Loss of Matrix Metalloproteinase-9 or Matrix Metalloproteinase-12 Protects Apolipoprotein E–Deficient Mice Against Atherosclerotic Media Destruction but Differentially Affects Plaque Growth

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Background—Epidemiological and histological evidence implicates proteinases of the matrix metalloproteinase (MMP) family in atherosclerosis and aneurysm formation. We previously indicated a role for urokinase-type plasminogen activator in atherosclerotic media destruction by proteolytic activation of MMPs. However, the role of specific MMPs, such as MMP-9 and MMP-12, in atherosclerosis remains undefined.

Methods and Results—MMP-9– or MMP-12–deficient mice were crossed in the atherosclerosis-prone apolipoprotein E–deficient background and fed a cholesterol-rich diet. Mice were killed at 15 or 25 weeks of diet to study intermediate and advanced lesions, respectively. Loss of MMP-9 reduced atherosclerotic burden throughout the aorta and impaired macrophage infiltration and collagen deposition, while MMP-12 deficiency did not affect lesion growth. MMP-9– or MMP-12 deficiency conferred significant protection against transmedial elastin degradation and ectasia in the atherosclerotic media.

Conclusions—This study is the first to provide direct genetic evidence for a significant involvement of MMP-9, but not of MMP-12, in atherosclerotic plaque growth. In addition, deficiency of MMP-9 or MMP-12 protected apolipoprotein E–deficient mice against atherosclerotic media destruction and ectasia, mechanisms that implicate the involvement of these MMPs in aneurysm formation. (Circulation. 2004;109:1408-1414.)

Key Words: atherosclerosis ■ aneurysm ■ metalloproteinases ■ collagen ■ hypercholesterolemia

Matrix metalloproteinase (MMP) family members are thought to be involved in atherogenesis, atherosclerotic plaque rupture, and aneurysm formation; however, their role is deduced mainly from indirect evidence. The promoter region of several MMP genes (MMP-1, -3, -9, and -12) contains polymorphisms, some of which are associated with coronary heart disease and abdominal aortic aneurysms (AAA). Increased MMP-9 levels were detected in plasma of coronary heart disease and abdominal aortic aneurysms. 

In addition, aneurysm and atherosclerotic tissues overexpress MMP-1, -2, -3, -7, -8, -9, -12, -13, -14, and -17 (Carrell et al4 and references therein).

MMP inhibitor studies have strengthened the link between MMPs and atherosclerosis. Transient overexpression of tissue inhibitor of metalloproteinase-1 (TIMP-1) reduced atherosclerotic lesion growth in apolipoprotein E–deficient (apoE−/−) mice and protected against aneurysm rupture in a xenograft model. Conversely, apoE−/−:TIMP-1−/− mice developed more media destruction than their apoE−/−:TIMP-1+/+ littermates. In addition, tetracycline derivatives and synthetic MMP inhibitors suppressed experimental AAA. We showed that urokinase-type plasminogen activator, in part by activation of MMPs, was essential for atherosclerotic media destruction. However, none of these studies clarified a direct role for individual MMPs.

Recently, MMPs were evaluated for their role in atherosclerosis. MMP-3 deficiency in apoE−/− mice increased plaque size but protected against media destruction. MMP-1 overexpression in apoE−/− mice resulted in smaller plaques, but the effect on the underlying vessel wall was not reported. MMP-9–deficient but not MMP-12–deficient mice were protected against elastase-induced AAA. Finally, MMP-2 and MMP-9 were shown to cooperate in mediating AAA formation after perivascular calcium chloride application.

In this study we analyzed the role of MMP-9 and MMP-12 in atherosclerosis and media destruction using the apoE−/− model. We found that apoE−/−:MMP-9−/− plaques were
smaller and contained fewer macrophages and collagen than apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> plaques, whereas MMP-12 deficiency did not significantly affect lesion growth. These findings indicate a differential role in atherosclerotic plaque growth. Importantly, MMP-9 or MMP-12 deficiency protected against atherosclerotic media destruction and ectasia.

