Compensatory Vascular Remodeling During Atherosclerotic Lesion Growth Depends on Matrix Metalloproteinase-9 Activity

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Objective—The compensatory arterial remodeling associated with atherosclerotic plaques is thought to rely on the activity of matrix metalloproteinases (MMP). To assess the role of MMP-9, we analyzed the effect of MMP-9 genetic deficiency on the development and remodeling of experimental atherosclerotic lesions induced in the apolipoprotein E (apoE) knockout (−/−) mouse model.

Methods and Results—We analyzed remodeling parameters and cellular composition of experimental carotid artery atherosclerotic lesions in apoE−/− and apoE−/− MMP-9−/− double-knockout (DKO) mice at 0, 3, 7, and 14 days after induction by flow cessation. Morphometric image analysis of arterial tissue sections indicated that overall artery size, measured as area encompassed by the external elastic lamina, increased 3.1-fold in the apoE−/− mice but only 1.6-fold in the DKO mice (P<0.0001) by 14 days. At the same time, the net lesion area occupied by macrophages was similar. Statistical analysis indicated that the overall expansion of the artery was 2.5-fold less sensitive to macrophage content in DKO compared with apoE−/− mice. No compensatory increase in other gelatinolytic activities was detected in the DKO.

Conclusions—MMP-9 deficiency significantly impaired compensatory vessel enlargement during carotid artery lesion development in the apoE−/− mouse, without altering macrophage content of lesions. (Arterioscler Thromb Vasc Biol. 2004;24:2123-2129.)

Key Words: matrix metalloproteinases ■ vascular remodeling ■ atherosclerosis ■ macrophages ■ transgenic mouse models

Vascular remodeling, defined as a lasting change in the diameter of a mature blood vessel, requires coordinated degradation and deposition of extracellular matrix to preserve the general architecture of the arterial wall while allowing the blood vessel to change in overall diameter. The outward remodeling of arteries provides a compensatory mechanism for preserving blood flow in the face of atherosclerotic lesion growth.1 Advances in vascular imaging have allowed the recent appreciation of the prevalence of compensatory enlargement in coronary artery disease.2 Because of this phenomenon, sizable plaques, which appear to be more likely to trigger acute clinical events, can elude regular angiography.3,4 Plaque instability has been associated clinically with increased macrophage content.5 We proposed that increased proteolytic activity, specifically caused by the action of matrix metalloproteinases (MMP), may provide a molecular mechanism for creating plaque vulnerability.

A substantial body of evidence suggests a key role for MMP in vascular remodeling.6 MMP belong to a family of zinc-dependent endopeptidases capable as a class of degrading all the components of the extracellular matrix. Untimely or unbalanced MMP activity has been associated with pathological remodeling. We previously demonstrated that formation of vascular lesions in a mouse blood flow cessation model is accompanied by increased gelatinolytic activity provided by MMP-2 and MMP-9.7,8 By comparing the extent of geometric remodeling in carotid arteries having smooth muscle cell-driven lesions, as seen in wild-type C57Bl/6 mice, to that in arteries that harbor macrophage-rich lesions, typical for apolipoprotein E (apoE) knockout (−/−) mice, we were able to support a role for macrophages in outward arterial remodeling. Based on these observations, we proposed that the differences in vascular remodeling resulted from significantly higher levels of MMP-9 secreted by infiltrating macrophages in the apoE−/− model. We also observed a positive statistical correlation between the macrophage content of lesions and the extent of outward carotid artery remodeling in the apoE−/− mouse and proposed that infiltrating macrophages may enable arterial expansive remodeling via release of MMP.7 Specifically, pro–MMP-9,

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secreted constitutively by macrophages in vitro and in vivo in both experimental and human atherosclerotic lesions, could facilitate macrophage-driven geometric remodeling. To examine the role of MMP-9 in vascular remodeling during formation of macrophage-rich lesions, we developed apoE/MMP-9 double-knockout (DKO) mice in the atherosclerosis-prone C57Bl/6 background and compared formation of carotid artery lesions in DKO mice and apoE−/− mice.

