ARTICLE

doi:10.1016/j.ymthe.2004.02.020

Potent Inhibition of Arterial Intimal Hyperplasia by *TIMP1* Gene Transfer Using AAV Vectors

Genaro A. Ramirez Correa,[1] Serena Zacchigna,[1] Nikola Arsic,[1] Lorena Zentilin,[1] Alessandro Salvi,[2] Gianfranco Sinagra,[2] and Mauro Giacca[1,3,*]

[1] *Molecular Medicine Laboratory, International Center for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy*
[2] *Cardiology Unit, Ospedale Maggiore, Trieste, Italy*
[3] *Scuola Normale Superiore and Istituto di Fisiologia Clinica, CNR, Pisa, Italy*

**To whom correspondence and reprint requests should be addressed. Fax: +39-040-226555. E-mail: giacca@icgeb.org.*

Available online 15 April 2004

Seminal to the process of arterial restenosis after balloon angioplasty is extracellular matrix degradation by metalloproteinases (MMPs); activity of these proteins is strongly inhibited by the tissue inhibitors of MMPs (TIMPs). Here we exploit gene transfer using an adeno-associated virus (AAV) for *TIMP1* gene delivery in a rat model of intimal hyperplasia. High-titer AAV-Timp1 efficiently transduced human coronary artery smooth muscle cells (SMCs) *in vitro* and inhibited the capacity of these cells to migrate through a Matrigel barrier. In injured rat carotid arteries, AAV vectors were found to transduce SMCs efficiently and to maintain transgene expression for several weeks *in vivo*. In AAV-Timp1-transduced animals, the intima:media ratio of injured carotids was significantly reduced by 70.5% after 2 weeks, by 58.5% after 1 month, and by 52.4% after 2 months from treatment. The decrease in intimal hyperplasia was paralleled by a significant inhibition of collagen accumulation and by increased elastin deposition in the neointima, two findings that relate to the inhibition of MMP activity. These results indicate that AAV vectors are efficient tools for delivering genes to the arterial wall and emphasize the importance of MMPs for the generation of intimal hyperplasia. Local *TIMP1* gene transfer might thus represent an efficient strategy to prevent restenosis.

INTRODUCTION

Restenosis of the arterial lumen after percutaneous angioplasty still represents a major challenge in interventional cardiology [1]. Luminal loss is a complex biological process determined by remodeling of both the arterial wall and the intimal hyperplasia. In particular, intimal thickening relies on vascular smooth muscle cell (SMC) migration across the internal elastic lamina and subsequent proliferation within the neointima [2]. While stent placement has significantly decreased the restenosis rate, by blocking early elastic recoil and inward remodeling [3], in several cases lumen gain improvement is later relapsed by the exacerbation of intimal hyperplasia [4].

The remodeling of the extracellular matrix by metalloproteinases (MMP) is essential for neointima formation in postangioplasty restenosis and in aortocoronary vein graft vasculopathy [1,5]. MMPs are zinc- and calcium-dependent proteases that degrade collagen as well as other matrix proteins such as elastin and proteoglycans [6,7]. In particular, MMP-2 and MMP-9 are expressed early after endothelial injury and are required for local extracellular matrix breakdown and subsequent migration of vascular SMCs from the media to the intima [7–9]. In the later phase of the arterial response, SMCs stop their migration and proliferation and start accumulating extracellular matrix [10].

Tissue inhibitors of metalloproteinases (TIMPs) belong to a family of low-molecular-weight proteins that bind to MMPs at high affinity and that stoichiometrically inhibit their enzymatic activity. The expression of *TIMP1* has been shown to modulate a number of biological events that involve extracellular matrix remodeling, including intimal hyperplasia [11–14], intravascular thrombus formation [15], and atherosclerosis [16,17]. Thus, the transfer and expression of the *TIMP1* gene in the arterial wall, concomitant with balloon angioplasty or stent implantation, emerges as an appealing possibility for the prevention of restenosis [18].

In vivo gene transfer to the arterial wall is a powerful experimental approach in the investigation of the role of individual genes in the development of arterial disease, as

1525-0016/03 $30.00

well as in the interference with local molecular pathways for therapeutic purposes. During the past few years, vectors based on the adeno-associated virus (AAV) have continued to gain popularity in the gene therapy community, due to a number of appealing features. These vectors can be purified and produced at high titers, they are not immunogenic nor do they induce an inflammatory reaction, they can be safely injected *in vivo*, and they maintain transgene expression for very long periods of time [19,20].

Here we assess the potential of AAV vectors to transduce arterial smooth muscle cells both *in vitro* and *in vivo* and demonstrate the efficacy of single endovascular delivery of an AAV vector expressing human TIMP1 to prevent intimal hyperplasia in a rat model of postangioplasty restenosis.

RESULTS

Permissivity of Human Coronary Artery Smooth Muscle Cells to AAV Transduction and the Functional Effect of AAV-Timp1

We obtained a high titer preparation of an AAV vector expressing the green fluorescent protein (*GFP*) reporter gene (AAV-GFP) and used this vector to assess the *in vitro* permissivity of vascular SMCs. Fig. 1A shows the flow cytometry profile of primary SMCs from human coronary artery at day 5 after transduction with AAV-GFP, indicating transgene expression by ~20% of the cells.

Next, we obtained an AAV vector expressing the human TIMP1 cDNA (AAV-Timp1) under the control of the CMV promoter, as schematically shown in Fig. 1B. We evaluated the efficiency of the transduction with AAV-Timp1 in transduced SMCs. By Western blot analysis, we demonstrated TIMP1 secretion into the supernatant of these cells as a 28-kDa band, showing proper glycosylation (data not shown). We also assessed TIMP1 expression in the same cells by immunocytochemistry with an antibody against TIMP1, which showed intense cytoplasmic positivity (Fig. 1C).

We assessed the effects of TIMP1 expression in SMCs by a chemoinvasion assay, measuring the capacity of the cells to degrade a Matrigel membrane and migrate in response to chemoattraction. As shown in Fig. 1D, the transduction of SMCs with AAV-Timp1 significantly inhibited their ability to invade the Matrigel barrier, inducing a 46% reduction in the number of migrated cells (23.5 ± 2.55 vs 41 ± 4.24, $P < 0.001$, $n = 3$). The same vector also proved to inhibit efficiently angiogenesis in the chorioallantoic membrane of embryonated eggs induced by rhVEGF (data not shown).

Together, these results indicate that primary human vascular SMCs are permissive to AAV transduction *in vitro* and that the AAV-Timp1 vector is highly active in preventing cell migration and invasion.

