Suppression of Atherosclerotic Plaque Progression and Instability by Tissue Inhibitor of Metalloproteinase-2

Involvement of Macrophage Migration and Apoptosis

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Background—Matrix metalloproteinase (MMP)–associated extracellular matrix degradation is thought to contribute to the progression and rupture of atherosclerotic plaques. However, direct evidence of this concept remains elusive. We hypothesized that overexpression of tissue inhibitor of metalloproteinase (TIMP)-1 or TIMP-2 would attenuate atherosclerotic plaque development and instability in high fat–fed apolipoprotein E–knockout (apoE^{-/-}) mice.

Methods and Results—Seventy male apoE^{-/-} mice (n=10/group) fed a high-fat diet for 7 weeks were injected intravenously with first-generation adenoviruses expressing the gene for human TIMP-1 (RAdTIMP-1) or TIMP-2 (RAdTIMP-2) or a control adenovirus (RAd66) and were fed a high-fat diet for a further 4 weeks. Analysis of brachiocephalic artery plaques revealed that RAdTIMP-2 but not RAdTIMP-1 infection resulted in a marked reduction (48±13%, P<0.05) in lesion area compared with that in control animals. Markers associated with plaque instability, assessed by smooth muscle cell and macrophage content and the presence of buried fibrous caps, were significantly reduced by RAdTIMP-2. Effects on lesion size were not sustained with first-generation adenoviruses, but murine TIMP-2 overexpression mediated by helper-dependent adenoviral vectors exerted significant effects on plaques assessed 11 weeks after infection. In an attempt to determine the mechanism of action, we treated macrophages and macrophage-derived foam cells with exogenous TIMP-2 in vitro. TIMP-2 significantly inhibited migration and apoptosis of macrophages and foam cells, whereas TIMP-1 failed to exert similar effects.

Conclusions—Overexpression of TIMP-2 but not TIMP-1 inhibits atherosclerotic plaque development and destabilisation, possibly through modulation of macrophage and foam cell behavior. Helper-dependent adenovirus technology is required for these effects to be maintained long term. (Circulation. 2006;113:2435-2444.)

Key Words: adenovirus ■ atherosclerosis ■ gene therapy ■ metalloproteinases ■ plaque

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The proteolytic potential of the MMPs is tightly regulated by their endogenous tissue inhibitors (TIMPs), of which 4 have been identified to date. TIMP-1 and TIMP-2, synthesized by both SMCs and macrophages, are equally effective in binding to the catalytic site and inhibiting the active forms of stromelysins, collagenases, and gelatinases. Although TIMPs have been detected in atherosclerotic plaques, the presence of net MMP activity suggests that the endogenous levels of TIMPs are insufficient for complete inhibition.

Consistent with this finding, previous studies have demonstrated that gene transfer to overexpress TIMP-1 can reduce MMP activity and reduce intimal thickening in various models. Rouis and colleagues examined adenoavirally mediated TIMP-1 overexpression on atherosclerotic plaque.
formation in the aortic sinuses of apolipoprotein E–knockout (apoE<sup>−/−</sup>) mice. TIMP-1 delivery resulted in a 30% reduction in atherosclerotic lesion area measured 4 weeks later, with histological evidence for a more stable plaque phenotype. However, direct measures of plaque stability were unavailable for that study. Furthermore, neither the long-term effects of TIMP-1 nor the effect of any other TIMP was reported. Fat-fed apoE<sup>−/−</sup> mice develop spontaneous atherosclerosis and develop acute plaque disruption in the brachiocephalic artery, as evidenced by discontinuity of the fibrous cap and intraplaque hemorrhage. Complex plaques also have buried fibrous caps associated with fibrin deposition, which is analogous to the healed plaque ruptures frequently observed in human atherosclerotic lesions. In the present study, we used first-generation adenoviruses to compare the short-term effects of elevated circulating levels of TIMP-1 and TIMP-2 on the development and stability of atherosclerotic lesions in this model. We then used helper-dependent (HD) virus technology to examine the effects of prolonged elevation of TIMP-2. In vitro studies on macrophages and foam cells were used to gain additional insights into potential mechanisms.

