FEBS Letters 531 (2002) 122–126 FEBS 26574

Systemic tissue inhibitor of metalloproteinase-1 gene delivery reduces neointimal hyperplasia in balloon-injured rat carotid artery

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Received 19 July 2002; revised 23 August 2002; accepted 1 September 2002

First published online 10 October 2002

Edited by Barry Halliwell

Abstract Metalloproteinases (MMP)-2 and MMP-9 play a role in smooth muscle cell (SMC) migration from the media to the intima following arterial injury. Intravenous administration of adenovirus encoding tissue inhibitor of metalloproteinase-1 (TIMP-1) into balloon-injured rat arteries (3×10^{11} viral particles/rat; $n=7$) resulted in a transient expression of TIMP-1 and a significant inhibition of neointima thickening within 16 days (~40% vs. control; $P=0.012$). Three days after injury, the number of intimal SMCs was decreased by ~98% in TIMP-1-treated rats. However, no alteration was seen in intimal SMC proliferation after 13 days of injury. Therefore, our results show that systemic gene transfer of TIMP-1 is a promising approach in early restenosis treatment.

Key words: Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Restenosis; Gene therapy

1. Introduction

Studies on animal models and in vitro systems have indicated that vascular smooth muscle cell (SMC) migration from the media to the intima and proliferation are important in the development of neointima [1]. Several growth and chemotactic factors as well as plasminogen/plasmin systems and matrix metalloproteinases (MMPs) have been reported to play a role in this phenomenon [2–6].

MMPs are a family of enzymes which together degrade all components of extracellular matrix [7]. The activity of MMPs is tightly controlled by endogenous specific inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), a family of four members [7,8]. Imbalance of MMP activity over TIMPs in the vasculature promotes migration and proliferation of vascular SMCs [7]. Thus, expression of gelatinase A (MMP-2) and gelatinase B (MMP-9), both produced by a variety of cells including vascular cells, is enhanced in the aorta of atherogenic diet-fed rabbits [9] and in perivascular electric injury in the mouse [10], rat [4,11] and pig [12] models of carotid angioplasty. Therefore, inhibition of MMP activity by pharmacological compounds or by gene transfer of TIMPs could arrest SMC migration and neointima formation. Indeed, local overexpression of TIMP-1 or TIMP-2, using adenoviral vectors, has been reported to inhibit neointima formation in the rat model of angioplasty restenosis and in an in vitro human model of vein graft [12]. Similarly, local adenovirus-mediated overexpression of TIMP-3 at the luminal surface of human and pig saphenous vein promotes apoptosis in the neointima and medial layer leading to a significant reduction of neointima formation [13]. However, local delivery of genes to sites of neointima formation is problematic due to the local difficulties of using infusion devices to deliver genes to the vessel wall. Therefore, increasing appropriate gene products in plasma following intravenous adenovirus injection could be a promising new approach to the treatment of vascular diseases. Indeed, Carmeliet and colleagues [14] have reported that systemic overexpression of tissue-type plasminogen activator, using adenovirus vector, augments thrombolysis in tissue-type plasminogen activator-deficient and plasminogen activator inhibitor-1-overexpressing mice. In this study, we show, for the first time, a significant inhibition of new intima thickening in balloon-injured rat carotid artery by increasing the plasma TIMP-1 level through intravenous administration of either adenovirus TIMP-1 vector or recombinant human TIMP-1 protein.

2. Materials and methods

2.1. Generation of the recombinant adenovirus

Recombinant vectors Ad.RSV.βGal and Ad.RSV.TIMP-1 were constructed as described earlier [15]. High-titer stocks of viral particles (vp) (5.8×10^{12}, 1.2×10^{12}) were produced in 293 cells for rAd.RSV.-TIMP-1 and rAd.RSV.βGal respectively, and were purified on CsCl gradients.

2.2. Rat model of balloon injury

The rat carotid artery balloon injury model was based on a model described by Clowes et al. [16]. Adult male Sprague–Dawley rats weighing 400–600 g were subjected to a distending, de-endothelializing injury with an inflated 2F Fogarty embolectomy catheter inserted through the external carotid artery.

