Modulation of the Extracellular Matrix in Advanced Atherosclerosis

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3 Lesional Overexpression of Matrix Metalloproteinase-9 Promotes Intraplaque Hemorrhage in Advanced Lesions, but not at Earlier Stages of Atherogenesis

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Abstract

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 overexpression on plaque stability in apoE deficient mice at different stages of plaque progression. Atherosclerotic lesions were elicited in carotid arteries by placement of a perivascular collar. MMP-9 overexpression in intermediate or advanced plaques was effected by intraluminal incubation with an adenovirus (Ad.MMP-9). A subset was co-incubated with Ad.TIMP-1. Mock virus served as a control. Two weeks later, 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, the prevalence of vulnerable plaque morphology tended to be increased (P=0.05 and 0.10 respectively). Half of MMP-9 treated lesions displayed intraplaque hemorrhage, whereas in controls and the Ad.MMP-9/Ad.TIMP-1 group this was only 8 and 16% respectively (P=0.007). Co-localization with neo-vessels may point to neo-angiogenesis as a source for intraplaque hemorrhage.
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.

Introduction

Matrix metalloproteinase (MMP) family members are enzymes with activity against extracellular matrix (ECM) constituents and have been linked to atherosclerotic plaque progression, rupture and aneurysm formation. Since atherosclerotic plaque rupture is a frequent cause of acute coronary syndromes[1,2,3] and MMPs are believed to degrade the ECM in the fibrous cap[4,5], these enzymes might prove to be relevant targets for therapeutic intervention.

However, the evidence that links these proteases to plaque destabilization is largely circumstantial and based on retrospective observations. Elevated mRNA and protein levels of several MMPs, among which MMP-9, were found in unstable and ruptured areas in carotid endarterectomy specimens.[6,7] Promoter polymorphisms, leading to enhanced MMP expression, are correlated to coronary artery disease and to the complexity of the lesions.[8,9] Also, elevated MMP-9 plasma levels can be detected in patients with acute coronary syndromes.[10,11]
Taken together, this suggests that MMP-9 is causally involved in plaque destabilization, although the underlying mechanism remains unclear. One report showed that MMP-9 overexpression might lead to thrombosis by stimulating release of matrix bound tissue factor in balloon injured coronaries.[12] Conversely, targeted gene disruption of MMP-9 in mice impaired SMC migration and led to interstitial collagen accumulation.[13] In vitro, MMP-9 deficiency impaired the contracting capacity of collagen gels, indicating that MMP-9 not only is important for SMC migration and matrix degradation, but also plays a pivotal role in ECM organization.[13]

Notwithstanding these observations, direct evidence for a causal role of MMP-9 in plaque rupture is still lacking. The ability of this protease to promote SMC migration and consolidation of collagens might even suggest the opposite. Indeed, Jackson and colleagues showed that in ApoE/MMP-9 double knockout mice the absence of this protease promotes, rather than prevents, vulnerable plaque morphology, suggesting a stabilizing effect of MMP-9.[14] However, in this model MMP-9 is lacking from all stages of atherogenesis, while in physiological conditions it is deemed to exert its adverse effects at later stages of lesion progression. Conceivably, the pathophysiological actions of MMP-9 could very well differ at various stages of plaque progression.
In this study we analyzed the effects of MMP-9 overexpression in moderately progressed (intermediate) and advanced atherosclerotic plaques in the carotid artery of apolipoprotein E (apoE) deficient mice. We found that in intermediate plaques, MMP-9 overexpression did not evoke adverse events, but caused outward remodeling, whereas in advanced lesions this was accompanied by an increased incidence of intraplaque hemorrhage (IPH), 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), 10-12 weeks of age, were obtained from our own breeding stock. Mice were placed on a western type diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). High fat diet and water were provided ad libitum. 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 perivascular collar placement as previously described.[15] Briefly, a constricting silastic manchette was placed on both carotids causing atherosclerotic lesions proximal to the collar within four to six weeks. High-fat diet started 14 days prior to collar placement. Previous time-related experiments in our lab showed a different stage of lesion progression at 21 vs 35 days after collar placement, with smaller plaques (~40,000 μm^2) containing fewer macrophages and displaying less outward remodeling in the former lesions compared to advanced lesions (~80,000 μm^2) (unpublished data). This can also be appreciated from the differences between both control groups in the present study. Intermediate (n=27) or advanced plaques (n=39) were incubated intraluminally with an adenovirus suspension 25 or 38 days after collar placement. Adenoviral vectors carried a human proMMP-9 (Ad.MMP-9) or an empty transgene (Ad.Empty) under control of the CMV promoter.[16] In order to verify that MMP-9 related effects could indeed be associated with increased proteolytic activity, we also included a subset (n=12) in the advanced group which was co-transduced with a human TIMP-1 transgene ($1.0 \cdot 10^{10}$ pfu/mL Ad.MMP-9 and $1.0 \cdot 10^{10}$ pfu/mL Ad.TIMP-1).[16] To exclude cytotoxicity or immunological effects, the virus load was equalized in all groups, thus the MMP-9 treated group (intermediate n=13; advanced n=15) was infected with $1.0 \cdot 10^{10}$ pfu/mL Ad.MMP-9 and $1.0 \cdot 10^{10}$ pfu/mL Ad.Empty and the control group (intermediate n=14; advanced n=12) was incubated with $2.0 \cdot 10^{10}$ pfu/mL Ad.Empty. Fourteen days after gene transduction, lesions were analyzed histologically with regard to morphology and composition.

