Cardiovascular, Pulmonary and Renal Pathology

Loss or Inhibition of uPA or MMP-9 Attenuates LV Remodeling and Dysfunction after Acute Pressure Overload in Mice

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Left ventricular (LV) hypertrophy is a natural response of the heart to increased pressure loading, but accompanying fibrosis and dilatation may result in irreversible life-threatening heart failure. Matrix metalloproteinases (MMPs) have been invoked in various cardiac diseases, however, direct genetic evidence for a role of the plasminogen activator (PA) and MMP systems in pressure overload-induced LV hypertrophy and in heart failure is lacking. Therefore, the consequences of transverse aortic banding (TAB) were analyzed in mice lacking tissue-type PA (*t-PA*^{-/-}), urokinase-type PA (*u-PA*^{-/-}), or gelatinase-B (*MMP-9*^{-/-}), and in wild-type (WT) mice after adenoviral gene transfer of the PA-inhibitor *PAI-1* or the MMP-inhibitor *TIMP-1*. TAB elevated LV pressure comparably in all genotypes. In WT and *t-PA*^{-/-} mice, cardiomyocyte hypertrophy was associated with myocardial fibrosis, LV dilatation and dysfunction, and pump failure after 7 weeks. In contrast, in *u-PA*^{-/-} mice or in WT mice after *PAI-1*- and *TIMP-1*-gene transfer, cardiomyocyte hypertrophy was moderate and only minimally associated with cardiac fibrosis and LV dilatation, resulting in better preservation of pump function. Deficiency of *MMP-9* had an intermediate effect. These findings suggest that the use of u-PA- or MMP-inhibitors might pre-

serve cardiac pump function in LV pressure overload-ing. (Am J Pathol 2005, 166:15–25)

Over ten percent of the industrialized population suffers severe hypertension, which predisposes to left ventricular (LV) hypertrophy, myocardial infarction (MI), arrhythmias, and congestive heart failure.¹ Despite improvements in anti-hypertensive treatment, therapy-resistant hypertension often leads to cardiac failure.

LV hypertrophy is a natural adaptation of the heart to increased pressure loading. This response may, however, progress to maladaptation and congestive heart failure when complicated by cardiomyocyte degeneration, LV fibrosis, and dilatation.^{2–7} The structural changes in myocardial extracellular matrix (ECM) during LV remodeling constitute a milestone in the progression to heart failure. At the myocyte level, laminin, collagen type-IV, and fibronectin in the basement membrane not only attach the cardiomyocyte to the interstitial ECM but also induce transmembrane signaling through integrin receptors.⁸ At the tissue level, interstitial collagen type-I and -III fibers interconnect, align, and organize myocytes and muscle fibers, allowing optimal force generation and transmission in the myocardium. When collagen fibrils and struts are lost, myocyte slippage, LV dilation, and progressive contractile dysfunction may occur.⁹

Remodeling of the myocardial ECM requires proteolysis. Support for the importance of the activity of matrix metalloproteinases (MMP) in the development of chronic heart failure has been demonstrated both in animal models of heart disease and in humans. Numerous papers indeed described the altered expression of interstitial

Supported in part by the European Union (Biomed BMH4 CT98 3380), by FWO, Belgium grant G0125.00, by an unrestricted Bristol-Myers-Squibb grant, by a Concerted Research Activities, Belgium grant GOA2001/09, by a Belgian Science Policy grant IAP-P5/02 and by a grant of the Netherlands Heart Foundation (NHS 2003To36).

Accepted for publication September 14, 2004.

