Gene Transfer of the Urokinase-Type Plasminogen Activator Receptor–Targeted Matrix Metalloproteinase Inhibitor TIMP-1.ATF Suppresses Neointima Formation More Efficiently Than Tissue Inhibitor of Metalloproteinase-1


Abstract—Proteases of the plasminogen activator (PA) and matrix metalloproteinase (MMP) system play an important role in smooth muscle cell (SMC) migration and neointima formation after vascular injury. Inhibition of either PAs or MMPs has previously been shown to result in decreased neointima formation in vivo. To inhibit both protease systems simultaneously, a novel hybrid protein, TIMP-1.ATF, was constructed consisting of the tissue inhibitor of metalloproteinase-1 (TIMP-1) domain, as MMP inhibitor, linked to the receptor-binding amino terminal fragment (ATF) of urokinase. By binding to the u-PA receptor this protein will not only anchor the TIMP-1 moiety directly to the cell surface, it will also prevent the local activation of plasminogen by blocking the binding of urokinase-type plasminogen activator (u-PA) to its receptor. Adenoviral expression of TIMP-1.ATF was used to inhibit SMC migration and neointima formation in human saphenous vein segments in vitro. SMC migration was inhibited by 65% in Ad.TIMP-1.ATF–infected cells. Infection with adenoviral vectors encoding the individual domains, Ad.TIMP-1 and Ad.ATF, reduced migration by 32% and 52%, respectively. Neointima formation in saphenous vein organ cultures infected with Ad.TIMP-1.ATF was inhibited by 72% compared with 42% reduction after Ad.TIMP-1 infection and 34% after Ad.ATF infection. These data show that binding of TIMP-1.ATF hybrid protein to the u-PA receptor at the cell surface strongly enhances the inhibitory effect of TIMP-1 on neointima formation in human saphenous vein cultures. (Circ Res. 2002;91:945-952.)

Key Words: neointima formation ■ matrix metalloproteinases ■ plasmin(ogen) ■ urokinase-type plasminogen activator ■ adenovirus

Injury to the vessel wall resulting from vascular intervention triggers neointima formation and vascular remodeling, which together have been implicated as an important cause of (re)stenosis and graft failure after balloon angioplasty. Various studies have demonstrated that neointimal thickening involves smooth muscle cell (SMC) migration and proliferation as well as extracellular matrix (ECM) deposition. SMCs synthesize many of the major components of the vessel wall ECM and have the ability to digest all of these components through production and secretion of degradative enzymes. This feature enables SMCs to invade and migrate, and to remodel the vessel wall, thereby contributing to the pathological process of (re)stenosis. Among these degradative enzymes, the matrix metalloproteinases (MMPs) and the plasminogen activation (PA) system play key roles in vascular remodeling.

The MMPs are a family of enzymes that can be divided into four subclasses: collagenases, gelatinases, stromelysins, and membrane-type MMPs. Increased MMP activity after vascular injury was demonstrated in various model systems, both in vitro and in vivo. MMP activity is controlled by specific inhibitors: tissue inhibitors of metalloproteinases (TIMPs). To date, four members of the tissue inhibitor family have been identified: TIMP-1, -2, -3, and -4. They are expressed by a variety of cells and are present in most tissues and body fluids. TIMP-1 and TIMP-2 have the broadest substrate specificity, acting against all members of the MMP family. Studies using the rat balloon injury model have demonstrated that TIMP-1 and TIMP-2 overexpression reduces or delays, respectively, neointimal development.