Tissue harvesting and preparation for histological analysis
Mice were sacrificed two weeks after infection. One day prior to sacrifice phenylephrin (8 µg/kg i.v.; Sigma Diagnostics, St. Louis, MO) was administered to all mice to assess the effect on plaque integrity by means of hemodynamic challenge. Before harvesting, the arterial bed was perfused with phosphate buffered saline (PBS) and formaldehyde.
Transverse, serial cryosections were prepared from OCT-embedded carotid artery and routinely stained with hematoxylin (Sigma) and eosin (Merck) or Masson's trichrome (Accustain kit, Sigma). Collagen staining was performed by picro Sirius Red (Direct red 80, Sigma) and elastin was visualized by accustain elastic staining (Sigma). Perl's staining was applied to detect intralesional iron deposits.
Corresponding sections were stained immunohistochemically with antibodies directed at mouse metallophilic macrophages (monoclonal mouse IgG_{2a}, clone MoMa2, dilution 1:50; Sigma), α-SM-actin (monoclonal mouse IgG_{2a}, clone 1A4, dilution 1:500; Sigma) and CD31 (BD Pharming). To assess intimal cell death, sections were subjected to TUNEL staining using protocols provided by the manufacturer (In Situ Cell Detection Kit, Roche Diagnostics).

Morphometry
In hematoxylin-eosin stained sections, the site of maximal plaque size was selected for morphometry. Images were digitized and analyzed as previously described.[15] Briefly, luminal, intimal and medial are 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. The stage of lesion progression was assessed with classification criteria defined by Virmani et al.[17] Of six defined categories (i.e 1: fibrous lesion, 2: atheromatous lesion, 3: thin cap fibroatheroma (TCFA), 4: healed rupture, 5: plaque rupture or intraplaque hemorrhage and 6: plaque erosion) the first two classes are considered stable, whereas lesions in classes 3 to 6 are perceived as plaques with characteristics of vulnerability. Lesions were blindly allocated to the different classes using observations of subsequent sections with a maximal interval of 100 µm. TCFA is defined as lesions with a thin fibrous cap (≤3 cell-layers) accompanied by a large necrotic core (>40%). Plaque erosion was defined as thrombus formation without apparent plaque rupture. Macrophage, smooth muscle cell (SMC), collagen and MMP-9 positive areas were determined by computer-assisted color-gated measurement, and related to the total intimal surface area.

MMP-9 activity in vitro and in vivo
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 $CaCl_2$, 250 mM NaCl, 1 µM $ZnCl_2$, 0.02% NaN_3 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).
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 $1.0 \cdot 10^{10}$ 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 $CaCl_2$ for 18 hours at 37°C and 5% CO_2. 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.

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 one-tailed Student's *t*-test. Gelatinolytic activity, collagen, elastin, TUNEL positivity, SMC and macrophage content were compared using the two-tailed Student's *t*-test. Differences in the occurrence of adverse events, iron depositions and in classification were analyzed with the Yate's corrected two-sided Fisher's exact test (two groups, i.e. intermediate lesions) or with the χ^2-test of independence (three 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 required for effective MMP-9 inhibition in vivo, MMP-9 activity was tested in media of transduced SMCs in vitro. Ad.MMP-9 resulted in a twofold increase of MMP-9 activity from 0.05±0.01 to 0.11±0.02 rfu/s (P=0.002). Co-transduction with Ad.TIMP-1 at a 1:1 titer ratio inhibited this effect by 45% to 0.08±0.02 rfu/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 lab [18], carotid plaques incubated intraluminally with Ad.CMV.LacZ revealed that the adenovirus principally targets the endothelium and SMCs of the fibrous cap (Fig. 1A). Similarly, intraluminal incubation with Ad.MMP-9 suspension at $1.0 \cdot 10^{10}$ pfu/mL resulted in a clear expression of the transgene, particularly at sites of SMC accumulation (Ad.Empty: 2.0±3.0 % vs. Ad.MMP-9: 17.0±5.0 %, P=0.007) (Fig.1B-D). In situ zymography was applied to assess lesional proteolytic activity. Ratio of intimal to adventitial 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 co-overexpression attenuated this to 0.87±0.27 (P=0.1, Fig 1E-I). Intracarotid virus instillation was well tolerated. No adverse effects were noted including any changes in bodyweight or plasma cholesterol levels.