**Methods**

**Mice and Gene Analysis**

apoE<sup>−/−</sup> mice were intercrossed with MMP-9<sup>−/−</sup> or MMP-12<sup>−/−</sup> mice (gift from Dr S.D. Shapiro, Washington University School of Medicine, St Louis, Mo), yielding apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> mice and apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> offspring. Breeding of these compound heterozygous mice generated apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> mice (a genetic background of 68.75% C57Bl/6, 12.5% 129SvJ, 12.5% CD1, and 6.25% 129SvJ), apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> mice (84.37% C57Bl/6, 12.5% 129SvJ, and 3.13% 129SvEvTac), and their apoE single-deficient littermates. Mice (all female) were maintained on regular chow for 5 weeks, after which they were fed a cholesterol-rich diet. Quantitative reverse transcription–polymerase chain reaction was performed as described.17

**Morphometric Analysis and Bone Marrow Transplantations**

Mice were anesthetized (60 mg/kg pentobarbital; Nembutal, Abbott Laboratories) and perfused with saline followed by 1% phosphate-buffered paraformaldehyde. Aortas and hearts were harvested and postfixed in the same fixative, dehydrated, and embedded in paraffin. Plaque burden was determined on a separate batch of unfixed, Oil Red O-stained aortas, as described.18 Seven-micrometer cross sections of the descending aorta and aortic origin or 5-μm longitudinal sections of the aortic arch were prepared. Bone marrow transplantation and cross-sectional plaque measurements in descending aorta and aortic origin were performed as described (Data Supplement).16 Elastin degradation was analyzed on at least 50 plaques (in 6 mice). Each plaque was analyzed over its entire length on serial cross sections of the descending aorta, each 70 μm apart. The number of ruptures and the mean cross-sectional rupture length were measured and normalized for plaque dimensions (plaque surface area and mean cross-sectional plaque base length, respectively), as illustrated in the Data Supplement Figure, and the percentage of ruptures in each elastic layer was determined (Data Supplement Figure). Aortic dilatation was determined as described (Data Supplement Figure).11 Sirius red (collagen)17 and Verhoeff–van Gieson (VVG) staining (elastin)11 were performed as described. To detect synthetic smooth muscle cells (SMCs),19 aortic arch sections were stained with mouse anti-rat osteopontin (DSHB). Macrophage content was analyzed on rat anti-mouse Mac-3–stained sections (Pharmingen).11 Morphometric analyses on sections stained with Mac-3, Sirius red, osteopontin, or VVG were performed with a Zeiss Axiosplan2 microscope, a 3CCD video camera (DXC-930P, Sony), and KS300 software.

**Statistical Analysis**

All data, expressed as mean±SEM, in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> and apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> mice were analyzed with the use of the unpaired Student t test and the Mann-Whitney U test, yielding similar results. Data were considered statistically significant at *P*<0.05.

**Results**

**MMP-9 but Not MMP-12 Deficiency Reduces Atherosclerosis**

apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> mice, apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> mice, and their apoE single-deficient littermates, fed a cholesterol diet, developed hypercholesterolemia (MMP-9 background: 2100±110 mg/dL; MMP-12 background: 1400±120 mg/dL; *n=10*). 11 As previously shown,11 immunostaining revealed no MMP-9 or MMP-12 expression in normal aorta, but their expression was highly upregulated in atherosclerotic plaques of apoE<sup>−/−</sup>:MMP<sup>+/+</sup> mice, co-localizing with macrophages.

