Adenovirus-Mediated Gene Transfer of the Human Tissue Inhibitor of Metalloproteinase-2 Blocks Vascular Smooth Muscle Cell Invasiveness In Vitro and Modulates Neointimal Development In Vivo

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Background—Endovascular injury induced by balloon withdrawal leads to the increased activation of matrix metalloproteinases (MMPs) in the vascular wall, allowing smooth muscle cells (SMCs) to digest the surrounding extracellular matrix (ECM) and migrate from the media into the intima. The objective of this study was to examine the effects of a replication-deficient adenovirus carrying the cDNA for human tissue inhibitor of metalloproteinase-2 (AdCMV.hTIMP-2) on SMC function in vitro and neointimal development in the injured rat carotid artery.

Methods and Results—Infection of cultured rat aortic SMCs at a multiplicity of infection of 100 with AdCMV.hTIMP-2 resulted in high-level expression of hTIMP-2 mRNA and protein secretion into the medium. Conditioned media (CM) from AdCMV.hTIMP-2–infected but not control virus (AdCMV.null or AdCMV.βgal)–infected SMCs inhibited MMP-2 activity on gelatin zymograms as well as the chemoattractant-directed migration of SMCs across reconstituted basement membrane proteins in the Boyden chamber assay. In contrast, AdCMV.hTIMP-2 CM had no effect on chemoattractant-directed migration of SMCs occurring in the absence of an ECM barrier or on the proliferation of cultured neointimal SMCs. Delivery of AdCMV.hTIMP-2 (2.5 × 10^9 pfu) to the carotid artery wall at the time of balloon withdrawal injury inhibited SMC migration into the intima by 36% (P<0.05) at 4 days and neointimal area by 53% (P<0.01) at 8 days and by 12% (P=NS) at 21 days after injury. AdCMV.hTIMP-2 had no effect on medial area.


Key Words: genes ■ viruses ■ metalloproteinases ■ restenosis

Balloon injury of the vascular wall initiates a cascade of biological events that may lead to vascular stenosis. In the rat carotid artery model of balloon withdrawal injury, medial smooth muscle cell (SMC) proliferation begins immediately after injury, reaching a peak ∼2 days later. SMCs are not normally present in the intima of rat arteries but begin to appear there at ∼4 days after injury, presumably as a result of their migration from the medial cell layer. Thereafter, the accumulation of SMCs in the neointima is thought to occur primarily from the proliferation of the SMCs that had previously migrated into the intima.1

Under normal conditions, SMCs in the media are quiescent and are embedded in a network of different extracellular matrix (ECM) components that act as barriers to both SMC migration and proliferation.2,3 Digestion and remodeling of the ECM occur early after vascular injury4-6 and are related to the activation of different proteases, including the matrix metalloproteinases (MMPs).7-9 All of the active MMPs are inhibited by a class of low-molecular-weight proteins known as tissue inhibitors of metalloproteinases (TIMPs).10 The TIMPs are ubiquitous and form a 1-to-1 complex with MMPs. It has been shown that in the rat carotid artery, the 92-kDa type IV collagenase/gelatinase, MMP-9, is not constitutively expressed and that both the mRNA and the catalytic activity of this enzyme increase 1 to 3 days after injury and rapidly decrease thereafter. The 72-kDa type IV collagenase/gelatinase, MMP-2, is constitutively expressed. Its mRNA shows a slight decrease 1 day after balloon injury,
whereas the relative activation of MMP-2 increases during the 5- to 14-day period after injury.5–8

Therefore, the time frame of the changes in MMP-2 are consistent with a role in the migration of SMCs into the intima. More recently, injury has been shown to substantially increase the expression of another MMP, membrane-type MMP.10 This increase precedes the changes in MMP-2 expression, consistent with its potential role as a cell-surface activator of MMP-2.11,12 Neither MMP-1 nor MMP-3 appears to play a role in neointima formation in the rat model of carotid injury.