**Methods**

**Development of ApoE MMP-9 DKO Mice**

To examine the role of MMP-9 deficiency in atherosclerotic lesion formation, we first backcrossed MMP-9−/− mice, originally developed by Vu et al13 in the 129/SvEv background, into the atherosclerosis-susceptible C57Bl/6 genetic background for 8 generations. The resulting MMP-9−/− mice were then intercrossed with apoE−/− mice in the C57Bl/6 background (B6.129P2-Apoem1Wts/J, Jackson Labs, Bar Harbor, Me). The heterozygous offspring were mated to produce double homozygous deficient in both apoE and MMP-9. Genotyping protocols are included in the online data supplement (please see http://atvb.ahajournals.org).

**Experimental Model of Carotid Artery Lesions**

Carotid artery lesion formation was induced in male apoE−/− or apoE−/−MMP-9−/− mice at 6 to 8 weeks of age by ligation of the left common carotid artery under ketamine/xylazine anesthesia, as previously described.7,8 combined with an atherogenic diet containing 1.25 wt% cholesterol, 0.5 wt% cholate, and 35 kcal% fat (Research Diets). This procedure induced formation of macrophage-rich lesions in the apoE−/− mouse. Heparinized blood samples were obtained by retroorbital puncture on the day of tissue harvest for total plasma cholesterol (Cholesterol CII assay kit; Wako). Lesions were characterized by morphometric, immunocytochemical, and biochemical analyses after euthanizing mice by carbon dioxide asphyxiation at 3, 7, and 14 days after carotid ligation, with unligated mice serving as day 0 controls. All mice were perfused at physiological pressure with normal saline via the left ventricle. All procedures involving animals were approved by the Emory University Institutional Animal Care and Use Committee.

**Morphological and Immunohistochemical Analysis**

Mice to be used for morphometry were additionally perfused with 10% neutral buffered formalin (n=3 to 7 each strain at each time point). Carotid artery specimens embedded vertically in paraffin blocks were sectioned, following previously described methods, selecting for morphometric and immunohistological analysis the lesion region with the greatest cross-sectional neointimal area (lesion “apex”). Consecutive 5-μm sections were de-paraffinized and stained for macrophages, using rat anti–Mac-3 monoclonal antibody (1:500; Pharmingen), biotinylated goat antirat IgG (Jackson ImmunoResearch), and HRP-labeled streptavidin; or for smooth muscle cells (SMC) with polyclonal rabbit antibody to smooth muscle α-actin (SMα) (1:300; LabVision), followed by biotinylated goat antirabbit IgG, HRP-labeled streptavidin, and diaminobenzidine substrate kit (Vector). Collagen I was detected with rabbit antimouse antirabbit IgG, HRP-labeled streptavidin, and diaminobenzidine substrate kit (Vector). We performed quantitative image analysis to determine areas encompassed by the lumen, internal elastic lamina, and external elastic lamina (EEL) using ImagePro Plus software (Media Cybernetics). Neointimal lesion area was calculated as the difference between internal elastic lamina area and lumen area. For immunostained sections, we acquired images under standardized exposure conditions and measured peroxidase-positive area by color segmentation, using a negative control section to set the lower limit of detection.

**Biochemical Analyses of Tissue**

Carotid artery specimens were also separately harvested at 0, 3, 7, and 14 days after ligation for biochemical analyses, using 4 to 5 mice of each strain per time point. After saline perfusion, left common carotid arteries were excised between the aortic arch and the carotid artery bifurcation, carefully trimmed of periadventitial fat, and snap-frozen in liquid nitrogen (LN) until use. Frozen tissues were homogenized on ice in cell lysis buffer and used for SDS-PAGE zymography and Western blotting as previously described.10 MMP-2 and MMP-9 levels were quantified by densitometry using a Gel-Doc camera system (Bio-Rad).