2.3. Local delivery of adenoviruses

On day 0, the left injured segments of arteries of eight rats were incubated in the presence of rAd.RSV.TIMP-1 (1×10^{11} vp) and the

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Abbreviations: SMC, smooth muscle cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; vp, viral particle; rAd, recombinant adenovirus; RSV, Rous sarcoma virus

0014-5793/02/$22.00
PII: S0014-5793(02)03388-4

right injured segments of arteries of the same eight rats were incubated in the presence of rAd.RSV.βGal (1×10^{11} vp) in a total volume of 100 μl saline for 15 min according to the previous described protocol [17]. On day 14, the rats were killed with an intraperitoneal injection of pentobarbital (100 mg/kg). The balloon-injured segments of the arteries from the proximal edge of the omohyoid muscle to the carotid bifurcation were perfused with saline and dissected. The tissues were then fixed with 100% methanol and embedded in paraffin. Histological images of 4 μm cross-sections from the center of the injured segment were stained with hematoxylin-eosin or elastic-trichrome and used to determine the intimal, medial, and luminal areas by quantitative morphometric analysis with a computerized sketching program (MACMEASURE version 1.9). Three sections from each artery were evaluated by an investigator who was blinded to the identities of the treatment groups. The experimental protocol was approved by the Institutional Animal Care and Use Committee of St. Elizabeth's Medical Center and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.4. Intravenous administration of adenoviruses

On day 0, a group of seven rats received an injection in the tail vein with rAd.RSV.TIMP-1 (3×10^{11} vp) and blood was drawn for plasma TIMP-1 evaluation. On day 3, a carotid balloon injury was performed on all animals and blood was drawn. On days 6 and 12, only blood was drawn and finally, on day 16, blood was collected and vessels were harvested for histology analysis.

2.5. Vascular smooth muscle cell proliferation in systemic rAd.RSV.TIMP-1- or rAd.RSV.βGal-infused rats

First, the rats were treated with rAd.RSV.TIMP-1 (3×10^{11} vp; $n=3$) or rAd.RSV.βGal (3×10^{11} vp; $n=3$) on day 0. Second, the rat carotid arteries were balloon-injured on day 3. Third, in order to label all cells entering S-phase during the last 24 h before sacrificing rats on day 13 after balloon injury, subcutaneous injections (25 mg/kg body weight) of bromodeoxyuridine (Boehringer Mannheim) were performed at 19, 9 and 1 h before death by pentobarbital injection as described earlier [11]. The rats were then killed and the vessels were fixed and immersed in 4% paraformaldehyde for 1 h according to Bendeck et al. [11]. Thereafter, 5 mm lengths of the carotid arteries were cut and embedded in paraffin, and histological cross-sections were made for the evaluation of intimal SMC proliferation as described [18].

2.6. Vascular smooth muscle cell migration in systemic rAd.RSV.TIMP-1- or rAd.RSV.βGal-infused rats

First, rats were injected on day 0 with rAd.RSV.TIMP-1 (3×10^{11} vp; $n=4$) or rAd.RSV.βGal (3×10^{11} vp; $n=4$). Second, the rat carotid arteries were balloon-injured on day 3. Third, 3 days after balloon injury, a 1 cm length of vessel was excised from the middle of the fixed common carotid and used to determine SMC migration as described earlier [19]. Briefly, migration was determined by staining the nuclei of intimal cells using an antibody against histone H1 (mAb 1276, Chemicon International) and by counting the number of cells in the intima.

2.7. SDS–PAGE zymography

Proteins were extracted from control and injured rat carotid artery as described by Olsen et al. [20]. SDS–polyacrylamide (Millipore, France) gel containing 1 mg/ml gelatin (Bio-Rad) was used to visualize gelatinolytic activity for MMP-2 and MMP-9 [21]. Clear bands indicated the presence of proteolytic activity. To analyze the activity of human TIMP-1 against arterial rat MMPs, a similar gel was employed and incubated with recombinant human TIMP-1 (a kind gift from G. Murphy).