Furthermore, in 14-day organ cultures of saphenous vein TIMP-1, -2, and -3 were found to be potent inhibitors of neointima formation, whereas only TIMP-3 overexpression inhibited stenosis in a porcine vein graft model in vivo. The second degradative enzyme system, the plasminogen activation system, revolves around the serine protease plasminogen activator.
min, which cannot only degrade ECM directly but also indirectly, by activation of MMP proenzymes. The proenzyme plasminogen can be converted to active plasmin by urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). u-PA plays a major role in local pericellular proteolysis, because it can bind to a specific cell-surface receptor, the u-PA receptor (u-PAR).22 u-PA consists of three distinct domains: a growth factor domain, followed by a kringle domain, together known as the amino terminal fragment (ATF), and a third region containing the serine protease domain. Binding of u-PA to u-PAR is mediated by its growth factor domain23,24 and brings u-PA in close contact to plasminogen on its receptor, allowing efficient activation of plasminogen to plasmin on the cell surface. A role for u-PA–mediated plasminogen activation has been implicated in vascular cell migration25–27 and neointima formation.28–30

Given the established role for both protease systems in cell migration and subsequent neointima formation, an approach to therapy is to overexpress inhibitors of these protease families.19,21,31–34 To inhibit both systems simultaneously, we have constructed a novel hybrid protein TIMP-1.ATF, consisting of the MMP inhibitor TIMP-1 linked to the ATF domain of u-PA.

The present study demonstrates the efficacy of an adenoviral vector encoding this novel hybrid protein, Ad.TIMP-1.ATF, as an inhibitor of cell migration and neointima formation, utilizing an in vitro model for human neointima development. TIMP-1.ATF is expected to cause several effects. First, effective MMP inhibition is realized on the cell surface, preventing degradation of cell-matrix contacts and matrix components.39 Second, TIMP-1.ATF prevents u-PA from binding to its receptor by competition, resulting in decreased activation of plasminogen and thus reduced activation of pro-MMPs by plasmin. To determine the additional effects of linking TIMP-1 to ATF, adenoviral vectors encoding the individual domains TIMP-1 (Ad.TIMP-1) and ATF (Ad.ATF) were also tested.

### Materials and Methods

#### Cell Culture

Human saphenous vein smooth muscle cells (HSVSMCs) were isolated as described36 and cultured in Dulbecco’s modified Eagle’s medium (DMEM), containing 2 mmol/L l-glutamine, 5% fetal calf serum (FCS), 5% human serum, and streptomycin/penicillin (100 μg/mL, 100 U/mL). Cells were used for experiments between passages 2 to 6.

#### Adenoviral Construction

For construction of adenoviral vectors, TIMP-1, ATF, ATF.TIMP-1, and TIMP-1.ATF were cloned into pCMV adenovirus shuttle vectors. Adenoviral vectors were generated by cotransfection of the pCMV constructs with pJM17 in HER-911 cells. A control vector, Ad.P10, was constructed from pCMV without insert.

Adenoviral vectors were propagated on HER-911 cells, purified and subsequently transferred to a nylon membrane filter (Hybond-NX Amersham Pharmacia Biotech). Hybridization was performed with [32P]cDNA probes for TIMP-1 or ATF.

TIMP-1 activity was determined as described previously,38 using 16 ng/mL purified MMP-9 and modified prourokinase, containing a general MMP-specific activation site.39

Cell surface binding of TIMP-1.ATF was verified by flow cytometry. HSVSMCs were incubated with TIMP-1.ATF-containing conditioned medium before incubation with the rabbit polyclonal anti-human u-PA40 and/or the mouse monoclonal anti-human TIMP-1 (MAB196 Oncogene Research). Immunoreactivity was detected by FITC-labeled rabbit anti-mouse and phycoerythrin-labeled mouse anti-rabbit (Becton-Dickinson). Cells were analyzed by flow cytometry (FACScalibur, BD Biosciences), whereby cells not loaded with TIMP-1.ATF, and not stained with antibodies, were used to set the quadrants.

