

ORIGINAL ARTICLE

Overexpression of PAI-1 prevents the development of abdominal aortic aneurysm in mice

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Vessel wall inflammation and matrix destruction are critical to abdominal aortic aneurysm (AAA) formation and rupture. We have previously shown that urokinase plasminogen activator (uPA) is highly expressed in experimental AAA and is essential for AAA formation and expansion. In this study, we examined the effects of overexpression of a natural inhibitor of uPA, plasminogen activator inhibitor-1 (PAI-1), on the development of angiotensin (Ang) II-induced AAA in ApoE-deficient (ApoE^{-/-}) mice. Mice were treated with recombinant adenovirus containing either the human PAI-1 gene (Ad5.CMV.PAI-1) or the luciferase gene (Ad5.CMV.Luc) delivered either locally by intra-adventitial injection or systemically by tail vein

injection. Our results show that local delivery of the PAI-1 gene completely prevented AAA formation (0 vs 55.6% in Ad5.CMV.Luc controls, P < 0.05). In contrast, systemic delivery of the PAI-1 gene did not affect AAA incidence (78 vs 90% in Ad5.CMV.Luc controls, P = 0.125). Local delivery of the PAI-1 gene 2 weeks after Ang II infusion prevented further expansion of small aneurysms, but had no significant effect on the progression of larger aneurysms. These data suggest that local PAI-1 gene transfer could be used to stabilize small AAA and reduce the rate of expansion and risk of rupture.

Gene Therapy (2008) 15, 224–232; doi:10.1038/sj.gt.3303069; published online 22 November 2007

Keywords: PAI-1; adenovirus; vascular gene transfer; abdominal aortic aneurysm; bioluminescence imaging

Introduction

Abdominal aortic aneurysm (AAA), the most common form of aneurysm, is characterized by weakening of the vessel wall, followed by progressive expansion and eventual rupture of the diseased aortic segment. This generally occurs at sites where there is atherosclerotic plaque.^{1–3} Previously, we have demonstrated that proteases, especially urokinase plasminogen activator (uPA) are highly expressed in a mouse model of AAA,⁴ and that uPA deficiency decreased the incidence of AAA.⁵

Vascular inflammation is a prominent feature of atherosclerotic AAA,^{6–8} characterized by the infiltration of monocytes/macrophages and lymphocytes into the arterial wall.^{9,10} Monocytes/macrophages are a major source of uPA in atherosclerotic lesions. uPA hydrolyzes plasminogen to form plasmin, a trypsin-like proteolytic enzyme capable of directly degrading components of the extracellular matrix and of activating matrix metalloproteinases (MMPs). MMPs degrade extracellular matrix proteins including collagen and elastin, thus impairing the structural integrity of the vascular wall and contributing to AAA formation and progression.¹¹

Atherosclerotic lesions in the aorta of ApoE^{-/-} mice fed with a high cholesterol diet show fragmentation of the elastic lamellae and rupture of the media,¹² resulting in aneurysm formation.^{4,13} These pathological changes were not observed in mice deficient in both ApoE and uPA (ApoE^{-/-}/uPA^{-/-} mice),⁵ suggesting that uPA plays a critical role in matrix destruction and aneurysm formation. Plasminogen activator inhibitor-1 (PAI-1) is the primary endogenous inhibitor of uPA and tPA. It not only regulates the proteolytic activity of uPA, but also determines the level of uPA bound to uPAR by promoting endocytosis of the trimolecular uPA–PAI-1–uPAR complex.^{14,15} PAI-1 has also been implicated in modulating cell migration by non-uPA-dependent mechanisms,^{16,17} blocking the interaction between vitronectin, uPAR and integrins;^{18–20} PAI-1 may also induce cell detachment from the extracellular matrix and thereby affect cellular migration. These data suggest that PAI-1 may have a direct role in modulating tissue inflammation.

Clinical data have demonstrated a correlation between PAI-1 gene expression and AAA frequency in patients. A polymorphism within the promoter region of PAI-1 has been described that modifies PAI-1 expression.²¹ The 4G homozygous variant is associated with increased PAI-1 expression and the 675 5G insertion is associated with reduced PAI-1 expression. It has been reported that the 5G homozygous polymorphism is two times more frequent in patients with AAA and a strong family history compared to control population (26 vs 13%).²² AAA expansion is also more rapid in patients with the

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Received 16 March 2007; revised 4 September 2007; accepted 10 October 2007; published online 22 November 2007

5G homozygous genotype.²³ This is consistent with other observations that human aneurysm lesions have increased tPA and uPA gene expression which could result in enhanced fibrinolytic and proteolytic activity in the AAA wall.²⁴

In a series of experiments, we tested the hypothesis that inhibition of uPA by overexpression of human PAI-1 prevents the development and progression of AAA. The PAI-1 gene was delivered locally to the vascular wall by injecting adenoviral human PAI-1 (Ad5.CMV.PAI-1) directly into the perivascular layer of the abdominal aorta. In a separate experiment, the PAI-1 gene was delivered systemically by injecting adenoviral human PAI-1 into the mouse through tail vein. In order to assess the effect of PAI-1 on AAA formation, gene transfer was performed at the time of angiotensin (Ang) II infusion. The effect of PAI-1 on AAA progression was investigated by performing gene transfer after AAA formation following 2 weeks of an infusion of Ang II. Our results show that local overexpression of PAI-1 prevents AAA formation and slows progression of small aneurysms.