**Statistical Analysis**

We analyzed differences between mouse strains at the same time points using 2-factor ANOVA followed by the Tukey test for pair-wise comparisons. We performed ANOVA of sample sets with unequal replication using a general linear model in Minitab 13.0 software (Minitab). We compared the slopes of regression lines using the modified t test described by Zar14 P<0.05 was considered significant. All data are reported as mean±SEM.

**Results**

**Development of ApoE MMP-9 DKO Mice**

The successful breeding of apoE−/− MMP-9−/− mice in the C57Bl/6 background was confirmed by polymerase chain reaction genotyping of tail tip DNA (Figure I, available online at http://atvb.ahajournals.org). Plasma total cholesterol levels in the DKO mice maintained on a standard chow diet were ≈30% lower than in apoE−/− mice (244 ± 22 mg/dL [n=5] versus 350 ± 35 mg/dL [n=12]; P=0.02), but after 14 days on the high-fat diet levels in the 2 strains were not significantly different (1024 ± 100 mg/dL [n=10] versus 1203 ± 180 mg/dL [n=7]; P=0.41).

**MMP-9 Deficiency Impairs Compensatory Vascular Remodeling During Atherosclerotic Lesion Development**

We compared the extent of vascular remodeling after lesion induction in the apoE−/− and apoE−/−MMP-9−/− by measuring the area enclosed by the EEL. As shown by representative histology (Figure 1), apoE−/−MMP-9−/− mice demonstrated impaired outward remodeling compared with apoE−/− mice. EEL area in the apoE−/−MMP-9−/− mice increased by 1.6-fold over baseline by 14 days after lesion induction, compared with a 3.1-fold increase in apoE−/− mice (P<0.0001) (Figure 2, top). Although ligated carotid arteries of MMP-9−/− mice demonstrated a modest increase in EEL area between 0 and 7 days, this change was not statistically significant. In contrast, EEL area in the apoE−/−MMP-9−/− mice increased significantly throughout the course of lesion development, both from 0 to 7 days and from 7 to 14 days. In both mouse strains, lesion growth led to near-total occlusion of the left carotid artery by 14 days, whereas the contralateral carotid remained lesion-free at all time points (data not shown). There were no significant
MMP-9 Deficiency Does Not Change Macrophage Accumulation in Carotid Lesions

Macrophage foam cells are a prominent cellular component of apoE−/− mouse atherosclerotic lesions, including those induced by carotid ligation.15,16 Macrophage accumulation in the apoE−/− mouse originate primarily from recruitment and proliferation of circulating monocytes.16 In vitro studies showed that inhibition of MMP can impair leukocyte migration through artificial membrane barriers.17,18 To investigate whether MMP-9 deficiency also affects macrophage accumulation in vivo, we identified macrophages in carotid lesions using immunohistochemistry (Figure 1, lower). In nonligated mice, macrophage staining was evident only in the adventitia (data not shown). Macrophage-positive area in the neointima of carotid lesions increased with time on diet in mice of both genotypes, with both strains having similar macrophage accumulation at 14 days, as confirmed by quantitative image analysis (Figure 3, top). Macrophage area as a percentage of total intimal area tended to be greater in the apoE−/− MMP-9−/− mice than in apoE−/− mice at the same time points, although the differences did not reach statistical significance (Figure 3, center). These data suggest that MMP-9 deficiency in vivo does not affect neointimal macrophage accumulation. Lack of MMP-9 expression in the macrophage-rich areas of 14-day carotid lesions in apoE−/− MMP-9−/− mice was confirmed by double immunohistochemistry (Figure 4, upper), further indicating that MMP-9 activity is unnecessary for macrophage accumulation in this model. Analyzing the correlation between vessel expansion and net macrophage content of lesions, we found that the slope of the regression line for apoE−/− mice was 2.5-fold greater than for apoE−/− MMP-9−/− mice (3.6±1.0 versus 1.5±0.3, P<0.05) (Figure 3, bottom), indicating that the outward remodeling response was enhanced by the production of MMP-9 by macrophages in the lesions.