**Methods**

**Construction of First-Generation and HD Adenoviral Vectors**

The construction of first-generation replication-defective recombinant adenoviruses (RAds) RAd66, RAdlacZ, RAdTIMP-1, and RAdTIMP-2 have been described previously. An HD adenoviral (HD-Ad) vector expressing mouse TIMP-2 was constructed as described in the online Data Supplement.

**Animals**

Homozygous C57BL/6;129 male apoE<sup>−/−</sup> mice (strain background: 71% C57BL/6 and 29% 129 by microsatellite analysis) were bred within the Animal Unit of the University of Bristol. The housing and care of the animals and all procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office.

**Optimization of First-Generation Adenoviral Gene Delivery In Vivo**

Gene transfer of first-generation RAd was based on the method of Tao et al., as outlined in the online Data Supplement.

**Infection With RAd**

To induce formation of complex atherosclerotic lesions, 70 male 8-week-old apoE<sup>−/−</sup> mice were fed a high-fat rodent diet containing 21% (wt/wt) fat from lard supplemented with 0.15% (wt/wt) cholesterol (Special Diets Services, Witham, UK) for a period of 7 weeks. First-generation and HD adenoviruses were administered (n=10/group) as shown in Figure 1 and as outlined in the online Data Supplement.

**Quantification of Plasma TIMP-1, TIMP-2, and Lipid Concentrations**

Plasma samples collected 2, 5, 8, 14, and 28 days after RAd administration were assayed for the presence of human TIMP-1 or TIMP-2 by ELISA (Amersham International, Amersham, UK), as previously described. Endogenous mouse TIMP-2 but not TIMP-1 is detected by these TIMP-2 and TIMP-1 ELISA kits, respectively. Plasma lipid profiles were determined in samples as described previously.

**MMP Inhibition Assay**

As an estimate of TIMP biological activity, the activity of exogenously added MMP-2 was measured in plasma by a fluorescence assay, as described in the online Data Supplement.
Termination

Animals were anesthetized, and the brachiocephalic artery was removed from each animal as outlined previously.19

Histology

Brachiocephalic arteries were embedded in paraffin or optimum cutting temperature compound (OCT; BDH Laboratory Supplies, Poole, UK). Serial sections were cut at 3 mm for paraffin-embedded sections and at 7 mm for OCT-embedded sections for 60 mm ft the proximal artery and were stained with hematoxylin and eosin, Miller’s elastin/van Gieson’s stain, and picrosirius red stain for fibrillar collagens. Plaque elastin and picrosirius red staining (under polarized light) were quantified by color thresholding of maximal and minimal signals through an image analysis system and expressed as percentage lesion area.

Immunohistochemistry

SMCs, macrophages, and apoptotic cells were identified by immunohistochemistry for α-smooth muscle actin, Mac2, and cleaved caspase-3, as described in the online Data Supplement.

In Situ End-Labeling

Apoptotic cells were identified by in situ end-labeling (ISEL), as described previously.10

Identification of Buried Fibrous Caps

Serial sections stained for elastin and α-smooth muscle actin were examined for the presence of structures rich in elastin and SMCs, and these were identified as buried fibrous caps, as previously described.19

In Situ Zymography

Gelatinolytic activity was localized in brachiocephalic arteries removed 5 days after infection by using a modification of the in situ zymography method24 previously described25 and detailed in the online Data Supplement.

In Vitro Studies on Macrophages and Macrophage-Derived Foam Cells

The effect of recombinant TIMP-1 and TIMP-2 on migration, proliferation, and apoptosis were determined in rabbit experimental foam cells and differentiated THP-1 cells (see online Data Supplement).

In Vitro Studies on SMCs

Human aortic SMCs were cultured in serum-free Dulbecco’s modified Eagle’s medium supplemented with 5 nmol/L recombinant human TIMP-1 or TIMP-2 and 200 ng/mL recombinant Fas ligand. Apoptosis was measured by immunocytochemistry for cleaved caspase-3 (R&D Systems, Abingdon, UK), as described in the online Data Supplement.