Results

In vivo detection of gene expression

To measure the distribution and time course of gene expression *in vivo*, we employed a bioluminescence imaging system equipped with a highly sensitive cooled charged-couple device (CCCD) camera.^{25,26} To validate this approach, we injected increasing doses (1×10^7 – 1×10^{11} viral particles) of the luciferase gene (Ad5.CMV.Luc) into the tail vein of BALB/C mice ($n=8$ per group). Four days following gene delivery, mice were anesthetized and bioluminescence assessed with the CCD camera. Animals were then immediately killed

and liver homogenates assayed for luciferase activity. The *in vivo* bioluminescence signal from the liver was highly correlated with *in vitro* hepatic luciferase enzyme activity ($R^2=0.998$) (Figures 1a–d).

Effects of PAI-1 on AAA Formation

Expression, distribution and duration of luciferase and human PAI-1 in the abdominal aorta following adventitial gene delivery. Intravenous infusion of Ang II (1.44 mg kg^{-1} per day) in ApoE^{-/-} mice resulted in reproducible AAA formation in the suprarenal abdominal aortic region as reported in previous studies.^{4,27} The expression pattern and time course of luciferase after local delivery of 1×10^{11} viral particles of Ad5.CMV.Luc in 200 μl of phosphate buffer solution (PBS) into the perivascular layer of the suprarenal region of the aorta (between the diaphragm and the renal artery) is shown in Figure 2a. Expression of luciferase in the aorta was maximum on day 1, and still detectable on day 4. Expression of luciferase in the liver was observed as early as on day 1, peaked on day 7 and was still detectable on day 28 (Figure 2b).

The expression pattern of human PAI-1 in the suprarenal region of the aorta was detected by western blot after intra-adventitial injection of 1×10^{11} viral particles of Ad5.CMV.PAI-1. PAI-1 expression was observed beginning on day 4, and was still evident on day 14 (Figures 2c and d). The observed differences in the time course between luciferase and PAI-1 expression could be due to the differences in transcription, RNA stability, RNA translation or post-translational modification of the two proteins.

Adventitial gene delivery of PAI-1 prevents AAA formation. Our previous study demonstrated that

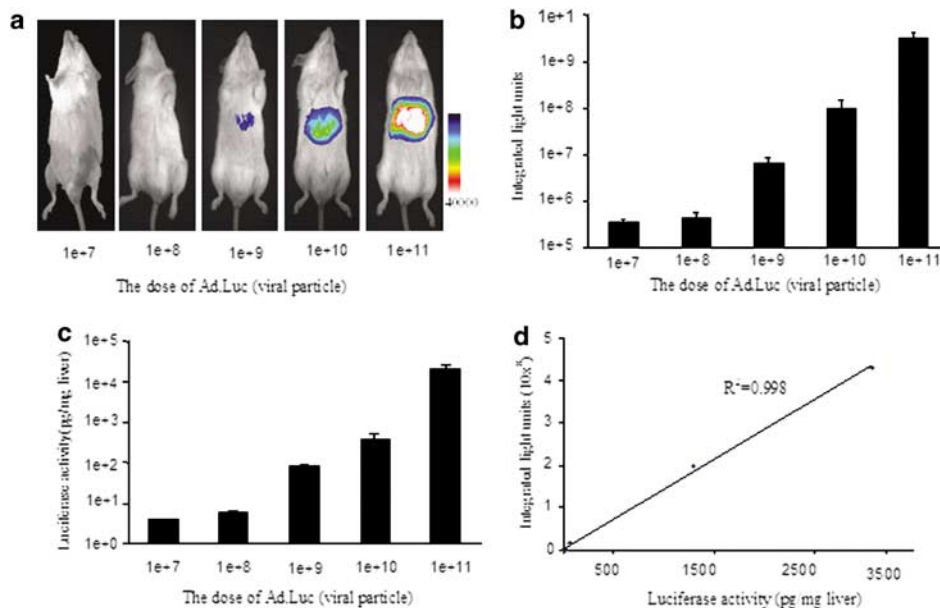


Figure 1 Bioluminescence imaging of luciferase expression in living mice and luciferase activity from liver extracts after intravenous injection of Ad5.CMV.Luc. Different doses of Ad5.CMV.Luc were injected into C57BL/6J mice through tail vein. Four days postsystemic gene delivery, the levels of *in vivo* luciferase expression were followed by bioluminescence imaging system in live animal described in Materials and methods (a and b). Four hour later, the animals were killed, the livers were harvested, *in vitro* luciferase assay was performed to test luciferase activity (c) and its correlation with *in vivo* bioluminescence imaging (d).