MMP-9 Deficiency Leads to Reduced Collagen Accumulation in Carotid Lesions

Macrophage accumulation and concomitant expression of MMPs in atherosclerotic plaques have been linked to decreased collagen content and thinning of the fibrous cap.19,20 Therefore, we asked whether MMP-9 deficiency alters collagen accumulation in mouse carotid lesions.

We analyzed collagen I accumulation in the neointima of mouse carotid arteries by immunohistochemistry and image analysis. In nonligated mice of both genotypes, collagen I was evident primarily in the adventitia. Fourteen days after lesion induction, immunoreactive collagen I was evident in...
the adventitia and the neointima of ligated vessels (Figure 4, bottom). Intimal collagen I content increased as a function of time after lesion induction in both apoE−/− and apoE−/−MMP-9−/− mice, with no difference between strains at any time point. Surprisingly, the carotid lesions of apoE−/−MMP-9−/− mice at 14 days had diminished collagen I content, as a percentage of neointimal area, compared with those of apoE−/− mice (28.7 ± 5.6% versus 52.3 ± 6.1%, P < 0.05) (Figure 5, top).

Because intimal SMC are the likely source of newly deposited collagen I in the neointima, we examined smooth muscle cell alpha actin (SMA) expression in lesions of apoE−/− and apoE−/−MMP-9−/− mice using immunohistochemistry. Net area of immunopositive SMA increased as a function of time after ligation in carotid lesions of both mouse strains (Figure 5, bottom). However, SMA-positive area was significantly greater in apoE−/−MMP-9−/− mice than in apoE−/− mice at 14 days after ligation, and SMA-positive cells accounted for a significantly larger fraction of the total neointimal area in the DKO mice (42.1 ± 9.5% versus 10.2 ± 5.0%, P = 0.02). Furthermore, there was no correlation between EEL area and intimal SMA area in apoE−/− mice (r²=0.18, P>0.05), suggesting that the difference in outward remodeling between the 2 mouse strains cannot be explained by changes in intimal SMC accumulation.
MMP-2 Expression Does Not Compensate for MMP-9 Deficiency in Carotid Lesions of ApoE⁻/⁻ Mice

The observation that macrophage content of lesions was not significantly altered in the DKO mouse, whereas expansive remodeling was impaired, further supported the importance of MMP-9 action. To verify that there was no compensatory upregulation of gelatinolytic enzymes, specifically MMP-2, a closely related gelatinase with similar substrates, we analyzed gelatinolytic activity in carotid tissue lysates using SDS-PAGE zymography.

Pro–MMP-9 expression increased after carotid ligation in apoE⁻/⁻ mice; as expected, pro–MMP-9 was absent in apoE⁻/⁻, MMP-9⁻/⁻ mice, as demonstrated by a representative zymogram (Figure 6A). In apoE⁻/⁻ mice, pro–MMP-9 levels in the ligated vessel increased 24-fold over baseline by 3 days after surgery and gradually declined to a level 8.5-fold over baseline by 14 days (Figure 6B). We confirmed the identity of the gelatinolytic band for MMP-9 by Western blotting of carotid lysates (data not shown). In both mouse strains, pro–MMP-2 and active MMP-2 levels in the left carotid increased to a similar extent after lesion induction, with pro–MMP-2 reaching levels 2-fold greater than baseline by 14 days (Figure 6C). These results support the specific role of MMP-9 in expansive arterial remodeling in vivo.

Discussion

Compensatory vascular remodeling occurs in response to atherosclerotic lesion growth. Several lines of evidence implicate MMP as key effectors of this process. Pasterkamp et al reported increased levels of MMP-2 and MMP-9 in expansively remodeled segments of human coronary arteries compared with constrictively remodeled segments. MMP-9 levels are higher in specimens of human abdominal aortic aneurysm, which we view as an extreme manifestation of expansive vascular remodeling, than in normal vessels. Pyo et al reported that MMP-9 knockout mice were resistant to development of experimental aortic aneurysms. Conversely, apoE⁻/⁻ mice genetically deficient in tissue inhibitor of metalloproteinase-1 showed increased medial degradation and formation of pseudo-microaneurysms during atherosclerotic lesion development, whereas overall lesion size and composition did not differ from those of control apoE⁻/⁻ mice. Studies in several animal models have shown that treatment with synthetic MMP inhibitors can limit flow-mediated expansive remodeling.