Statistical Analysis

Values are expressed as mean±SEM. Data were analyzed by ANOVA for multiple comparisons, followed by Tukey’s post test. Differences were considered statistically significant when P<0.05.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Effect of Transient Overexpression of TIMP-1 or TIMP-2 With First-Generation Adenoviruses

Optimization of In Vivo Gene Transfer

Intravenous injection (Figure 1) of 8×1010 viral particles per mouse of an empty replication-deficient adenovirus (RAd66), 4 hours before infection with the reporter adenovirus RAd-lacZ, substantially increased the number of hepatocytes expressing β-galactosidase, as expected on the basis of published data.23 This was apparent macroscopically (Data Supplement Figure I). No microscopic abnormalities were detected within the livers of infected animals (data not shown). Consequently, this predosing scheme was used in all subsequent experiments wherein first-generation RAds were administered.

Plasma Concentrations of TIMP-1 and TIMP-2

Plasma concentrations of human TIMP-1 protein were detected in RAdTIMP-1–infected animals (n=6), whereas elevated TIMP-2 levels were detected in RAdTIMP-2–infected animals (n=6; Figure 2A and 2B). Maximum concentrations were reached at 5 days (Figure 2A and 2B). Levels were significantly greater than in animals infected with RAd66 at 2, 5, and 8 days after infection (n=6 per time point).

MMP Inhibitory Activity

Plasma samples from mice infected with RAdTIMP-1 or RAdTIMP-2 demonstrated significant inhibition of exogenous MMP-2 activity at 5 and 8 days after infection, compared with plasmas from RAd66-infected control animals. This inhibitory effect was lost 14 days after adenovirus administration (Figure 2C).

Plasma Lipid Analysis

There were no statistically significant differences in plasma lipid and lipoprotein levels between treatment groups (data not shown).
Effect on Atherosclerosis Development

Plaque cross-sectional area was reduced by ≈50% (P<0.05) at 4 weeks after infection with RAdTIMP-2 compared with RAd66-infected controls or animals that had received the high-fat diet alone (n=10/group; Table 1 and Figure 3). However, infection with RAdTIMP-1 (n=10) did not affect plaque area (Table 1 and Figure 3). The incidence of buried SMC-rich fibrous caps, heavily invested with elastin, was reduced by 90% in animals infected with RAdTIMP-2 (P<0.05) but was unaffected by RAdTIMP-1 infection (Table 1 and Figure 3). The mean medial area was not affected by infection with RAdTIMP-1 or RAdTIMP-2.

Mice infected with RAdTIMP-1 or RAdTIMP-2 had a significantly higher elastin content than did RAd66-infected controls (>2-fold increase, P<0.05; Table 1). No effect on collagen deposition or lipid content was observed (Table 1).

As shown in Table 1 and Figure 3, animals infected with RAdTIMP-2 had a 45% increase (P<0.01) in the content of α-smooth muscle actin–positive cells within their lesions compared with RAdTIMP-1 and control mice. Conversely, a 42% (P<0.01) decrease in macrophage content was observed, relative to RAdTIMP-1 and control animals. Additionally, in brachiocephalic lesions 5 days after infection, significantly fewer apoptotic cells were detected in macrophage-rich regions of TIMP-2–infected animals (5±1%) compared with control and TIMP-1–infected animals (17±1% and 24±4% respectively; Figure 4).

No significant differences in any of the parameters measured, except elastin content, were observed between first-generation RAdTIMP-1, RAd66, and high-fat-only mice 11 weeks after adenoviral administration (Table 2).

In Situ Zymography

Proteolytic activity was reduced in brachiocephalic arteries from animals infected 5 days previously with RAdTIMP-2.