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collagenase (MMP-1), stromelysin (MMP-3), 72-kd gelatinase (MMP-2), 92-kd gelatinase (MMP-9), TIMP-1, and TIMP-2, while also neutrophil-elastase (MMP-8), membrane-type-1 MMPs, and TIMP-4 seem substantially up-regulated during cardiac hypertrophy, remodeling, and failure.^{7,10–32} The functional importance of the MMP system in LV remodeling has been evidenced by genetic and pharmacological studies. Direct genetic evidence for a role in cardiac remodeling has been provided for MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-3. Chronic myocardial overexpression of *MMP-1* in mice produced loss of interstitial collagen associated with compensatory cardiac hypertrophy and cardiac dysfunction at older age,³³ while *MMP-9* as well as *MMP-2* deficiency in mice prevented cardiac rupture, and decreased cardiac dilatation and dysfunction after acute myocardial infarction.^{34–36} In addition, we recently demonstrated that increased MMP-2 activity in absence of *TSP-2* predisposed to cardiac rupture and dilated cardiomyopathy during hypertension.³⁷ Instead, gene deletion of *TIMP-1* caused spontaneous LV dilatation³⁸ and amplified the hypertrophic response and adverse LV remodeling after myocardial injury, thereby emphasizing the importance of local endogenous control of cardiac MMP activity by TIMP-1.^{39,40} Also, loss of *TIMP-3* function triggered spontaneous LV dilatation, cardiomyocyte hypertrophy, and contractile dysfunction.⁴¹ Broad-spectrum pharmacological inhibition of MMPs significantly attenuated myocardial remodeling associated with chronic volume overload, hypertension, myocardial infarction, or other conditions in animal models,^{26,42–50} while selective MMP inhibition, eg, sparing MMP-1, attenuated LV remodeling after MI or pacing.^{51–54}

Also, other non-MMP proteinases, including serine elastase, the cysteine protease, caspase-1, the major serine proteases released by mast cells, chymase and tryptase, and the lysosomal cysteine peptidase, cathepsin, have been implicated in cardiac ECM remodeling in response to myocardial infarction or during viral myocarditis.^{55–62} Particularly relevant is the plasminogen system, with its plasminogen activators (PA), eg, tissue-type PA (t-PA) and urokinase-type PA (u-PA) and its PA-inhibitor (PAI-1), as plasmin activates pro-MMPs in the cardiovascular system.^{34,63} The implication of this system in myocardial tissue degradation, leading to congestive heart failure, has received little attention thus far.^{34,64,65} Although u-PA activity in the LV is increased after pressure overload,^{66,67} a functional implication in LV remodeling has not been demonstrated. A single study provided circumstantial evidence that reduced inactivation of PAI-1 in myeloperoxidase-deficient mice diminished LV remodeling after myocardial infarction,⁶⁸ while another recent paper showed that increased expression of *PAI-1* contributes to myocardial fibrosis after myocardial infarction.⁶⁹ Thus, the role of t-PA or u-PA in the progression of the initially adaptive to maladaptive cardiac response to acute pressure overload, and the potential of *PAI-1* to attenuate LV remodeling, remain to be elucidated. Similarly, while LV remodeling after myocardial infarction has been studied in *MMP-9*-deficient mice, the role of MMP-9 or TIMP-1 in LV remodeling after acute pressure overload has not been studied at the genetic

level. We therefore addressed these outstanding issues in the present study.

Materials and Methods

Mice

Ten-to fourteen-week-old male C57Bl6(75%)/129Sv(25%) mice lacking *u-PA* or *t-PA*, CD1(50%)/129Sv(50%) mice lacking *MMP-9* (gift from Dr. S.D. Shapiro, Washington University School of Medicine, St. Louis, MO) and their genetically identical wild-type (WT) mice, weighing 27 to 30 g, were used. Animals were maintained in an open animal facility and experiments were performed according to the guidelines for the care and use of laboratory animals approved by the institutional ethical animal care committee.

Transverse Aortic Banding

Transverse aortic banding (TAB) was performed as previously described.⁷⁰ Briefly, after anesthetizing by isoflurane inhalation (induction with 3% isoflurane in 100% O₂ and maintenance with 1.5% isoflurane), mice were intubated, ventilated, and a sternotomy was performed. The transverse aortic arch was ligated between the innominate and left common carotid arteries with an overlying 27-gauge needle, which after removal of the needle left a reproducible discrete region of stenosis. Sham-operation included all procedures, except constriction of the aorta. Perioperative (24 hours) and 1-week mortalities were each ~20% in all genotypes.