Efficiency of AAV Vector Transduction of Rat Carotid Arteries after Balloon Injury

We proceeded to assess the capacity of AAV vectors to transduce the damaged arterial wall *in vivo*. We injured rat carotid arteries with a Fogarty catheter and exposed them to either PBS or AAV-LacZ (1×10^{11} viral particles) for 40 min before restoring blood flow. We analyzed AAV-LacZ-transduced arteries for β-galactosidase (β-gal) expression by X-gal staining at 2 and 4 weeks after treatment. Marker gene expression was already high at 2 weeks after vector delivery and persisted afterward. β-Gal expression was distributed in all three layers of the vessel wall with a degree of preference for the cells of the neointima, followed by media and adventitia (shown at 2 weeks after transduction, in Fig. 2A, right). Cells that showed positive for β-gal in the neointima also reacted to an antibody against α-actin of SMCs, indicating their SMC phenotype (Fig. 2A, left).

In the injured carotid arteries, we observed a remarkable neointimal hyperplasia at 2, 4, and 8 weeks after damage. Morphometric examination of the arterial cross sections at 2 and 4 weeks revealed that the hyperplastic response was not statistically different in arteries treated with either PBS ($n = 6$) or AAV-LacZ ($n = 12$; data not shown), thus ruling out any nonspecific effect of AAV transduction per se on the hyperplastic process.

To ascertain the long-term expression of TIMP1 after AAV vector transduction *in vivo*, we exposed 12 rats to a high dose of AAV-Timp1 (1×10^{11} viral particles) immediately after balloon injury. We studied expression of TIMP1 in the treated animals by immunohistochemistry and by real-time RT-PCR quantification of human TIMP1 mRNA. Similar to AAV-LacZ transduction, cells that were positive for human *TIMP1* transgene expression were mostly found in the neointima and media layers (Fig. 2B). As shown in Fig. 2C, transgene expression was already detectable at 1 week after transduction and persisted at comparable levels for at least 8 weeks.

Inhibition of Rat Arterial MMPs by TIMP1

To visualize the production of active MMPs by injured rat carotid arteries, we performed an *in vitro* zymography. Three days after balloon injury, we harvested a rat carotid artery and kept it in culture for 3 additional days. We resolved the concentrated conditioned medium by SDS–PAGE on a 0.1% gelatin gel and incubated it at 37°C in collagenase buffer for 16 h. Fig. 3A shows the appearance of two major areas of proteolysis, corresponding to the molecular weight of active MMP9 and MMP2, indicating the active secretion of these MMPs by the injured rat carotid artery.

To assess the effects of human TIMP1 on rat MMP2 and MMP9 activity, we performed a reverse zymography using the supernatants of balloon-injured rat ca-

doi:10.1016/j.ymthe.2004.02.020

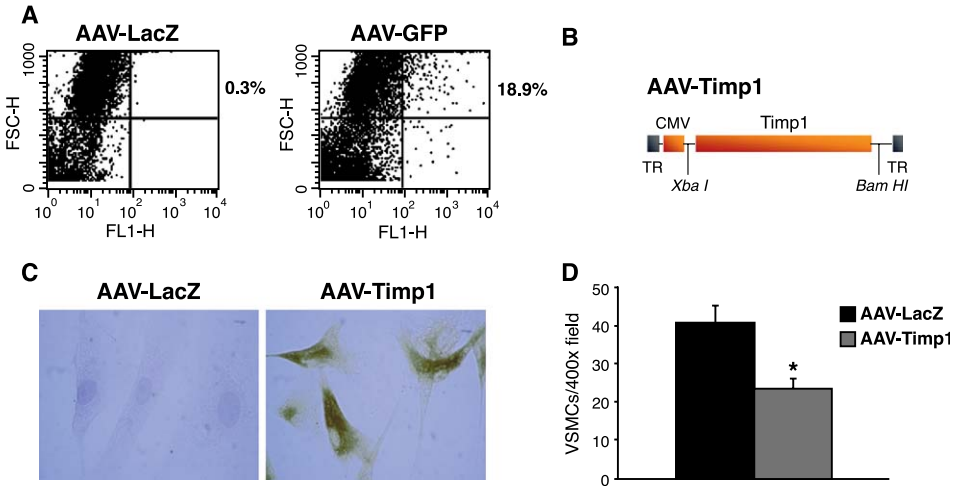

FIG. 1.

rotid arteries (obtained as above) as a source of MMPs. Serum-free media conditioned for 48 h were collected from *TIMP1*- and mock-transfected 293 cells, concentrated, resolved by 0.6% gelatin SDS–PAGE, and incubated in the presence of MMPs. A clear band at 28 kDa (Fig. 3B), absent in the control lane, indicated that the secreted human TIMP1 efficiently inhibited the gelatinolytic activity of the rat MMPs.

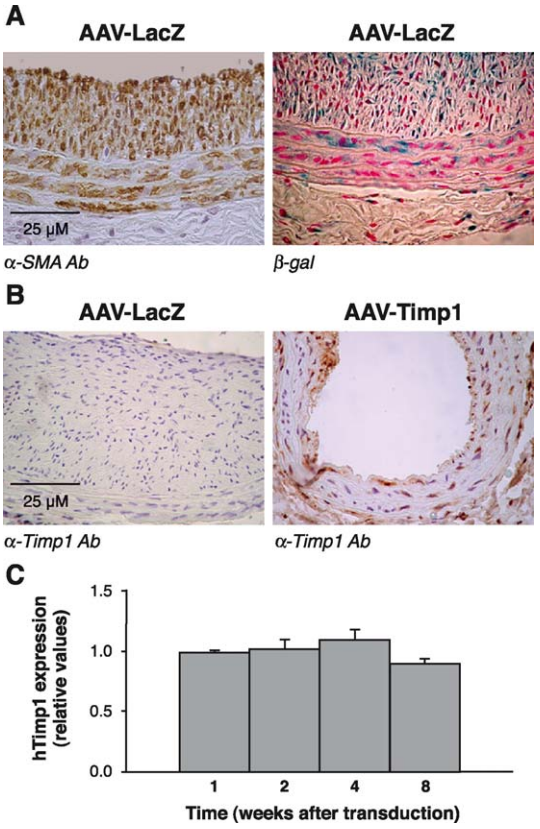

FIG. 2.