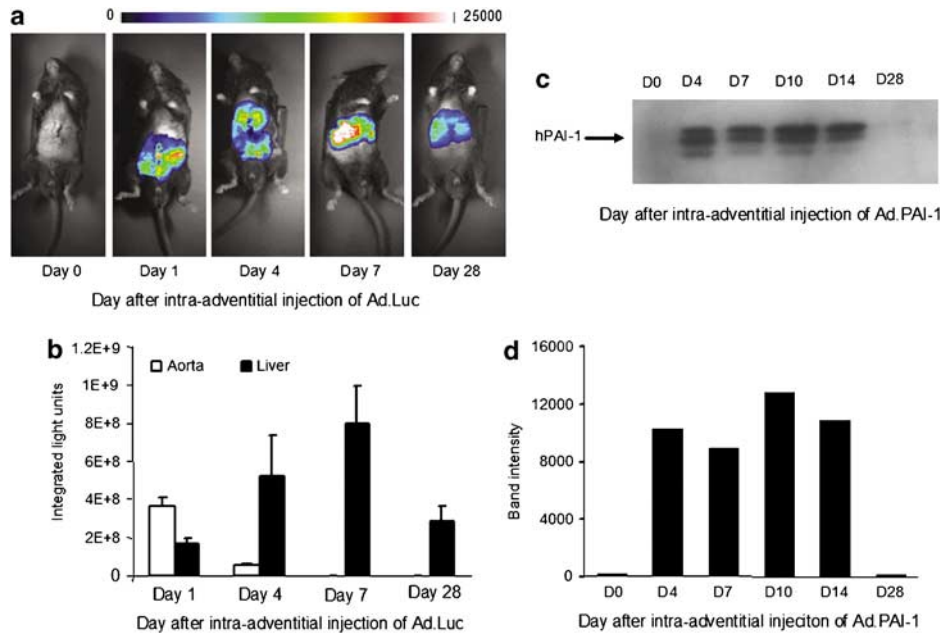


Figure 2 Expression, distribution and duration of luciferase and human PAI-1 in aorta and liver following adventitial delivery of adenovirus. (a) 1×10^{11} viral particle of Ad5.CMV.Luc in 200 μ l of PBS was intra-adventitially injected into the aorta of the suprarenal region. The images were captured by *in vivo* bioluminescence imaging system before injection and at day 1, 4, 7 and 28 after virus injection for the detection of luciferase gene expression levels. (b) Luciferase activity from liver and aorta extracts in above animals. (c) 1×10^{11} viral particle of Ad5.PAI-1 in 200 μ l of PBS was injected into the peri-adventitial aorta of the suprarenal region in ApoE^{-/-} mice. Aortas were collected at day 4, 7, 10, 14 and day 28 after virus injection. Aorta lysates were prepared and used for the western blot analysis of human PAI-1 protein expression. The blot was incubated with a primary polyclonal antibody (rabbit anti-human PAI-1 antibody), detected with horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG (1:10 000), and then developed with Amersham ECL kit. (d) The intensity of the bands was quantitated on Storm 840 PhosphorImager System (Molecular Dynamics).

uPA plays a critical role in formation of experimental AAA.^{4,5} We therefore hypothesized that local overexpression of PAI-1 would decrease uPA activity and attenuate inflammation thereby preventing AAA formation. ApoE^{-/-} mice were infused with Ang II subcutaneously, and then 1×10^{11} viral particles of Ad5.CMV.PAI-1 in 200 μ l of PBS were injected into the perivascular tissue of the aorta. AAA formation was completely prevented in the Ad5.CMV.PAI-1 treated group ($n = 8$); whereas, 5 of 9 (55.6%) mice developed AAA in the Ad5.CMV.Luc control group ($P = 0.012$) (Figure 3). These results demonstrate that overexpression of PAI-1 in the vascular wall prevents AAA development induced by Ang II in ApoE^{-/-} mice.

Systemic delivery of Ad5.CMV.Luc and Ad5.CMV.PAI-1 in mouse. Adventitial delivery of Ad5.CMV.Luc into the abdominal aorta resulted in some systemic transgene distribution as evidenced by detectable gene expression in the liver (*in vivo* bioluminescence imaging). In order to evaluate systemic transgene expression, duration, and determine if prevention of AAA formation by adventitial PAI-1 gene delivery was due to local or systemic gene expression, 1×10^{11} viral particles of Ad5.CMV.Luc or Ad5.CMV.PAI-1 in 100 μ l of PBS, was injected into the tail vein at the time of Ang II infusion. The pattern and time course of luciferase expression measured by *in vivo* bioluminescence imaging system are shown in Figure 4. All of the luciferase expression detected by CCD camera was located in the liver, which is consistent with the result of our *in vivo* bioluminescence imaging and

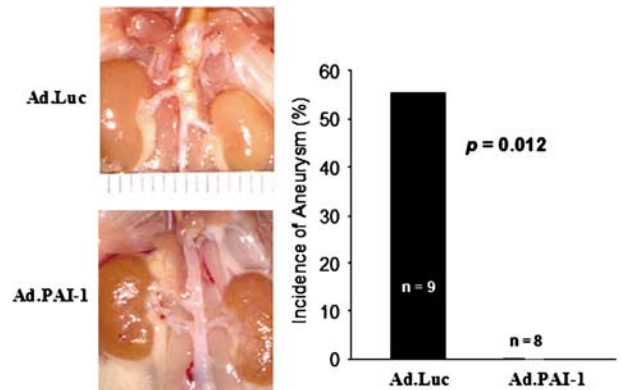


Figure 3 Incidence of aneurysm formation in Ang II-induced AAA model treated with local delivery of Ad5.CMV.PAI-1 and Ad5.CMV.Luc. Representative images (left panel) showing incidence of AAA formation in Ang II-infused ApoE^{-/-} mice treated with 1×10^{11} viral particle of Ad5.CMV.PAI-1 ($n = 9$) or Ad5.CMV.Luc ($n = 8$) by peri-adventitia injection. Total incidence rate was shown in right panel ($P = 0.012$).

confirmed by *in vitro* luciferase assay. As expected, systemic gene delivery leads to an earlier peak liver expression compared to adventitial gene delivery (at day 4 vs day 7, Figure 4).

There was no significant difference in the incidence of aneurysm formation between the Ad5.CMV.PAI-1 treated group and the Ad5.CMV.Luc control group ($P = 0.125$) (Figure 5). Thus, prevention of AAA

formation by adventitial PAI-1 gene delivery was due to local but not systemic gene expression.

