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Adenovirus-Mediated Overexpression of Tissue Inhibitor of Metalloproteinase-1 Reduces Atherosclerotic Lesions in Apolipoprotein E–Deficient Mice

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Background—To define the role of metalloproteinases (MMPs) in the development of lipid-rich atherosclerotic lesions in relation to the balance of proteolytic and antiproteolytic activities, we investigated the impact of adenovirus-mediated elevation in the circulating levels of human tissue inhibitor of MMP (TIMP-1) in atherosclerosis-susceptible apolipoprotein E–deficient (apoE−/−) mice.

Methods and Results—Infusion of apoE−/− mice fed a lipid-rich diet with rAd.RSV.TIMP-1 (1×10^11 viral particles) resulted in high hepatic expression of TIMP-1. At 2 weeks after injection, plasma TIMP-1 levels ranged from 7 to 24 μg/mL (mean 14.8±6.8). Marked overexpression of TIMP-1 was transient, with levels of TIMP-1 decreasing to 2.5 to 8 μg/mL (mean 4.3±2.1) at 4 weeks. Plasma lipid and lipoprotein levels in mice treated with rAd.RSV.TIMP-1 were similar to those treated with rAd.RSV.βGal. However, rAd.RSV.TIMP-1–infused mice displayed a marked reduction (∼32%; P<0.05) in mean lesion area per section (512±121 μm²×10³; n=12 sections from 4 animals) as compared with rAd.RSV.βGal–infused mice (750±182 μm²×10³; n=12 sections from 4 animals). Similarly, marked reduction in macrophage deposition as well as MMP-2, MMP-3, and MMP-13 antigens was observed.

Conclusions—Histological and immunohistochemical analyses of atherosclerotic lesions revealed increases in collagen, elastin, and smooth muscle α-actin content in mice treated with rAd.RSV.TIMP-1. These qualitative and quantitative features were the consequence of TIMP-1 infiltration from plasma to arterial intima, as immunohistochemical analyses revealed an abundance of TIMP-1 specifically in lesions of rAd.RSV.TIMP-1–treated mice. (Circulation. 1999;100:533-540.)

Key Words: atherosclerosis ■ metalloproteinases ■ apolipoproteins ■ genes

Atherosclerosis is a multifactorial, complex pathological process. Cell-cell and cell-matrix interactions involving monocyte-derived macrophages, vascular smooth muscle cells (SMC), endothelial cells, and T-lymphocytes are involved. Extracellular matrix degradation is a key factor in clinical thromboembolic events. Indeed, sudden death caused by acute myocardial infarction frequently results from the rupture of coronary atheroma, particularly at sites of thinning of the lesion’s fibrous cap; such rupture results in hemorrhage into the plaque, resulting in a prothrombotic response followed by rapid occlusion of the artery. The accumulation of macrophage-derived foam cells in rupture-prone areas, such as the vulnerable shoulder regions of plaques, correlates with increased local release of matrix-degrading metalloproteinases (MMPs) and weak fibrous cap tissue. These findings suggest a potential role of macrophage-derived MMPs in the fragilization and ultimate rupture of plaque structure.

MMPs are a family of enzymes secreted in a latent zymogen form. Interactions between cysteine residues in the propeptide domain and the zinc atom present at the catalytic site of all MMPs are responsible for maintaining the propeptide over the catalytic site. Metalloproteinase activation can occur in vitro or in vivo when the propeptide is cleaved by proteases or when the zinc-cysteine bond is disrupted.

The proteolytic capacity of these MMPs together facilitates degradation of many if not all extracellular components present within the fibrous areas of atheromatous plaques. The activity of MMPs can be controlled by specific tissue inhibitors or TIMPs. Three TIMPs (TIMP-1, TIMP-2, and TIMP-3) have been characterized and a fourth (TIMP-4) has been cloned.
TIMP-1 is produced by virtually all mesenchymal cells; its cDNA has been cloned and sequenced. TIMP-1 is a secreted glycoprotein with a molecular weight of ~28 kDa stabilized by 6 disulfide bridges. TIMP-1 inhibits all MMPs but has high affinity for MMP-1, MMP-2, MMP-3, and MMP-9. 

Increased expression of MMPs has been detected in injured arteries and atherosclerotic plaques. Indeed, stromelysin mRNA localize to focal areas enriched in macrophages in human lesions, whereas immunoreactive interstitial collag enase (MMP-1), the 72-kDa gelatinase (MMP-2), stromelysin-1 (MMP-3), and the 92-kDa gelatinase (MMP-9) colocalize to lesional macrophages. In addition, human lipid-laden macrophages prominently express matrix metalloproteinase (MMP-7) and metalloelastase (MMP-12). These MMPs have a potent capacity to degrade proteoglycans, insoluble elastin, and fibronec tin, suggesting that such matrix components are major targets of macrophage-mediated tissue destruction.