Efficacy of AAV-Timp1 in the Reduction of Intimal Hyperplasia

Next we evaluated the efficacy of TIMP1 to prevent neointima formation. Immediately after balloon injury, we exposed the rat carotid arteries of 15 rats to 1×10^{11} viral particles of AAV-Timp1 for 40 min. A matched group of controls received either AAV-LacZ or PBS. We quantitated the stenotic response after 2, 4, and 8 weeks by measuring the intima and media areas of at least four sections per animal. The thickness of the media of injured rat carotids was similar in the control and AAV-Timp1-treated groups (Fig. 4B), whereas the neointimal area of the treated group was reduced by 55.3, 51.9, and 55% at 2, 4, and 8 weeks, respectively (0.132 ± 0.01 vs 0.059 ± 0.006 mm^2, $P < 0.0001$ at 2 weeks; 0.179 ± 0.012 vs 0.086 ± 0.013 mm^2, $P < 0.0001$ at 4 weeks; 0.198 ± 0.03 vs 0.089 ± 0.04 mm^2, $P < 0.05$ at 8 weeks; Fig. 4A). The intima-to-media ratio, which is a more sensitive parameter for assessing relative changes in intima and media thickness, is reported in Fig. 4C. This ratio was significantly lower in the TIMP1 group at 2 weeks (0.46 ± 0.16 vs 1.56 ± 0.22; $P = 0.0001$), 4 weeks (0.86 ± 0.22 vs 2.08 ± 0.3; $P = 0.003$), and 8 weeks (2.0 ± 0.33 vs 0.91 ± 0.49, $P < 0.05$) after treatment. These values correspond to 70.5, 58.5, and 54.5% reduction in the intima-to-media ratio, respectively. The higher efficacy of treatment at the earlier time might be indicative of a specific effect of TIMP1 in the early phases of the stenotic process, when MMPs are overexpressed. Two representative cross sections of *LacZ*-

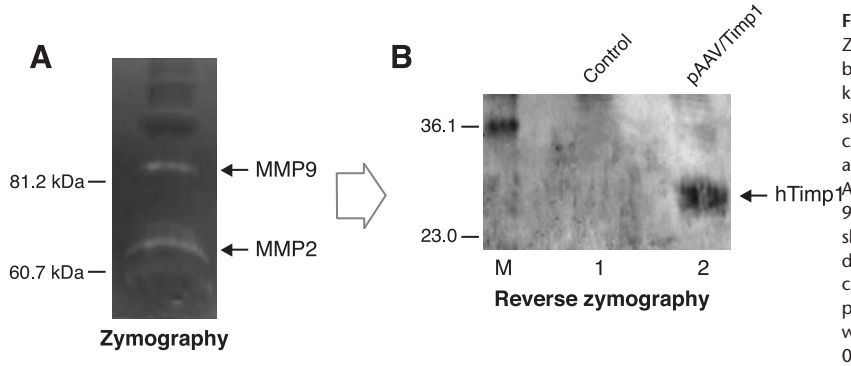

FIG. 3. Inhibition of rat MMPs by human TIMP1. (A) Zymography of a rat carotid artery. Three days after balloon injury, a rat carotid artery was harvested and kept in organ culture. After 3 days, the concentrated supernatant was loaded in a 10% SDS–PAGE gel containing 0.1% gelatin. Clear bands indicate MMP activity in a background of gelatin. MMP2, gelatinase A, 72-kDa type IV collagenase; MMP9, gelatinase B, 94-kDa type IV collagenase. (B) Reverse zymography showing human TIMP1-mediated inhibition of gelatin degradation by rat arterial MMPs. Concentrated cell culture supernatants from 293 cells transfected with pAAV-Timp1 (lane 2) or a control plasmid (lane 1) were resolved on an 11% SDS–PAGE gel containing 0.6% gelatin. The gel was then washed and incubated in collagenase buffer mixed 1:1 with the supernatants of balloon-injured rat carotid arteries—obtained as in (A)—as a source of rat gelatinases. After Coomassie staining, a dark band of ~28 kDa was evident in the supernatants of pAAV-Timp1-transfected cells, corresponding to the area in which gelatin degradation by rat arterial MMPs was prevented by secreted human TIMP1 (indicated by an arrow). M, molecular weight markers.

and *TIMP1*-transduced arteries at two weeks after transduction are shown in Fig. 4D.

Extracellular Matrix Content after AAV-Timp1 Transduction

Given the reported effects of MMP blockage by nonspecific inhibitors on collagen synthesis and degradation [10] and the effects of TIMP1 overexpression on elastin metabolism [21], we set out to explore the extracellular matrix composition in the AAV-Timp1-treated and control animals. For this purpose, we analyzed cross sections of rat carotid arteries at 2 and 4 weeks after injury by staining with the Azan–Mallory and Weigert–Van Gieson procedures, which reveal collagen and elastin content, respectively (Figs. 5A and 5C).

We found that, in AAV-LacZ-treated animals, collagen occupied 83.5% ± 1.28 of the neointimal layer at 2 weeks after transduction; collagen deposition did not change significantly over a 2-week period in this control group (73.7% ± 6.49 at day 28; *P* nonsignificant). In contrast, collagen content in the AAV-Timp1-transduced arteries was decreased by 22.9 and 42.6% at 2 and 4 weeks, respectively (83.5% ± 1.28 vs 64.8% ± 3.92; $P = 0.009$ and 73.7% ± 6.49 vs 42.2% ± 7.64; $P = 0.03$; the decrease between 2 and 4 weeks in the AAV-Timp1 group was also statistically significant; $P < 0.01$). These results demonstrate an important effect in the modulation of collagen deposition by AAV-Timp1 transduction (Fig. 5B). We found no significant differences in collagen content in the medial layers (data not shown).

Contrary to collagen, elastin fibers, as evaluated after staining with Weigert–Van Gieson, were more abundant in the neointimal areas of the AAV-Timp1-transduced arteries compared to those treated with AAV-LacZ at both 2 (16.5% ± 1.36 vs 26.9% ± 4.46 of neointima; $P = 0.01$) and 4 weeks (23% ± 4.4 vs 42.3% ± 10.59; $P = 0.04$; 63.1

FIG. 1. Transduction of vascular SMCs with AAV vectors. (A) Transduction of vascular SMC from human coronary artery with high-titer AAV-GFP [43]. Cell fluorescence was analyzed by flow cytometry, showing 18.9% positive cells at 5 days after infection (counts in the upper right). (B) Schematic representation of the AAV-Timp1 expression vector used in this study. TR, AAV terminal repeat sequences; CMV, constitutive cytomegalovirus immediate early promoter; pA, polyadenylation site. Cloning restriction sites are indicated. (C) Immunocytochemistry on transduced vascular SMCs showing expression of TIMP1. (D) Effect of TIMP1 overexpression on SMC migration across a Matrigel barrier. A suspension of SMCs, transduced with either AAV-LacZ or AAV-Timp1, was placed in the upper chamber and medium with chemoattractants was placed in the lower compartment. The bars show the number of cells that migrated in response to chemoattraction. The invasion activity is expressed as the mean number of cells that had migrated per microscopic field (400×) ± SEM for triplicate determinations. The asterisk denotes statistical significance ($P = 0.0005$) using the Student *t* test, one tailed.