Receptor-bound TIMP-1 activity was determined on plates coated with 5 μg/mL human soluble u-PAR.41 MMP activity was measured as described,42 using 0.05 nmol/L active MMP-13 (kindly provided by Dr P. Mitchell, Pfizer Central Research, Groton, Conn) and the fluorogenic MMP substrate TNO211.43

Inhibition of cell surface–localized plasminogen activation by TIMP-1.ATF was assessed using a plasmin activity assay on cultured HSVSMCs. Diluted samples of conditioned medium from Ad.TIMP-1.ATF–infected Chinese hamster ovary (CHO) cells were added to the cells together with 25 ng/mL u-PA (Ukidan, Serono Laboratories). After 2 hours of incubation, media were replaced by 110 nmol/L plasminogen (Roche) and 0.27 mmol/L S2251 chromogenic substrate (Chromogenix) in 0.05 nmol/L Tris-HCl buffer (pH 7.4) supplemented with 0.03% human serum albumin. Culture plates were placed at 37°C, and absorbance was monitored at 405 nm.

#### Migration and Proliferation Assays

For migration assays, HSVSMCs were seeded onto 12-well plates. Adenoviral infection was postponed until 2 days after confluency when MMP expression is maximal.44 Cells were infected 2 hours with 106 and 107 pfu/mL. After 24 hours, cultures were mechanically wounded by detaching cells with Millipore MF filters (0.2-μm filters). Cultures were incubated with DMEM/1% FCS, supplemented with 200 kIU/mL aprotinin (Bayer), 10 μg/mL BB94 (British Biotech), or no additives. After 4 days, cells were fixed and stained with Crystal violet. Migration was determined by image analysis (QWin, Leica Imaging Systems).

Cell proliferation was analyzed by [3H]thymidine incorporation as described previously.46

#### Saphenous Vein Organ Culture and Adenoviral Infection

Segments of saphenous vein were kindly provided by Prof R. Dion (Department of Thoracic Surgery, Leiden University Medical Center, Leiden, the Netherlands) according to guidelines of the local ethics committee. Veins from 9 individual patients were divided into four segments and infected with Ad.P10, Ad.TIMP-1, Ad.TIMP-1.ATF (2×109 pfu/mL, 1 hour, 37°C) or mock-infected and cultured for 4 weeks as previously described.43,46

For RT-PCR, infected veins were cultured for 4 days, after which RNA was extracted. First-strand cDNA synthesis was performed using Oligo(dT) and Superscript reverse transcriptase (RT, Life Technologies). Primers were designed to amplify recombinant TIMP-1 and TIMP-1.ATF and not endogenous TIMP-1. The expected sizes of the amplification products were 763 bp (Ad.TIMP-1) and 1213 bp (Ad.TIMP-1.ATF). Negative controls were Ad.P10-infected or an incubation without RT or template. RNA quality was checked using human β-actin cDNA-specific primers.

After a 4-week culture period, veins were fixed, paraffin-embedded, and cross-sectioned. From each of the 9 segments per group, 6 sections were used for quantification of neointimal areas by image analysis on multiple Weigert’s elastin and Alcian Blue–stained sections using Leica QWin and compared with untreated counterparts.
Statistical Analysis
Statistical analysis was performed using one-way analysis of variance (ANOVA) and Fisher’s least significant difference test. For neointimal areas, the paired samples t test was applied. Statistical significance was accepted at P<0.05.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
Recombinant mRNA Production
HSVSMCs infected with Ad.ATF, Ad.TIMP-1, Ad.ATF.TIMP-1, Ad.TIMP-1.ATF, and control vector Ad.P10 were subjected to Northern blot analysis (Figure 1). Hybridization with a TIMP-1 probe detected the presence of endogenous TIMP-1 (0.9 kb) in all RNA samples; larger recombinant TIMP-1 mRNA (1.1 kb) in Ad.TIMP-1–infected cells; and a 1.7-kb band, the expected size for TIMP-1.ATF and ATF.TIMP-1 mRNA. The 1.7-kb bands also hybridized with the ATF probe confirming the presence of both domains of the hybrid protein. ATF mRNA (1.1 kb) was present in Ad.ATF–infected cells. A second signal of 1.4 kb was detected in these cells, a size compatible with a read-through to the pIX polyadenylation signal located downstream of the ATF expression cassette. This phenomenon was also described by Li et al47 for adenoviral expression of murine ATF.