Effects of PAI-1 on the progression of AAA

Treatment with Ad5.CMV.PAI-1 and progression of established aneurysm. Experiments were conducted to determine if Ad5.CMV.PAI-1 could prevent further expansion of an established aneurysm compared to Ad5.CMV.Luc. Male ApoE^{-/-} mice were implanted with subcutaneous osmotic mini-pumps and infused with Ang II (1.44 mg kg⁻¹ per day). Two weeks later, the abdominal aorta of each animal was surgically exposed,

photographed and injected with 1 × 10¹¹ viral particles containing either Ad5.CMV.Luc or Ad5.CMV.PAI-1 into the perivascular sheath surrounding the suprarenal region of the abdominal aorta. Three weeks later, the animals were killed, and the suprarenal segments of the abdominal aorta were harvested for evaluation. Representative pictures on day 14 (before PAI-1 treatment) and day 35 (21 days after PAI-1 treatment) are shown in Figure 6a. The diameters of the suprarenal aorta of the ApoE^{-/-} mice, before (2 weeks) and after (5 weeks) treatment with Ad5.CMV.PAI-1 or Ad5.CMV.Luc, were measured. The diameter ratios (5 weeks vs 2 weeks) are shown in Figure 6b. The mean ratio was not

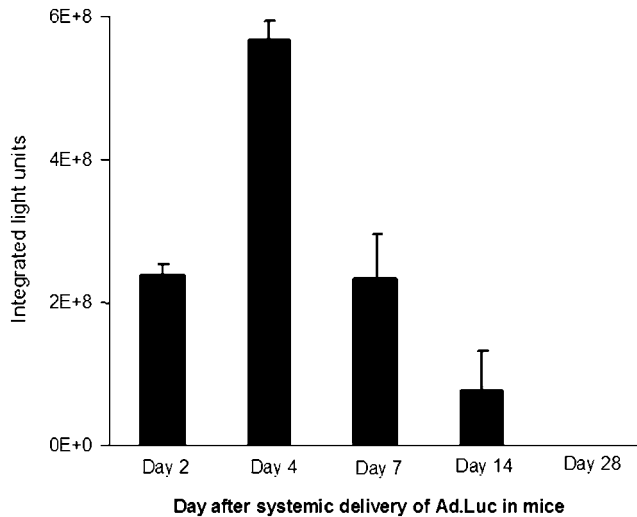


Figure 4 Gene expression and duration following systemic delivery of Ad5.CMV.Luc. Eight to ten month old ApoE^{-/-} male mice were injected with 1 × 10¹¹ viral particle of Ad5.CMV.Luc in 100 μl of PBS through tail vein. The images were captured by CCD camera at day 2, 4, 7, 14 and 28 after virus injection for the detection of luciferase expression.

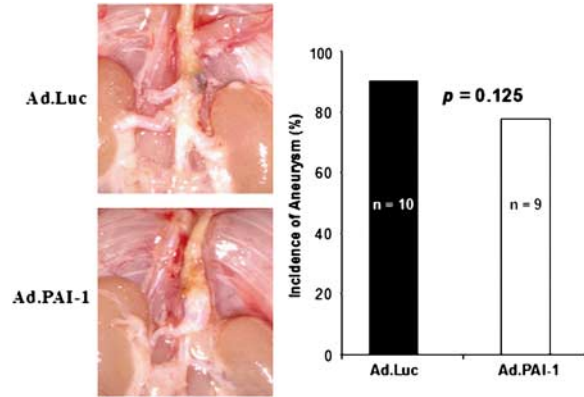


Figure 5 Incidence of aneurysm formation in Ang II-induced AAA model after systemic delivery of Ad5.CMV.PAI-1 and Ad5.CMV.Luc. Ad5.CMV.PAI-1 was systemically delivered by tail vein injection into ApoE^{-/-} mic. After gene transfer, each mouse was implanted subcutaneously an osmotic minipump containing Ang II. At 28 days, mice were killed for the incidence of AAA. Left panel shows representative image of AAA, and right panel shows incidence of AAA after treatment with Ad5.CMV.PAI-1 (n = 9) or Ad5.CMV.Luc (n = 10) (P = 0.125).

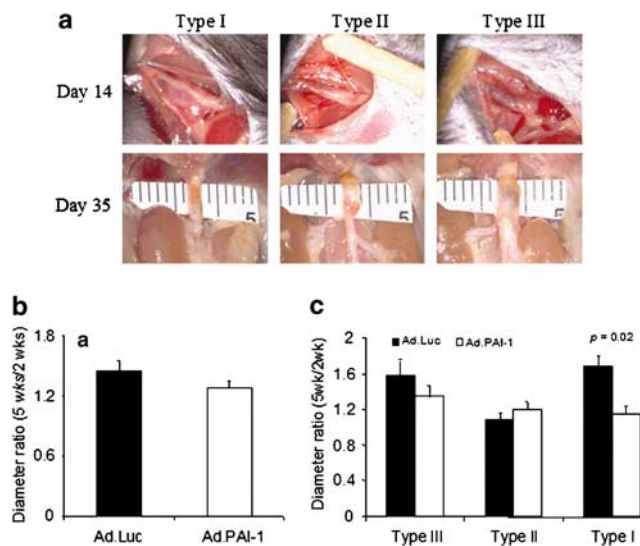


Figure 6 Incidence of aneurysm formation in the treatment study. The representative aneurysm image at day 14 (before PAI-1 treatment) and day 35 (21 days after PAI-1 treatment) were shown in (a). The reduction of the overall diameter ratio (5w/2w) was not statistically significant between treatment with Ad5.CMV.PAI-1 and Ad5.CMV.Luc (b). When the abdominal aortic aneurysms were subgrouped according to their initial size at week 2 (type I, II and III), Ad5.CMV.PAI-1 treatment did significantly reduce the progression of the type I aneurysm, and had no effect on the progression of type II and III aneurysm as compared to Ad5.CMV.Luc treatment (c).

statistically significant between groups. However, when the aneurysms were subgrouped according to their initial size at week 2 (type I, II and III), Ad5.CMV.PAI-1 treatment significantly reduced the progression of type I aneurysms, but had no significant effect on the progression of type II and III aneurysms compared to Ad5.CMV.Luc treatment (Figure 6c). These results indicate that PAI-1 modulates AAA progression during the early stages of the disease but less effective in more advanced stages.