After 15 weeks of diet, cross-sectional plaque area in the descending aorta was not different between apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> and apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> lesions (Table 1). However, apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> mice developed fewer lesions in the descending aorta than their apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> littermates, and the aortic area covered by Oil Red O–stained lipid lesions was significantly reduced in the absence of MMP-9 (Table 1; Figure 1a, 1b). After 25 weeks of diet, MMP-9 deficiency...
After 25 weeks of diet, analysis of collagen content on Sirius red–stained cross sections of the descending aorta revealed significantly less fibrillar collagen in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> lesions than in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions (Sirius red area: 13 000 ± 2000 versus 50 000 ± 11 000 µm²; n = 7; P < 0.05; Figure 1c, 1d). Collagen density was, however, comparable (37.4 ± 3% versus 39.6 ± 2%, respectively). Lower collagen content was due to a decreased collagen synthesis by SMCs, as evidenced by the reduced transcript levels of osteopontin, a marker of collagen-producing SMCs (number of mRNA copies per 1000 hprt copies: 110 ± 18 in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> lesions versus 212 ± 35 in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions; n = 5; P < 0.05). Correspondingly, osteopontin protein expression in the aortic arch was significantly reduced in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> mice compared with their apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> littermates (osteopontin area: 77 000 ± 26 000 µm² or 2.5 ± 0.9% versus 218 000 ± 33 000 µm² or 6.5 ± 1.1%, respectively; n = 7; P < 0.05). In addition, 1α (I) procollagen transcript levels in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> lesions were only 42% of those in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions. In addition, fewer Mac-3–positive macrophages were present in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> than in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions; the Mac-3 area (expressed as density) in plaques of the thoracic aorta was 3700 ± 1100 µm² (5.4 ± 1.0%) in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> lesions versus 13 700 ± 4100 µm² (13.7 ± 3.8%) in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions, respectively (n = 6; P < 0.05; Figure 1e to 1h). While macrophages penetrated into the entire plaque in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions, they were confined mainly to the surface in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> lesions (Figure 1g, 1h). Collagen content at 25 weeks of diet was not altered by MMP-12 deficiency: the Sirus red area in the descending aorta was 46 000 ± 8000 µm² (34.2 ± 3%) in apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> lesions versus 48 000 ± 13 000 µm² (37.5 ± 5%) in apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> lesions (n = 6; P = NS). Macrophage content was similar: the Mac-3 area was 12 400 ± 4800 µm² (9.1 ± 3.5%) in apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> versus 12 400 ± 5500 µm² (7.0 ± 1.1%) in apoE<sup>−/−</sup>:MMP-12<sup>+/−</sup> lesions; n = 4; P = NS).

**Media Destruction and Ectasia Are Reduced in Absence of MMP-9 or MMP-12**

After 25 weeks of diet, the proportion of plaques featuring fragmentation of at least the internal elastic lamina was comparable in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> mice and apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> mice (n = 93%). Quantification of elastin ruptures in 69 apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> and in 50 apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> plaques throughout the descending aorta revealed ruptures occurring in all elastic layers with varying lengths and frequencies. Mean cross-sectional length of the ruptures (normalized for the mean cross-sectional plaque base length [Data Supplement Figure]) and the number of ruptures (normalized for plaque base area [Data Supplement Figure]) were comparable in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> and apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> mice (Table 2). To analyze rupture depth, the percentage of ruptures in each elastic layer (from internal elastic lamina to external elastic lamina; Data Supplement Figure) was calculated. In apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> and apoE<sup>−/−</sup>:MMP-12<sup>−/−</sup> mice, the majority of ruptures was located in the internal elastic lamina, but a substantial percentage was found in the deeper elastic layers (Table 2; Figure 2a, 2c). In 26 ± 6% of the apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> plaques, all elastic layers were disrupted, and the plaque bulged out into the adventitia (ectasia), covered by a

