive for SMα-actin (data not shown). Cell migration from the explants of MMP-2 KO mouse aortas significantly decreased compared with those of wild-type. Gelatin zymographic analysis of the conditioned medium 7 days after explantation revealed that major bands with gelatinolytic activity were observed at molecular masses of 72 kDa and 62 kDa, which corresponded to the latent and active forms of MMP-2, respectively (data not shown). In some tissue explants, a faint band of 92 kDa, which seemed to be MMP-9, was also detected in the conditioned medium. In contrast, 72 and 62 kDa bands were not observed in the medium from explants of MMP-2 KO mice. As in explants from wild-type mice, 92 kDa of MMP-9 was also observed in some cases.

To examine the ability of PDGF-BB–directed SMCs to migrate across a Matrigel thin-coated filter, three clones of cultured SMCs derived from either wild-type or MMP-2 KO mice were used. There were no differences in the number of cells that passed through the filters in the presence of a gradient of chemoattractant between the SMCs from wild-type and those from MMP-2 KO mice (Figure 6C). In contrast, SMC invasion through the Matrigel barrier was significantly reduced in SMCs from MMP-2 KO (Figure 6D). There was no difference in the PDGF-BB–induced proliferative response of cultured SMCs derived from wild-type and those derived from MMP-2 KO mice (Figure 6D). It should be noted that the lack of the effect of MMP-2 deficiency on SMC motility and proliferation was also observed when filter or plate was coated with other matrixes, including fibronectin and gelatin (data not shown).

Discussion

The interruption of blood flow by ligating the mouse common carotid artery results in a marked reduction in luminal area...
through a combination of neointimal formation and arterial constriction. In this model, we observed that MMP-2 and MMP-9 were upregulated after ligation with different time courses in the carotid arteries of wild-type mice, which is in agreement with the previous report. MMPs have overlapping substrate specificities, so the effect of the loss of MMP-2 can be compensated for to some degree by another gelatinase, MMP-9. In fact, the higher MMP-9 level was observed in MMP-2 KO mice during follow-up periods after ligation compared with wild-type mice, although there was no significant difference in the MMP-3 expression. In addition, we also found the decreased expression of TIMPs mRNA in MMP-2 KO mice. Although the mechanism of this down-regulation of TIMPs in MMP-2 KO is not known, MMP-2 may play a role in regulating the expression of TIMPs.

Despite the fact that compensatory increase of MMP-9 expression and the reduction of TIMPs in MMP-2–deficient mice, the intimal area and the ratio of the intimal to the medial area of the ligated arteries of MMP-2 KO mice were much smaller than those in wild-type mice, suggesting that MMP-2 contributes to intimal hyperplasia, at least in this model. No difference of PCNA labeling index in intimal lesion and media region between wild-type and MMP-2 KO mice indicated that SMC proliferation was not involved in the reduction of intimal hyperplasia in KO mice. It is possible that the influence of MMP-2 deficiency on the inflammatory reactions in ligated arteries may contribute to the intimal hyperplasia reduction. However, the lack of MMP-2 did not affect the inflammatory cell infiltration, since no difference in CD45-positive cell infiltration around the ligated arterial wall was observed between wild-type and MMP-2 KO mice at day 3 after ligation.

To elucidate the effect of MMP-2 deficiency on SMC behaviors, we used ex vivo explants and in vitro SMC migration models. The SMC migration from arterial explants reflects the intrinsic ability of cells to migrate and degrade the local extracellular matrix proteins. Previous reports have suggested that this SMC migration from explants was independent of cell proliferation but dependent on matrix-degrading proteases such as MMPs and plasminogen/plasminogen activator families. The observed differences in the migratory properties of SMC from controls and SMC from MMP-2 KO arterial explants and the presence of MMP-2 as a main gelatinolytic activity in medium conditioned with control explants suggested that MMP-2 plays a critical role in SMC migration from arterial explants. We also demonstrated that the invasion of MMP-2–deficient SMCs through the reconstituted basement membrane protein barrier was reduced in comparison with that of control SMCs. It should be noted that no difference was observed in SMC migration across the filter without a matrix proteins barrier between control and MMP-2–deficient SMCs, suggesting that MMP-2 has no effect on SMC motility itself. These findings and the observed absence of effect of MMP-2 deficiency on SMC proliferation indicated that the reduction of the intimal hyperplasia in MMP-2 KO mice appears to be related mainly with the attenuation of the migration of SMCs from the medial to the intimal region through the reduction of proteolytic activities resulting from MMP-2 deficiency.

It has been demonstrated that the interruption of flow caused by carotid artery ligation induces a reduction in vessel diameter in accordance with intimal hyperplasia. However, these two events that are related to arterial remodeling are independent of each other. Our observation of the absence of differences in EEL length after ligation between wild-type and MMP-2 KO mice indicated that MMP-2 was not involved in the mechanism of vessel diameter reduction.

In the present study, we demonstrated that MMP-2 participates in intimal hyperplasia in a mouse carotid artery ligation model but is not involved in the reduction of vessel diameter. The lack of MMP-2 did not affect the early inflammatory phase, which leads to subsequent neointimal hyperplasia but did in fact attenuate the SMC migration from the media into the intima by degrading and breaching the extracellular matrix proteins that surround each cell and basement membrane barrier. However, other MMPs, including MMP-9, serine proteases such as plasminogen/plasminogen activator families may also contribute to intimal hyperplasia in this model.

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