Effects of PAI-1 on vascular histopathology

Ang II resulted in atherosclerotic lesions that were fibrotic and contained significant numbers of macrophages in Ad5.CMV.Luc treated sections. Similar histological changes were also observed in the established aneurysms subsequently treated with Ad5.CMV.PAI-1. Areas of hemorrhage were also noted in many instances. In some cases, there was break down of the medial layer with true aneurysm formation (Figure 7a). The overall extent of atherosclerosis and medial elastin lysis was not affected by PAI-1 treatment in the established aneurysm. However, a significant reduction was seen ($P = 0.023$) in adventitial lesions with the PAI-1 treatment (Figure 7b), as shown by a decrease in inflammatory cells.

Effects of PAI-1 on aortic matrix metalloproteinase activity

Zymographic analysis of MMP activities in the lysates of suprarenal aortas showed that activities of pro-MMP-9 and activated MMP-2 were decreased in animals treated locally with Ad5.CMV.PAI-1 compared to Ad5.CMV.Luc. There were no differences in pro-MMP-2 activity between groups (Figures 8a and b).

Discussion

In this study we assessed the role of PAI-1 in Ang II-induced AAA formation and progression in ApoE^{-/-} mice. The major findings were (1) Local aortic overexpression of PAI-1 prevented AAA formation and further expansion of early stage aneurysms and (2) PAI-1 overexpression reduced the number of inflammatory cells and MMPs activity in the aorta of ApoE^{-/-} mice before and after early AAA formation. These results indicate that PAI-1 plays an important role in the formation and early progression of AAA in this mouse model probably by inhibiting inflammation and protease activity in the vascular wall. However, the protective effects of PAI-1 on AAA were no longer apparent once larger, more complex aneurysms had formed.

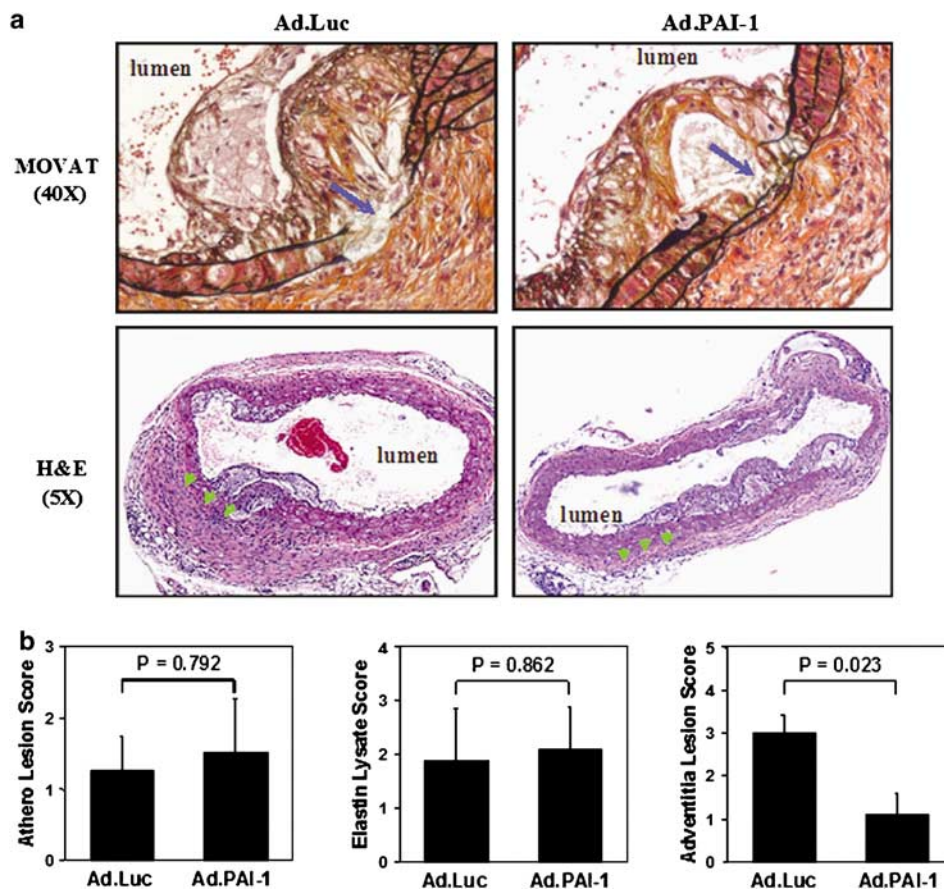


Figure 7 Histological analysis from week 5 suprarenal aortas in Ang II-induced AAA model after adventitial injection of Ad5.CMV.PAI-1 and Ad5.CMV.Luc in treatment study. Animals were given Ang II for 2 weeks before injection of Ad5.CMV.PAI-1 and Ad5.CMV.Luc. Representative images were shown in (a). Twenty-one days after treatment with PAI-1 or Luc, suprarenal segment of the abdominal aorta were sectioned, and stained with H&E, typical elastin degradation was indicated in MOVAT-stained sections. Perivascular infiltration was indicated in H&E-stained sections (a). Atherosclerosis scores, elastin lysate score and adventitial lesion score (see Materials and methods) were shown in (b).