### Table 1. Effects of Overexpression of TIMP-1 or TIMP-2 on ApoE−/− Mouse Brachiocephalic Artery Characteristics 4 Weeks After Infection (n=10/Group)

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>RAd66</th>
<th>RAdTIMP-1</th>
<th>RAdTIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque area, ×10^3 μm²</td>
<td>118±15</td>
<td>125±19</td>
<td>102±21</td>
<td>64±17†</td>
</tr>
<tr>
<td>Medial area, ×10^3 μm²</td>
<td>78±5</td>
<td>85±4</td>
<td>87±4</td>
<td>91±13</td>
</tr>
<tr>
<td>Buried fibrous caps</td>
<td>0.80±0.25</td>
<td>1.00±0.21</td>
<td>0.73±0.19</td>
<td>0.10±0.10†</td>
</tr>
<tr>
<td>Plaque elastin, %</td>
<td>4.3±0.7</td>
<td>3.2±0.7</td>
<td>6.9±1.8*</td>
<td>6.8±1.3†</td>
</tr>
<tr>
<td>Plaque collagen, %</td>
<td>6.1±0.2</td>
<td>9.6±1.8</td>
<td>6.1±1.6</td>
<td>7.2±2.5</td>
</tr>
<tr>
<td>Plaque lipid, %</td>
<td>37.7±7.2</td>
<td>41.5±5.3</td>
<td>52.7±4.1</td>
<td>43.9±2.9</td>
</tr>
<tr>
<td>Plaque SMCs, %</td>
<td>38.6±1.8</td>
<td>34.5±2.9</td>
<td>35.5±4.5</td>
<td>54.8±4.6†</td>
</tr>
<tr>
<td>Plaque macrophages, %</td>
<td>31.2±2.9</td>
<td>29.8±4.6</td>
<td>29.9±3.1</td>
<td>18.0±3.2†</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
*P<0.05, RAdTIMP-1 vs RAd66 and uninfected.
†P<0.05, RAdTIMP-2 vs RAd66 and uninfected.

Overexpression of TIMP-2 by HD Adenoviruses

To determine the effects of prolonged TIMP-2 expression, HD adenovirus technology was used, which provides long-term gene expression in mice.26

MMP Inhibitory Activity

In contrast to animals infected with first-generation RAds, plasma taken from HD-AdTIMP-2–infected mice retained significant MMP-2 inhibitory activity at all time points during the 11-week study period, relative to plasmas from HD-AdEmpty–infected control mice (Figure 6). Addition of the MMP inhibitors BB94 (Figure 5) and EDTA (data not shown) abolished the proteolytic activity, indicating the presence of gelatinolytic MMPs. Addition of other protease inhibitors (E64, pepstatin A, and aminophenylmethylsulfonyl fluoride) did not affect the observed activity (data not shown).

Effect on Atherosclerosis Development

Exactly as found with first-generation adenoviruses, plaque area and the number of buried fibrous caps were significantly reduced by HD-AdTIMP-2 (P<0.05) relative to HD-AdEmpty–infected mice 4 weeks after infection (n=10/group; Table 2). Furthermore, after 11 weeks of infection, a 60% (P<0.05) reduction in plaque area was maintained in animals infected with HD-AdTIMP-2 compared with HD-AdEmpty–infected controls and uninfected animals (n=10/group; Table 2 and Figure 7). Additionally, the frequency of buried fibrous caps was significantly decreased by >90% relative to control animals (P<0.001; Table 2 and Figure 7). As found with RAd-TIMP-2 after 4 weeks of infection (Table 1) and 11 weeks of infection (Table 2), lesion elastin content was 2-fold higher in HD-AdTIMP-2 compared with HD-AdEmpty–infected controls. A 3-fold increase in collagen content was observed in HD-AdTIMP-2–infected mice compared with HD-AdEmpty and high-fat-only control mice (Table 2 and Data Supplement Figure II). No difference in the proportion of lipid was observed in lesions in any group.

A 2-fold increase (P<0.01) in the content of α-smooth muscle actin–positive cells was detected in HD-AdTIMP-2 lesions compared with HD-AdEmpty–infected animals and uninfected animals (Table 2 and Figure 7). This number was comparable to that in RAdTIMP-2–infected mice 4 weeks after infection (HD-AdTIMP-2, 59.2±5.1% and RAdTIMP-2, 54.8±4.6%). Conversely, a 2-fold decrease (P<0.01) in the macrophage content was detected in HD-AdTIMP-2 lesions compared with HD-AdEmpty–infected animals and uninfected animals (Table 2 and Figure 7). Furthermore, a dramatic reduction (P<0.0001) in the number of lesional ISEL-positive apoptotic cells was detected in HD-AdTIMP-2–infected mice compared with HD-AdEmpty and high-fat-only control mice (Table 2 and Data Supplement Figure II). No difference in the proportion of lipid was observed in lesions in any group.