FIG. 2. Transduction of rat carotid artery by AAV vectors. (A) Transduction of rat carotid arteries with AAV-LacZ at day 14 after balloon injury. The left shows immunohistochemistry with an antibody against α-actin of VSMCs to show the SMC phenotype of neointima cells. The right shows β-galactosidase reactivity of the transduced smooth muscle cells in the media and the neointima. Few adventitial cells also appeared positive for β-galactosidase expression. (B) Immunohistochemistry of carotid arteries at 2 weeks after transduction with either AAV-LacZ (left) or AAV-Timp1 (right) using an antibody against TIMP1, to show expression of the transgene in the transduced cells. (C) Quantification of hTIMP1 expression *in vivo* by real-time PCR. Balloon-injured rat carotid arteries (three animals per time point) were transduced with AAV-Timp1. At the indicated time points, total RNA was extracted and subjected to real-time PCR amplification. In each sample, the expression of transduced hTIMP1 was normalized to the levels of the housekeeping 18s gene. The results are expressed relative to the expression at 1 week; shown are the means ± SEM. No statistical difference was detected among the different time points.

doi:10.1016/j.ymthe.2004.02.020

and 84% increase, respectively). As for collagen, the elastin content within the media was similar at both time points in both treated and control arteries (data not shown).

Taken together, these observations suggest that the reduction of neointimal thickness determined by MMP blockage after AAV-Timp1 transduction is paralleled by the inhibition of extensive collagen accumulation and the promotion of elastin deposition in the neointimal lesion.

DISCUSSION

In this work we demonstrate the *in vitro* and *in vivo* efficacy of recombinant AAV vectors for vascular SMC transduction and show the therapeutic potential of *TIMP1* gene transfer for the prevention of restenosis. After balloon injury of rat carotid arteries followed by AAV-LacZ transduction, the expression of the β-gal transgene was visualized through the arterial wall, with special intensity in the media and neointima layers and with particular localization at the sites of maximal damage, in agreement with a recent study [22]. In our experiments, the overall efficiency of transduction varied widely from 10 to 50% of the entire vascular wall. This variability may be secondary to the unequal conditions in intravascular delivery, the extent of balloon injury, and the cell types infected at the moment of injury when neointimal cells are not present. On average, the efficiency of AAV vectors for gene transfer to the damaged arterial wall is lower than that obtained using first-generation adenoviral vectors, but this is largely outweighed by the lack of undesirable inflammatory effects and the long-term expression of the therapeutic genes [19,22]. In addition, this rate of *in vivo* transduction appears satisfactory, especially when the transferred gene encodes a secreted protein (such as TIMP1), which exerts effects not only on transduced cells, but also on neighboring cells, which may not have undergone gene transduction. This paracrine action may be of great importance in achieving a significant therapeutic effect.

AAV vectors are of interest not only for their potential therapeutic applications, they also represent valuable tools to assess the *in vivo* effects of gene overexpression. The possibility of delivering and expressing a suitable gene within the arterial wall for prolonged periods of time might facilitate the molecular understanding of the disease process and the implementation of new therapeutic strategies without systemic side effects. In this context, the inhibition of intimal hyperplasia in injured rat carotid arteries by AAV-Timp1 underscores the importance of extracellular matrix remodeling in the pathogenesis of this process (reviewed in Refs. [23,24]). This outcome is consistent with biochemical studies that have revealed MMP overexpression after coronary angioplasty [25] or other procedures that have damaged the vascular endothelium [26,27], with recent genetic studies that have highlighted the role of MMP2 and MMP9 polymorphisms on the rate of restenosis [28], with the outcome of studies performed in MMP2- [29] and MMP9-knockout animals [30–32], and with the therapeutic role of pharmacological inhibition of MMP activity on the stenotic process [8,33,34]. Contrary to systemic therapy with nonspecific inhibitors of MMP activity in the minipig model—which were shown to affect constrictive arterial remodeling but not neointima formation [8]—in our experiments the local overexpression of TIMP1 in the arterial wall markedly reduced the neointimal area. This effect might be related to the advantages of local gene delivery and expression compared to systemic treatment.

What is the actual molecular mechanism by which local overexpression of TIMP1 in the arterial wall reduces neointimal thickness? This outcome correlates with the modulation of extracellular matrix content and with a marked reduction in the number of proliferating cells migrated to the neointima. Much experimental evidence indicates that MMPs are overexpressed in the early phases of the stenotic process and that their activity is essential

FIG. 4. Inhibition of intimal hyperplasia by AAV-Timp1. (A–C) Inhibition of neointima formation by AAV-Timp1 at different times after injury. Arterial morphometric analysis was performed in control animals at 2, 4, and 8 weeks after injury ($n = 11$, 6, and 5, respectively) and in animals treated with AAV-Timp1 at the same time points ($n = 5$ for all groups). Shown are the mean values and SEM of the (A) neointima and (B) media areas and of the (C) intima:media ratio; at least three sections per animal were evaluated. The asterisk denotes statistical significance between AAV-Timp1 and AAV-LacZ ($P < 0.01$) using the Student *t* test, one tailed. (D) Neointima formation in two carotid arteries transduced with AAV-LacZ (left) or AAV-Timp1 (right) at 2 weeks after injury. The arteries were visualized by staining with nuclear fast red.

FIG. 5. Modification of collagen and elastin content of the arterial wall after AAV-Timp1 transduction. (A) Effects of AAV-Timp1 transduction on collagen content as assessed by Azan–Mallory staining. Shown are enlargements of the arterial wall at 4 weeks after transduction with AAV-Timp1 or AAV-LacZ. Collagen is visualized by blue staining; muscle cells appear in orange. (B) Quantification of collagen content at 2 and 4 weeks after transduction. The histogram shows the percentage of collagen in the neointima expressed as number of pixels per area (see Materials and Methods). Shown are the mean values and SEM. Quantification was performed in four animals per group, analyzing at least three sections per animal. The asterisks indicate statistical significance ($P < 0.05$). (C) Effects of AAV-Timp1 transduction on elastin content as visualized by Weigert–Van Gieson staining. Shown are the images of two arteries and of the respective enlargements at 4 weeks after transduction with AAV-Timp1 or AAV-LacZ. Elastin is visualized as dark fibers by staining with Weigert resorcin fuchsin; collagen stains in red. (D) Quantification of elastin content at 2 and 4 weeks after transduction. The histogram shows the percentage of elastin in the neointima expressed as number of pixels per area (see Materials and Methods). Shown are the mean values and SEM. Quantification was performed in four animals per group, analyzing at least three sections per animal. The asterisks indicate statistical significance ($P < 0.05$).