Recombinant Protein Functionality
To assess the functionality of recombinant TIMP-1, ATF.TIMP-1, and TIMP-1.ATF, an MMP-9 immunocapture activity assay was performed. As shown in Figure 2A, TIMP-1– and TIMP-1.ATF–conditioned medium inhibited MMP-9 activity by 70% and 78%, respectively, compared with Ad.P10 medium. On the contrary, ATF.TIMP-1 showed no inhibitory effect. This indicates that the tertiary structure of ATF.TIMP-1 does not form a correct MMP-inhibiting moiety. This is not unexpected, because the first amino acid residues of TIMP-1 are part of the active site of the molecule.48 For this reason, Ad.ATF.TIMP-1 was not further tested.

Cell surface binding of TIMP-1.ATF was demonstrated by FACS analysis of HSVSMCs after incubation with conditioned medium from Ad.TIMP-1.ATF–infected CHO cells (Figure 3). The majority of cells (>90%) exhibited cell
surface labeling with either the TIMP-1 or ATF antibody. In addition, when cells were immunolabeled with both antibodies, >81.3% exhibited positive immunoreactivity. Treating the cells at pH 3.0 before immunolabeling, a known treatment that could disrupt the pH-sensitive binding of ATF to u-PAR, decreased the percentage of labeled cells to 29%.

To certify that the MMP inhibitory activity of TIMP-1.ATF was retained upon binding to u-PAR, conditioned medium from CHO cells infected with the various adenoviral constructs was incubated in u-PAR–coated wells. TIMP-1.ATF–conditioned medium inhibited MMP-13 activity by 47% compared with its P10 counterpart (Figure 2B), whereas TIMP-1– and ATF-conditioned medium had no inhibitory effect.

Apart from its effects on MMP activity, TIMP-1.ATF is also expected to inhibit plasminogen activation by competing for the u-PA receptor. Indeed, this could be demonstrated in a plasmin activity assay on HSVSMCs. In the presence of 25 ng/mL u-PA, conditioned medium from Ad.TIMP-1.ATF–infected CHO cells inhibited plasmin activity by 55% compared with its P10 counterpart (Figure 2B), whereas TIMP-1– and ATF-conditioned medium had no significant effect.

Taken together, these data demonstrate that both domains of the hybrid protein TIMP-1.ATF are functional, thereby achieving u-PAR–bound MMP inhibition as well as inhibition of plasminogen activation.

Effect of Ad.TIMP-1.ATF on SMC Migration In Vitro

SMC migration requires degradation of the ECM by cellular proteases, in particular MMPs and plasmin. Indeed, addition of the MMP inhibitor BB-94 and the plasmin inhibitor aprotinin significantly inhibited SMC migration by 64.1% and 64.0%, respectively, compared with control SMCs (Figure 4).

Infection of SMCs with the control adenovirus Ad.P10 slightly increased cell migration at 10⁶ pfu/mL and decreased cell migration by 28.3% at 10⁷ pfu/mL (Figure 4). Compared with Ad.P10 infection, Ad.TIMP-1 and Ad.ATF reduced migration by 26–32% and 33–52%, respectively, depending on the adenovirus concentration. Infection of SMCs with Ad.TIMP-1.ATF inhibited SMC migration most potently by 64–65%. These results indicate that adenoviral delivery of the natural MMP inhibitor TIMP-1, as well as the receptor-binding domain of u-PA–ATF, can inhibit cell migration. Localization of TIMP-1 to the cell surface u-PAR (TIMP-1.ATF) can strongly enhance this inhibitory effect.