Apolipoprotein E (apoE)-deficient mice (apoE−/−) are hypercholesterolemic and develop atherosclerosis spontaneously even when fed a chow diet. Both marked hypercholesterolemia and atherosclerosis are manifest more rapidly in these mice when fed a high-fat, Western-type diet. In such animals, the atherosclerotic lesions present at advanced stages of progression display necrotic cores and are located at the same sites of predilection as human lesions. ApoE−/− mice are therefore a relevant animal model in which to identify the complex molecular and cellular mechanisms involved in plaque progression and fragilization.

To define the role of MMPs in relation to the balance between proteolytic and antiproteolytic activities in atherosclerotic lesions, we investigated the impact of elevated circulating levels of human TIMP-1 on the severe atherosclerosis characteristic of hypercholesterolemic apoE−/− mice. Overexpression of human TIMP-1 was achieved by adenovirus-mediated gene transfer.

Methods

Generation of Recombinant Adenovirus
Recombinant vectors were constructed on the basis of the pAd.RSV.βGal vector by the use of standard techniques. The full-length TIMP-1 cDNA was generated by reverse transcription–polymerase chain reaction from the total RNA of the HL-60 cell line. To optimize initiation of translation, hybrid intron (HI), untranslated sequence (UTR), and Kozak consensus sequence were linked downstream of TIMP-1 cDNA.

Cell Transfection Analysis
Human 293 kidney cells (American Type Culture Collection, Rockville, Md) of low passage number were transfected with rAd.RSV.TIMP-1 by use of the calcium phosphate procedure, and TIMP-1 was identified in the culture media by use of an ELISA kit (Amersham) and by immunoblotting with a TIMP-1–monospecific antibody. In addition, use of SDS-PAGE zymography showed human TIMP-1 to inhibit murine MMP activity.

SDS-PAGE Zymography
Tissue from 6-month-old atherosclerotic mouse aortas were used as a source of MMPs. Mouse aortas were homogenized in 1 mL cold buffer containing 0.2% Triton X-100, 50 mmol/L Tris, 10 mmol/L CaCl2, and 2 mol/L guanidine hydrochloride (pH 7.5). The homogenate was centrifuged for 5 minutes at 14 000 g.
TIMP-1, lipids, and lipoproteins. Four weeks after treatment with either adenoviruses or PBS, the mice were killed and tissues analyzed.

**Plasma Lipid and Lipoprotein Analyses**

Total cholesterol and triglyceride levels were assayed on fasting plasma (10 μL) with the use of enzymatic kits (Biomerieux); HDL cholesterol was measured with dextran sulfate precipitation (Biomerieux) according to the manufacturer’s specifications. Plasma lipoproteins were analyzed by fast protein liquid chromatography with 2 Superose 6 columns in series (Pharmacia Biotechnology). Lipoproteins were eluted at a constant flow rate (0.4 mL/min) with PBS buffer containing 1 mM EDTA and 0.02% sodium azide.\(^28\)

**Quantification of Plasma TIMP-1 in Mice by ELISA Procedure**

Human TIMP-1 in mouse plasma was quantified by a sandwich ELISA kit (Amersham) containing 2 monoclonal antibodies specific to human TIMP-1 antigen.

**Quantitative and Qualitative Analysis of Atherosclerotic Plaques**

Mice were killed, and freshly-removed hearts were fixed in 10% buffered formalin and then either embedded in paraffin for histological analysis (collagen and elastin staining) or frozen for immunohistochemical studies. Lesion quantification was performed on paraffin sections.

**Histological Analysis**

Serial paraffin-embedded sections (5 μm) were stained with Sirius red or orcein for collagen and elastin visualization, respectively, or stained with hematoxylin, eosin, and safron for lesion area measurement and the mean lesion area per animal quantified (three 5-μm aortic sections, each separated by 100 μm).\(^29\)