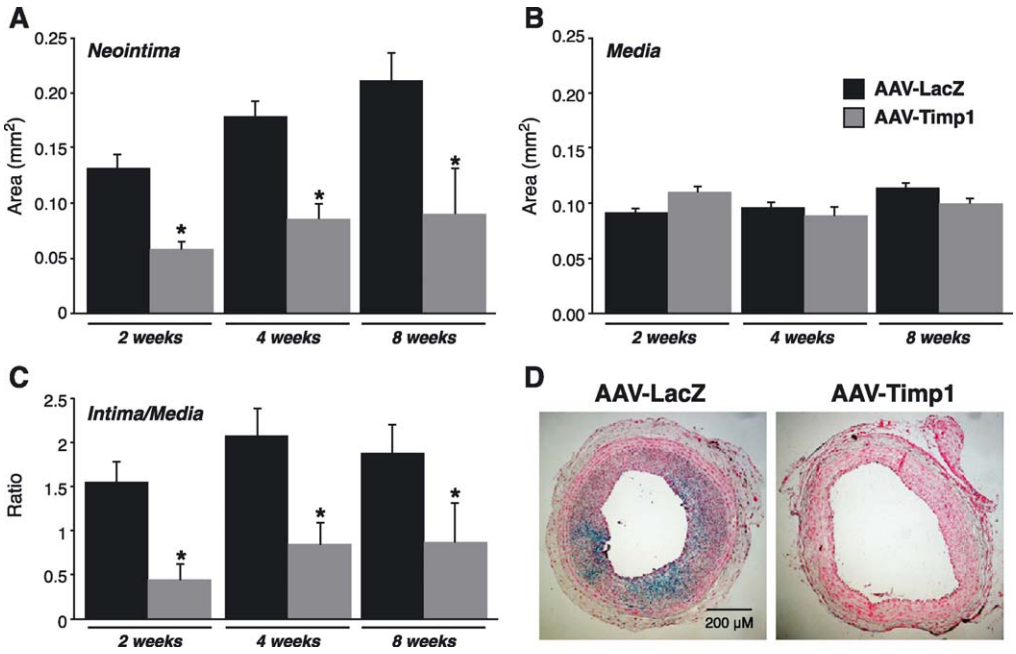

FIG. 4.

for neointima formation [7–9]. In this respect, it is worth noting that restenosis and constrictive remodeling are positively correlated with endothelial dysfunction and collagen accumulation, whereas elastin density is inversely correlated with neointimal growth [35]. Consistent with these findings, other groups have also observed that col-

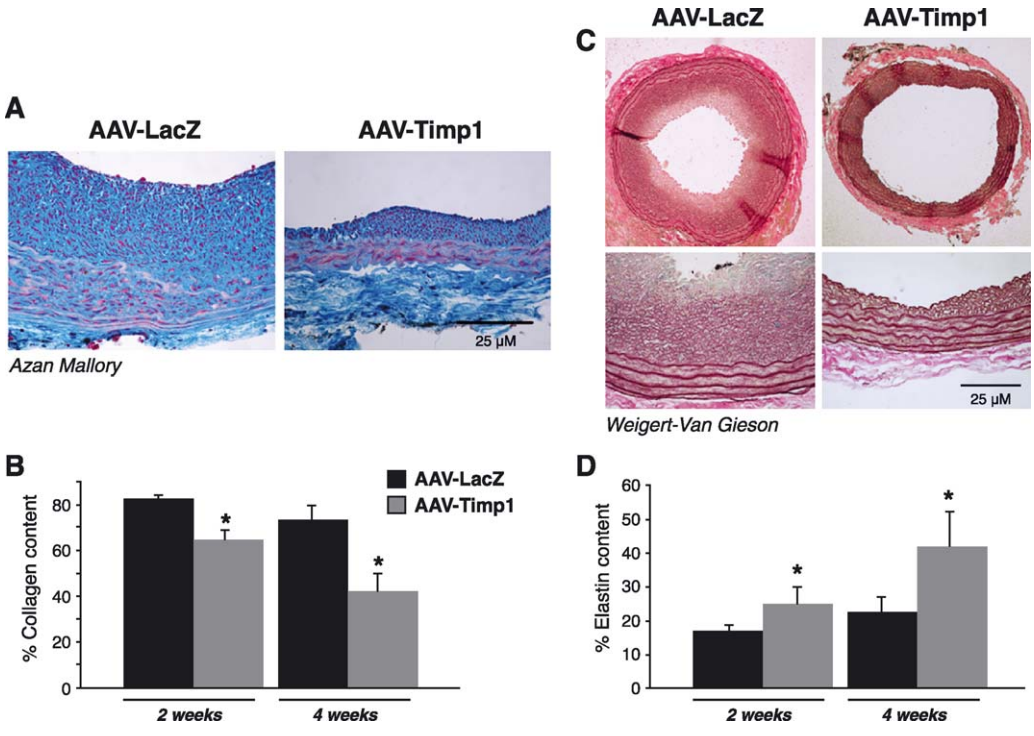

FIG. 5.

doi:10.1016/j.ymthe.2004.02.020

lagen content was altered after balloon injury in normolipemic rabbits and pigs and that pharmacological inhibition of MMPs resulted in diminished collagen accumulation [10,36]. Furthermore, Johnson *et al.* have found that MMP9 is necessary for collagen organization by SMCs [32]. Our findings, showing decreased collagen deposition and increased elastin content after AAV-mediated *TIMP1* gene transfer, are in perfect agreement with these observations, as well as with the conclusions of the work of Forough *et al.*, who have shown that local overexpression of TIMP1 increases elastin accumulation through a posttranslational processing mechanism [21]. Most likely, the overall outcome of the modifications induced by TIMP1 overexpression is the reduction of passive tensile strength (less collagen) and the maintenance of the elastic properties of the arterial wall (more elastin).