TABLE 2. Effect of Adenovirus Infection on ApoE<sup>−/−</sup> Mouse Brachiocephalic Artery Characteristics 11 Weeks After Infection (n=10/Group)  

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Uninfected</th>
<th>RAd66</th>
<th>RAdTIMP-2</th>
<th>HD-AdEmpty</th>
<th>HD-AdTIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque area, ×10&lt;sup&gt;3&lt;/sup&gt; μm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>105.1±3</td>
<td>106.1±9</td>
<td>93.1±14</td>
<td>106.2±20</td>
<td>41±10†</td>
</tr>
<tr>
<td>Medial area, ×10&lt;sup&gt;3&lt;/sup&gt; μm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>70.3±3</td>
<td>72.3±3</td>
<td>73.5±5</td>
<td>71.2±2</td>
<td>67±3</td>
</tr>
<tr>
<td>Buried fibrous caps</td>
<td>1.3±0.2</td>
<td>1.3±0.17</td>
<td>1.0±0.24</td>
<td>1.1±0.30</td>
<td>0.11±0.11†</td>
</tr>
<tr>
<td>Plaque elastin, %</td>
<td>8.3±1.2</td>
<td>11.7±1.6</td>
<td>20.1±2.8*</td>
<td>8.8±1.8</td>
<td>16.6±3.6†</td>
</tr>
<tr>
<td>Plaque collagen, %</td>
<td>4.8±0.7</td>
<td>4.5±0.7</td>
<td>7.2±0.7</td>
<td>8.4±1.4</td>
<td>19.7±2.6†</td>
</tr>
<tr>
<td>Plaque lipid, %</td>
<td>40.2±7.1</td>
<td>36.9±5.5</td>
<td>36.7±6.5</td>
<td>39.2±7.9</td>
<td>35.8±12.6</td>
</tr>
<tr>
<td>Plaque SMCs, %</td>
<td>24.3±3.4</td>
<td>29.5±3.3</td>
<td>29.8±2.0</td>
<td>21.9±3.0</td>
<td>59.2±5.1†</td>
</tr>
<tr>
<td>Plaque macrophages, %</td>
<td>46.1±7.0</td>
<td>46.6±4.9</td>
<td>52.9±7.8</td>
<td>41.9±7.3</td>
<td>22.7±4.9†</td>
</tr>
<tr>
<td>Plaque apoptosis, %</td>
<td>7.28±5.06</td>
<td>4.40±0.57</td>
<td>5.06±2.72</td>
<td>5.17±3.28</td>
<td>0.14±0.14†</td>
</tr>
</tbody>
</table>

Values are mean±SEM.  
*P<0.05, RAdTIMP-2 vs RAd66 and uninfected.  
†P<0.05, HD-AdTIMP-2 vs HD-AdEmpty and uninfected.  

Figure 4. Effect of RAdTIMP-1 or RAdTIMP-2 infection on macrophage and apoptotic cell colocalization. Representative brachiocephalic artery atherosclerotic lesions 5 days after infection are shown. Scale bar in C represents 100 μm and is applicable to B–F. White arrowheads indicate areas of immunopositivity. A–C, Macrophage immunohistochemistry. D–F, Cleaved caspase-3 immunohistochemistry. A and D, Mice infected with RAd66. B and E, Mice infected with RAdTIMP-1. C and F, Mice infected with RAdTIMP-2.
HD-AdTIMP-2 lesions compared with HD-AdEmpty–infected animals and uninfected animals (Table 2). Mice that had been infected with HD-AdTIMP-2 for 11 weeks exhibited plaque areas and incidences of buried fibrous caps similar to those of RAdTIMP-2 animals 4 weeks after infection (Tables 1 and 2). No significant effects on medial area were observed between treatment groups and controls (Table 2).

