Expression of Tissue Inhibitor of Matrix Metalloproteinases 1 by Use of an Adenoviral Vector Inhibits Smooth Muscle Cell Migration and Reduces Neointimal Hyperplasia in the Rat Model of Vascular Balloon Injury

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Background—Cell migration is a major contributor to injury-induced neointimal hyperplasia and depends on alteration of the proteolytic balance within the arterial wall toward matrix breakdown. This is partly mediated by the matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

Methods and Results—An increase in expression of biologically active and immunoreactive TIMP-1 was seen in vitro after infection of rat smooth muscle cells (SMCs) with Av1.TIMP1 (an adenoviral vector containing the human TIMP1 cDNA). Infection of rat SMCs with Av1.TIMP1 reduced migration in vitro by 27% compared with control virus–infected cells (37.6 ± 4.34 versus 51 ± 5.01 cells per high-power field, P = 0.05). The adenoviral vector was delivered to the injured rat carotid artery, and 4 days later, immunoreactive protein was identified and migration of SMCs reduced by 60% (5.2 ± 0.5 versus 12.8 ± 1.5 cells per section, P < 0.05, n = 5). Neointimal area 14 days after injury showed a 30% reduction in the animals receiving the Av1.TIMP1 virus compared with controls (0.09 ± 0.01 versus 0.14 ± 0.01 mm², P = 0.02, n = 14).

Conclusions—The response to arterial balloon injury involves MMP-dependent SMC migration and can be attenuated in vivo by the transmural expression of TIMP-1 by adenoviral gene transfer. (Circulation. 1999;99:3199-3205.)

Key Words: restenosis ■ genes ■ muscle, smooth ■ cells

Extracellular matrix stability is important in maintaining the integrity of the vessel wall, and recent studies have focused on its role in the postprocedural vasculopathies, such as angioplasty restenosis.1–4 The restenotic process after vascular injury is a result of normal but unwanted healing causing lumen loss and return of symptoms in 25% of patients.5

After arterial injury, vascular smooth muscle cells (VSMCs) proliferate and migrate, forming a neointima that later accumulates extracellular matrix.6 VSMCs secrete proteases, which facilitate migration by plasminogen-dependent and -independent pathways.7 Expression of both plasminogen activators and inhibitors after rat arterial balloon injury correlates with migration of VSMCs.7 Vascular injury has been shown to cause early upregulation of both gelatinase A and B.1,3,4 but the tissue inhibitors of the matrix metalloproteinases (MMPs), the TIMPs, appear to be upregulated 48 to 72 hours after the peak of the MMPs.1,3,4 It is ultimately the balance between the MMPs and their inhibitors that determines the focal proteolysis around the SMCs of the arterial media. Thus, in the first 1 to 4 days after balloon injury, a rapid alteration of the proteolytic balance toward matrix breakdown occurs concurrently with proliferation and migration of SMCs. This process is established by 7 days after injury, when TIMP2 and plasminogen activator inhibitor-1 are upregulated.5,8 The later phase of the arterial response to injury is characterized by the cessation of SMC migration and commencement of matrix accumulation.6

It was our hypothesis in this study that adenoviral gene transfer of the cDNA encoding TIMP immediately after injury would result in increased levels of TIMP1 and its earlier expression. This rapid inhibition of matrix breakdown might then reduce the early phases of SMC migration and attenuate neointima formation. An adenoviral vector was used because it allows efficient direct in vivo gene transfer to quiescent native SMCs. Accordingly, the purpose of this study was to investigate the effects of increased TIMP1 expression on proliferation and migration of SMCs both in vitro and in vivo within the arterial wall.