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**Figure 1. Reduced atherosclerosis in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> mice.**

a, b, En face Oil Red O–stained apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> (a) and apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> aortas (b) reveal significant reduction in plaque burden in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> mice (b). Thoracic (T), abdominal (A), and femoral (F) segments are shown. c, d, Sirius red–stained cross sections through the descending aorta of apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> (c) and apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> mice (d) reveal less collagen in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> lesions (d) (25 weeks of diet). e, f, Mac-3 staining on cross sections through the descending aorta shows fewer macrophages in apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> (f) than in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions (e) (25 weeks of diet). g, h, Mac-3 staining (higher magnification than e, f) reveals that macrophages are present mainly in the superficial layers of apoE<sup>−/−</sup>:MMP-9<sup>−/−</sup> plaques (arrowheads) (h) compared with apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions (g) (25 weeks of diet). L in all panels indicates vessel lumen. Bar = 100 µm in c to f; bar = 50 µm in g and h.
MMP-9 and MMP-12 deficiency protected against media destruction. The proportion of plaques with ruptures was reduced to 68±8% and 53±7% in apoE−/−:MMP-9+/− and apoE−/−:MMP-12+/− mice, respectively. Mean cross-sectional rupture length and number of ruptures were decreased 2-fold in the absence of MMP-9 (Table 2; Figure 2a, 2b). Elastin degradation was decreased 5- to 8-fold by the loss of MMP-12 (Table 2; Figure 2c, 2d). Moreover, while a sub-
A substantial percentage of ruptures was detected in the deeper elastic layers in apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> and apoE<sup>−/−</sup>:MMP-12<sup>+/+</sup> mice, ruptures were largely confined to the internal elastic lamina in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> and apoE<sup>−/−</sup>:MMP-12<sup>+/−</sup> mice (Table 2, Figure 2a to 2d). The incidence of ectasia was decreased 6.5- and 16-fold in the absence of MMP-9 or MMP-12, respectively. Accordingly, aortic dilatation was attenuated significantly by MMP-9 or MMP-12 deficiency (Table 2). Macrophages under apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> and apoE<sup>−/−</sup>:MMP-12<sup>+/−</sup> plaques were confined to the internal elastic layers compared with their apoE single-deficient littermates (Table 2).

**Discussion**

This is the first study to evaluate the role of MMP-9 and MMP-12 in primary atherosclerosis with the use of gene-inactivated mice. Deficiency of these MMPs had a different effect on plaque growth: MMP-9 deficiency reduced and MMP-12 deficiency did not affect lesion growth and composition. These observations, together with recent findings that MMP-3 deficiency increased and MMP-1 overexpression reduced plaque growth and collagen content, argue for specific roles for MMPs in plaque development.

A possible explanation for their distinct roles may relate to different substrate specificities. While collagenolytic MMPs (ie, MMP-1 or MMP-3, which can activate MMP-1) may reduce plaque growth by degrading fibrillar collagen, other MMPs (such as MMP-9) with proteolytic activities against the basement membrane collagens may stimulate lesion growth and collagen accumulation, since basement membrane breakdown seems a prerequisite for the migration, proliferation, and phenotypic switch of SMCs to their synthetic phenotype. The decreased collagen content in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> lesions seems to result from reduced collagen synthesis. The lower abundance of osteopontin, a marker of synthetic collagen-producing SMCs, in the absence of MMP-9 is in agreement with this hypothesis. In turn, the lower amounts of osteopontin may have adversely affected collagen deposition or reduced plaque growth by decreasing macrophage infiltration. Paradoxically, collagen content was increased in the absence of MMP-9 after mouse carotid ligation. We speculate that these apparently discrepant results may be related to model differences, more specifically to the absence (the former study) or presence (our study) of macrophages, which have been documented to induce SMCs to switch to a synthetic phenotype in an MMP-dependent manner. Finally, MMPs, mainly degrading elastin (such as MMP-12), may be particularly critical for atherosclerotic media destruction by destroying the elastic laminae, without significantly affecting plaque growth.