There was no significant difference in the size of the plaques 11 weeks after infection with HD-AdTIMP-2 or after 8 weeks of the high-fat diet alone (Figure 7). Plaques measured 11 weeks after infection with HD-AdTIMP-2 had a more stable phenotype, with an increased SMC content and a reduced macrophage number than did 8-week high fat–fed mice (Table 2 and Figure 7).

Effect of Exogenous TIMP-1 and TIMP-2 on Macrophage and SMC Behavior

Recombinant TIMP-1 and TIMP-2 failed to affect both macrophage and macrophage-derived foam cell proliferative rates (Table 3). Recombinant human TIMP-1 failed to significantly affect the migration of THP-1 macrophages or experimental macrophage-derived foam cells through a Matrigel-coated micropore filter in response to the chemoattractant monocyte chemotactic protein-1 compared with untreated cells (Table 3). In contrast, addition of recombinant human TIMP-2 significantly inhibited the migration of both THP-1 macrophages (66%) and macrophage-derived foam cells (38%).

The rate of lipopolysaccharide-induced apoptosis, as detected by cleaved caspase-3 immunocytochemistry, was unchanged in both THP-1 macrophages and macrophage-derived foam cells by treatment with recombinant human TIMP-1, compared with untreated cells (Table 3 and Figure 8). Additionally, Western blotting revealed that protein levels for cleaved Poly(ADP-ribose) polymerase (PARP-1) were also unaffected by TIMP-1 in THP-1 macrophages treated with lipopolysaccharide (Figure 8). Conversely, apoptosis was significantly inhibited in both THP-1 macrophages (76%) and macrophage-derived foam cells (93%) after recombinant human TIMP-2 addition, compared with untreated cells (Table 3 and Figure 8). Furthermore, cleaved PARP-1 protein levels were dramatically reduced (45%) in THP-1 macrophages treated with lipopolysaccharide, relative to control cells (Figure 8).

The rate of Fas ligand–induced apoptosis was unchanged in SMCs treated with recombinant human TIMP-1 relative to untreated cells (Table 3). Conversely, apoptosis was significantly inhibited in SMCs (36%) after recombinant human TIMP-2 addition (Table 3).

Discussion

In these studies, we have shown that gene transfer of TIMP-2 significantly inhibits the development of atherosclerotic lesions in the brachiocephalic arteries of fat-fed apoe–/– mice. Furthermore, the lesions are of a more stable phenotype, as assessed by the SMC and macrophage content, and the...
development of buried fibrous caps. Furthermore, these beneficial effects on plaque expansion and stability were maintained by long-term overexpression of TIMP-2 by an HD adenovirus.

With first-generation adenoviruses, the plasma concentrations of TIMP-1 and TIMP-2 peaked 5 days after infection and then returned to baseline within 28 days, in agreement with previous observations.27 However, infection with the HD adenovirus resulted in elevated plasma levels of TIMP-2 throughout the study period of 11 weeks, confirming the ability of these vectors to maintain long-term gene expression in mice.28 Use of an MMP activity bioassay confirmed the biological activities of the transferred genes.

One of the most striking findings of this study was the observation that plaques measured 11 weeks after infection with HD-AdTIMP-2 were of a comparable size to those in mice fed the high-fat diet for 8 weeks, suggesting that plaque progression was virtually abolished. Support for this idea comes from the finding that plaques measured 4 weeks after infection with RAdTIMP-2 were also of a similar size, suggesting that both methods of viral delivery were equally effective in the short term. The lack of effect of RAdTIMP-2 at 11 weeks after infection highlights the requirement for maintenance of elevated TIMP-2 levels.

We observed dramatic changes in lesion cellular content, a decrease in macrophage number, and an increase in SMC number. These changes may have been the result of altered cellular migration and proliferation, processes that are known to be modulated by MMPs.22,29–31 Indeed, the results from recombinant TIMP proteins suggested that TIMP-2, but not TIMP-1, profoundly inhibited the migration of macrophages and macrophage-derived foam cells, whereas no effect on proliferation was observed. Thus, we suggest that reduced migration of macrophages may contribute to their reduced numbers observed in plaques from TIMP-2–infected animals. With regard to effects on SMC behavior, TIMP-2 has previously been shown to inhibit SMC migration and proliferation.22 Thus, it is unlikely that these mechanisms participated in the increased SMC content observed.