MMP-9 mildly increases size of intermediate, but not of advanced plaques

Because this study aims to assess the effect of MMP-9 on pre-existing plaques, it was important to induce lesions prior to 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 two weeks later carotids were harvested for further analysis.
Plaque size was approximately 2-fold larger in advanced than in intermediate lesions (Fig.2A). In the latter, MMP-9 overexpression led to a marginal, but not significant, increase in plaque size (Ad.MMP-9: 44,000±12,000 μm^2 vs. Ad.Empty: 35,000±13,000 μm^2; P=0.06). This was accompanied by a modest increase of the intima: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) (Fig.2B). In advanced lesions no difference in lesion size 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 (Fig.2C-D). Media size increased by 30% from 28,000±950 μm^2 in controls to 36,500±3,500 μm^2 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±5,300 μm^2 vs. Ad.Empty: 130,000±5,400 μm^2; P=0.02) indicating pronounced outward remodeling.
In advanced plaques, no such differences could be detected. Media size amounted 39,000±8,000 μm^2 in controls, whereas in the MMP-9 and MMP-9:TIMP-1 treated group this was 38,500±5,500 μm^2 and 34,500±3,000 μm^2 respectively. Also, total vessel area did not differ between groups in arteries with advanced plaques (Ad.Empty: 237,000±26,500 μm^2 vs. Ad.MMP-9: 250,000±16,500 μm^2 and AdMMP-9:Ad.TIMP-1: 230,000±14,500 μm^2) (Fig.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 (Fig.3A-D). Although, in intermediate plaques, significantly more vessels showed characteristics of vulnerability in the Ad.MMP-9 group (85% vs. 43% in the controls; P=0.046), no effect of MMP-9 overexpression on the occurrence of adverse events was observed (Ad.Empty: 2/14 vs. Ad.MMP-9: 2/13) (Table 1).
In advanced plaques, 87% of MMP-9 overexpressing lesions displayed features of a vulnerable plaque morphology as compared to only 50% in controls and 58% in the TIMP-1 co-transduced group (Table 1). Although this indicates that TCFAs are the predominant lesion type in MMP-9 overexpressing plaques, it did not reach statistical significance (P=0.1). However, MMP-9 transduction led to a significant increase of intraplaque hemorrhage (IPH), defined as presence of extravasated erythrocytes within the intima accompanied by iron deposits. In controls and TIMP-1 co-treated group 8% and 16% of such events were observed, whereas MMP-9 overexpressing plaques displayed an incidence of 53% (P=0.007) (Fig.3E-F). 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 (Fig.3C-D) and all of these events were accompanied by iron depositions suggesting presence of intramural thrombi (Fig.3B).