Additional support for the role of MMPs in SMC migration to the intima comes from in vivo studies on the inhibition of these proteinases.5,13,14 Given the particular importance of MMP-2 to vessel injury and the fact that a unique association occurs between MMP-2 and TIMP-2,15 we constructed a replication-deficient adenovirus encoding human TIMP-2 to infect cultured SMCs as well as SMCs in vivo and assess the effects of its expression on SMC function in cell culture and neointimal development after balloon withdrawal injury.

Methods

SMC Isolation and Culture
Rat aortic medial SMCs (RASMCS) were isolated from the thoracic aortas of 3-month-old Wistar rats, and rat carotid neointimal SMCs were isolated from the neointima of carotid arteries 14 days after injury, as previously described.4

Recombinant Adenovirus Construction
A replication-deficient adenovirus (Ad) carrying the cDNA for hTIMP-216 under the transcriptional control of the cytomegalovirus (CMV) immediate early promoter was constructed and amplified as previously described.17 An Ad vector expressing the bacterial (CMV) immediate early promoter was constructed and amplified as AdCMV.null–infected cells. In the case of AdCMV.hTIMP-2 CM, sufficient CM was added to give a final concentration of hTIMP-2 of 200 ng/mL. The concentration of hTIMP-2 in the CM was determined by quantitative Western blotting using purified hTIMP-2 (Oncogene Science) as a standard. Gels were stained with Coomassie blue R-250, and metalloproteinase produced clear areas of lysis in the gel.

SMC Migration and Invasion Assays
RASM C migration and invasion were assessed with a modified Boyden chamber and platelet-derived growth factor-BB (PDGF-BB; Collaborative Research) as the chemoattractant18 (10 ng/mL in 0.1% BSA/DMEM). For invasion assays, filters (Nucleopore; 8-µm pore size) were coated with a 10-µm barrier of reconstituted basement membrane proteins (Matrigel). For migration assays, filters were coated with a dilute mixture of fibronectin and collagen type I (50 μg/mL; Collaborative Research). After 4 hours of incubation, cells on both sides of the filter were fixed and stained with hematoxylin/ eosin. The average number of cells from 4 randomly chosen high-power (×400) fields on the lower side of the filter was counted.

SMC Proliferation Assay
Rat carotid neointimal SMCs (5×10^4 cells/well) were plated into individual wells of a 6-well tissue culture plate and then infected with either AdCMV.null or AdCMV.hTIMP-2 at 100 MOI. Relative cell numbers per well were estimated at 1, 5, 7, 10, and 15 days after infection with the MTT colorimetric assay.20

Rat Carotid Injury
Six-month-old male Wistar rats were used for all studies. Injury of the left common carotid artery was performed in the standard fashion.21 Immediately after injury, 200 μL of a solution containing 2.5×10^9 pfu of either AdCMV.hTIMP-2 or AdCMV.null was introduced into the 1.5-cm segment of the injured carotid artery with a 24-gauge intravenous catheter. Total dwell time for the solution containing the Ad vector in the injured carotid artery was 7 minutes. Thereafter, the external carotid artery was ligated, and blood flow through the internal carotid artery was reestablished.

Measurement of SMC Migration In Vivo
SMC accumulation in the denuded intima at 4 days after injury was measured by the en face technique as previously described.3

Immunohistochemistry of Rat Carotid Arteries
Dissected carotid segments were embedded in OCT compound (Miles Laboratory). Frozen sections 5 µm thick were fixed in acetone, and 0.3% H2O2 in methanol was used to block endogenous peroxidases. Sections were incubated with 1 µg/mL anti-hTIMP-2 (Oncogene Science) for 2 hours. Subsequent incubation for 30 minutes in biotinylated goat anti-mouse IgG was followed by a 30-minute incubation with streptavidin-peroxidase. Peroxidase was visualized with 3-aminobenzidine (Zymed Corp). Sections were counterstained with Mayer’s hematoxylin.