A reduction in apoptosis could also contribute to the changes in plaque cellular content. In support of this concept, we observed significantly less apoptosis in plaques from TIMP-2–infected animals. Interestingly, we observed that recombinant TIMP-2, but not TIMP-1, profoundly inhibited apoptosis of macrophages and macrophage-derived foam cells. Furthermore, macrophages/foam cells within TIMP-1 and control plaques appeared to have undergone apoptosis, although this feature was rarely observed in plaques from TIMP-2–infected animals. Attenuation of macrophage-de-

TABLE 3. Effect of Exogenous TIMP-1 and TIMP-2 on Macrophage, Foam Cell, and SMC Behavior

<table>
<thead>
<tr>
<th>Behavior/Cell Type</th>
<th>Control</th>
<th>TIMP-1</th>
<th>TIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferation, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages (n=3)</td>
<td>100 ± 0</td>
<td>78 ± 2</td>
<td>87 ± 9</td>
</tr>
<tr>
<td>Foam cells (n=6)</td>
<td>100 ± 0</td>
<td>63 ± 13</td>
<td>81 ± 16</td>
</tr>
<tr>
<td><strong>Migration, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages (n=3)</td>
<td>100 ± 0</td>
<td>116 ± 34</td>
<td>34 ± 10*</td>
</tr>
<tr>
<td>Foam cells (n=3)</td>
<td>100 ± 0</td>
<td>92 ± 6</td>
<td>62 ± 11*</td>
</tr>
<tr>
<td><strong>Apoptosis, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages (n=3)</td>
<td>100 ± 0</td>
<td>86 ± 6</td>
<td>24 ± 8*</td>
</tr>
<tr>
<td>Foam cells (n=3)</td>
<td>100 ± 0</td>
<td>68 ± 15</td>
<td>7 ± 4*</td>
</tr>
<tr>
<td>SMCs (n=3)</td>
<td>100 ± 0</td>
<td>95 ± 3</td>
<td>64 ± 7*</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P<0.05, TIMP-2 vs TIMP-1 and control.
rived foam cell apoptosis retards the accumulation of extracellular lipid and proinflammatory debris\textsuperscript{32} and thereby reduces further macrophage recruitment,\textsuperscript{33} resulting in a reduction of macrophage number and an increase in plaque stability. This is supported by 2 previous studies\textsuperscript{34,35} wherein apoptosis was induced in advanced atherosclerotic lesions by gene transfer; nonthrombotic rupture, intraplaque hemorrhage, and buried caps were observed, indicative of enhanced plaque instability. In addition, we demonstrated that recombinant TIMP-2 modestly but significantly inhibited SMC apoptosis, suggesting this may have contributed to the increase in SMC density observed. Additionally, SMC apoptosis has been shown to induce macrophage recruitment.\textsuperscript{36} In summary, we suggest that TIMP-2 reduces macrophage migration into the lesion and retards further macrophage recruitment by inhibiting macrophage/foam cell apoptosis. Furthermore, TIMP-2 inhibits SMC apoptosis, and as a result, reduces additional macrophage recruitment. These effects increase the number of SMCs, decrease the number of macrophages, and therefore substantially affect the progression and stability of the atherosclerotic plaque.

In addition to changes in cellular composition, we observed reduced levels of MMP activity and changes in the content of elastin and collagen in the plaque. As predicted, MMP activity (detected by in situ zymography) was reduced by overexpression of either TIMP-1 or TIMP-2. This supports the hypothesis that changes in plaque development were the result of reduced MMP activity. However, we cannot rule out the involvement of MMP-independent direct effects of TIMPs on vascular cell behavior.\textsuperscript{37} The increased elastin content in both TIMP-1– and TIMP-2–overexpressing mice suggests that elevated TIMP levels result in inhibition of elastinolytic MMPs. It is interesting to note that although the beneficial effects of RAdTIMP-2 infection on plaque size and stability were lost 11 weeks after infection, there was still an increase in elastin content at this time point. This result suggests either that elastin degradation was still retarded or that plaques are slower to recover their elastinogenic capacity than they do other aspects of their growth and development.