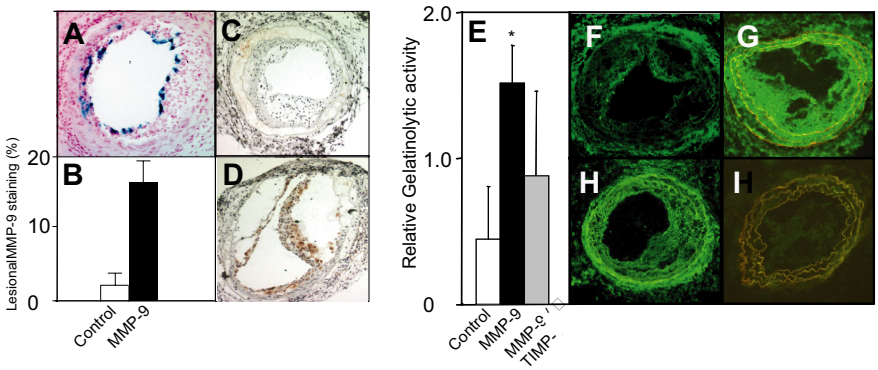

Figure 1. Adenoviral expression pattern of LacZ and MMP-9 (n=9) **A.** Carotid lesions (n=3) were incubated with $1{\cdot}10^{10}$ pfu/mL Ad.LacZ and harvested one week later. β-Galactosidase staining revealed that the adenoviral vectors principally targets the vascular endothelium and SMCs. **B.** Carotid lesions were incubated with $1{\cdot}10^{10}$ pfu/mL Ad.Empty (**C**) or Ad.MMP-9 (**D**) for ten minutes. After one week, staining for MMP-9 revealed significant expression of the transgene (P=0.007). **E.** In situ zymography showed a modest endogenous gelatinase activity in Ad.Empty treated plaques relative to adventitial gelatinase activity (**F**) in contrast to MMP-9 overexpression (**G**), which led to increased intimal gelatin degradation (P=0.05), and was attenuated by TIMP-1 co-overexpression (P=0.10) (**H**). The MMP inhibitor phenantroline completely inhibited gelatinase activity at 1mM (**I**). Values are mean±SEM. * P=0.05

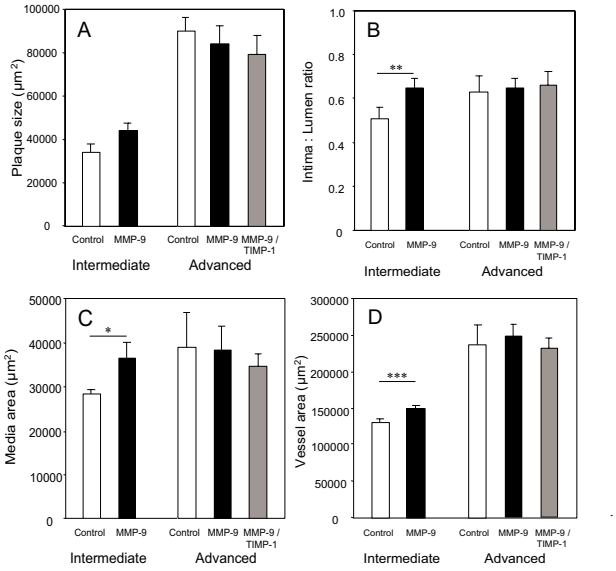

Figure 2. Morphometric parameters of intermediate or advanced carotid lesions (n=12-15/group). **A.** Plaque size (μm^2) had not changed. However, in intermediate lesions, MMP-9 tended to increase lesion size (P=0.06). **B.** MMP-9 overexpression increased intima:lumen ratio in intermediate lesions. **C.** Medial surface area (μm^2) was increased by 30% after MMP-9 transduction in intermediate lesions. **D.** Total vessel area expanded in intermediate plaques, suggesting outward remodeling. Values are mean ± SEM. * P=0.04, ** P=0.03, *** P=0.02

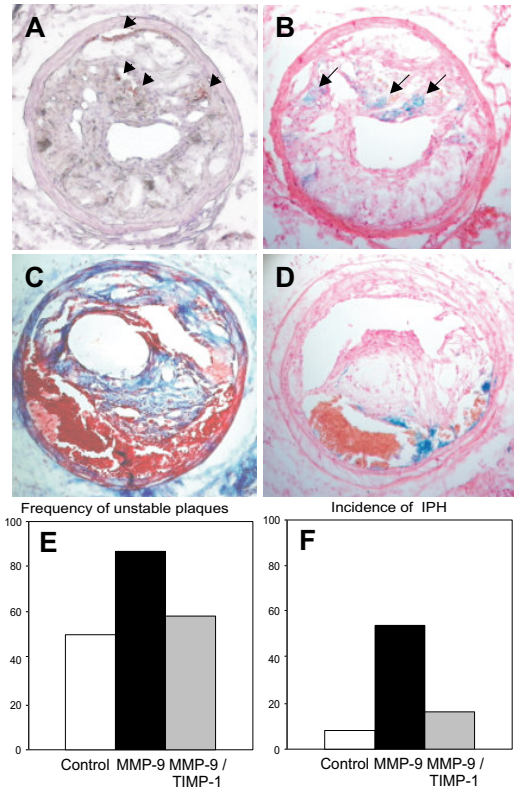

Figure 3. MMP-9 overexpression in advanced plaques led to an increased incidence of adverse events.
A. Intraplaque hemorrhage. **B.** Iron deposits on Perl's staining, suggesting presence of intramural thrombi. **C and D.** Massive intraplaque hemorrhage with the appearance of an incomplete intima-media dissection.
E. Prevalence of unstable plaques (i.e. TCFA or IPH) as was classified by a blinded observer. **F.** Incidence of intraplaque hemorrhage, which was defined as presence of extravasated erythrocytes within the intima accompanied by iron deposits.