The activity of MMPs is essential to permit SMC migration and invasion of the neointima, as also confirmed by the results of our *in vitro* chemoinvasion assay. Although TIMP1 does not seem able to affect SMC proliferation directly [11,37], recent experimental evidence indicates that the composition of the extracellular matrix regulates the responsiveness of SMCs to mitogenic stimuli, allowing their transition from a contractile to a proliferative phenotype [38–41]. Accordingly, both SMC migration and proliferation are significantly impaired in MMP9-knockout mice [31]. Thus, TIMP1 might affect SMC proliferation indirectly by interfering with the remodeling of the extracellular matrix. In a consistent manner, in our *in vivo* experiments we observed that *TIMP1* gene transfer determined a marked reduction in the total number of cells in the neointima and lowered the fraction of proliferating cells, as shown by reactivity to PCNA immunostaining (data not shown). The overall result of the earlier organization of the neointimal matrix and the inhibition of SMC migration and proliferation is the reduction in neointimal thickness. This conclusion is consistent with the higher efficacy of AAV-Timp1 gene transfer in the early phases of the stenotic response (2 weeks compared to 1 or 2 months), when MMPs are overexpressed [9]. The slightly reduced effect of TIMP1 at the later time points could also be ascribed to the development of an immune response against the transferred human TIMP1. However, the high similarity between the human and the rat proteins, the persistence of human TIMP1 expression over time, and the absence of inflammation at histological examination indicate indirectly that the animals did not raise an important immune response against the transgene-expressing cells. Nevertheless, we cannot exclude the presence of neutralizing antibodies against the human TIMP1, which might account for a partial reduction in the therapeutic effect over time.

Taken together, the results described in this work suggest that MMPs contribute to the restenosis process favoring SMC migration from the media to the intima and inducing extensive remodeling of extracellular matrix, which in turn might be essential for SMC accommodation within the neointima. Up-regulation of MMP expression—especially of MMP2 and MMP9—was shown to be even higher after stent implantation than after balloon angioplasty [7]. Thus, AAV-Timp1 gene delivery might find an application as an approach to avoid neointima formation in the process of in-stent restenosis, which depends mostly on intimal hyperplasia. The development of a gene therapy strategy for this condition might be of great interest as an alternative approach to the implantation of drug-coated stents.

MATERIALS AND METHODS

Production, purification, and characterization of rAAV vectors. pAAV-Timp1 was constructed by cloning the human TIMP1 cDNA, which was obtained by RT-PCR amplification from HeLa cell total RNA, to substitute for the GFP and neomycin-resistance genes in plasmid pTR-UF5, kindly provided by N. Muzyczka (University of Florida, Gainesville, FL, USA). pAAV-LacZ was again obtained by inserting the *LacZ* gene from plasmid pCH110 (Pharmacia, Uppsala, Sweden) [42] into the pTR-UF5 backbone.

The cloning and propagation of AAV plasmids was carried out in the JC 8111 *Escherichia coli* strain. Infectious AAV2 vector particles were generated and titered exactly as described previously [42,43]. Viral preparations used for this work had titers of $\sim 1 \times 10^{12}$ viral genome particles per milliliter.

Evaluation of smooth muscle cell transduction by AAV vectors. To define the permissivity of smooth muscle cells to AAV vectors *in vitro*, a preparation of AAV-GFP (obtained from plasmid pTR-UF5; 1×10^{11} genome particles) was used to transduce 1×10^5 coronary artery smooth muscle cells (purchased from Clonetics, BioWhittaker, Walkersville, MD, USA). After 5 days, flow cytometric analysis of GFP-expressing cells was performed using a FACScalibur instrument (Becton–Dickinson, San Jose, CA, USA) acquiring 1×10^4 events for each sample.

The efficiency of the AAV transduction of the arterial wall *in vivo* was evaluated by intraluminal delivery of rAAV-LacZ for 40 min. After 2 and 4 weeks from treatment, the carotid arteries were harvested, fixed, and stained for X-gal reactivity as described [22]. Five to eight 5-μm sections were counterstained with nuclear fast red.

Chemoinvasion assay. To assay the ability of SMCs to invade matrix, 1×10^5 human coronary artery SMCs were transduced with either AAV-Timp1 or AAV-LacZ (1×10^{11} genome particles) for 48 h and then seeded in serum-free medium into the cell culture inserts of 8-μm pore size invasion chambers coated with Matrigel (BioCoat Matrigel Invasion Chambers; Becton–Dickinson). The same medium supplemented with serum and chemoattractants (hEGF 0.5 ng/ml, insulin 2.5 μg/ml, hFGF-B 1 ng/ml, FBS 5%) was placed in the lower chamber. After 22 h incubation, cells that migrated to the lower side of the filter were fixed in methanol, stained with Giemsa solution, and counted in eight fields per membrane at 400× magnification. Each assay was carried out in triplicate. Results are given as the mean number of migrated cells ± standard error of mean (SEM).

Balloon injury and AAV application. Animal care and treatment were conducted in conformity with institutional guidelines issued in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, December 12, 1987). Male Wistar rats weighing

300–400 g were anesthetized and the right common and external carotid arteries were exposed and isolated. A 2F Fogarty catheter (Baxter–Edwards Healthcare, Irvine, CA, USA) was introduced into the common carotid artery through an arteriotomy in the external carotid artery and inflated to 1.5–2 atm. Injury was induced as described by Clowes *et al.* [2], covering a total length of 1 cm. After balloon removal, rAAV vectors were injected through an Intramedic PE50 (Becton–Dickinson) polyethylene cannula and allowed to incubate in the injured segment in the absence of flow for 40 min. The external carotid was then tied and the blood flow was restored through the common carotid artery.

The AAV-Timp1-treated group consisted of 15 animals, whereas the control group was composed of 22 animals; animals were randomly exposed to AAV-Timp1, PBS, or AAV-LacZ after an appropriate curve of learning on a previous set of animals. The treated group received 100 μl of rAAV-Timp1 (1×10^{11} total viral particles); the control group received 100 μl of rAAV-LacZ (1×10^{11} particles) or PBS ($n = 16$ and $n = 6$, respectively). Animals were sacrificed after 2, 4, and 8 weeks (Timp1 group, $n = 5$ at each time point; control group, $n = 11$, $n = 6$, $n = 5$ at 2, 4, and 8 weeks, respectively).

Quantification of transduced hTIMP1 by real-time RT-PCR. Total RNA from injured arteries at different times after transduction was extracted according the guanidinium thiocyanate–phenol–chloroform extraction procedure [44]. After reverse transcription with random hexameric primers, real-time quantitative PCR was performed on a 7000 ABI Prism instrument (Applied Biosystems, Foster City, CA, USA). In the same tubes, a multiplex reaction was carried out for the simultaneous amplification of the hTIMP1 cDNA and the housekeeping gene 18s as a standard. The TaqMan probe and primers were purchased from Applied Biosystems as an Assay-on-Demand for hTIMP1 and as a Predevelopment Assay Reagent for the 18s.

