Lesional Overexpression of Matrix Metalloproteinase-9 Promotes Intraplaque Hemorrhage in Advanced Lesions But Not at Earlier Stages of Atherogenesis


**Background**—Matrix metalloproteinase-9 (MMP-9) is involved in atherosclerosis and elevated MMP-9 activity has been found in unstable plaques, suggesting a crucial role in plaque rupture. This study aims to assess the effect of MMP-9 on plaque stability in apolipoprotein E-deficient mice at different stages of plaque progression.

**Methods and Results**—Atherosclerotic lesions were elicited in carotid arteries by perivascular collar placement. MMP-9 overexpression in intermediate or advanced plaques was effected by intraluminal incubation with an adenovirus (Ad.MMP-9). A subset was coincubated with Ad.TIMP-1. Mock virus served as a control. Plaques were analyzed histologically. In intermediate lesions, MMP-9 overexpression induced outward remodeling, as shown by a 30% increase in media size ($p=0.03$). In both intermediate and advanced lesions, prevalence of vulnerable plaque morphology tended to be increased. Half of MMP-9–treated lesions displayed intraplaque hemorrhage, whereas in controls and the Ad.MMP-9/Ad.TIMP-1 group this was 8% and 16%, respectively ($p=0.007$). Colocalization with neovessels may point to neo-angiogenesis as a source for intraplaque hemorrhage.

**Conclusion**—These data show a differential effect of MMP-9 at various stages of plaque progression and suggest that lesion-targeted MMP-9 inhibition might be a valuable therapeutic modality in stabilizing advanced plaques, but not at earlier stages of lesion progression. *(Arterioscler Thromb Vasc Biol. 2006;26:340-346.)*

**Key Words:** adenovirus • atherosclerosis • metalloproteinases • remodeling • vulnerable plaque
in the carotid artery of apoE-deficient mice. We found that in intermediate plaques, MMP-9 did not evoke adverse events, but caused outward remodeling, whereas in advanced lesions this was accompanied by an increased incidence of intraplaque hemorrhage, a sign of plaque vulnerability. These findings provide important new insights into the role of MMP-9 throughout atherosclerotic lesion development and identify a target for plaque stabilizing therapy in advanced lesions.

Materials and Methods

Animals
Female apoE-deficient mice on a C57Bl/6 background (n = 66), 10 to 12 weeks of age, obtained from our own breeding stock, were placed on a high-fat diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). All animal work was approved by the regulatory authority of Leiden University and performed in compliance with the Dutch government guidelines.

Carotid Collar Placement and Transgene Expression
Carotid atherosclerotic lesions were induced by collar placement as previously described. Briefly, a constricting manchette was placed on both carotids causing atherosclerotic lesions proximal to the collar within 4 to 6 weeks. Previous time-related experiments showed a different stage of lesion progression at 21 versus 35 days after collar placement, with smaller plaques (>40 000 μm²) displaying fewer macrophages and less remodeling compared with advanced lesions (>80 000 μm²) (unpublished data). Plaques were incubated intraluminally with an adenovirus suspension 25 or 38 days after collar placement. Vectors carried human proMMP-9 (Ad.MMP-9) or an empty transgene (Ad.Empty). To verify that MMP-9–related effects are associated with increased proteolytic activity, we included a TIMP-1 overexpressing subset in the advanced group. The virus load was equalized in all groups to exclude toxicity or any immunologic effects (for details please see http://atvb.ahajournals.org).

Tissue Harvesting and Preparation for Histological Analysis
Two weeks after infection and 1 day before euthanization, phenyl-ephrine (8 μg/kg intravenous; Sigma) was administered to assess plaque integrity by means of hemodynamic challenge. Before harvesting, the arteries were perfused with formaldehyde.