Effect of Ad.TIMP-1.ATF on SMC Proliferation In Vitro

[³H]Thymidine incorporation, after serum stimulation of adenovirally transfected HSVSMCs, was performed to determine whether inhibitory effects of the various adenoviral constructs on SMC mitogenesis occurred. Ad.ATF and Ad.TIMP-1.ATF had no significant effect on cell proliferation compared with uninfected SMCs (Figure 5). Infection with control adenovirus Ad.P10 slightly increased cell proliferation at 5 × 10⁶ and 5 × 10⁷ pfu/mL compared with uninfected cells.

Effects of Ad.TIMP-1.ATF on Neointima Formation in Cultured Human Saphenous Veins

To demonstrate the efficacy of TIMP-1.ATF as an inhibitor of neointima formation in blood vessels, segments of human saphenous vein were infected with 2 × 10⁹ pfu/mL Ad.TIMP-1, Ad.TIMP-1.ATF, or control vector Ad.P10. This adenovirus concentration showed maximal effective trans-
TIMP-1.ATF and recombinant TIMP-1 mRNA were detected only in the TIMP-1.ATF– and TIMP-1–infected vein segments (Figure 6). Both vein segments and the Ad.P10-infected control segment gave comparable signals for /H9252-actin.

After a 4-week culture period, uninfected control segments developed a multilayered, cell-rich neointima, which is clearly distinguished by Weigert’s elastin and Alcian Blue staining (Figure 7). Whereas infection with Ad.P10 did not affect the development of this neointima, Ad.TIMP-1– and Ad.TIMP-1.ATF–infected vein segments clearly developed smaller neointimal structures. Quantitative analysis of multiple cross sections (>6) of uninfected control and Ad.P10-infected vein segments revealed an average intimal area of 0.42±0.06 mm² and 0.39±0.06 mm² per microscopic field, respectively, corresponding to a reduction in neointimal area of ~7% as a result of adenovirus infection per se. Ad.TIMP-1– and Ad.TIMP-1.ATF–infected veins developed 0.24±0.04 mm² and 0.12±0.03 mm², respectively. This corresponds to a reduction in neointima development of 38–42% by TIMP-1 overexpression and 69–72% by expression of the hybrid protein TIMP-1.ATF compared with uninfected and Ad.P10-infected controls (Figure 8). In parallel experiments (not shown), Ad.ATF inhibited neointima formation by 34±16% compared with uninfected controls.34

Discussion

In the present study, we report on the effects of adenoviral delivery of a novel cell surface–directed hybrid protein TIMP-1.ATF on SMC migration and neointima formation. Infection of HSVSMCs with Ad.TIMP-1.ATF inhibited migration of these cells in wounded cultures more efficiently than Ad.TIMP-1 or Ad.ATF individually. Moreover, Ad.TIMP-1.ATF was found to be a more potent inhibitor of neointima formation in cultured saphenous veins than Ad.TIMP-1.

Many studies have demonstrated increased MMP activity after vascular injury.7,11,12,49,50 In an attempt to counteract

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**Figure 4.** Inhibition of SMC migration by Ad.TIMP-1.ATF. The migration of HSVSMCs infected with 10⁶ and 10⁷ pfu/mL Ad.ATF, Ad.TIMP-1.ATF, Ad.TIMP-1, and Ad.P10 as a control, was determined by quantification of the number of cells that migrated into the wounded area of SMC cultures. As additional controls, 200 kIU/mL aprotinin and 10 μg/mL BB94 were used. The results are presented as percentage coverage of uninfected controls±SEM. *P<0.05 compared with control (no virus); #P<0.05 compared with the corresponding Ad.P10 virus concentration.

**Figure 5.** Effects of the various adenoviral vectors on SMC proliferation. The proliferation of HSVSMCs infected with Ad.ATF, Ad.TIMP-1.ATF, Ad.TIMP-1, and Ad.P10 as a control vector was determined by measuring [³H]thymidine incorporation. As an additional control, the proliferation of noninfected SMCs was measured (no adenovirus). Results are expressed as counts per minute (cpm)±SEM. *P<0.05 compared with uninfected control SMCs; n=3.