Morphometric Analysis of Rat Carotid Arteries
Eight and 21 days after balloon injury, the rats were euthanized with an overdose of sodium pentobarbital. Paraffin sections of vessel segments were stained with elasin–van Gieson and hematoxylin-eosin. Morphometric analyses were performed on 3 to 4 cross sections for each vessel, and the cross-sectional area of the intima and media was measured.15

Gelatin Zymography
Recombinant human MMP-2 (rhMMP-2) was generously provided by Dr Rafi Fridman (Wayne State University, Detroit, Mich) and was converted from its latent to active form with 4-aminophenylmercuric acetate as previously described. Several dilutions of rhMMP-2 (500 to 2000 pg) were denatured in SDS sample buffer minus dithiothreitol, without boiling, and then subjected to electrophoresis on 10% SDSPolyacrylamide gels containing 0.1% (wt/vol) gelatin (Novex Chemical). Gels were washed with 2.5% Triton X-100 for 30 minutes at room temperature and then incubated at 37°C for 18 hours in 5 mL of a solution of the following composition: 50 mmol/L HEPES, 0.2 mol/L NaCl, 5 mmol/L CaCl2, 20 μmol/L ZnCl2, and 0.02% Brij-35 (pH 7.5) containing CM from AdCMV.null- or AdCMV.hTIMP-2-infected cells. SDS-polyacrylamide gels containing 0.1% (wt/vol) gelatin (Novex Chemical). Gels were washed with 2.5% Triton X-100 for 30 minutes at room temperature and then incubated at 37°C for 18 hours in 5 mL of a solution of the following composition: 50 mmol/L HEPES, 0.2 mol/L NaCl, 5 mmol/L CaCl2, 20 μmol/L ZnCl2, and 0.02% Brij-35 (pH 7.5) containing CM from AdCMV.null- or AdCMV.hTIMP-2-infected cells. In the case of AdCMV.hTIMP-2 CM, sufficient CM was added to give a final concentration of hTIMP-2 of 200 ng/mL. The concentration of hTIMP-2 in the CM was determined by quantitative Western blotting using purified hTIMP-2 (Oncogene Science) as a standard. Gels were stained with Coomassie blue R-250, and metalloproteinase produced clear areas of lysis in the gel.

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Evaluation of TIMP-2 Plasma Levels

To examine whether the injection of AdCMV.hTIMP-2 into the arterial circulation was associated with increased TIMP-2 plasma levels, anesthetized male Wistar rats were injected into the cardiac left ventricle either with AdCMV.hTIMP-2 or with AdCMV.null (2.5 \times 10^9 pfu in 200 \mu L). Blood was withdrawn from the left ventricle 2 and 4 days after infection, and TIMP-2 plasma levels were analyzed by ELISA (Amersham Corp).

Statistical Analysis

Results are expressed as the mean±SEM. ANOVA combined with the Student-Newman-Keuls test was used to examine the statistical significance of continuous variables among different experimental groups. A value of \( P < 0.05 \) was considered significant.

Results

Adenovirus-Mediated Human TIMP-2 Gene Transfer to RASMCs

Proliferating RASMCs were infected either with AdCMV.hTIMP-2, AdCMV.\beta\text{gal}, or AdCMV.null and then maintained in serum-free media. Total RNA was isolated at various times after infection and probed for hTIMP-2 and rat GAPDH mRNA. Figure 1A shows that AdCMV.hTIMP-2-infected cells expressed hTIMP-2 mRNA as early as 4 hours after infection, and very high levels of expression were observed thereafter. At 4 hours, the predominant transcript was \( \approx 1 \) kb in length, as expected from the cDNA size. At later times, an \( \approx 3.5 \) -kb transcript could also be seen. This higher-weight band may represent long, unprocessed hTIMP-2 transcripts due to inefficient recognition of the SV40 termination and polyadenylation signal. Total RNA isolated from cells infected with the control virus, AdCMV.\beta\text{gal}, contained no hTIMP-2 mRNA, as expected.

CM was collected at times up to 21 days after infection to determine whether hTIMP-2 protein was synthesized and secreted by the infected RASMCs and how long hTIMP-2 secretion persisted after a single infection. Figure 1B shows the results of Western blotting analysis of the CM using a human-specific antibody to TIMP-2. Human TIMP-2 protein was seen in the CM within the first 24 hours after infection, and the amount of TIMP-2 protein secreted over the 24-hour collection period remained relatively constant up to 2 weeks. Human TIMP-2 protein was not detected in CM from uninfected or AdCMV.\beta\text{gal}-infected RASMCs (data not shown).