Transverse, serial cryosections were prepared from carotid artery and stained with hematoxylin (Sigma) and eosin (Merck) or Masson’s trichrome (Accustain kit; Sigma). Collagen was stained by picro Sirius Red (Direct red 80; Sigma) and elastin was visualized by accustain elastic staining (Sigma). Perl’s staining was applied to detect intraluminal iron deposits. Corresponding sections were immunostained with antibodies directed at mouse metalloproteinase inhibitors such as α-SM-actin (clone 1A4; Sigma), α-SM-actin (clone 1A4; Sigma), and CD31 (BD Pharmingen). To assess cell death, sections were terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) stained using protocols provided by the manufacturer (In-Situ-Cell-Detection-Kit; Roche Diagnostics).

Morphometry
The site of maximal plaque size was selected for morphometry. Images were digitized and analyzed as previously described. Briefly, luminal, intimomedial, and media were directly measured using Leica QWin software. The area circumscribed by the external elastic lamina was calculated from these values and designated as total vessel area. Stage of lesion progression was assessed with Virmani’s classification criteria. Of 6 defined categories (i.e., 1: fibrous lesion; 2: atheroma; 3: thin cap fibroatheroma [TCFA]; 4: healed rupture; 5: rupture or intraplaque hemorrhage; and 6: plaque erosion), the first 2 classes are considered stable, whereas lesions in classes 3 to 6 are perceived as vulnerable plaques. Lesions were allocated to different classes using observations of subsequent sections with a maximal interval of 100 μm. TCFA is defined as lesions with a thin cap (≤3 cell layers) accompanied by a large necrotic core (>40%). Macrophage, smooth muscle cell (SMC), collagen and MMP-9 positive areas were determined by computer-assisted color-gated measurement, and related to total intimal surface area.

MMP-9 Activity in Vitro and in Vivo
The inhibitory action of Ad.TIMP-1 on MMP-9 was tested in vitro. For this, SMCs were transduced with: (1) Ad.Empty; (2) Ad.MMP-9; and (3) Ad.MMP-9/Ad.TIMP-1.

To confirm that MMP-9 gene transduction also increases MMP-9 expression and activity in vivo, we performed MMP-9 immunohistochemistry and in situ zymography. A subset of carotid plaques was incubated with Ad.LacZ to map the cells that are targeted by this vector system by β-galactosidase staining. For a detailed description please see http://atvb.ahajournals.org.

Statistics
All values are displayed as mean±SEM. Differences in plaque size were statistically analyzed for significance using the Mann-Whitney U test. Human MMP-9 overexpression was assessed with a 1-tailed Student t test. Gelatinolytic activity, collagen, elastin, TUNEL positivity, SMC, and macrophage content were compared using the 2-tailed Student t test. Differences in the occurrence of adverse events, iron depositions, and classification were analyzed with the Yate’s corrected 2-sided Fisher’s exact test (2 groups, i.e., intermediate lesions) or with the χ² test of independence (3 groups, i.e., advanced lesions).

Results
Adenoviral Expression Pattern
To confirm MMP-9 expressing activity of Ad.MMP-9 and validate the Ad.MMP-9:Ad.TIMP-1 ratio, MMP-9 activity was tested in media of transduced SMCs in vitro. Ad.MMP-9 resulted in a 2-fold increase of MMP-9 activity from 0.05±0.01 to 0.11±0.02 rfu/s (P = 0.002). Cotransduction with Ad.TIMP-1 at a 1:1 titer ratio inhibited this effect by 45% to 0.08±0.02 relative fluorescent units/s (P = 0.02).

To establish the efficiency and distribution of vascular transduction, initial studies were performed with Ad.MMP-9. In line with earlier studies in our laboratory, carotid plaques incubated intraluminally with Ad.CMV.LacZ revealed that this principally targets the endothelium and SMCs of the fibrous cap (Figure 1A). Similarly, incubation with Ad.MMP-9 resulted in a clear expression of the transgene, particularly at sites of SMC accumulation (P = 0.007) (Figure 1B to 1D). In situ zymography was applied to assess proteolytic activity. Ratio of intimomedial fluorescent staining, reflecting gelatinase activity, was raised from 0.45±0.20 in controls to 1.51±0.26 in MMP-9 overexpressing plaques (P = 0.05). TIMP-1 overexpression attenuated this to 0.87±0.27 (P = 0.1; Figure 1E to 1I). As in situ zymography is a semiquantitative method, these borderline-significant results suggest that the vector is functional in vivo, which encouraged us to continue with further experiments regarding plaque stability. In all experiments, intracarotid virus instillation was well-tolerated. No adverse effects were noted including any changes in bodyweight or cholesterol levels.