Our own work has demonstrated that both MMP-2 and MMP-9 are upregulated during vascular remodeling in the mouse carotid ligation model. The carotid ligation model provides a rapid and reproducible pattern of lesion development. In combination with a hypercholesterolemic diet, carotid ligation in the apoE⁻/⁻ mouse results in development of complex lesions, which reproduce many of the features of advanced “spontaneous” plaques in this animal model. Although this model does not precisely replicate spontaneous atherogenesis, because of differences in the hemodynamic environment in which lesions develop, it serves as a useful starting point to evaluate the effects of genetic deficiencies on cell migration and vascular remodeling. We previously observed that levels of gelatinolytic activity were much higher in ligated carotid arteries of the apoE⁻/⁻ mouse, which underwent dramatic compensatory remodeling, than in arteries of background-matched wild-type mice, which showed minimal remodeling.

Based on our previous results using the apoE⁻/⁻ mouse model of macrophage-rich carotid artery lesions, we suggested that the observed correlation between neointimal macrophage content and compensatory enlargement reflected the contribution of macrophage-derived MMP to remodeling of the vessel structure. Macrophage foam cells in atherosclerotic lesions secrete MMP-9, which has elastolytic as well as collagenolytic activity. MMP-9 production by infiltrating macrophages could contribute to vascular remodeling by partially breaking-down the structure of the elastic lamellae, thus allowing the vessel to expand. In the present study, we have provided evidence in support of this hypothesis using apoE⁻/⁻ mice that were genetically deficient in MMP-9. We used a transgenic mouse model to investigate the role of MMP-9 in compensatory remodeling to separate the individual contributions of the 2 gelatinases, MMP-2 and MMP-9. Most available pharmacological inhibitors of MMP activity are relatively nonspecific in their action, as are the native tissue inhibitors of metalloproteinases. Therefore, use of exogenously supplied tissue inhibitors of metalloproteinases or pharmacological agents to inhibit MMP activity cannot distinguish the unique roles of MMP-2 and MMP-9 during vascular remodeling.

We and others have previously demonstrated that MMP-9 deficiency impairs SMC migration in vitro. This effect was also observed in vivo, resulting in reduced neointima formation in the smooth muscle cell-driven carotid lesions of normocholesterolemic MMP-9⁻/⁻ mice. In that model, macrophages were absent from the neointima, whereas in...
the current model, both macrophages and SMC contribute to lesion growth. In vitro studies of mononuclear cell migration across ECM-coated membranes have suggested that gelatinase expression may enhance leukocyte migration, as well.\(^1,7,18\) Both MMP-2 and MMP-9 can degrade the nonfibrillar collagens of the endothelial basement membrane, potentially facilitating leukocyte entry into the arterial wall. However, our results indicate that macrophage content of atherosclerotic lesions in the apoE\(^{-/-}\) mouse model was not diminished by MMP-9 deficiency. Because most macrophage foam cells in carotid lesions of the apoE\(^{-/-}\) mouse originate from circulating monocytes,\(^{16}\) our observations suggest that monocyte diapedesis can occur in the absence of MMP-9 expression. However, the present study does not rule out the possibility that macrophage proliferation, which also contributes to the growth of atherosclerotic lesions,\(^{16}\) could make up for any difference in monocyte migration rates resulting from MMP-9 deficiency. In addition, MMP-2 secretion by vascular cells may be sufficient to aid monocyte migration into the arterial wall. In fact, T lymphocytes migrating through an endothelial monolayer increased their expression of MMP-2 rather than MMP-9.\(^{18}\)