	Intermediate			Advanced			
	Control N=14	Ad.MMP-9 N=13	P-value	Control N=12	Ad.MMP-9 N=15	Ad.MMP-9 / Ad.TIMP-1 N=12	P-value
Lesion size (mm^2)	0.035 ±0.004	0.044 ±0.004	NS	0.090 ±0.006	0.085 ±0.008	0.079 ±0.009	NS
Unstable plaque	6	11	0.05	6	13	7	0.10
IHP	2	2	NS	1	8	2	0.007

Table 1. Plaque size and distribution of collar induced lesions showing vulnerable plaque morphology or presence of intraplaque hemorrhage.

Because plaque stability could also be influenced by fibrous cap integrity, we measured fibrous cap thickness. Mean cap thickness, measured from twelve different sites per section (Fig.4A), was decreased by 41% in MMP-9 overexpressing intermediate lesions (Ad.Empty: 34.5±8.7 μm vs. Ad.MMP-9:

20.5±2.6 µm; P=0.04). Cap thinning was also observed in advanced plaques (Ad.Empty: 21.1±2.03 µm vs. Ad.MMP-9: 15.9±1.24 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±1.57µm) (Fig.4B). The same effects were observed for fibrous cap area (data not shown).

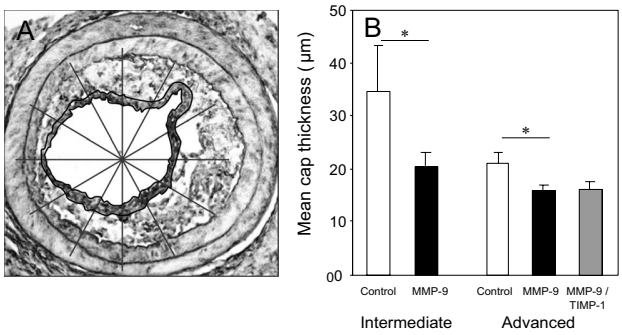

Figure 4. A. Fibrous cap thickness was measured at twelve evenly spaced intervals radiating from the luminal center within the section showing maximal plaque size. This was repeated in all sections to find mean cap thickness. **B.** MMP-9 moderately decreased mean cap thickness (µm) in both types of lesion. Values are mean ± SEM. * P=0.04

Plaque composition is not affected at both stages of development

Because the chance of adverse events, such as IPH or plaque rupture, is increased in plaques with high accumulation of infiltrated leukocytes, macrophage specific immunostaining was performed and showed more macrophages in advanced compared to intermediate plaques. However, MMP-9 overexpression did not affect intimal macrophage content in both intermediate (macrophage:intima ratio: 0.22±0.15 vs. 0.27±0.17 in the controls) (data not shown) and advanced lesions (macrophage:intima ratio: 0.52±0.17 vs. 0.44±0.15 in the control and 0.41±0.17 in the Ad.TIMP-1 co-incubated group) (Fig.5A).
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 (SMC:intima ratio: Ad.Empty: 0.34±0.10; Ad.MMP-9: 0.36±0.15;Ad.MMP-9/Ad.TIMP-1: 0.36±0.15) (Fig.5B).
Intimal collagen content 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 (collagen:intima ratio: 0.33±0.16 vs. 0.20±0.16 in the controls, P=0.07), but this was not significant and unaffected by co-transduction with Ad.TIMP-1 (collagen:intima ratio: 0.23±0.14) (Fig.5C). Also intimal elastin remained unaffected by MMP-9 overexpression (Fig.5D)
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 significant changes in apoptotic rate (Fig.5E). Besides ECM weakening, risk of IPH may be enhanced by increased neo-angiogenesis. CD31 staining did not reveal any differences in density of neo-vessels between groups. However, it did

show that such vessels not only are present in collar-induced lesions, but also in the media suggesting that neo-vessels most likely originate from the adventitial side of the plaque, penetrating the elastic lamina (Fig.6A-B). Incidental co-localization with sites of extravasated erythrocytes may point to neo-angiogenesis as a source for intraplaque hemorrhage.