MMP-9 Does Not Significantly Affect Size of Intermediate or Advanced Plaques
Because this study aims to assess the effect of MMP-9 on pre-existing plaques, it was important to induce lesions before
gene transfer. In this way, neither lesion size nor site could influence plaque composition or stability. Therefore, we applied the collar model for rapid atherogenesis. When plaques had developed to the desired stage, gene transduction was performed and 2 weeks later carotids were harvested for further analysis.

Plaque size was ≈2-fold larger in advanced than in intermediate lesions (Figure 2A). In the latter, MMP-9 did not significantly affect plaque size, but moderately increased intima-to-lumen ratio, reflecting the degree of stenosis, from 0.51±0.05 in controls to 0.65±0.04 in MMP-9 overexpressing vessels (P=0.04) (Figure 2B). In advanced lesions no difference in lesion size or intima-to-lumen ratio could be detected between groups.

MMP-9 Overexpression Leads to Outward Remodeling in Intermediate Lesions

Although plaque size had not changed significantly, MMP-9 overexpression did affect other vessel dimensions in intermediate lesions (Figure 2C and 2D). Media size increased by...
30% from 28,000±950 μm² in controls to 36,500±3500 μm² in MMP-9 overexpressing mice (P=0.03). Also, total vessel area was increased in Ad.MMP-9 treated vessels (Ad.MMP-9: 150,000±5300 μm² versus Ad.Empty: 130,000±5400 μm²; P=0.02), indicating initiation of outward remodeling.

In advanced plaques, no such differences could be detected. Media size amounted to 39,000±8000 μm² in controls, whereas in the MMP-9 and MMP-9:TIMP-1–treated group this was 38,500±5400 μm² and 34,500±3000 μm² respectively. Also, total vessel area did not differ between groups in arteries with advanced plaques (Ad.Empty: 237,000±26,000 μm² versus Ad.MMP-9: 250,000±16,500 μm² and AdMMP-9:Ad.TIMP-1: 230,000±14,500 μm²) (Figure 2D).

**MMP-9 Overexpression Leads to Vulnerable Plaque Morphology in Advanced Lesions**

The main objective of this study was to assess the effect of MMP-9 overexpression on plaque stability. Therefore, lesions were categorized according to general morphological features, cap thickness, and the presence of adverse events. Fibrous lesions and atheroma, class 1 and 2, were perceived as stable. Plaques showing thin cap morphology or adverse events (classes 3 to 6) were considered unstable (Figure 3A to 3D). Although, in intermediate plaques, significantly more vessels showed characteristics of vulnerability in the Ad.MMP-9 group (85% versus 43% in the controls; P=0.046), no effect of MMP-9 overexpression on the occurrence of adverse events was observed (Table).

In advanced plaques, 87% of MMP-9 overexpressing lesions displayed features of a vulnerable plaque morphology as compared with only 50% in controls and 58% in the TIMP-1 cotransduced group. Although this indicates that TCFAs are the predominant lesion type in MMP-9 overexpressing plaques compared with controls, it did not reach statistical significance (P=0.10) (Table). However, MMP-9 transduction led to a considerable increase in the incidence of intraplaque hemorrhage (IPH), defined as presence of extravasated erythrocytes within the intima. In controls and TIMP-1 cotreated group a mere 8% and 16% of such events were observed, whereas MMP-9 overexpressing plaques displayed an incidence of 53% (P=0.007) (Table). No effect on incidence of elastic lamina rupture was observed in both types of lesions. Two events of IPH had the appearance of an incomplete intima-media dissection (Figure 3C and 3D), and all of these events were accompanied by iron depositions suggesting presence of intramural thrombi (Figure 3B).