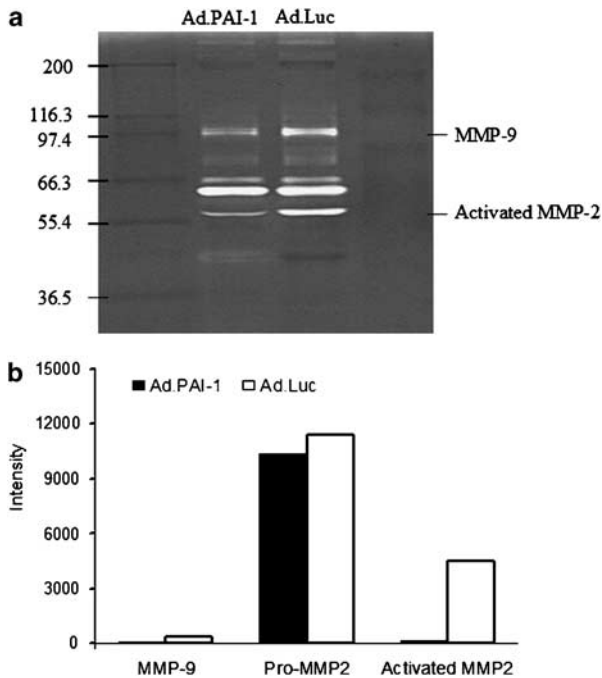


Figure 8 Gelatin zymographic analysis of MMP2 and MMP9 activities in aorta. Abdominal aortic segments isolated from Ad5.CMV.PAI-1 and Ad5.CMV.Luc treated mice were used for protein extraction and gelatin zymography analysis. Proforms and activated MMP-2 and MMP-9 were visualized by staining with 0.01% Coomassie blue R-250. The intensity was quantitated on a Storm 840 PhosphorImager.

The effects of PAI-1 on the response to vascular injury in experimental models reported in the literatures are not consistent. Carmeliet *et al.*^{11,29} demonstrated increased neointima formation after electrical or mechanical injury in PAI-1^{-/-} mice, which was prevented by overexpression of PAI. Consistent with these results, de Waard *et al.*³⁰ reported an increase in neointima formation in PAI-1^{-/-} mice after carotid artery ligation; and Allaire *et al.*³¹ showed that local overexpression of PAI-1 prevented aneurysm development in a xenograft model. In contrast to these findings, Ploplis and Castellino³² reported that copper-cuff-induced neointima formation was decreased in PAI-1^{-/-} mice compared to wild type controls and DeYoung *et al.*³³ reported that overexpression of PAI-1 gene in the carotid artery enhanced neointima growth following balloon injury in rats. In a recent study, transplantation of wild type bone marrow in PAI-1 deficient mice significantly suppressed neointima formation after vascular injury with ferric chloride.³⁴ In this nonatherosclerotic models, PAI-1 inhibited neointimal proliferation after arterial injury. In the present study, we used the atherosclerotic ApoE^{-/-} model with infusion of Ang II. Ang II has been shown in this model to promote progression of atherosclerotic lesions and to induce AAA formation.^{13,35} The aneurysms observed in this study were similar to those described previously in this model⁴ and similar to those occurring in humans.

The adventitial of vascular wall has been shown to participate in remodeling and neointima formation after arterial injury.^{36,37} The adventitial also may influence atherosclerosis and vascular hypertrophy secondary to hypertension.³⁸ Because of the advantages that adventitial gene transfer avoids interruption of blood flow and

disruption of the endothelium, as a target for site-specific vascular wall therapy, adventitial-specific gene transfer through the delivery of adenoviral vectors into the peri-arterial sheath has been demonstrated in different animal models.^{37,39,40} Adventitial delivery of vectors for gene transfer may be achieved easily in large peripheral arteries by direct application during the surgery.³⁸

Local overexpression of PAI-1 inhibited progression of type I aneurysms and reduced the degree of adventitial inflammation (Figures 6 and 7). Although PAI-1 inhibited progression of type I aneurysms, there was no effect on type II or III aneurysms (Figure 6c) and also no effect on the degree of vascular wall inflammation. Interestingly, although PAI-1 overexpression slowed progression of type I AAA, it did not significantly affect the degree of atherosclerosis and medial elastin degradation in the more advanced aneurysms. However the overall adventitial inflammatory score was significantly reduced in the PAI-1 treated mice compared to the Ad5.CMV.Luc treated controls ($P=0.0263$) (Figure 7). These data are consistent with our previous observation that the inflammatory infiltration appeared starting from the adventitia and migrating inward to the media.⁴ The Ad5.CMV.PAI-1 viral particles should have infected the fibroblasts and smooth muscle cells to form a barrier for inflammatory infiltration. Once such infiltration was abundant, the PAI-1 gene delivery would be less effective. This hypothesis is also in agreement with the observation made in the xenograft model, that pretreatment of the artery with PAI-1 gene blocked the development of aneurysm.³¹ In more advanced aneurysmal lesions, the vascular wall remodeling may also influence local PAI-1 gene expression and distribution after viral delivery, affecting PAI-1 efficacy. Taken together, these observations suggest that the anti-inflammatory properties of PAI-1 play an important role in preventing AAA formation and progression of early aneurysm lesions.