2.8. Quantification of human TIMP-1 in rat plasma or culture media by the ELISA procedure

Human TIMP-1 in rat plasma or rat carotid culture media (following rAd.RSV.TIMP-1 transfection) was quantitated with a sandwich ELISA kit (Amersham, France), containing two monoclonal antibodies specific to human TIMP-1 antigen.

2.9. Statistical analysis

The intima/media ratio was compared between groups by Student's *t*-test.

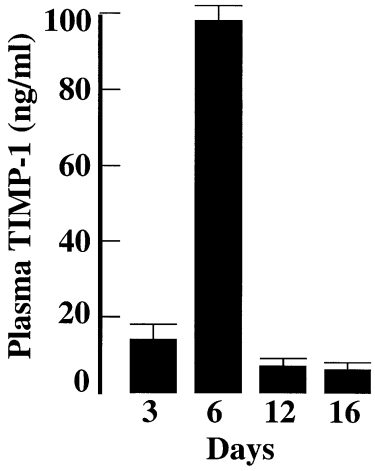

Fig. 1. Kinetic expression of human TIMP-1 in rat plasma following intravenous injection of rAd.RSV.TIMP-1 adenovirus. Adult male Sprague–Dawley rats ($n=7$) received an injection of rAd.RSV.-TIMP-1 (3×10^{11} vp) in the tail vein. Blood was drawn and plasma TIMP-1 evaluated using a sandwich ELISA kit. Values are means ± S.E.M.

3. Results

Infusion of rats with rAd.RSV.TIMP-1 at 3×10^{11} vp/rat resulted in a transient expression of TIMP-1 (Fig. 1). On day 6 after injection, plasma TIMP-1 levels reached ∼100 ± 12 ng/ml and they decreased to ∼8 ± 0.9 ng/ml by 12 days. The endogenous plasma TIMP-1 could not be evaluated due to the absence of a specific murine ELISA kit. This administration procedure of rAd.RSV.TIMP-1 (3×10^{11} vp/rat) resulted in a significant inhibition of neointima thickening in 16 days (intima/media (I/M) ratio = 0.56 ± 0.45; $P=0.012$ vs. control) (Figs. 2B and 3). In addition, kinetic analysis of in vivo TIMP-1 production following local delivery of rAd.-RSV.TIMP-1 (1×10^{11} vp/rat) on balloon-injured arteries of three animals was performed. The result showed that the plasma TIMP-1 level was merely detectable while the TIMP-1 concentration in homogenized transfected artery indicated a maximum concentration of ∼3 ± 0.7 ng/10^6 intimal SMCs following 6 days of transfection (not shown). In addition, local delivery of rAd.RSV.TIMP-1 into rat balloon-injured left carotid resulted in a significant reduction of the I/M ratio after 14 days of transfection as compared to the balloon-injured right carotid of the same animal transfected with rAd.RSV.βGal. The I/M was 1.17 ± 0.20 for the control and 0.54 ± 0.19 for the rAd.RSV.TIMP-1-treated rats ($P=0.038$ vs. the control group). This inhibition represents ∼49% vs. the control (Fig. 2A). Furthermore, intravenous rAd.RSV.-TIMP-1 administration ($n=3$) was found to reduce intimal SMC migration by day 3 after injury. The number of intimal SMCs was decreased from 114 ± 21 cells/mm^2 in rAd.RSV.-βGal-treated rats to 2.2 ± 2 cells/mm^2 in rAd.RSV.TIMP-1-treated rats ($P=0.005$) (Fig. 4A). However, no significant alteration in intimal SMC proliferation occurred at 13 days after injury between the control and rAd.RSV.TIMP-1-treated rats (14.3 ± 4% for the control group ($n=4$) and 17 ± 4% for the rAd.RSV.TIMP-1-treated rats ($n=4$)) (Fig. 4B). It is most likely that these effects were due to MMP inhibition since zymography study showed that human TIMP-1 was able to inhibit the activity of rat MMPs (Fig. 5).