Because plaque stability could also be influenced by fibrous cap integrity, we measured fibrous cap thickness. Mean cap thickness, measured from 12 different sites per section, was decreased by 41% in MMP-9 overexpressing intermediate lesions (Ad.Empty: 34.5±8.7 μm versus Ad.MMP-9: 20.5±2.6 μm; P=0.04). Cap thinning was also observed in advanced plaques (Ad.Empty: 21.1±2.0 μm versus Ad.MMP-9: 15.9±1.2 μm; P=0.02), but TIMP-1 treatment did not significantly alter cap thickness in MMP-9–treated advanced lesions (Ad.MMP-9/Ad.TIMP-1: 16.2±1.6 μm) (Figure 3E). Similar effects were observed for fibrous cap area (data not shown).

**Plaque Composition Is Not Affected at Both Stages of Development**

Because the chance of adverse events is expected to increase in plaques with high accumulation of infiltrated leukocytes, mac-
rophage-specific immunostaining was performed and showed more macrophages in advanced compared with intermediate plaques. However, MMP-9 overexpression did not affect intimal macrophage content in both intermediate (macrophage/intima, 0.22 ± 0.15 versus 0.27 ± 0.17 in controls) and advanced lesions (macrophage/intima, 0.52 ± 0.17 versus 0.44 ± 0.15 in controls and 0.41 ± 0.17 with TIMP-1 cooverexpression) (Figure IA, available online at http://atvb.ahajournals.org).

Because MMP-9 may promote SMC migration into the intima and SMCs play a central part in ECM homeostasis, sections were stained for α-SM-actin. No difference in α-SM-actin staining could be detected suggesting that intimal SMC content had not been affected (Figure IB).

Intimal collagen was comparable between both stages of plaque progression. In intermediate lesions, no clear effect of MMP-9 on collagen content could be detected. In advanced plaques, however, intimal collagen tended to diminish after MMP-9 gene transfer, but this was not significant and unaffected by cotransduction with Ad.TIMP-1 (Figure IC). Also intimal elastin remained unaffected by MMP-9 overexpression (Figure ID).

Because MMP-9 might have affected cell death through release of matrix-bound pro-apoptotic factors, apoptosis was quantified by TUNEL staining, which did not reveal any changes in apoptotic rate (Figure IE).

Besides ECM weakening, risk of IPH may be enhanced by increased neo-angiogenesis. CD31 staining did not reveal any differences in density of neovessels between groups. However, it did show that such vessels not only are present in collar-induced lesions but also in the media, suggesting that neovessels most likely originate from the adventitial side of the plaque, penetrating the elastic lamina (Figure 4A and 4B). Incidental colocalization with sites of extravasated erythrocytes may point to neo-angiogenesis as a source for IPH.

**Discussion**

Atherosclerotic plaque rupture is a major cause of acute ischemic events. Several mechanisms, including apoptosis

### Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Intermediate</th>
<th>Advanced</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td></td>
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</table>

Values are mean±SEM. NS indicates not significant.
and matrix degradation, have been implicated in this process. Several studies demonstrated a role of MMPs in atherosclerosis and plaque stability. MMP-9 plasma levels are raised in patients with acute coronary syndromes and high-expressing MMP-9 polymorphisms are associated with increased risk for cardiovascular events. Conversely, apoE−/−/MMP-9−/− mice displayed more characteristics of plaque vulnerability than their MMP-9−/− littermates.

In this study, MMP-9 was overexpressed in pre-existing plaques at an intermediate and advanced stage of progression to evaluate the effect on both plaque morphology and stability. The results show, in a prospective manner, that MMP-9 can destabilize advanced plaques and that TIMP-1 overexpression is able to attenuate this effect. In advanced lesions, MMP-9 overexpression increased the incidence of IPH, a common manifestation of plaque destabilization in mouse models of advanced atherosclerosis and thought to further aggravate plaque vulnerability. In intermediate lesions, MMP-9 overexpression promoted morphological characteristics of vulnerability, ie, TCFA, but this was not accompanied by more adverse events, indicating that fibrous cap thinning is not causally related to IPH. Furthermore, in contrast to advanced plaques, intermediate lesions showed a modest increase in outward remodeling resulting from 2 weeks of MMP-9 overexpression. This study is the first to our knowledge to demonstrate a differential effect of MMP-9 during plaque development.