Zymography and reverse zymography. Three days after balloon injury, rat carotid arteries were harvested and kept in culture in serum-free medium. The 3-day conditioned medium was used for substrate gel electrophoresis under nonreducing conditions on a 0.1% gelatin and 10% SDS–polyacrylamide gel. After extensive washing in 2.5% Triton to remove SDS, the gel was incubated for 16 h at 37°C with shaking in collagenase buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, and 0.2% Triton X-100). Finally, the gel was stained in 0.1% Coomassie brilliant blue; clear zones against the blue background indicate the presence of gelatinolytic activity [45].

The activity of human TIMP1 on rat MMP activity was assessed by reverse gel zymography [45]. 293 cells transfected with pAAV-Timp1 or a control plasmid were cultured for 24 h in serum-free medium; supernatants were harvested, filtered, and concentrated prior to loading onto a 0.6% gelatin and 12% polyacrylamide gel. The gel was then incubated for 2 h in the medium of cultured rat carotid arteries (used as a source of rat MMPs) mixed 1:1 with the collagenase buffer. Functional TIMP1 appeared as a dark band at approximately M_r 28,000.

Histology and immunohistochemistry. For histological analysis, a segment of each harvested artery was fixed in 2% formaldehyde, embedded in paraffin, sectioned at 5 μm, and processed for microscopic examination after hematoxylin staining.

The intima and media areas and the intima-to-media ratio were calculated by digital planimetry of tissue sections, by an investigator blinded for treatment regimen. For each animal, at least four individual sections (at 350-μm intervals), obtained from the middle portion of the treated segment, were analyzed.

Protocols for immunohistochemistry were undertaken according to the Vectastain Elite ABC Kit (universal or goat) from Vector Laboratories using primary antibodies against TIMP1 (NeoMarkers, Fremont, CA, USA) and α-SMA (1A4; Sigma Chemical Co., St. Louis, MO, USA). After treatment, slides were rinsed in PBS and signal was developed using 3,3′-diaminobenzidine as substrate for the peroxidase chromogenic reaction (Lab Vision Corp., Fremont, CA, USA).

Collagen and elastin content quantification. The collagen and elastin content was assessed by Azan–Mallory and Weigert–Van Gieson staining. Briefly, for Azan–Mallory staining rehydrated sections were consecutively stained in Azocarmin and Azan solutions, using phosphotungstic acid as a mordant, and a careful differentiation was completed in anilin. For Weigert–Van Gieson staining, rehydrated sections were first stained in Weigert resorcin fuchsin solution at 60°C, rinsed in water, differentiated in acid alcohol, and counterstained in Van Gieson solution.

For each animal analyzed, at least three individual sections (at 350-μm intervals) were stained with Azan–Mallory or Weigert–Van Gieson to evaluate collagen (cyan) and elastin (black), respectively. Histological quantification was performed by transforming the respective colors into monochrome with a 255-level gray scale (NIH Image J 1.29 software, Bethesda, MD, USA), followed by the evaluation of the relative number of pixels after adjustment of the individual thresholds for each color, thus permitting a binary analysis [35]. Results are expressed as the number of pixels per area classified as collagen or elastin divided by total number of pixels in each area.

Statistical analysis. Results are expressed as means ± SEM. The Student *t* test was used to compare cell migration, media (M) and neointima (I) mean areas, and I/M ratios. The nonparametric Mann–Whitney test was used to compare collagen and elastin content in treated and control groups. Values were considered statistically different when $P < 0.05$. Two occluded arteries were excluded from the AAV-Timp1 group at 14 days and one from AAV-LacZ group at 28 days.

ACKNOWLEDGMENTS

This work was supported by grants from the Progetto Finalizzato "Genetica Molecolare" of the Italian National Research Council and from the Fondazione Cassa di Risparmio of Trieste, Italy. The authors are grateful to Sara Tomasi for technical support and to Suzanne Kerbavcic for editorial assistance.

RECEIVED FOR PUBLICATION JULY 4, 2003; ACCEPTED FEBRUARY 29, 2004.

REFERENCES

1. Topol, E. J., and Serruys, P. W. (1998). Frontiers in interventional cardiology. *Circulation* **98:** 1802–1820.
2. Clowes, A. W., and Schwartz, S. M. (1985). Significance of quiescent smooth muscle migration in the injured rat carotid artery. *Circ. Res.* **56:** 139–145.
3. Serruys, P. W., *et al.* (1994). A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. *N. Engl. J. Med.* **331:** 489–495.
4. Hoffmann, R., *et al.* (1996). Patterns and mechanisms of in-stent restenosis: a serial intravascular ultrasound study. *Circulation* **94:** 1247–1254.
5. Loscalzo, J. (2000). Vascular matrix and vein graft failure: is the message in the medium? *Circulation* **101:** 221–223.
6. Libby, P., and Lee, R. T. (2000). Matrix matters. *Circulation* **102:** 1874–1876.
7. Feldman, L. J., *et al.* (2001). Differential expression of matrix metalloproteinases after stent implantation and balloon angioplasty in the hypercholesterolemic rabbit. *Circulation* **103:** 3117–3122.
8. de Smet, B. J., *et al.* (2000). Metalloproteinase inhibition reduces constrictive arterial remodeling after balloon angioplasty: a study in the atherosclerotic Yucatan micropig. *Circulation* **101:** 2962–2967.
9. Bendeck, M. P., Zempo, N., Clowes, A. W., Galardy, R. E., and Reidy, M. A. (1994). Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circ. Res.* **75:** 539–545.
10. Strauss, B. H., *et al.* (1996). In vivo collagen turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. *Circ. Res.* **79:** 541–550.
11. George, S. J., Johnson, J. L., Angelini, G. D., Newby, A. C., and Baker, A. H. (1998). Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. *Hum. Gene Ther.* **9:** 867–877.
12. Lijnen, H. R., Soloway, P., and Collen, D. (1999). Tissue inhibitor of matrix metalloproteinases-1 impairs arterial neointima formation after vascular injury in mice. *Circ. Res.* **85:** 1186–1191.
13. Dollery, C. M., Humphries, S. E., McClelland, A., Latchman, D. S., and McEwan, J. R. (1999). 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. *Circulation* **99:** 3199–3205.

doi:10.1016/j.ymthe.2004.02.020

14. Turunen, M. P., *et al.* (2002). Peptide-retargeted adenovirus encoding a tissue inhibitor of metalloproteinase-1 decreases restenosis after intravascular gene transfer. *Mol. Ther.* **6:** 306–312.