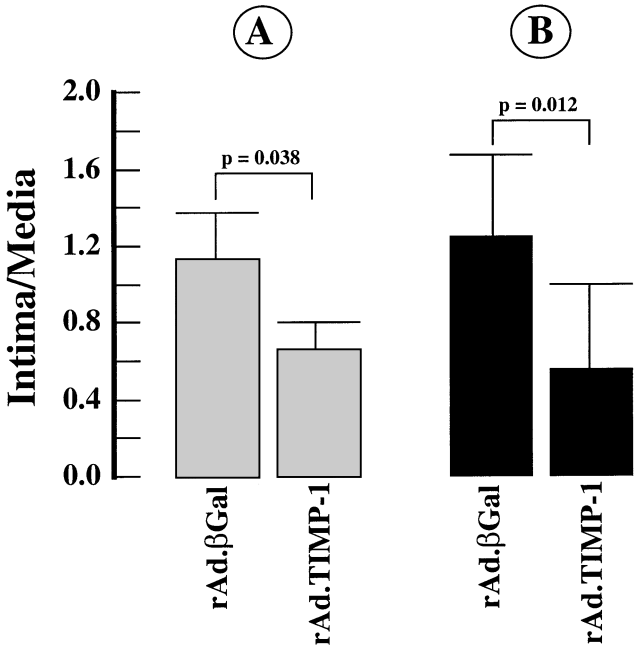

Fig. 2. Histological analysis of balloon-injured rat carotid arteries. Following rAd.RSV.βGal (control) or rAd.RSV.TIMP-1 treatment, adult rats were subjected to a distending, de-endothelializing injury. A: Local delivery of adenoviruses was performed on day 0. The left and right injured segments of arteries of eight rats were incubated in the presence of rAd.RSV.TIMP-1 (1×10^{11} vp) (left) or rAd.RSV.-βGal (1×10^{11} vp) (right). On day 14, rats were killed and the intimal, medial, and luminal areas were quantitated. B: Intravenous administration of adenoviruses was performed on 14 rats divided into two groups ($n=7$ each). On day 0, each group received an injection in the tail vein of either rAd.RSV.TIMP-1 (3×10^{11} vp) or rAd.RSV.-βGal (3×10^{11} vp) (control) and blood was drawn for plasma TIMP-1 evaluation. On day 3, a carotid balloon injury was performed on all animals and blood was drawn. On days 6 and 12, only blood was drawn and finally, on day 16, blood was collected and vessels were harvested for histology analysis. Values are means ± S.D.
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4. Discussion

Increased matrix-degrading MMP activity has been reported in several arterial pathologies such as intimal hyperplasia after angioplasty or vein graft [22]. Vascular SMC migration from the media and proliferation in the neointima are events believed to be the consequence, at least in part, of the action of MMPs. Indeed, enhanced SMC migration and neointima proliferation have been reported when rat SMCs are transfected with MMP-9 and seeded into the luminal surface