Because MMP-9 exerts pleiotropic effects including ECM degradation, release of matrix-bound factors, and adhesion molecule shedding, we speculate that various stages of atherogenesis feature a different aspect from this wide array of physiological capacities. Depending on its context, source and abundance MMP-9 could, directly or indirectly, by activating other proteases or releasing matrix-bound effectors, affect matrix homeostasis, cell recruitment, and apoptosis. Although the vector system applied in the present study principally targets SMCs and endothelial cells, it is conceivable that the secreted zymogen diffuses throughout the plaque and is activated elsewhere in the lesion. This is illustrated by the medial SMC proliferation that clearly lies at the base of the observed vessel remodeling in intermediate plaques, a process that requires basal membrane degradation by proteolytic activity. Conversely, in advanced plaques MMP-9 overexpression did not affect α-actin positive SMC content or apoptotic rate, suggesting that intimal SMC turnover remained unchanged in these lesions.

Previous evidence suggested that MMP-9 might directly degrade the collagens type I and III. Notwithstanding the fact that, in the present study, intimal collagen content remained unchanged in intermediate and only moderately decreased in advanced lesions, it is possible that MMP-9 overexpression may have affected collagen organization, therefore changing structural integrity of the plaque. Galis et al showed that MMP-9 deletion in the arterial wall resulted in accumulation of adventitial collagen without showing tissue constrictive and that MMP-9 deficiency impairs the capacity of SMCs to contract collagen gels in vitro.

Furthermore, degradation of ECM constituents can result in release of several matrix bound bioactive molecules, such as FGF-2 and transforming growth factor (TGF)-β. Together with digestion of ground matrix and the basal membrane, this can result in migration and activation of inflammatory cells. Moreover, MMP-9 may facilitate neo-angiogenesis via basal membrane degradation and release of VEGF. Newly formed leakier vessels can contribute to persistent inflammation by conveying blood cells into the plaque. However, our findings do not show an increase of intimal macrophages, indicating that, in this model, plaque destabilization was not so much induced by increased influx of inflammatory cells, but by direct proteolytic action, modulating the extracellular or pericellular matrix.

Induction of lesional neo-angiogenesis may enhance the risk of IPH as well. Although, no clear effect of MMP-9 on the extent of intimal neovessels was observed, CD31 staining did indeed reveal incidental colocalization of neovessels and sites of extravasated and degraded erythrocytes, pointing to intimal neo-angiogenesis as a feasible source for IPH.

Finally, it should be noted that the pleiotropic actions of MMP-9, its diffuse distribution and ability to activate other proteolytic enzymes within the plaque are limiting factors in providing an interpretable topological evaluation of MMP-9 activity in relation to the different cell types. Additional studies may be required to further map the cell or location specific actions of MMP-9 with regard to remodeling, cap thinning, and intraplaque hemorrhage.

In conclusion, MMP-9 promotes atherosclerotic plaque progression, cap thinning, and outward remodeling in intermediate lesions, but does not affect the incidence of adverse events, such as IPH, rupture, or thrombosis. In advanced complex lesions, it promotes vulnerable plaque morphology accompanied with a high incidence of IPH. Concomitant TIMP-1 gene transfer prevented these adverse events. This indicates that selective MMP-9 inhibition could certainly be a valuable therapeutic modality. However, as at the onset of atherogenesis MMP-9 appears to play a more protective role, and in intermediate lesions MMP-9 may preserve lumen patency through outward remodeling, MMP-9 inhibition might not be desirable in every stage of lesion progression making a systemic therapeutic approach less appropriate. Therefore, a lesion-targeted strategy toward advanced, complex plaques may be more beneficial in patients with coronary artery disease and selection of these target lesions should be approached with utmost care.