Methods

Generation of Adenovirus Vectors

Two adenoviral vectors were used, Av1.LacZ4 and Av1.TIMP1. Av1.LacZ4 is a replication-deficient first-generation adenoviral vec-
tor encoding β-galactosidase. The cDNA encoding human TIMP1 was the kind gift of Prof Gillian Murphy (University of East Anglia). The TIMP1 cDNA was excised from pBluescript TIMP1 with BamHI and HindIII, and the restriction sites were filled with Klenow fragment. The blunt-ended fragment was cloned into the EcoRV-digested pAVS6A vector. Correct insertion and orientation were confirmed by restriction enzyme analysis and sequence analysis. Recombinant viruses were generated by calcium phosphate cotransfection of pAVS6A TIMP1 with the Ccl4 fragment of d 327 adenovirus type 5 on 293 cells. Recombinant adenovirus clones were screened for the TIMP1 cDNA by Southern analysis and plaque-purified. Viral stocks were generated in 293 cells and concentrated by cesium chloride ultracentrifugation. The resultant bands were dialyzed against 10 mmol/L Tris (pH 7.4), 1 mmol/L MgCl₂, and 10% glycerol. Plaque titration on 293 cells showed titers of 1×10⁸ and 1×10¹⁰ pfu/mL for Av1.LacZ4 and Av1.TIMP1, respectively.

Cell Culture and Adenovirus Infection
Primary VSMCs were isolated from male Wistar rats and used at passage 4 to 6. Cells were incubated at 37°C in a humidified 5% CO₂/95% O₂ incubator in RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, 100 U/mL streptomycin, and 1% transferrin. Immunohistochemistry using an anti-α-smooth muscle actin antibody (Sigma) confirmed staining in 98% of cells. Eighty percent confluent monolayers were transferred to serum-free RPMI 24 hours before infection. Cells were incubated with Av1.TIMP1 or Av1.LacZ4 for 1 hour. The infection media were then replaced with serum-free RPMI. Conditioned media harvested 48 hours later were concentrated by use of Centricon-3 (Amicon Inc) filters and assayed for protein content.

Western Blot Analysis and Gelatin and Reverse Zymography
For Western analysis, protein was loaded and run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (Amer sham). Mouse anti-human TIMP-1 monoclonal antibody (the kind gift of Dr A. Docherty, Celltech Ltd) was added at 1:1000 for 2 hours and probed with 1:1000 horseradish peroxidase-conjugated rabbit anti-mouse monoclonal antibody (Sigma). Enzyme-linked chemiluminescence detection was used to visualize the signal (Amer sham). MMP and TIMP activities were assayed by gelatin and reverse zymography, respectively, as previously described. Briefly, samples in nonreducing buffer were run on a 10% gel containing 1 mg/mL of gelatin (Sigma) for zymography plus 0.1 mL of hamster gelatinase A solution/mg gelatin for reverse zymography (UTI Inc). Gels were washed in 2.5% Triton X-100 and incubated for 16 hours at 37°C before staining with Coomassie blue.

Invasion Assay and Proliferation of SMCs In Vitro
Biocoat Matrigel invasion chambers were used according to the manufacturer’s instructions for invasion studies (Becton Dickinson). Briefly, SMCs were infected as described above and 24 hours later which the sections came.

Statistical Analysis
All values are expressed as mean±SEM. Student’s t test was used to examine the difference between experimental groups.

Results
Expression and Activity of TIMP-1 In Vitro
Expression of the transgene human TIMP1 in rat VSMCs infected with Av1.TIMP1 was demonstrated by Western analysis of conditioned media and compared with media from uninfected cells or cells infected with a control virus (Av1.LacZ4) (Figure 1). Immunoreactive TIMP1 increased with the multiplicity of infection (MOI) of the virus. Human TIMP1 was seen to inhibit rat gelatinases (data not shown). The biological activity of the TIMP1 was demonstrated by quantifying [3H]thymidine incorporation and normalized to the total protein content per well to correct for unequal seeding density.
reverse zymography (Figure 2). In addition to a major band of activity at 28 kDa in Av1.TIMP1-infected cells, the gels also showed smaller bands at the same molecular weight in controls. These bands are likely to represent constitutively secreted rat TIMP-1, which was not detected by Western analysis for human TIMP1. At higher MOIs of Av1.TIMP1, a lower-molecular-weight band was seen by both Western analysis and reverse zymography; this is likely to be partially glycosylated human TIMP1.