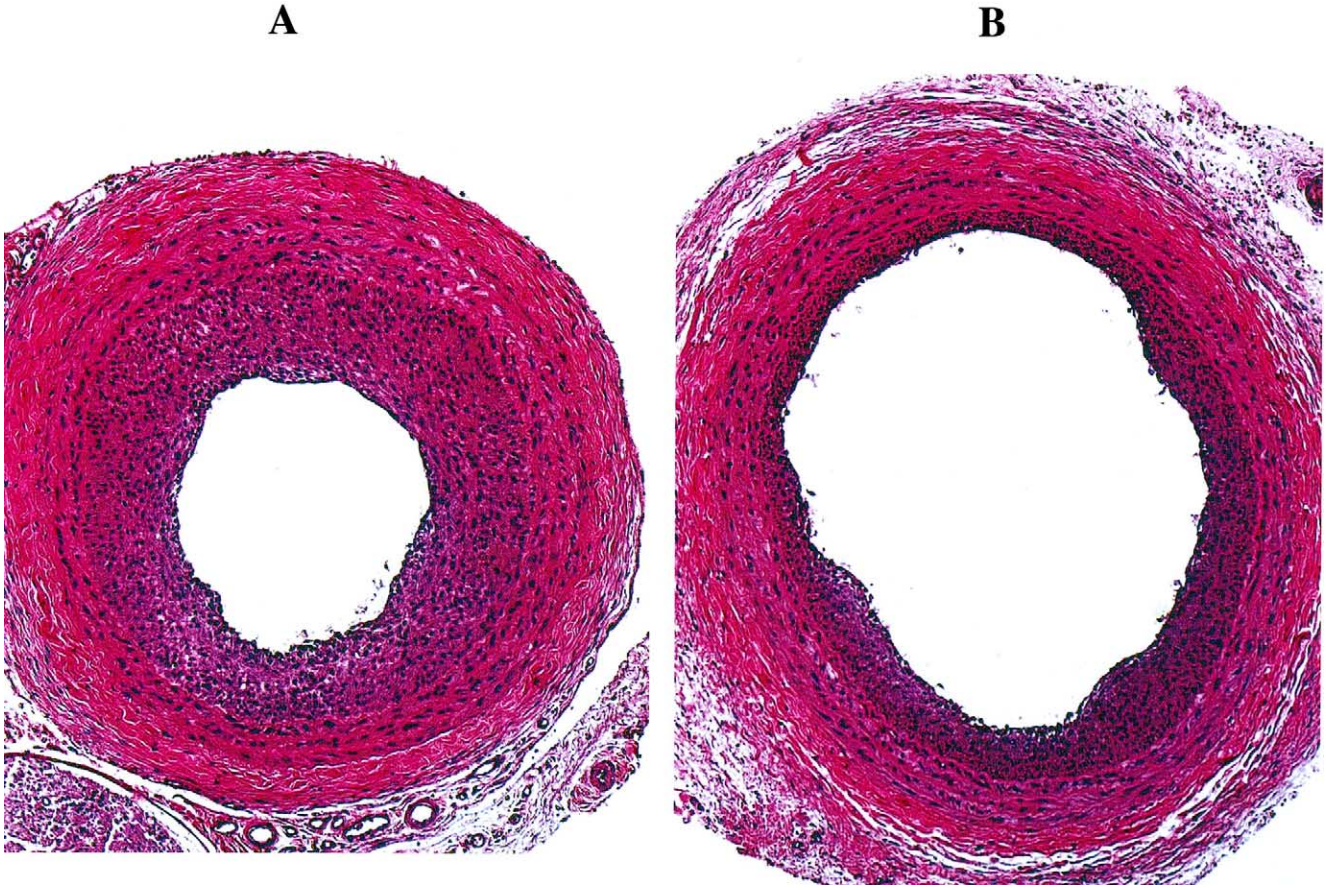

Fig. 3. The balloon-injured segments of the arteries were perfused with saline and dissected. The tissues were then fixed and embedded in paraffin. Histological images of 4 μm cross-sections from the center of the injured segment were used to determine the intimal, medial, and luminal areas by quantitative morphometric analysis. A: rAd.RSV.βGal-treated rats (control). B: rAd.RSV.TIMP-1-treated rats.