PAI-1 and TIMP-1 Gene Transfer

Two days before constriction of the transverse aorta, 100 μ l of 1.3×10^9 plaque-forming units (pfu) of the replication-deficient adenovirus expressing human *PAI-1* (Ad-*PAI-1*),⁷¹ Ad-*TIMP-1*,⁷² or control Ad-*RRS5*⁷³ virus was injected in the tail vein of C57BL6(75%)/129Sv(25%) WT mice. Five days after virus injection, PAI-1 or TIMP-1 plasma levels were measured in 100 μ l blood, sampled from the retro-orbital plexus, using a murine monoclonal antibody-based enzyme-linked immunosorbent assay for PAI-1,⁷¹ or using a commercially available ELISA for TIMP-1 (Amersham Biosciences, Amersham, UK).

Morphology and Histology

After the study period, constricted or sham-operated mice were anesthetized and perfused via the abdominal aorta with a 0.9% saline solution until the blood was removed. Lungs, left, and right ventricle were dissected, blotted dry, and weighed. To measure heart weight/body weight ratio, actual left ventricular mass was normalized to body mass just before TAB. Left and right ventricles were then post-fixed in 1% phosphate-buffered paraformaldehyde overnight, dehydrated, and embedded in paraffin. Four- μ m thick transverse sections were made for histological analysis or for immunostaining as de-

scribed.⁷⁴ Immunostaining was performed using antibodies against thrombomodulin (gift from Dr. R. Jackman, Department of Health Sciences, Boston University, Boston, MA), CD45 (Pharmingen, Becton Dickinson, Aalst, Belgium), ANF (Santa Cruz Biotechnology, Telri Bio, Bouchout, Belgium), and laminin (Sigma, Bornem, Belgium) as previously described.³⁴ Morphometric analysis was performed using a Quantimet Q 600 image analysis system (Leica, Brussels, Belgium). The average cardiomyocyte cross-sectional area was evaluated in the subendocardial layer, the central and the subepicardial layer of the mid-septum, and the mid-free wall of the left ventricle, on laminin-stained sections. Suitable cross sections of myocytes were defined as containing nuclei and having nearly circular capillary profiles. Approximately 300 cells were counted per sample and the average was used for analysis. Thrombomodulin-stained capillaries and CD45-stained leukocytes were counted in different layers through the left ventricle in at least 20 optical fields at $\times 40$ magnification. Collagen type-I and -III was stained using Sirius red, and the amount of collagen was quantified as percentage Sirius red stained area per total cardiac area, excluding fibrosis around large cardiac vessels.³⁴ Ultrastructural analysis was done as described.^{34,74}

Biochemical and Molecular Analysis

Levels of angiotensin-converting enzyme (ACE) activity were measured in serum and in left ventricular tissue extracts, using a spectrophotometric assay, with 3-(2-furylacryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate (Sigma). For ACE-activity measurement in cardiac tissue, the left ventricle was extracted in 400 μ l extraction buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4, 0.5% Triton-X 100, 25 μ mol/L ZnCl₂ and 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), and corrected for total protein level (Biorad). LV levels of latent and active TGF- β 1 were measured with a commercially available ELISA (R&D Systems).³⁴

Quantitative RT-PCR for atrial natriuretic factor (*ANF*) and α 1 pro-collagen type I (*Col 1a1*) was performed on RNA isolated from left ventricular tissue, as described.^{34,75} The following forward (F) and reverse (R) oligonucleotides, and probes (P) were used for real-time RT-PCR of *ANF*: (F: 5' GTG TAC AGT GCG GTG TCC AA 3'; R: 5' TCC CAG TCT GTG TCC CAC TTC 3'; P: 5' TGG CCC TAC CTT GAA ATC CAT CAG ATC TG 3'), and of *pro-Coll-1*: (F: 5' GCT TTG TGG ATA CGC GGA CT 3'; R: 5' CGT ACT GAT CCC GAT TGC AA 3'; P: 5' CAG TAA CTT CGT GCC TAG CAA CAT GCC AAT 3'). The relative expression levels of these genes were calculated by dividing their signals by the signals obtained for the *HPRT* gene.