**Immunohistochemistry**

Frozen serial sections (7 μm) were treated with 0.3% H₂O₂ in PBS to block endogenous peroxidase activity, followed by blocking in 4% BSA (Sigma). Slides were incubated (2 hours at 20°C) with either purified rat monoclonal antibody against mouse macrophages (CD11b antigen = Mac-1) (Valbiotech) at 1:25 dilution (20 μg/mL), goat polyclonal antibody against mouse MMP-3 (a generous gift of Dr C. Peeters-Joris, Louvain, Belgium) at 1:300 dilution (40 μg/mL), goat polyclonal antibody against mouse MMP-13 (a generous gift of Prof Y. Eeckhout, Louvain, Belgium) at 1:50 dilution (40 μg/mL), sheep polyclonal antibody against human MMP-2, which cross-reacts with mouse MMP-2 (Valbiotech) at 1:100 dilution (8 μg/μL), and a rabbit polyclonal antibody against human TIMP-1 (Euromedex) at 1:1000 dilution (1 μg/mL); for α-smooth muscle actin studies, a kit (Sigma, ref. IMMH-2) was used. As a negative control, we used either a purified nonimmune IgG from rat, goat, sheep, or rabbit. A second biotinylated antibody was added, either anti-rat at 1:50 dilution, anti-goat, anti-sheep, or anti-rabbit at 1:100, respectively. Thereafter, peroxidase-labeled or alkaline phosphatase–labeled streptavidin was added. The activity of peroxidase was revealed with diaminobenzidine (Merck) as a substrate, yielding a yellow-brown deposit; the substrate for alkaline phosphatase was the fast red substrate system (Dako) yielding an insoluble red reaction product.

**Statistical Analysis**

The mean surface area of lesions was compared between groups by ANOVA.

**Results**

Plasma lipoprotein profiles were determined by fast protein liquid chromatography and revealed that HDL predominated with minor amounts of VLDL and LDL in control mice. In
Plasma Lipids and Lipoproteins After Adenovirus-Mediated Gene Transfer of TIMP-1 in ApoE⁻/⁻ Mice

<table>
<thead>
<tr>
<th>TC, mg/dL (n=4)</th>
<th>TG, mg/dL (n=4)</th>
<th>HDL-C, mg/dL (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>Day 14</td>
<td>Day 21</td>
</tr>
<tr>
<td>PBS</td>
<td>rAd.CMV</td>
<td>rAd.RSV.βGal</td>
</tr>
<tr>
<td>820±140</td>
<td>806±154</td>
<td>823±171</td>
</tr>
<tr>
<td>124±71</td>
<td>121±74</td>
<td>122±69</td>
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<tr>
<td>6.1±0.8</td>
<td>9.5±3.5</td>
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<tr>
<td>937±170</td>
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<td>135±69</td>
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<td>4.8±1.0</td>
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<td>1031±211</td>
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<td>1080±199</td>
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<td>289±102</td>
<td>283±109</td>
<td>283±109</td>
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<tr>
<td>13.6±5.1</td>
<td>7.6±1.5</td>
<td>7.6±1.5</td>
</tr>
</tbody>
</table>

TC indicates total cholesterol; TG, triglycerides; and HDL-C, HDL cholesterol.

Discussion

The present findings reveal, for the first time, that elevated circulating levels of human TIMP-1 significantly reduce atherosclerotic lesions in atherosclerosis-susceptible hypercholesterolemic apoE⁻/⁻ mice.
A rapid (4 weeks) and significant reduction of lesion area (32% vs control; \( P < 0.05 \)) occurred in rAd.RSV.TIMP-1–treated mice (Figure 5). This reduction appears to result from the local inhibition of the activity of MMPs by human TIMP-1, a protein detected only in the lesions of mice treated with rAd.RSV.TIMP-1 (Figure 7H). Human TIMP-1 inhibited murine MMPs in vitro (Figure 4), a finding consistent with previous work showing that murine MMPs were inhibited in transgenic mice overexpressing human TIMP-1. 32,33 In addition, MMP-3, MMP-13 (Figure 7, C through F), and MMP-2 (data not shown) have been immunolocalized in lesions of our apoE \(^{−/−} \) mice: one consequence of MMP inhibition might be the limitation of the proteolysis of elastin, collagen, and \( \alpha \)-actin (Figure 6, A through F). Indeed, inhibition of MMPs by local overexpression of TIMP-1 increases elastin accumulation in rat carotid artery intima. 34

Overexpression of TIMPs in neoplastic human 35 and murine 36 cells is associated with decreased MMP activity and reduction in the malignant behavior of these cells. Indeed, MMP overexpression might lead to proliferation and migra-