Acknowledgments

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References


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MMP-9 activity in vitro

To validate the Ad.MMP-9:Ad.TIMP-1 ratio to be used in vivo, the inhibitory action of Ad.TIMP-1 on MMP-9 was tested in vitro at a titer ratio of 1:1. For this, SMCs were incubated with (1) 300 m.o.i. Ad.Empty, (2) 150 m.o.i. Ad.MMP-9 and 150 m.o.i. Ad.Empty or (3) 150 m.o.i. Ad.MMP-9 and 150 m.o.i. Ad.TIMP-1. Cells were incubated with virus for 16 h after which cells were washed and incubated for an additional 24 h. MMP activity was measured using the internally quenched fluorogenic peptide substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys(Fluorescein)-Ala-Lys-NH2). Culture medium samples were 8-fold diluted in MMP buffer (50 mM Tris, 5 mM CaCl₂, 250 mM NaCl, 1 μM ZnCl₂, 0.02% NaN₃ and 0.01% Brij-35, pH 7.5). EDTA-free Complete(tm) (serine and cysteine protease inhibitors, Roche, Mannheim, Germany; 1 tablet in 50 ml) was added to all conditions. Conversion of TNO211-F (5 μM) was assessed in the presence or absence of 5 μM BB94 (a broad spectrum MMP inhibitor). The difference in the initial rate of substrate conversion between samples with or without BB94 addition was used as a measure of MMP activity. Fluorescence was monitored real-time for 4 hrs at 30°C using a Cytofluor 4000 apparatus (Applied Biosystems, Foster City, CA).

MMP-9 activity in vivo

To confirm that MMP-9 gene transduction also increases MMP-9 expression and activity in vivo we performed MMP-9 immunohistochemistry (antibody was a kind gift from Dr. Hanemaaijer, TNO-PG, Leiden, Netherlands) and in situ zymography on plaques 1 week after incubation with Ad.MMP-9, Ad.MMP-9/Ad.TIMP-1 or with mock virus in a separate experiment (n=9). A subset of carotid plaques (n=3) was incubated with 1.0 ·10¹⁰ pfu/mL Ad.CMV.LacZ to map the cells that are targeted by this vector system by β-galactosidase staining (X-gal, 1 mg/mL; Eurogentec, Belgium). Non-fixed cryosections were washed in PBS and incubated with 0.05% DQ-gelatin (Molecular Probes, Netherlands) in 50mM Tris-HCL pH 7.6 and 5mM CaCl₂ for 18 hours at 37°C and 5% CO₂. Gelatinolytic activity was visualized as green fluorescent staining under a 465-495 nm excitation filter. Auto-fluorescence was suppressed with 0.5% Chicago Sky Blue (Sigma). The protease inhibitor 1.10-phenantrolin (1 mM) was added to the buffer as a negative control. Intimal gelatinolytic activity was expressed relative to adventitial activity, which is unaffected by luminal adenoviral incubation.
Experimental setup intraluminal adenoviral transductions

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<td>Ad.TIMP-1</td>
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Intermediate (n=27) or advanced plaques (n=39) were incubated intraluminally with an adenovirus suspension 25 or 38 days after collar-placement, respectively. Vectors carried human proMMP-9 (Ad.MMP-9) or an empty transgene (AdEMPTY) under control of a CMV promotor. To verify that MMP-9 related effects could be associated with increased proteolytic activity, we included a TIMP-1 overexpressing subset (n=12) in the advanced group. To exclude toxicity or immunological effects from the adenovirus itself, the virus load was equalized in all groups. Fourteen days after gene transduction, lesions were analyzed histologically with regard to morphology and composition.
Figure I. Composition of advanced lesions. A. Ratio of macrophage to intimal staining area. MMP-9 overexpression did not alter macrophage content. B. SMC:Intima ratio. MMP-9 gene transfer did not affect α-SM-actin immunostaining. C. Collagen:Intima ratio. MMP-9 transduction tended to decrease intimal collagen in advanced lesions (P=0.07). D. Elastin:Intima ratio remained unaffected by MMP-9 overexpression with or without TIMP-1 co-transduction. E. TUNEL staining for apoptosis expressed as percentage of positive cells. Values are mean ± SEM.