TIMP1 Does Not Affect DNA Synthesis in VSMCs but Inhibits Migration Across a Basement Membrane Barrier

No significant change in DNA synthesis was seen in Av1.TIMP1-infected cells compared with uninfected or Av1.LacZ4-infected cells (all, P > NS) (Figure 3). In contrast, cells stimulated with bFGF and infected with higher MOIs of Av1.TIMP1 (5, 50, and 100) showed significantly greater proliferation than cells infected with 100 MOI of Av1.LacZ4 (all, P < 0.05). Comparison of Av1.TIMP1-infected cells stimulated with FGF with the more appropriate control of uninfected FGF-stimulated cells showed no significant difference. The Av1.LacZ4-infected cells showed reduced DNA synthesis compared with control cells in both quiescent and stimulated conditions, as previously reported. The invasion assay revealed a 27% reduction in migration through a Matrigel barrier in cells infected with Av1.TIMP1 at an MOI of 100 compared with Av1.LacZ4 (Figure 4) (P < 0.05, n = 4).

Presence of Human TIMP1 Protein After In Vivo Gene Transfer

Successful transgene expression in vivo was demonstrated by widespread transmural human TIMP1 immunostaining,
which was greatest in the media (Figure 5). No immunoreactivity was seen in arteries infected with Av1.LacZ4. Western analysis of arterial extracts for human TIMP1 confirmed a 28-kDa band in Av1.TIMP1-infected arteries that was absent in Av1.LacZ4-infected arteries (Figure 5).

**Av1.TIMP1 Infection Inhibits Neointimal Hyperplasia In Vivo**

The effect of TIMP1 expression on neointimal hyperplasia was quantified by histomorphometric analysis of arterial cross sections from 28 animals 14 days after vascular injury. A 32% reduction in neointimal area in the Av1.TIMP1-infected arteries was seen compared with Av1.LacZ4-infected arteries (0.14 ± 0.01 and 0.09 ± 0.01 mm² in the Av1.LacZ4 and Av1.TIMP1 groups, respectively, P < 0.02) (Figure 6). Similarly, the ratio of neointima to media was 27% less in Av1.TIMP1 arteries compared with Av1.LacZ4 arteries (P < 0.04) (Figure 6). To assess changes in overall vessel wall size, the area within the external elastic lamina (EEL) was calculated and showed a mean area of 0.448 ± 0.02 cm² in the Av1.LacZ4 group and 0.403 ± 0.01 cm² in the Av1.TIMP1 group (P = NS).

**Role of DNA Synthesis and Cell Number, SMC Migration, and Matrix Accumulation in Reducing Neointimal Hyperplasia**

The relative contributions of cellular migration and proliferation and matrix accumulation in the observed reduction in neointimal hyperplasia were assessed. Ki 67 immunoreactivity 2 days after balloon injury showed no significant difference in the Av1.TIMP1-infected arteries compared with the Av1.LacZ4 at either time point (Ki 67 labeling index: day 2, 19.5 ± 5.3 versus 19.2 ± 3.3, P = NS; day 14, 0.35 ± 0.16 versus 0.22 ± 0.10, P = NS). The total cell number within the EEL 4 days after injury in the Av1.TIMP1-infected arteries was 40% less than in the Av1.LacZ4-treated arteries, suggesting significant cell loss (260 ± 36 compared with 434 ± 56, P = 0.048, n = 5). Previous studies in our laboratory show that medial cell number in uninjured artery of animals 72 to 96 hours after contralateral arterial injury does not differ from Av1.LacZ4-treated arteries nor from sham-operated, saline-irrigated vessels (data not shown).

In vivo migration was assessed by counting all cells within the internal elastic lamina in 20 sections taken at 100-μm intervals in each artery. A highly significant difference of 5.2 ± 0.5 versus 12.8 ± 1.5 cells per section was seen between the Av1.TIMP1 and Av1.LacZ4 arteries (P = 5 × 10⁻⁶) (Figure 7).