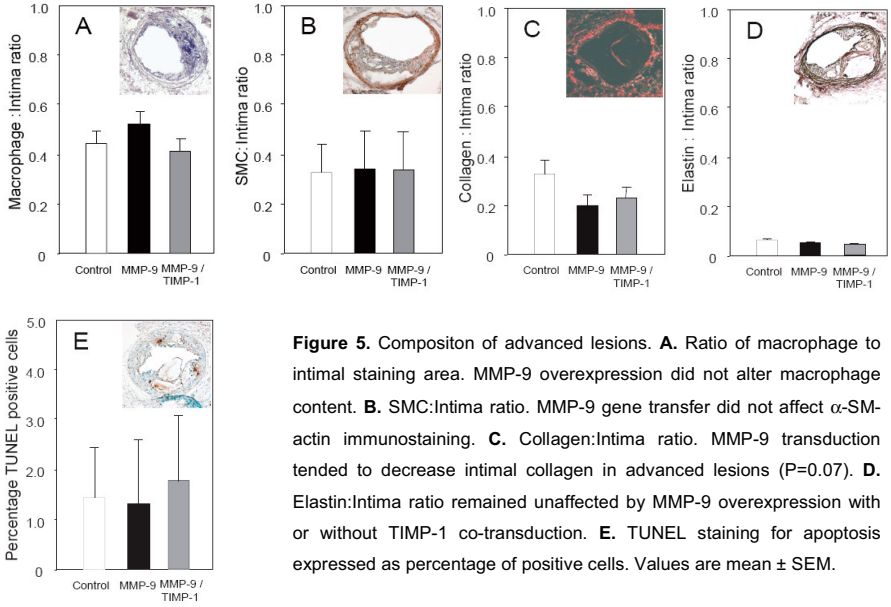

Figure 5. Compositon of advanced lesions. **A.** Ratio of macrophage to intimal staining area. MMP-9 overexpression did not alter macrophage content. **B.** SMC:Intima ratio. MMP-9 gene transfer did not affect α-SM-actin immunostaining. **C.** Collagen:Intima ratio. MMP-9 transduction tended to decrease 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.

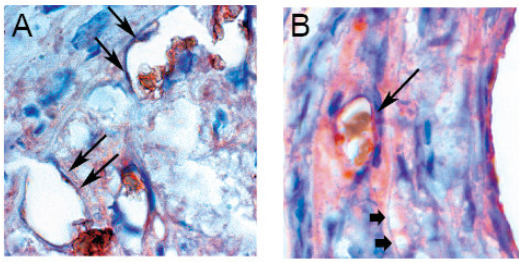

Figure 6. CD31 staining revealed presence of neo-vessels within the intima (open arrows), adjacent to the internal elastic lamina **(A)** and in the media **(B)** suggesting that these vessels originate from the abluminal, adventitial side (closed arrows: internal elastic lamina). These neo-vessels often showed presence of luminal erythrocytes and incidentally co-localized with extravasated blood cells pointing to neo-angiogenesis as a source for IPH.

Discussion

Atherosclerotic plaque rupture is a major cause of acute ischemic events.[3,19] Several mechanisms, like apoptosis and matrix degradation, have been implicated in this process.[20,21,22,23,24] A number of studies have pointed to a role of MMPs in atherogenesis[25] and plaque stability.[20,26] MMP-9 plasma levels are raised in patients with acute coronary syndromes[10,27] and patients with high expressing MMP-9 polymorphisms showed increased risk for cardiovascular events.[9,28] Conversely, ApoE$^{-/-}$/MMP-9$^{-/-}$ mice displayed more characteristics of plaque vulnerability than their MMP-9$^{+/+}$ littermates.[14]

In this study, MMP-9 was overexpressed in pre-existing plaques at an intermediate (~40,000 μm^2) and advanced (~80,000 μm^2) stage of progression to evaluate the effect on both plaque morphology and stability. The results show, for the first time 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 led to an increased incidence of adverse events, i.e. IPH, which is a common manifestation of plaque destabilization in mouse models of advanced atherosclerosis and believed to further aggravate plaque vulnerability.[29] In intermediate lesions, MMP-9 overexpression promoted morphological characteristics of vulnerability, i.e. 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 lesion size and outward remodeling as a result of MMP-9 transduction. Therewith, 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 such as ECM degradation, release of matrix bound factors and adhesion molecule shedding [30,31,32], 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, via activation of other proteases or releasing matrix bound effectors, affect matrix homeostasis, cell recruitment and apoptosis.[33,32,34]

Although the adenoviral vector system applied in the present study mainly targets intimal SMCs and endothelial cells[18], it is conceivable that the secreted zymogen diffuses throughout the plaque and is activated elsewhere in the lesion. This is illustrated by the fact that medial SMC proliferation clearly lies at the base of the observed vessel remodeling in intermediate plaques, a process that requires degradation of the basal membrane (BM) by proteolytic activity. Conversely, in advanced plaques MMP-9 overexpression did not affect α-actin positive SMC content or apoptotic rate, indicating that intimal SMC turnover remained unchanged in these lesions.