To determine whether the TIMP-2 secreted by AdCMV.hTIMP-2-infected RASMCs was functionally active, the ability of CM to inhibit MMP-2 activity was determined. MMP-2 activity was measured by gelatin zymography of recombinant hMMP-2 that had been preactivated by incubation with 4-aminophenylmercuric acetate. Increasing amounts of recombinant hMMP-2 were loaded onto the gel, and their activity was measured in the presence of a constant amount of CM. Figure 2 shows the results of Western blotting analysis of the CM using a human-specific antibody to TIMP-2. Human TIMP-2 protein was seen in the CM within the first 24 hours after infection, and the amount of TIMP-2 protein secreted over the 24-hour collection period remained relatively constant up to 2 weeks. Human TIMP-2 protein was not detected in CM from uninfected or AdCMV.\beta\text{gal}-infected RASMCs (data not shown).

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Effects of AdCMV.hTIMP-2 on Cultured SMC Function

The effects of CM from uninfected, AdCMV.hTIMP-2–infected, and AdCMV.null-infected cells on SMC migration, invasion, and proliferation were examined. Figure 3A shows the effects of CM from the different sources on the ability of RASMCs to migrate toward PDGF-BB across a 10-μm ECM barrier constituted by Matrigel (invasion assay). Although few cells migrated in the absence (bar 1) and in presence (bar 2) of PDGF-BB in bottom chamber, PDGF-BB–directed chemotaxis in presence of CM from AdCMV.hTIMP-2–infected (bar 4) or AdCMV.null-infected (bar 5) RASMCs is also shown. A peptide previously shown to inhibit MMP-2 activity was used as a positive control (bar 3). When RASMCs were incubated with CM containing Ad-expressed hTIMP-2 (bar 4), they lost their ability to invade Matrigel coating filter and move into lower chamber (P<0.05). B, Migration assay. Experimental conditions are same as for A. RASMC migration in presence of a fibronectin/collagen I mixture coating of filter was not affected by presence of CM from AdCMV.hTIMP-2–infected cells. Results shown in both panels represent average of 5 independent experiments performed in triplicate in each condition and were analyzed by ANOVA.

Effects of AdCMV.hTIMP-2 on proliferation of intimal SMCs: rat carotid intimal SMCs either were infected with AdCMV.hTIMP-2 or AdCMV.null (100 MOI) or were not infected. Cell proliferation was assessed by MTT assay at 1, 5, 7, 10, and 15 days after infection. No differences were observed among 3 groups. Results represent average of 3 independent experiments performed in triplicate.

Figure 4. Effect of AdCMV.hTIMP-2 on proliferation of intimal SMCs: rat carotid intimal SMCs either were infected with AdCMV.hTIMP-2 or AdCMV.null (100 MOI) or were not infected. Cell proliferation was assessed by MTT assay at 1, 5, 7, 10, and 15 days after infection. No differences were observed among 3 groups. Results represent average of 3 independent experiments performed in triplicate.

Figure 5. Adenovirus-mediated hTIMP-2 expression in intact rat carotid artery. Blood vessels were immunostained with a monoclonal antibody to human TIMP-2 at 5 days after balloon injury and infection (2.5×10^9 pfu). A, Cross section of a representative carotid artery infected with AdCMV.hTIMP-2. Media (M) exhibits intense reddish staining indicative of hTIMP-2 expression. There is no evidence of hTIMP-2 expression in media. Adventitia shows nonspecific staining. B, Cross section of a representative carotid artery infected with AdCMV.null. There is no evidence of hTIMP-2 expression in media. Adventitia shows nonspecific staining. L indicates lumen; arrow, internal elastic lamina. Magnification ×400.
significant reduction in cell number up to 15 days after infection with AdCMV.hTIMP-2 versus either AdCMV.null-infected or uninfected control cells.