Interactions with elastin contribute to maintenance of a quiescent, contractile phenotype in vascular SMC.\(^{33}\) Modulation to a synthetic phenotype results in increased production of ECM proteins, important for reshaping the vessel structure during remodeling, and decreased expression of smooth muscle \(\alpha\)-actin.\(^{34,35}\) Because MMP-9 is an elastolytic enzyme, we would expect impaired elastin degradation during remodeling in the DKO mice. In 14-day carotid lesions, we observed significantly more SMA-positive area in the apoE\(^{-/-}\) MMP-9\(^{-/-}\) mice than in apoE\(^{-/-}\) mice, together with a significant decrease in collagen I deposition. Because SMC that have converted to a synthetic phenotype can lose SMA expression,\(^{35}\) the observed reduction in SMA staining in the apoE\(^{-/-}\) should not be interpreted as evidence for impaired SMC migration. On the contrary, the dramatic increase in newly deposited collagen I in the apoE\(^{-/-}\) suggests that SMA-negative, synthetic-type SMC are present and are actively secreting matrix in these lesions. These results in combination suggest that neointimal SMC in the DKO mice tend to retain a contractile phenotype, but additional work is necessary to confirm this hypothesis.

Expansive remodeling, inflammatory cell content, and loss of collagen I have all been associated with plaque vulnerability.\(^{2,5,19,20}\) We found that genetic MMP-9 deficiency reduces expansive arterial remodeling without appreciable effects on macrophage accumulation. Furthermore, MMP-9 deficiency led to diminished accumulation of newly synthesized collagen in developing arterial lesions. Although a reduction in outward remodeling might lead to increased plaque stability,\(^2\) the decreased collagen content of lesions in the DKO mice could offset this effect. Thus, the net effect of MMP-9 deficiency on the mechanical properties of atherosclerotic lesions, which determine their susceptibility to rupture, remains to be determined. Our findings suggest that clinical application of MMP inhibitors as a means of stabilizing atherosclerotic plaques may have both positive and negative effects. Although such a therapeutic approach has been shown to prevent outward remodeling, it might also lead to reduced collagen content of lesions.

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Methods
The mice were genotyped by polymerase chain reaction (PCR) analysis of tail tip DNA, isolated according to the manufacturer’s instructions using the Wizard Genomic DNA kit (Promega). To identify apoE knockout mice, we used the primers 5’-GCCTAGCGAGGGAGAGCCG-3’ and 5’-TGTGACTTGGGAGCTCTGCAGC-3’ to amplify a 155-bp band in wild type individuals, and the primers 5’-GCCTAGCGAGGGAGAGCCG-3’ and 5’-GCCGCCAGCTGCATCTGACTCT-3’ to amplify a 245-bp band in mice carrying the apoE deletion construct \(^1\). For MMP-9 genotyping, we used the primers 5’-GCATACTTGTACCGCTATGG-3’ (forward) and 5’-TGTGATGTTATGATGGTCCC-3’ (reverse) to amplify a 224-bp fragment of exon 2 in the wild type gene and the primers 5’-ATGATTGAACAAGATGGATTGCAC-3’ (forward) and 5’-TTGGTCCAGATCATCCTGATCGAC-3’ (reverse) to amplify a 479-bp fragment located within the neomycin resistance cassette of the MMP-9 deletion construct.

Figure I. PCR confirmation of apoE\(^{-/-}\) MMP-9\(^{-/-}\) genotype. PCR amplification of genomic DNA as described under Methods yields a 245-base pair (bp) band for apoE\(^{-/-}\) mice and a 155-bp band for mice carrying the wild-type allele. PCR using primers specific for the MMP-9 deletion construct yields a 479-bp band for MMP-9\(^{+/+}\) mice and a 224-bp band for the wild-type MMP-9 allele. Lane 1: B6.129P2-Apoel\(^{-/-}\) Unc (apoE\(^{-/-}\)) control; Lane 2: C57Bl/6 MMP-9\(^{-/-}\) control; Lane 3: apoE\(^{-/-}\) MMP-9\(^{-/-}\) double knockout, apoE primers; Lane 4: double knockout, MMP-9 primers; Lane 5: apoE\(^{+/+}\) MMP-9\(^{-/-}\) double heterozygote, apoE primers; Lane 6: double heterozygote, MMP-9 primers.

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