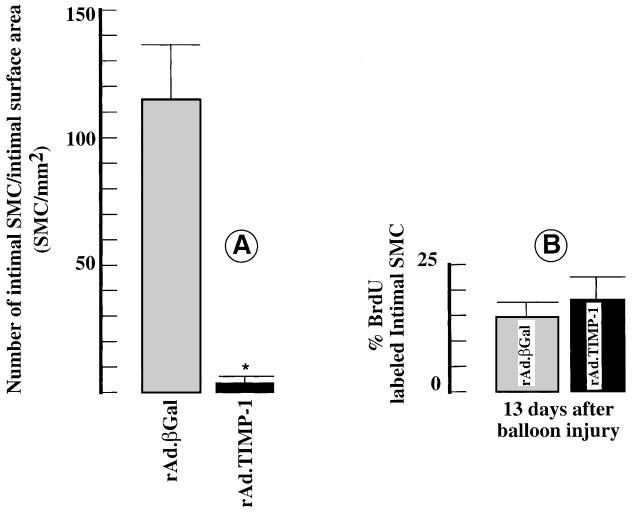

Fig. 4. A: Vascular SMC migration. Six rats were divided into two groups. On day 0, the first group ($n=3$) received an injection in the tail vein with rAd.RSV.βGal (control group) and the second group ($n=3$) received an injection with rAd.RSV.TIMP-1. On day 3, the left and right arteries of both groups were injured. On day 6 (or day 3 after balloon injury), cell migration was determined by counting the number of SMCs in the intima, expressed per unit of surface area. Data are mean ± S.D. B: Intimal SMC proliferation. Thirteen days after balloon injury (which correspond to 16 days after adenovirus injection), rats ($n=3$ for rAd.RSV.βGal and $n=3$ for rAd.RSV.TIMP-1) were used to evaluate the percentage of intimal SMC proliferation. Data are mean ± S.D.

of denuded artery [23]. Conversely, a deficiency of TIMP-1 in mice significantly enhances neointima formation following injury of the femoral artery, confirming the role of MMPs with respect to SMC migration [24]. In addition, inhibition of MMP by adenovirus-mediated overexpression of TIMP-1 has a beneficial effect on aneurysmal degeneration [25] and neointimal formation in several models [26]. However, these latter studies concerned local delivery of the TIMP-1 gene. Although these approaches clarify the role of TIMP-1 in the inhibition of neointima formation, they are not convenient for therapeutic treatment due to the local difficulties of using infusion devices to deliver genes to the vessel wall.

In this study, we evaluated the impact of elevated plasma TIMP-1 on neointima formation, following intravenous administration of rAd.RSV.TIMP-1 in rat injured carotid. In this approach, the liver is utilized as a source of production of TIMP-1 protein. A single injection of adenoviral vector (3×10^{11} vp/rat) significantly reduced neointima formation by approximately 40% after 16 days of balloon injury. The highest level of plasma TIMP-1 following adenovirus administration was approximately 100 ng/ml. Nevertheless, a comparison of this concentration with basic endogenous murine plasma TIMP-1 cannot be performed since, to the best of our knowledge, there is no ELISA kit available for murine TIMP-1 quantification. Similarly, local TIMP-1 delivery using rAd.RSV.TIMP-1 transiently increased TIMP-1 to reach $\sim$3 ng/10^6 intimal SMCs and reduced neointima formation by $\sim$48% after 14 days of balloon injury. This beneficial effect can be attributed to the local expression of TIMP-1. Plasma TIMP-1-derived local transfection is hardly detectable (data not shown) which supports the hypothesis that human TIMP-1 concentration in the microenvironment is sufficient to counterbalance the activity of local MMPs. In addition, rAd.RSV.TIMP-1 inhibited SMC migration from the media to the intima after 3 days of balloon injury. The cell migration phenomenon is mediated, at least in part, by the action of MMPs such as MMP-2 and MMP-9. Inhibition of the activity of these enzymes using human TIMP-1 blocked SMC migration. Indeed, human TIMP-1 was found to inhibit rat MMPs as shown by a zymography study (Fig. 5). However, there was no effect on SMC proliferation with rAd.RSV.TIMP-1 at 13 days after balloon injury indicating that a different process was involved in this phenomenon.