Previous evidence suggested that MMP-9 might directly degrade the collagens type I and III.[13] 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, therewith changing structural integrity of the plaque. Indeed, Galis and co-workers showed that MMP-9 deletion in the arterial wall resulted in accumulation of adventitial collagen without showing tissue constriction and that MMP-9 deficiency impairs the capacity of SMCs to contract collagen gels in vitro.[13]

Furthermore, degradation of ECM constituents can result in release of several matrix bound bioactive molecules, such as FGF-2, TGF-β and TNF-α.[35,36] Together with digestion of ground matrix and the BM this can result in migration and

activation of inflammatory cells.[32,37,38] Moreover, MMP-9 may facilitate neo-angiogenesis via BM degradation and release of VEGF.[39] Newly formed, leakier, vessels can contribute to persistent inflammation by conveying blood cells into the plaque.[40] 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. However, in this study there was no clear effect of MMP-9 on the extent of intimal neo-vessels, although CD31 staining did indeed reveal incidental co-localization of neo-vessels and sites of extravasated and partly 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 remodelling, cap thinning and intraplaque hemorrhage.

In summary, 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 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[14] 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 towards 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. In conclusion, these data point to an important and differential role for MMP-9 in plaque progression, vessel remodeling and plaque stability. MMP-9 can act as a culprit in destabilizing advanced plaques, making it a promising target for therapeutic intervention in advanced, complex atherosclerotic lesions.

References

1. Fuster V. Elucidation of the role of plaque instability and rupture in acute coronary events. *Am J Cardiol*. 1995;76:24C-33C.
2. Schoenhagen P, Tuzcu EM, Ellis SG. Plaque vulnerability, plaque rupture, and acute coronary syndromes: (multi)-focal manifestation of a systemic disease process. *Circulation*. 2002;106:760-2.
3. Haft JI. Multiple atherosclerotic plaque rupture in acute coronary syndrome. *Circulation*. 2003;107:e65-6; author reply e65-6.
4. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Libby P. Enhanced expression of vascular matrix metalloproteinases induced in vitro by cytokines and in regions of human atherosclerotic lesions. *Ann N Y Acad Sci*. 1995;748:501-7.
5. Shah PK. Role of inflammation and metalloproteinases in plaque disruption and thrombosis. *Vasc Med*. 1998;3:199-206.

6. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest*. 1994;94:2493-503.
7. Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, Thompson MM. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. *Stroke*. 2000;31:40-7.
8. Pollanen PJ, Karhunen PJ, Mikkelsson J, Laippala P, Perola M, Penttila A, Mattila KM, Koivula T, Lehtimaki T. Coronary artery complicated lesion area is related to functional polymorphism of matrix metalloproteinase 9 gene: an autopsy study. *Arterioscler Thromb Vasc Biol*. 2001;21:1446-50.
9. Morgan AR, Zhang B, Tapper W, Collins A, Ye S. Haplotypic analysis of the MMP-9 gene in relation to coronary artery disease. *J Mol Med*. 2003;81:321-6.
10. Kai H, Ikeda H, Yasukawa H, Kai M, Seki Y, Kuwahara F, Ueno T, Sugi K, Imaizumi T. Peripheral blood levels of matrix metalloproteases-2 and -9 are elevated in patients with acute coronary syndromes. *J Am Coll Cardiol*. 1998;32:368-72.
11. Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, Meyer J, Cambien F, Tiret L. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. *Circulation*. 2003;107:1579-85.
12. Morishige K, Shimokawa H, Matsumoto Y, Eto Y, Uwatoku T, Abe K, Sueishi K, Takeshita A. Overexpression of matrix metalloproteinase-9 promotes intravascular thrombus formation in porcine coronary arteries in vivo. *Cardiovasc Res*. 2003;57:572-85.
13. Galis ZS, Johnson C, Godin D, Magid R, Shipley JM, Senior RM, Ivan E. Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. *Circ Res*. 2002;91:852-9.
14. Johnson JL, George S, Newby A, Jackson C. Matrix metalloproteinases-9 and -12 have opposite effects on atherosclerotic plaque stability. *Atherosclerosis Supplements*. 2003;4:196.
15. von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation*. 2001;103:1164-70.
16. Baker AH, Wilkinson GW, Hembry RM, Murphy G, Newby AC. Development of recombinant adenoviruses that drive high level expression of the human metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and -2 genes: characterization of their infection into rabbit smooth muscle cells and human MCF-7 adenocarcinoma cells. *Matrix Biol*. 1996;15:383-95.
17. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2000;20:1262-75.
18. von der Thusen JH, van Vlijmen BJ, Hoeben RC, Kockx MM, Havekes LM, van Berkel TJ, Biessen EA. Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus-mediated transfer of p53. *Circulation*. 2002;105:2064-70.
19. Fuster V, Stein B, Ambrose JA, Badimon L, Badimon JJ, Chesebro JH. Atherosclerotic plaque rupture and thrombosis. Evolving concepts. *Circulation*. 1990;82:II47-59.
20. Molloy KJ, Thompson MM, Jones JL, Schwalbe EC, Bell PR, Naylor AR, Loftus IM. Unstable carotid plaques exhibit raised matrix metalloproteinase-8 activity. *Circulation*. 2004;110:337-43.
21. Shah PK. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol*. 2003;41:15S-22S.
22. Bennett MR. Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res*. 1999;41:361-8.
23. Newby AC, Zaltsman AB. Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc Res*. 1999;41:345-60.
24. Kolodgie FD, Narula J, Haider N, Virmani R. Apoptosis in atherosclerosis. Does it contribute to plaque instability? *Cardiol Clin*. 2001;19:127-39, ix.
25. Lemaitre V, O'Byrne TK, Borczuk AC, Okada Y, Tall AR, D'Armiento J. ApoE knockout mice expressing human matrix metalloproteinase-1 in macrophages have less advanced atherosclerosis. *J Clin Invest*. 2001;107:1227-34.
26. Nikkari ST, O'Brien KD, Ferguson M, Hatsukami T, Welgus HG, Alpers CE, Clowes AW. Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis. *Circulation*. 1995;92:1393-8.
27. Inokubo Y, Hanada H, Ishizaka H, Fukushi T, Kamada T, Okumura K. Plasma levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 are increased in the coronary circulation in patients with acute coronary syndrome. *Am Heart J*. 2001;141:211-7.
28. Jones GT, Phillips VL, Harris EL, Rossaak JI, van Rij AM. Functional matrix metalloproteinase-9 polymorphism (C-1562T) associated with abdominal aortic aneurysm. *J Vasc Surg*. 2003;38:1363-7.
29. Rekhter M. Vulnerable atherosclerotic plaque: emerging challenge for animal models. *Curr Opin Cardiol*. 2002;17:626-32.

30. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev*. 2000;14:163-76.
31. Komorowski J, Pasieka Z, Jankiewicz-Wika J, Stepien H. Matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases and angiogenic cytokines in peripheral blood of patients with thyroid cancer. *Thyroid*. 2002;12:655-62.
32. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood*. 2002;100:1160-7.
33. Bendeck MP, Conte M, Zhang M, Nili N, Strauss BH, Farwell SM. Doxycycline modulates smooth muscle cell growth, migration, and matrix remodeling after arterial injury. *Am J Pathol*. 2002;160:1089-95.
34. Camp TM, Tyagi SC, Senior RM, Hayden MR. Gelatinase B(MMP-9) an apoptotic factor in diabetic transgenic mice. *Diabetologia*. 2003;46:1438-45.
35. Dallas SL, Rosser JL, Mundy GR, Bonewald LF. Proteolysis of latent transforming growth factor-beta (TGF-beta)-binding protein-1 by osteoclasts. A cellular mechanism for release of TGF-beta from bone matrix. *J Biol Chem*. 2002;277:21352-60.
36. Guo XL, Lin GJ, Zhao H, Gao Y, Qian LP, Xu SR, Fu LN, Xu Q, Wang JJ. Inhibitory effects of docetaxel on expression of VEGF, bFGF and MMPs of LS174T cell. *World J Gastroenterol*. 2003;9:1995-8.
37. Sellebjerg F, Sorensen TL. Chemokines and matrix metalloproteinase-9 in leukocyte recruitment to the central nervous system. *Brain Res Bull*. 2003;61:347-55.
38. Kintscher U, Kon D, Wakino S, Goetze S, Graf K, Fleck E, Hsueh WA, Law RE. Doxazosin inhibits monocyte chemotactic protein 1-directed migration of human monocytes. *J Cardiovasc Pharmacol*. 2001;37:532-9.
39. Belotti D, Paganoni P, Manenti L, Garofalo A, Marchini S, Taraboletti G, Giavazzi R. Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation. *Cancer Res*. 2003;63:5224-9.
40. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med*. 2001;7:425-9.