**Adenovirus-Mediated hTIMP-2 Expression in the Vessel Wall**

To determine whether AdCMV.hTIMP-2 could infect and express hTIMP-2 in the balloon-injured rat carotid artery, either AdCMV.hTIMP-2 or AdCMV.null was delivered to the vessel wall immediately after balloon catheter injury. Five days after injury, carotid arteries infected with AdCMV.hTIMP-2 (Figure 5A) showed intense staining for human TIMP-2 that was localized to the medial cell layer, whereas arteries infected with AdCMV.null (Figure 5B) showed no significant staining. The adventitial area of both AdCMV.hTIMP-2– and AdCMV.null-infected vessels showed nonspecific staining, which was also seen when the primary anti–hTIMP-2 antibody was omitted (data not shown).

**In Vivo Effect of AdCMV.hTIMP-2 on SMC Migration and Neointima Development**

The effect of AdCMV.hTIMP-2 on in vivo SMC migration assessed by the en face technique was examined in infected carotid arteries 4 days after balloon injury (Figure 6). AdCMV.hTIMP-2 infection caused a 36% decrease in intimal cell accumulation ($P < 0.05$), with arteries infected with AdCMV.hTIMP-2 exhibiting $20.3 \pm 3.2$ intimal cells/mm$^2$ ($n = 6$) versus $32.3 \pm 4.1$ intimal cells/mm$^2$ in AdCMV.null-infected carotid arteries ($n = 6$).

To examine the effect of AdCMV.hTIMP-2 infection on neointimal accumulation, both neointimal and medial areas were measured 8 days after injury. Representative examples are shown in Figure 7 and average results are shown in Figure 8. There was a significant 53% reduction in neointimal area in AdCMV.hTIMP-2–infected vessels ($0.029 \pm 0.008$ mm$^2$; $n = 6$) compared with AdCMV.null-infected vessels ($0.062 \pm 0.005$ mm$^2$; $n = 6$; $P < 0.01$). No difference, however, was seen in medial area ($0.141 \pm 0.009$ versus $0.152 \pm 0.005$ mm$^2$ for AdCMV.hTIMP-2 and AdCMV.null-infected vessels, respectively; $P = NS$). As expected, the ratio of neointimal to medial areas showed a significant difference between AdCMV.hTIMP-2– and AdCMV.null-infected vessels ($0.200 \pm 0.043$ versus $0.413 \pm 0.045$; $P < 0.001$). These results show that adenovirus-mediated gene transfer of hTIMP-2 to the rat carotid artery immediately after injury causes a significant decrease in early cell accumulation and neointima development. Additional
experiments examined the effect of AdCMV.hTIMP-2 on neointima development 21 days after injury. At this time, there was a 12% inhibition in neointima accumulation between AdCMV.hTIMP-2–infected (0.269±0.011 mm²; n=9) and AdCMV.null-infected (0.237±0.023 mm²; n=10) carotid arteries, which was not statistically significant (P=NS). Medial area was similar in the 2 groups (0.133±0.003 mm² in AdCMV.hTIMP-2 versus 0.130±0.003 mm² in AdCMV.null). It is noteworthy that some inflammatory cells were observed on the adventitial side of infected blood vessels at 8 days but not at 21 days after infection (not shown).

It cannot be excluded that under the experimental conditions of the present study, some Ad vectors may enter the systemic circulation. In this case, early inhibition of SMC migration and neointima development may be due, at least in part, to systemic rather than localized infection with AdCMV.hTIMP-2. Therefore, additional experiments were aimed at excluding the presence of high circulating levels of TIMP-2 after systemic infection with AdCMV.hTIMP-2. In these experiments, either AdCMV.hTIMP-2 or AdCMV.null (2.5×10⁹ pfu in 200 μL) was injected directly into the left ventricle of the heart, and TIMP-2 plasma levels were determined 2 and 4 days after infection. In these experiments, circulating TIMP-2 was 173.1±35.6 ng/mL (AdCMV.hTIMP-2; n=7) and 156.5±29.7 ng/mL (AdCMV.null; n=7) at 2 days and 207.6±33.2 ng/mL (AdCMV.hTIMP-2; n=4) and 197.1±34.1 ng/mL (AdCMV.null; n=7) at 4 days. Because TIMP-2 plasma levels were similar in AdCMV.hTIMP-2– and AdCMV.null-infected animals, the inhibition of SMC migration at 4 days and neointima development at 8 days in AdCMV.hTIMP-2–infected carotid arteries was due to localized hTIMP-2 overexpression rather than to high levels of this protein in the bloodstream.