The mechanism for the reduction in adventitial inflammation by PAI-1 may be multifaceted. PAI-1 is known to block uPAR-mediated inflammation. We have shown previously that overexpression of uPAR promotes macrophage infiltration into the vascular wall of ApoE^{-/-} mice with Ang II-induced AAA.²⁷ Previous studies have shown that PAI-1 can complex with uPA, uPAR and low density lipoprotein-related protein 1 (LRP1) at the cell surface and mediate internalization of the complex.^{15,41} Thus, PAI-1 might modulate inflammatory responses by blocking uPAR-mediated macrophage infiltration into the vascular wall. It was known that MMPs played an important role in aneurysm development.^{42,43} As the matrix protein degradation and subsequent weakening of the aortic wall are the chief features of AAA, the association of MMP activities and aneurysm has been established by many studies both in animal models^{4,44} and in human.⁴⁵ MMPs are produced as zymogens mostly by activated macrophages in the vascular wall. Serine proteases uPA and plasmin was known to initiate the activation of proMMPs.⁴⁶ We showed in this study that the activation of both MMP-9 and MMP-2 was suppressed by the expression of PAI-1 (Figure 8). These observations demonstrated a complex role of PAI-1 in the development of aneurysm.

The results of this study suggest the possibility that local PAI-1 gene transfer could be used as a new

treatment paradigm to prevent progression of small aneurysms. Prevention of AAA expansion would be expected to reduce the risk of rupture. Local gene delivery would have the advantage of achieving efficacious levels of PAI-1 at the site of disease while minimizing potential systemic anti-fibrinolytic effects of PAI-1.

Materials and methods

Animals

Eight to ten month old male ApoE^{-/-} mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for the experiments. Animals were kept in rooms under controlled temperature (24 °C) and lighting (14:10 h light-dark cycle) conditions with free access to food (normal chow) and water. The experiments were conducted according to the protocols approved by the Animal Care and Use Committee at Berlex Biosciences, in agreement with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Mouse AAA model

Osmotic minipumps (model 2004, Alzet, Cupertino, CA) containing Ang II were implanted subcutaneously into male ApoE^{-/-} mice as reported previously.⁴ The rate of Ang II infusion was 1.44 mg kg⁻¹ per day.

In vivo bioluminescence imaging

In vivo luciferase expression, distribution and duration were determined noninvasively using a bioluminescence imaging system. The system consisted of a CCD camera (CCCD Model LN/CCD-1300EB, Roper Scientific, Princeton Instruments, Trenton, NJ, USA) equipped with a 50-mm Nikon lens, a light-tight specimen chamber, a camera controller (ST-133) and a computer for data analysis. This device can be used for repeated non-invasive measurement to detect and quantify the level of bioluminescent photons that are transmitted from internal organs of living small animals placed in a dark box.

For *in vivo* imaging, ApoE^{-/-} mice or 3 month old BABL/C mice were anesthetized with a 4:1 mixture of ketamine (80 mg kg⁻¹ body weight) and xylazine (10 mg kg⁻¹ body weight) intraperitoneally. After 5–10 min of anesthetic induction, an aqueous solution of the substrate, beetle luciferin (Promega Corporation, Madison, WI, USA) was injected into the peritoneal cavity (25 mg ml⁻¹ at a dose of 126 mg kg⁻¹ body weight). The animals were immediately placed in a light-tight chamber and baseline gray-scale body-surface images were taken. Afterwards, photons emitted from the firefly luciferase reaction within the animal were acquired repetitively (5–15 images per animal, 2 min acquisition per image) until passage of the peak relative light unit (RLU) was confirmed. Following imaging, ApoE^{-/-} mice were kept warm with lamps and allowed to recover before returning to the vivarium. To analyze the correlation between *in vivo* bioluminescence imaging and *in vitro* luciferase assay, all BABL/C mice were killed 4 h after imaging to test *in vitro* luciferase activity in liver.

In vitro luciferase assay

To quantitate expression of the reporter gene luciferase, mice were killed on day 4 after Ad5.CMV.Luc transduction. Livers were removed and homogenized in 4 ml g⁻¹ tissue of Reporter Lysis buffer (Promega Corporation). After freeze thawing three times at -80 °C for 15 min each, the homogenate was centrifuged at 14 000 rpm for 15 min. Firefly luciferase activity was measured using 20 µl supernatant with 100 µl Luciferase Assay Reagent (Promega). The results were normalized to RLU per milligram of protein as measured by the Bio-Rad Protein Assay System (Bio-Rad, Hercules, CA, USA). Results from the *in vitro* assays were correlated with the maximum RLU per minute obtained from the CCD camera for all mice.

Effects of PAI-1 on AAA formation

Adventitial gene delivery. To evaluate the effect of local perivascular overexpression of the PAI-1 gene in the ApoE^{-/-} mouse AAA model, Ad5.CMV.Luc or Ad5.CMV.PAI-1 (a kind gift from Dr Carmeliet)²⁹ was injected directly into the adventitial space on the same day as implantation of osmotic minipumps containing Ang II. The suprarenal region of the aorta was exposed through a middle abdominal incision. A 30-gauge needle was inserted into the aortic adventitial space between the diaphragm and the renal artery, and 1 × 10¹¹ viral particles of Ad5.CMV.Luc or Ad5.CMV.PAI-1 in 200 µl of PBS infused. As the solution was being injected, the needle was gently advanced, distending the perivascular sheath to a length of 0.5 cm. After the needle was withdrawn, the abdominal incision was closed.

Systemic gene delivery. To evaluate the effect of systemic delivery of the PAI-1 gene in the ApoE^{-/-} mouse AAA model, Ad5.CMV.Luc or Ad5.CMV.PAI-1 was injected intravenously, immediately following implantation of Osmotic minipump containing Ang II. A total of 1 × 10¹¹ viral particle of Ad5.CMV.Luc or Ad5.CMV.PAI-1 in 100 µl of PBS was injected through the tail vein. To evaluate transgene expression and duration, the real time expression of luciferase was measured by *in vivo* bioluminescence imaging system.

Four weeks later, the animals were euthanized, the diameter of the suprarenal segment of the abdominal aorta were measured to evaluate efficacy of systemic gene therapy with PAI-1.