Collagen content was assessed in 14-day lesions by densitometric analysis of picrosirius red–stained sections. The integral density of picrosirius red staining in the neointima and media of Av1.TIMP1-infected arteries was significantly lower than in Av1.LacZ4 arteries (2483 ± 171 versus 3161 ± 187, P < 0.01). The apparent decrease in total collagen staining in the Av1.TIMP1 arteries is likely simply to reflect the smaller neointimal area of the arteries. When the picrosirius red staining was expressed per cell, the results were not significantly different (Av1.TIMP1, 2.14 ± 0.13 per cell versus Av1.LacZ4, 2.12 ± 0.17 per cell).

**Discussion**

The results presented show that in vivo arterial gene transfer of TIMP1 attenuates neointimal hyperplasia after vascular injury, with a marked reduction in SMC migration but without altering proliferation. It is our hypothesis that the proteolytic state induced by vascular injury was prevented by increased levels of TIMP1 protein demonstrated by Western analysis and immunohistochemistry. In vitro experiments showed that secretion of biologically active TIMP1 after Av1.TIMP1 infection inhibits VSMC migration, and this was confirmed by a 60% reduction in migration of SMCs in vivo.
Both in vivo and in vitro analysis of DNA synthesis and proliferation showed no reduction in proliferation in response to TIMP1, and in stimulated cells in vitro, TIMP1 was mildly promitogenic. This study confirms that in vivo gene transfer of TIMP1 alters neointimal formation and presents the first in vivo evidence that attenuation of the response to vascular injury by direct gene transfer of TIMP1 alters SMC migration.

Our in vitro studies largely parallel the findings of Forough et al., who used retroviral vectors to express TIMP1 in SMCs that were transferred to the arterial wall. This technique prevents examination of migration of SMCs, because the arterial lumen is colonized by transferred cells. In contrast, the efficiency of adenoviral vectors allows direct in vivo gene transfer and yields high levels of episomal transgene expression, which is well suited to temporary genetic modification of postprocedural vasculopathy.

Our results show a 27% reduction in in vitro invasion of SMCs through a basement membrane barrier when infected with Av1.TIMP1 compared with control or Av1.LacZ4 cells. This is consistent with findings in retrovirally transduced cells. Inhibition of invasion by TIMPs has been demonstrated in cancer and angiogenesis. Migration of SMCs in vivo is difficult to quantify, but 2 methods have been described. Both techniques rely on the observation that SMCs first appear in the neointima 3 to 4 days after injury and that 96% of these cells result from migration and not proliferation. We counted intimal cells in 20 transverse sections taken at 100-μm intervals along the length of infected arteries and observed a 60% reduction in Av1.TIMP1-infected arteries compared with those infected with Av1.LacZ4.

We saw no evidence of an alteration in DNA synthesis in quiescent cells after Av1.TIMP1 infection, but cells stimulated with bFGF showed a small increase compared with Av1.LacZ4-infected cells but not with uninfected cells. The control vector, Av1.LacZ4, reflects the effects of adenoviral infection, which are unrelated to TIMP1 gene expression. TIMP1 is known to be a mitogen in some cell types. In our experiments, however, no increase in proliferation was observed compared with uninfected cells. Interestingly, retroviral gene transfer of TIMP1 in vitro is associated with a reduction in cell number compared with cells infected with a control retrovirus. Cell number, however, may be influenced by both the rate of DNA synthesis and loss of cells by necrosis or apoptosis.

The Ki-67 antibody reacts with cells at all stages of the cell cycle but not with cells in the G0 phase. With this antibody, no difference was detected between the Av1.LacZ4 and Av1.TIMP1 groups. This is not unexpected, because <10% of cells in the arterial wall are infected by this method of gene transfer. This result reflects predominantly a comparison between cells exposed to TIMP1 protein and uninfected cells: these exhibit similar proliferation in vitro. There was, however, a decrease in cell number in the media and neointima of the Av1.TIMP1-infected arteries 4 days after vascular injury. Neither in vitro nor in vivo results suggest that this is due to altered SMC proliferation. Av1.TIMP1-infected arteries, however, show a lower cell number than control groups. Adenoviral gene transfer can cause loss of medial cells at high titer but would have been detected in the Av1.LacZ4 group and would have increased neointima formation. There is a significant cell loss in the arteries from the Av1.TIMP1-treated group. This may be due to necrosis or apoptosis. It is known that extracellular matrix contact can mediate cell survival and that an imbalance in the matrix