Hemodynamic Measurements

Pressure measurements and M-mode echocardiography in mice were performed as described.^{34,76} Briefly, a 1.4 French high-fidelity catheter-tip micromanometer (SPR-671; Millar Instruments, Houston, TX) was inserted

through the right carotid artery into the left ventricular cavity to measure carotid and LV pressure. For M-mode echocardiography, animals were sedated with intraperitoneal ketamine (30 mg/kg) and diazepam (2.0 mg/kg) and transthoracic echocardiography was performed with a 12-MHz transducer (Hewlett Packard, Brussels, Belgium) on a Sonos 5500 (Hewlett Packard) echocardiograph. Views in the parasternal short-axis were obtained for guided M-mode measurements.

Statistical Analysis

All data are expressed as mean $\pm$ SEM. The Mann-Whitney *U*-test assessed statistical significant difference between groups, using two-sided *P* values.

Results

Loss of *u-PA* Attenuates LV Hypertrophy after Pressure Overload

Transverse aortic banding (TAB) was performed in wild-type (WT) mice and in mice lacking tissue-type plasminogen activator (*t-PA*^{-/-}), urokinase-type plasminogen activator (*u-PA*^{-/-}), or MMP-9 (*MMP-9*^{-/-}). Compared to sham-operated mice, left ventricular (LV) systolic pressure and right carotid arterial pressure increased by $\sim 40\%$ in all genotypes after TAB (Table 1). Increased loading resulted in significant LV hypertrophy in WT and *t-PA*^{-/-} mice, as evidenced by the $\sim 35\%$ increase of the LV/body weight ratio and the $\sim 40\%$ increase in the LV cardiomyocyte size (Figure 1, a and d; Table 2). Hypertrophy occurred primarily during the first 2 weeks and persisted for up to 7 weeks after TAB. Loss of *u-PA* did not abolish LV hypertrophy, but provided a significant protection against LV hypertrophy for at least 7 weeks, ie, the LV/body weight ratio increased by only $\sim 20\%$ and LV cardiomyocytes enlarged by only $\sim 22\%$ in *u-PA*^{-/-} mice (Figure 1, b and e; Table 2). LV hypertrophy was intermediate in hypertensive *MMP-9*^{-/-} mice (about 30% increase in cardiomyocyte size) (Figure 1, c and f; Table 2). Measurements of the thickness of the LV posterior and septal wall by M-mode echocardiography confirmed the genotypic differences in cardiac hypertrophy at 2 and 7 weeks after TAB (Table 3). Posterior and septal wall thickness increased in all genotypes after aortic banding but, compared to the other genotypes, the increase in wall thickness was the smallest in *u-PA*^{-/-} mice and intermediate in *MMP-9*^{-/-} mice (Table 3).

By ultrastructural analysis, cardiomyocytes appeared normal in WT and *u-PA*^{-/-} mice after sham-operation (Figure 2, a and b). At 4 days after TAB, the majority of LV cardiomyocytes in hypertensive WT mice appeared hypertrophied and contained prominent sarcomeres. There were signs of focal cardiomyocyte degeneration, atrophy, and myofibril disorganization, especially in the subendocardium and at the junction between the septum and anterior wall, ie, at sites of maximal mechanical stress (Figure 2, c, e, and f). ECM degradation and disorganization with interstitial edema were pronounced