**Figure 6.** Expression of TIMP-1.ATF and recombinant TIMP-1 in saphenous vein segments. Saphenous vein segments infected with 2×10⁹ pfu/mL Ad.TIMP-1.ATF and Ad.TIMP-1 were analyzed for recombinant gene expression by RT-PCR 4 days after infection. As negative controls, an Ad.P10-infected segment of the same vessel was used, as well as incubation without reverse transcriptase (−RT) and incubation without template (−control). Human β-actin–specific primers were used as a control for RNA quality. The expected sizes of the TIMP-1.ATF, TIMP-1, and β-actin transcripts are 1213, 763, and 652 bp, respectively.
these increases in MMP activity, efforts have been directed at inhibiting MMP activity. In cultured human saphenous veins and balloon-injured rat arteries, a reduction in neointima formation was observed when synthetic MMP inhibitors marimastat and doxycycline were used. Adenoviral overexpression of TIMP-1, -2, and -3 was also shown to reduce neointima formation in cultured saphenous veins at the end of a 14-day culture period. Overexpression of TIMPs in balloon-injured rat carotid arteries has produced varying results. Whereas TIMP-1 reduced neointimal area by 30%, TIMP-2 only delayed neointima formation. Seeding of TIMP-1-overexpressing SMCs at the time of injury reduced neointimal areas by 25%. Although TIMP overexpression was demonstrated to effectively reduce vascular SMC migration, the inhibitory effects on neointima formation after prolonged time periods have been discouraging.

In the present study, the effect of MMP inhibition was analyzed in a well-characterized long-term organ culture model of human saphenous veins. In this model, where vein segments are cultured for 4 weeks, a multilayered cell-rich neointima develops that resembles the early neointimal lesions formed in vivo after bypass surgery. The novel hybrid protein TIMP-1.ATF inhibits MMP activity equally well as TIMP-1, with the difference that it binds to the u-PA receptor. Infection of HSVSMCs with Ad.TIMP-1.ATF inhibited migration more efficiently than Ad.TIMP-1 or Ad.ATF individually or even high concentrations of either the MMP inhibitor BB94 or the plasmin inhibitor aprotinin (Figure 4).

In 4-week organ cultures of saphenous vein, 38% inhibition of neointima formation by TIMP-1 gene transfer was observed. The novel hybrid protein TIMP-1.ATF inhibited neointima formation more efficiently by reducing neointimal area by 69%. This enhanced effect of TIMP-1.ATF may be explained by the fact that the construct combines features of both the ATF domain of u-PA and the MMP inhibitor TIMP-1.

Various in vitro studies, including ours, have shown that inhibition of MMP activity can be reduced not only by inhibition of MMP activity but also by inhibition of the PA system, eg, by interfering with the binding of u-PA to its receptor. TIMP-1.ATF targets both of these systems by localizing MMP inhibition to the cell surface u-PA receptor. This allows inactivation of MMPs directly at the cell surface where proteolytic enzymes are actively degrading ECM. Second, the construct prevents u-PA from binding to its receptor by competition. Because ATF and urokinase bind with similar affinities, it is likely that TIMP-1.ATF also binds with similar affinity. Therefore, binding of TIMP-1.ATF to the u-PAR blocks u-PA binding and thus affects activation of plasminogen by u-PA and subsequent pro-MMP activation on the cell surface. This dual effect of the hybrid protein makes it a more potent inhibitor of cell migration neointima formation than ATF or TIMP-1 individually, as was confirmed in the migration assays and saphenous vein organ cultures.
In summary, our study demonstrates that the hybrid protein TIMP-1-ATF significantly reduces SMC migration and neo-intima formation in a human model for vein graft stenosis. TIMP-1-ATF targets both the plasminogen activation system and MMPs, suggesting that these regulators play a significant role in cell migration and the pathogenesis of intimal thickening. Moreover, these results suggest that adenoviral delivery of TIMP-1-ATF may prove efficacious for the treatment of intimal hyperplasia associated with uncontrolled cell migration and neointima development.

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References


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