**Figure 6.** Reduction in neointima in response to Av1.TIMP1 infection. A, Neointimal area in Av1.TIMP1- and Av1.LacZ4-infected arteries 14 days after injury and gene delivery. Data shown are mean ± SEM (n = 14). B, Intimal/medial ratio in Av1.TIMP1- and Av1.LacZ4-infected arteries. C, H-E–stained sections of Av1.TIMP1- and Av1.LacZ4-infected arteries (day 14).

**Figure 7.** In vivo SMC migration. Migration of SMCs was assessed in Av1.TIMP1- and Av1.LacZ4-infected arteries 4 days after injury by counting cells within internal elastic lamina in 20 sections from each artery taken at 100-μm intervals. Five arteries were studied in each group; mean and SEM are shown.
composition of the arterial wall might mediate the cell loss observed. TIMP3 has recently been shown to cause apoptosis in VSMCs, but TIMP1 does not appear to share this property in vitro.

The inhibition of the MMP enzymes is likely to result in early-onset matrix accumulation after vascular injury. However, no constrictive remodeling could be identified when the areas subtended by the EEL in Av1.TIMP1 and Av1.LacZ4 arteries were compared. We used a modification of the method of Coats et al to investigate the collagen content of vessels 14 days after gene transfer. Integral density of the media and neointima of picrosirius red–stained sections was measured, reflecting the collagen content. Unexpectedly, this showed more collagen in the Av1.LacZ4-treated group than in the Av1.TIMP1 group. However, when these results were normalized to the number of cells in the artery, the results showed no difference, suggesting the same cell-to-matrix ratio in both groups. These results reflect those found when a nonspecific pharmacological MMP inhibitor (GM6001) was used in the rabbit injury model, in which collagen content was reduced by MMP inhibition. In the rat, GM6001 reduced in vivo SMC migration by 97% 4 days after injury, but the final lesion was unchanged at 14 days. This has been explained by a phase of “catch-up” growth between 10 and 14 days after injury. One interpretation of these studies was that inhibiting SMC migration was not sufficient to inhibit lesion growth.

Our results show that in vivo transfer of the TIMP1 gene inhibition of migration of SMCs and reduces neointimal hyperplasia 14 days after injury. This supports previous analysis of the rat model suggesting that 50% of the neointimal cells are derived through proliferation and 50% through migration and may explain the incomplete inhibition of neointimal hyperplasia by inhibition of either proliferation or migration alone. The difference between pharmacological inhibitors and these studies of gene transfer may be due to the specificity or level of inhibition of the MMPs. In contrast to the studies discussed above, 1 report using a broad-spectrum MMP inhibitor, batimastat, in the rat carotid model showed reduced neointimal formation. This may be because batimastat is a less specific MMP inhibitor and may inhibit other metalloproteinases, such as the disintegrin and metalloproteinase (ADAMs) group, whose role as a TNF–metalloproteinases, such as the disintegrin and metalloproteinases.

In summary, this study shows that direct in vivo gene transfer of TIMP1 using an adeno viral vector inhibits neointimal development by 30% and that a similar reduction in SMC migration both in vivo and in vitro may be the underlying mechanism. We have also shown a reduction in cell number in the arterial wall after TIMP1 gene transfer in vivo. In this study, the use of TIMP gene transfer provides evidence of the role of MMPs in vascular SMC migration and the response to vascular injury. To date, the majority of gene transfer studies of the response to arterial balloon injury have focused on reducing proliferation of SMCs, but our results suggest that migration of these cells has an equally important role. Ultimately, the unwanted aspects of the response to vascular injury may be best targeted by inhibition of both migration and proliferation of SMCs. The MMPs and their inhibitors are a major regulatory component in these responses.

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