![Figure 6](image_url). Histological analysis of aortic lesions. Serial paraffin-embedded sections (5 \( \mu \)m thick) from rAd.RSV.\( \beta \)Gal-treated (A, C, and E) or rAd.RSV.TIMP-1–treated mice (B, D, and F) were stained with orcein or Sirius red for visualization of elastin (A, B) and collagen (C, D), respectively, or were immunostained for detection of \( \alpha \)-smooth muscle actin (E, F). A, B, C, and D, \( \times 40 \) magnification; E and F; \( \times 100 \) magnification.
Figure 7. Immunohistochemical analysis of aortic lesions. Frozen serial sections (7 μm) from rAd.RSV.βGal-infused (A, C, E, and G) or rAd.RSV.TIMP-1–infused mice (B, D, F, and H) were treated as described in Methods. Slides were incubated for 2 hours at 20°C with either a purified rat monoclonal antibody against mouse macrophage CD11b antigen (A and B), a goat polyclonal antibody against mouse MMP-3 (C and D), a goat polyclonal antibody against mouse MMP-13 (E and F), or a rabbit polyclonal antibody against human TIMP-1 (G and H). As a negative control, we used a purified nonimmune IgG from rat, goat, or rabbit according to the experiment.
tion, whereas overexpression of TIMP-1 might serve to maintain cells in the quiescent state. Inhibition of TIMP-1 expression with antisense oligonucleotides\(^2\) or through targeted gene disruption by homologous recombination\(^3\) enhances the invasive properties of tumor cells in vitro and their metastatic potential in vivo. In addition, adenovirus-mediated overexpression of TIMP-1 in cultured rat SMC inhibits SMC chemotaxis and invasion without affecting the number of cells.\(^9\) Similarly, adenovirus-mediated gene transfer of TIMP-1 inhibits SMC migration and neointimal formation in human saphenous vein segments in organ culture without effect on SMC proliferation.\(^40\) In this context, our data suggest that the beneficial effect resulting from the overexpression of TIMP-1 might also arise from the inhibition of SMC invasion. In addition, increase in lipid levels with time in rAd.RSV.TIMP-1–treated mice occurred in a manner similar to that in controls. This finding suggests that high levels of human TIMP-1 in murine plasma do not alter lipid metabolism in such a way as to inhibit progression or facilitate lesion regression. Because mice were continuously fed with a cholesterol-rich diet during the adenoviral treatment, the increasing level of TIMP-1 may inhibit the progression of arterial lesions rather than inducing their regression. The reduced number of foam cell–derived macrophages in the lesions of rAd.RSV.TIMP-1–treated mice, in addition to decrease in the dilation of the aortic area as a result of extracellular matrix remodeling, could account for this beneficial effect. A similar observation was reported recently by Carmeliet et al.,\(^10\) who established that deficiency of urokinase-type plasminogen in apoE\(^{-/-}\) mice protects against media destruction and aneurysm formation, probably by means of reduced plasmin-dependent activation of pro-MMPs. Equally, TIMP-1 may contribute to lesion regression through MMP-induced reduction in HDL-mediated cholesterol efflux from human macrophage foam cells after truncation of the carboxyl terminus of apoA-I.\(^41\) Therefore MMP inhibition by TIMP-1 may protect HDL particles and promote efficient reverse cholesterol transport.

The efficiency of intravascular gene delivery to atherosclerotic lesions may be low because lesions that are frequently rich in connective tissue contain limited numbers of transflectable cells.\(^42\) Nevertheless, high levels of TIMP-1 were observed in lesions treated by rAd.RSV.TIMP-1 in our animal model, and this despite the expression of the irrelevant \(\beta\text{Gal}\) gene mainly in the liver (data not shown). The localization of TIMP-1 protein in lesions is unlikely to result from transfection of vascular cells but more likely to be attributed to TIMP-1 infiltration across the endothelium. Indeed, TIMP-1 is a stable protein of low molecular weight that may readily infiltrate into the intimal space. Indeed, overexpression of apoIV in murine plasma exerts a protective effect against plaque formation by a mechanism that does not involve increase in HDL cholesterol,\(^43\) suggesting that apoIV infiltrates into the intimal space, where it exerts a protective effect. In addition, adenovirus-mediated overexpression of apoE induces regression of the atherosclerotic lipid core in plaques of apoE\(^{-/-}\) mice by a mechanism implicating its infiltration and association with lipid present within the intimal area (Duverger et al., personal communication).

In conclusion, our data prompt the hypothesis that local progression of established atherosclerotic lesions or vulnerable lipid-rich plaques can be retarded by gene transfer techniques. The adenovirus-mediated overexpression of human TIMP-1 in apoE\(^{-/-}\) mice, an animal model of atherosclerotic plaque formation, provides a convincing example of this therapeutic approach.

Acknowledgments

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