The plaque collagen content was unaffected in the short-term experiment by TIMP-2, despite beneficial effects on plaque stability. However, circulating levels of TIMP-2 were decreased 14 days after infection; therefore, any favorable effect may have been lost by 28 days after infection. The results from the longer-term experiments, which showed a significant increase in collagen content within lesions from HDAd-TIMP-2–infected mice, are in line with expectations for an MMP inhibitor. This increase in collagen content is also to be expected from the associated increase in SMC number.

Our observation that overexpression of TIMP-1 had no significant effect on brachiocephalic artery lesion size or stability appears to be in contrast to a previous study in the aortic sinus of apoE\textsuperscript{−/−} mice.\textsuperscript{16} Interestingly, plaque destabilization has not been observed in the mouse aortic sinus but has frequently been detected in the brachiocephalic artery.\textsuperscript{17–19,38} This discrepancy suggests differing susceptibilities to atherogenic modulator mechanisms at the 2 sites, as suggested in a recent review.\textsuperscript{39}

Our study shows that there are important differences between the biological effects of TIMP-1 and TIMP-2 in brachiocephalic artery plaques because the levels of overexpression were similar. Although TIMP-1 and TIMP-2 have similar structures, functional differences have been reported. For example, TIMP-1 poorly inhibits membrane-type MMPs, but these are completely inhibited by TIMP-2.\textsuperscript{40} Additionally, TIMP-2 is 7-fold more effective than TIMP-1 in inhibiting MMP-9.\textsuperscript{41} A quantitative comparison of the inhibitory efficacies of TIMPs against the complete range of MMPs could give clues as to the roles of these enzymes in atherogenesis and plaque destabilization.

In conclusion, this study shows that TIMP-2 is an effective inhibitor of atherosclerotic plaque growth and promotes a stable plaque phenotype in the fat-fed apoE\textsuperscript{−/−} mouse bra-
cholephic artery. We suggest that this is a result of modulation of SMC and macrophage behavior. TIMP-2 decreases both SMC apoptosis and macrophage migration, promoting a more stable phenotype. Furthermore, TIMP-2–reduced macrophage and SMC apoptosis may also induce a more stable phenotype by retarding further macrophage recruitment. Hence, the differential effects of TIMP-1 and TIMP-2 on plaque stability suggest that selective inhibition of specific MMPs may be beneficial.

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Disclosures
None.

References
Rupture of an atherosclerotic plaque and subsequent thrombosis are the major causes of stroke and myocardial infarction, resulting in increased morbidity and mortality. The structural integrity of the atherosclerotic plaque is maintained by a collagen-rich structure termed the fibrous cap that encapsulates the thrombogenic macrophage/lipid-rich core, thus preventing thrombosis. It has been proposed that the matrix-degrading matrix metalloproteinases (MMPs), a family of enzymes expressed within atherosclerotic lesions, reduce the strength of the fibrous cap and thus contribute to plaque rupture. Subsequently, numerous studies have determined the effects of synthetic MMP inhibitors on atherosclerosis in both animal models and human clinical trials. Unfortunately, the findings of these studies have been disappointing. Using gene therapy, we have discovered that overexpression of endogenous tissue inhibitor of metalloproteinase (TIMP)-2 dramatically reduced the incidence of atherosclerotic plaque rupture–related events and concomitantly induced a transition to a stable plaque phenotype. We suggest that the beneficial effects exerted by TIMP-2 are due to a reduction in macrophage migration and apoptosis, factors associated with increased plaque vulnerability. Indeed, in vitro studies have demonstrated that TIMP-2 favorably modulates macrophage behavior. Conversely, TIMP-1 failed to exert any beneficial effects on atherosclerotic plaque stability or macrophage function. These novel findings highlight the differential effects of TIMP-1 and TIMP-2 on plaque stability. Furthermore, the mechanistic results suggest TIMP-2 as a favorable candidate for therapeutic prevention of atherosclerotic plaque growth and rupture.
Suppression of Atherosclerotic Plaque Progression and Instability by Tissue Inhibitor of Metalloproteinase-2: Involvement of Macrophage Migration and Apoptosis

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