The importance of MMP-12 in elastin degradation and matrix invasion was documented in vitro and in pulmonary emphysema. Its role, however, was redundant in cardiac rupture and in postinfarction myocardial healing, presumably because of the lower elastin content of the heart than of the aorta. In the present study MMP-12 deficiency protected against elastin degradation in the atherosclerotic media. Although protection by MMP-12 deficiency seemed more dramatic than by MMP-9 deficiency, the different genetic background of both mouse strains did not allow direct comparison. This reduced elastin degradation and accompanying aortic dilatation by loss of MMP-12 is at variance with findings of Pyo et al. who found no effect of MMP-12 deficiency on the incidence and extent of elastolysis and aortic dilatation after elastase infusion. Differences in the models may explain this apparent discrepancy, ie, MMP-12 may be more important in elastin degradation in a more chronic inflammation model such as atherosclerosis-associated media destruction than in the more acute elastase model. However, in mice with a combined deficiency of MMP-9 and MMP-12, protection was somewhat increased compared with single deficiency of MMP-9, indicating that MMP-12 could enhance the effects of MMP-9 in the elastase model.

The mechanisms and the main cell source of MMP-9 responsible for the plaque phenotype remain to be determined. Although bone marrow transplantation restored the plaque phenotype, indicating that bone marrow–derived macrophages were an important source of MMP-9, this does not exclude a possible contribution from SMCs. Nevertheless, immunostainings of plaques revealed almost exclusive colocalization of MMP-9 within macrophages, indicating the latter as the cardinal cell source. Remarkably, in apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> mice, the number of lesions was reduced by half from 15 to 25 weeks of diet, whereas the number of plaques did not change in time in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> mice (Figure 3). Therefore, we speculate that merging of adjacent small plaques into larger plaques in apoE<sup>−/−</sup>:MMP-9<sup>+/+</sup> mice, but not in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> mice, in part explains the increased plaque growth in apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> compared with apoE<sup>−/−</sup>:MMP-9<sup>+/−</sup> mice at advanced stages of atherosclerosis (Figure 3). MMP-9–deficient plaques contained fewer macrophages confined to the superficial layers of the plaque, possibly unable to invade through the endothelial basement membrane. In addition, reduced recruitment of myeloid cells or progenitors may explain the decreased macrophage content. Finally, MMP-9 may indirectly affect myeloid cell recruitment by releasing vascular endothelial growth factor (VEGF) from the plaque matrix, as observed during bone development. In support of this, we demonstrated that blocking Flt1, a receptor for VEGF (and its homologues placental growth factor and VEGF-B), attenuated lesion growth by reducing myeloid progenitor mobilization into the blood and by impairing their differentiation/activation and infiltration into atherosclerotic plaques.

The protective effect of MMP-9 and MMP-12 deficiency against atherosclerotic media degradation and accompanying ectasia and aortic dilatation suggests that these particular MMPs may play a central role in aneurysm formation since elastin degradation and aortic diameter expansion are hallmarks of this disease. In regard to MMP-9, its involvement in aneurysm progression was additionally supported in 2 inflammatory models of AAA.
and possibly MMP-12, as potential targets to prevent aneurysm expansion before it reaches a critical size, leading to life-threatening ruptures. At present, no therapeutic alternatives exist for patients with aneurysms beyond careful monitoring until they become eligible for surgical repair of the aneurysm. Therefore, pharmacological inhibition of aneurysm expansion, such as specific MMP inhibition, would be of significant benefit for these patients. In addition, MMP-9 inhibition may also reduce atherosclerotic lesion progression.

Acknowledgments
This work was supported by the European Community (Bio-med BMH4-CT98-3380), GOA/2001/09, and IUAP/05/02, granted to Dr Carmeliet, and an FWO fellowship, granted to Dr Luttun. The authors thank A. Bouché, M. De Mol, S. Jansen, W.Y. Man, S. Terclavers, B. Vanwetswinkel, S. Wyns (CTG, Belgium), B. Wuyts (University Hospital, Ghent), and W. Landuyt (Radiobiology, KULeuven) for technical assistance and A. Vandenhoock (CTG, Belgium) for artwork.

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_Circulation_. 2004;109:1408-1414; originally published online March 1, 2004; doi: 10.1161/01.CIR.0000121728.14930.DE

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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