15. Morishige, K., *et al.* (2003). Overexpression of matrix metalloproteinase-9 promotes intravascular thrombus formation in porcine coronary arteries in vivo. *Cardiovasc. Res.* **57:** 572–585.

16. Silence, J., Collen, D., and Lijnen, H. R. (2002). Reduced atherosclerotic plaque but enhanced aneurysm formation in mice with inactivation of the tissue inhibitor of metalloproteinase-1 (TIMP-1) gene. *Circ. Res.* **90:** 897–903.

17. Lemaitre, V., Soloway, P. D., and D'Armiento, J. (2003). Increased medial degradation with pseudo-aneurysm formation in apolipoprotein E-knockout mice deficient in tissue inhibitor of metalloproteinases-1. *Circulation* **107:** 333–338.

18. Forough, R., *et al.* (1996). Overexpression of tissue inhibitor of matrix metalloproteinase-1 inhibits vascular smooth muscle cell functions in vitro and in vivo. *Circ. Res.* **79:** 812–820.

19. Monahan, P. E., and Samulski, R. J. (2000). AAV vectors: is clinical success on the horizon? *Gene Ther.* **7:** 24–30.

20. Arsic, N., *et al.* (2003). Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. *Mol. Ther.* **7:** 450–459.

21. Forough, R., *et al.* (1998). Metalloproteinase blockade by local overexpression of TIMP-1 increases elastin accumulation in rat carotid artery intima. *Arterioscler. Thromb. Vasc. Biol.* **18:** 803–807.

22. Rolling, F., Nong, Z., Pisvin, S., and Collen, D. (1997). Adeno-associated virus-mediated gene transfer into rat carotid arteries. *Gene Ther.* **4:** 757–761.

23. Ward, M. R., Pasterkamp, G., Yeung, A. C., and Borst, C. (2000). Arterial remodeling: mechanisms and clinical implications. *Circulation* **102:** 1186–1191.

24. Galis, Z. S., and Khatri, J. J. (2002). Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ. Res.* **90:** 251–262.

25. Hojo, Y., Ikeda, U., Katsuki, T., Mizuno, O., Fujikawa, H., and Shimada, K. (2002). Matrix metalloproteinase expression in the coronary circulation induced by coronary angioplasty. *Atherosclerosis* **161:** 185–192.

26. Kranzhofer, A., Baker, A. H., George, S. J., and Newby, A. C. (1999). Expression of tissue inhibitor of metalloproteinase-1, -2, and -3 during neointima formation in organ cultures of human saphenous vein. *Arterioscler. Thromb. Vasc. Biol.* **19:** 255–265.

27. Godin, D., Ivan, E., Johnson, C., Magid, R., and Galis, Z. S. (2000). Remodeling of carotid artery is associated with increased expression of matrix metalloproteinases in mouse blood flow cessation model. *Circulation* **102:** 2861–2866.

28. Cho, H. J., *et al.* (2002). Functional polymorphism in the promoter region of the gelatinase B gene in relation to coronary artery disease and restenosis after percutaneous coronary intervention. *J. Hum. Genet.* **47:** 88–91.

29. Kuzuya, M., *et al.* (2003). Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia. *Circulation* **108:** 1375–1381.

30. Galis, Z. S., *et al.* (2002). Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. *Circ. Res.* **91:** 852–859.

31. Cho, A., and Reidy, M. A. (2002). Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury. *Circ. Res.* **91:** 845–851.

32. Johnson, C., and Galis, Z. S. (2003). Matrix metalloproteinase-2 and -9 differentially regulate smooth muscle cell migration and cell-mediated collagen organization. *Arterioscler. Thromb. Vasc. Biol.*

33. Bendeck, M. P., Conte, M., Zhang, M., Nili, N., Strauss, B. H., and Farwell, S. M. (2002). Doxycycline modulates smooth muscle cell growth, migration, and matrix remodeling after arterial injury. *Am. J. Pathol.* **160:** 1089–1095.

34. Wentzel, J. J., *et al.* (2001). Shear-stress and wall-stress regulation of vascular remodeling after balloon angioplasty: effect of matrix metalloproteinase inhibition. *Circulation* **104:** 91–96.

35. Lafont, A., *et al.* (1999). Endothelial dysfunction and collagen accumulation: two independent factors for restenosis and constrictive remodeling after experimental angioplasty. *Circulation* **100:** 1109–1115.

36. Sierevogel, M. J., *et al.* (2002). Matrix metalloproteinase inhibition reduces adventitial thickening and collagen accumulation following balloon dilation. *Cardiovasc. Res.* **55:** 864–869.

37. Baker, A. H., Zaltsman, A. B., George, S. J., and Newby, A. C. (1998). Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro: TIMP-3 promotes apoptosis. *J. Clin. Invest.* **101:** 1478–1487.

38. Raines, E. W., Koyama, H., and Carragher, N. O. (2000). The extracellular matrix dynamically regulates smooth muscle cell responsiveness to PDGF. *Ann. N. Y. Acad. Sci.* **902:** 39–51, discussion 51–32.

39. Davenpeck, K. L., *et al.* (2001). Regional differences in integrin expression: role of alpha(5)beta(1) in regulating smooth muscle cell functions. *Circ. Res.* **88:** 352–358.

40. Stegemann, J. P., and Nerem, R. M. (2003). Altered response of vascular smooth muscle cells to exogenous biochemical stimulation in two- and three-dimensional culture. *Exp. Cell Res.* **283:** 146–155.

41. Ross, J. J., and Tranquillo, R. T. (2003). ECM gene expression correlates with in vitro tissue growth and development in fibrin gel remodeled by neonatal smooth muscle cells. *Matrix Biol.* **22:** 477–490.

42. Deodato, B., *et al.* (2002). Recombinant AAV vector encoding human VEGF165 enhances wound healing. *Gene Ther.* **9:** 777–785.

43. Zentilin, L., Marcello, A., and Giacca, M. (2001). Involvement of cellular double-strand DNA break-binding proteins in processing of recombinant adeno-associated virus (AAV) genome. *J. Virol.* **75:** 12279–12287.

44. Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.

45. Zacchigna, S., *et al.* (2004). AAV-mediated gene transfer of tissue inhibitor of metalloproteinases-1 inhibits vascular tumor growth and angiogenesis in vivo. *Cancer Gene Ther.* **11:** 73–80.