Effects of PAI-1 on progression of established AAA

Osmotic minipumps containing Ang II (1.44 mg kg⁻¹ per day) were implanted into the male ApoE^{-/-} mice for 2 weeks prior to gene delivery. The diameters at the maximal expanded portion of the suprarenal aorta were marked and measured to establish the baseline diameter. After the measurement was completed, a total of 1 × 10¹¹ viral particles of Ad5.CMV.Luc or Ad5.CMV.PAI-1 in 200 µl of PBS were immediately injected into the aortic adventitial space between the diaphragm and the renal artery.

Three weeks following gene transfer, the animals were euthanized, and the diameter of the suprarenal segment of the abdominal aorta measured. The aorta was harvested, placed in formalin and processed for histological evaluation.

Measurement of diameters of suprarenal aorta. In the ApoE^{-/-} mice, the cross-sectional diameters of the suprarenal aorta were measured as described previously.⁴ After the aorta was dissected free from the surrounding connective tissue, images were obtained with a digital camera. The image of the aorta was used to measure the outer diameter of the suprarenal aorta. In some groups, the outer diameters of the thoracic aorta immediately above the diaphragm and the abdominal aorta immediately below the right renal artery were also measured using the same method.

Classification of aneurysm. A four-point grading system was used to classify aneurysms as described in detail by Daugherty *et al.*¹³ type 0, no aneurysm; type I, dilated lumen in the suprarenal region of the aorta with no thrombus; type II, remodeled tissue in the suprarenal region that frequently contained thrombus; type III, a pronounced bulbous form of type II that contained thrombus; and type IV, a form in which there are multiple aneurysms containing thrombus. No animals were found to have a type IV aneurysm in the present study.

Western blot for human PAI-1. Mouse aneurysm segments were homogenized in tissue extraction buffer (50 mM phosphate buffer, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 tablet proteinase inhibitor/10 ml lysis buffer) to extract proteins. Protein concentrations were determined by BCA protein quantification kit from Pierce (Rockford, IL, USA). A total of 40 µg of proteins mixed with 2 × SDS sample buffer were loaded to each lane, and separated on 4–12% SDS-PAGE gel. The proteins were then transferred to polyvinylidene difluoride membrane (Invitrogen Corporation, Carlsbad, CA, USA). The membrane was blocked with 10% solution of nonfat dried milk in tris-buffered saline Tween 20 (TBST) (50 mM Tris-HCl, pH7.4, 150 mM sodium chloride, 0.05% Tween 20) for 1 h at room temperature, then incubated with rabbit anti-human PAI-1 antibody (2 µg ml⁻¹ in TBST, from Molecular Innovations Inc., Southfield, MI, USA) overnight at 4 °C. After wash three times, the membrane was subsequently incubated with horseradish peroxidase (HRP)-donkey anti-rabbit IgG (1/15 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blot was then developed by enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA). The intensity of the bands was quantitated with Storm 840 PhosphorImager System (Molecular Dynamics, Sunnyvale, CA, USA).

Gelatin zymography. Mouse AAA segments were pooled together (average of 10 mice) from each experimental group. Protein extracts were prepared from aortic tissues as described above. A total of 100 µg proteins were mixed with nonreducing 2 × SDS sample buffer, and subjected to separation on Novex 10% zymogram (precast with 0.1% gelatin) gel (Invitrogen Corporation). After electrophoresis, proteins were renatured by incubation with Triton X-100 for 30 min. The gels were then incubated overnight at 37 °C in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂. Pro and activated MMP-2 and MMP-9 were visualized by staining with 0.1% Coomassie blue R-250. The intensity of the bands was quantitated

with Storm 840 PhosphorImager System (Molecular Dynamics).

Histomorphometric evaluation. At the end of treatment study, animals were euthanized, and the suprarenal segment of the abdominal aorta was harvested, placed in 10% formalin. Following fixation, the samples were blocked into two or more cross-sectional segments. This was to ensure that at least one representative section would be sampled from the mid-lesion area. All samples from a single animal were placed together in a plastic tissue-processing cassette. The cassetted samples were dehydrated, cleared and infiltrated for paraffin embedding using Vacuum Infiltration Processor Program 2.5 µm sections were cut from the blocks and stained with Hematoxylin and Eosin Stain (H&E) and MOVAT. Quantitative analysis of pathological changes was evaluated using a graded histology scoring system as follows: *Atherosclerosis Score*: 1–4 = number of quadrants with atherosclerosis; 0.5 = focal cluster of macrophages. *Elastin lysate Score*: 4 = large breaks of elastic lamina with or without pseudo-lumen; 3 = focal areas of full thickness elastin lysis; 2 = one area of full thickness elastin lysis; 1 = partial elastin lysis; 0.5 = one elastin layer damaged; 0 = normal. *Adventitial Score*: 4 = severe fibrosis, inflammation and thrombosis with or without pseudo-lumen; 3 = severe fibrosis and inflammatory infiltration; 2 = moderate inflammatory infiltration; 1 = mild focal inflammatory infiltration; 0.5 minimal changes; 0 = normal.

Calculations and statistical analysis. All results are expressed as mean ± standard error of the mean. Statistical significance was evaluated using unpaired Student's test or analysis of variance (ANOVA) for comparisons between two means. A value of *P* < 0.05 was considered as statistically significant.

Acknowledgements

We express sincere thanks to Dr Peter Carmeliet (Center for Molecular and Vascular Biology, Department of Transgene Technology and Gene Therapy, KU Leuven, Belgium) for the gift of Ad5.CMV.PAI-1. We thank Ana Freay and Linda Cashion for technical assistance.

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