Discussion

This study describes the potential therapeutic efficacy of adenovirus-mediated gene transfer of an MMP inhibitor in an animal model of neointima development after endovascular injury.

A large body of evidence supports the notion that MMP expression and activation play a critical role in the remodeling of the vessel wall that occurs after experimental injury. In fact, both MMP-2 and MMP-9 expression and activation increase after balloon catheter injury to rat, pig, and rabbit vessels. Because TIMP-2 has been shown to be 10-fold and 7-fold more effective than TIMP-1 in inhibiting MMP-2 and MMP-9 activity, respectively, and because it has been shown to inhibit activation of MMP-2, TIMP-2 was selected to express the cDNA for TIMP-2. Infection with AdCMV.hTIMP-2 caused a significant reduction in SMC migration within the first 4 days after injury and in neointimal area 8 days after injury. However, 21 days after injury, the inhibition of neointima accumulation in AdCMV.hTIMP-2–versus AdCMV.null-treated carotid arteries was only 12% and did not achieve statistical significance. This result is in agreement with those of others that have shown that daily systemic administration of a peptide inhibitor of MMP activity blocked SMC cell movement to the intima in the injured rat carotid artery by >90% and inhibited neointimal thickening up to 10 days. However, neointimal area in the treated vessels was not significantly different from that of controls 14 days after injury, presumably because of persistent proliferation of neointimal SMCs in the treated vessels. In contrast, SMCs genetically engineered ex vivo with a retrovirus vector to overexpress TIMP-1 and subsequently implanted onto balloon-injured rat carotid artery inhibited neointima accumulation at 14 days by 40%. The results of these studies and of the present work are difficult to compare, because different strategies were used to inhibit MMP activity. Nevertheless, even if MMP inhibitors do not prevent neointimal accumulation in the rat late after balloon withdrawal injury, this would not exclude a potential role for them in the treatment of vascular disorders. This may be possible because there are many differences between balloon catheter injury to a naive vessel in an animal that rarely develops a vascular lesion resembling human atherosclerotic lesions and angioplasty of human diseased coronary and peripheral arteries. Indeed, recent studies in a double-injury model in the rabbit have revealed an unexpected effect of MMP peptide inhibitors, namely, their ability to inhibit ECM deposition. Such an effect would be of obvious significance to lesions that are composed primarily of ECM and are acellular, such as the second lesion in the rabbit model and primary human restenotic lesions. Therefore, it is possible that MMP inhibition in the vascular wall may diminish neointima formation after vascular injury via 2 different mechanisms: it may inhibit SMC migration from the media to the intima, and it may also decrease ECM accumulation by a direct effect on collagen synthesis and degradation. Because there is considerable redundancy in the mechanisms that cause SMCs to reenter the cell cycle, migrate from the media to the intima, and secrete ECM components, it seems unlikely that interfering with just 1 pathway would be adequate to prevent neointima development after injury. Chances for success would probably be higher if MMP inhibition were combined with other strategies to prevent restenosis. This is currently feasible, because gene transfer can prevent thrombus formation, accelerate reendothelialization, and induce cell death, and cell cycle arrest after vascular injury. Therefore, TIMP-2–mediated inhibition of SMC migration from the media to the intima may be combined with interventions aimed at diminishing the likelihood for thrombus formation and inducing cell cycle arrest. In contrast, because reendothelialization at the site of vascular injury probably requires endothelial cell migration, the combined use of TIMP-2 and of an endothelial cell mitogen may show no significant benefit. Nevertheless, the combined use of Ad vectors that code for different genes and the development of vectors with prolonged transgene expression that can be targeted to different cell types within the vascular wall may enhance the likelihood that Ad-mediated gene therapy may be successful in the treatment of restenosis after endovascular injury.

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References


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