It should be noted that GM6001, a non-specific synthetic MMP inhibitor, has been shown to impair SMC migration and to reduce the lesion size following continuous treatment for up to 10 days after a rat carotid artery had been injured

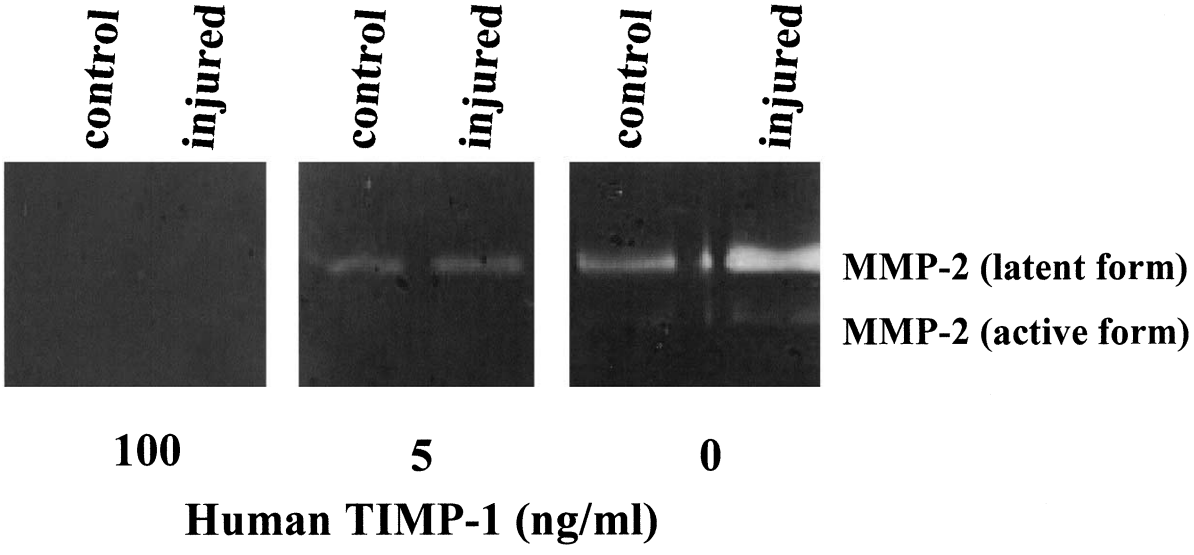

Fig. 5. Zymography. Proteins (10 μg) from control or balloon-injured rat carotid artery for 6 days were electrophoresed in 10% SDS–polyacrylamide gel containing 1 mg/ml gelatin. Clear bands indicate the presence of proteolytic activity. To analyze the activity of human TIMP-1 against arterial rat MMPs, two similar gels were made and incubated with recombinant human TIMP-1 at 5 and 100 ng/ml.

by balloon catheter [11]. In our study, systemic overexpression of human TIMP-1, using rAd.RSV.TIMP-1 vector, was efficacious in the rat carotid injury model for up to 16 days suggesting that TIMP-1 is functionally different from GM6001. For example, inhibition of multiple MMPs with synthetic inhibitors might lead to inactivation of stromelysin-3 (MMP-11) which has been reported to accelerate neointima formation after vascular injury in mice [5].

Circulating TIMP-1 could exercise a relatively prolonged protection against MMP-mediated degradation of specific extracellular molecules. This latter phenomenon is now well established as being responsible for SMC migration [23,24]. However, neither the available synthetic MMP inhibitors nor TIMP-1 affect SMC proliferation. This is a major drawback, since it is unlikely that neointima thickening could be reduced without blocking of the SMC proliferation. With this particular gene therapy approach, we hypothesize that a combination of Ad.TIMP-1 and Ad.TIMP-3 might give a better result, since TIMP-3 has been shown to promote apoptosis in the neointima and medial layer, leading to a significant reduction of neointima formation [13].

In conclusion, local vascular transfer of several genes has produced beneficial effects in the treatment of experimental restenosis. Nevertheless, intravenous injection of adenoviruses, a simple way to overexpress genes, could give a similar result. Unfortunately, several problems remain to be solved before gene transfer can be applied to clinical medicine and notably the use of a new generation of vectors, allowing long duration expression of MMP inhibitors.

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