Table 1. Hemodynamic Parameters after Aortic Banding (TAB)

	Arterial pressure (mmHg)	LV systolic pressure (mmHg)	Peak +dP/dt (mmHg/s)	Heart rate (beats/min)
Sham				
WT (u/t-PA)	86 ± 3	90 ± 4	7700 ± 670	560 ± 20
WT (MMP-9)	91 ± 4	89 ± 3	8100 ± 570	560 ± 30
<i>t-PA</i> ^{-/-}	89 ± 2	87 ± 4	7900 ± 510	540 ± 30
<i>u-PA</i> ^{-/-}	87 ± 6	86 ± 7	8200 ± 850	530 ± 40
<i>MMP9</i> ^{-/-}	93 ± 3	91 ± 4	8100 ± 540	530 ± 30
TAB 2 weeks				
WT (t/u-PA)	132 ± 5*	135 ± 5*	8100 ± 440	530 ± 10
WT (MMP-9)	134 ± 6*	134 ± 2*	8100 ± 310	530 ± 20
<i>t-PA</i> ^{-/-}	139 ± 4*	137 ± 6*	7900 ± 530	550 ± 10
<i>u-PA</i> ^{-/-}	136 ± 5*	138 ± 5*	11000 ± 890*†	550 ± 20
<i>MMP9</i> ^{-/-}	134 ± 3*	139 ± 3*	9300 ± 530	510 ± 30
TAB 7 weeks				
WT (u-PA)	127 ± 7*	123 ± 8*	6100 ± 610*	520 ± 20
WT (MMP-9)	126 ± 6*	124 ± 7*	6000 ± 590*	530 ± 10
<i>u-PA</i> ^{-/-}	134 ± 4*	136 ± 2*	10300 ± 70*†	540 ± 10
<i>MMP9</i> ^{-/-}	137 ± 3*	139 ± 4*	9200 ± 360*†	540 ± 20
TAB 2 weeks after gene transfer (WT mice)				
AdRR5	139 ± 5*	136 ± 4*	9700 ± 430	550 ± 10
AdPAI-1	138 ± 4*	137 ± 5*	11900 ± 450‡	560 ± 20
AdTIMP-1	145 ± 4*	142 ± 6*	12700 ± 790‡	550 ± 20

Sham, sham-operated mice; TAB, transverse aortic banding studied 2 and 7 weeks after operation; LV, left ventricular; WT, wild-type mice. *n* = 6 in sham-operated mice; *n* = 6 to 10 in banded mice. *, *P* < 0.05 in TAB as compared to sham mice; †, *P* < 0.05 in gene-inactivated mice as compared to wild-type mice after TAB; ‡, *P* < 0.05 in PAI-1 and TIMP-1-treated mice as compared to control RR5-treated mice after TAB.

in WT mice after TAB (Figure 2, e and f). In contrast, in banded *u-PA*^{-/-} mice, moderate cardiomyocyte hypertrophy but only minimal degenerative changes were detectable (Figure 2d).

Attenuation of cardiac hypertrophy in *u-PA*^{-/-} mice was also evidenced at 4 days after TAB by the reduced up-regulation of transcript levels of atrial natriuretic factor (ANF), a marker of cardiac hypertrophy,⁷⁷⁻⁷⁹ (number of mRNA copies/1000 *hprt* copies: 43 ± 3 in sham-operated WT hearts and 492 ± 69 in banded WT hearts *versus* 32 ± 5 in sham-operated *u-PA*^{-/-} hearts and 283 ± 25 in banded *u-PA*^{-/-} hearts; *n* = 5, *P* < 0.05).

Loss of *u-PA* Attenuates LV Inflammation and Fibrosis after Pressure Overload

Cardiac remodeling after TAB is associated with infiltration of inflammatory cells.^{80,81} Very few CD45⁺ leukocytes (<5 per mm²) were present in the sham-operated LV of WT and *u-PA*^{-/-} mice (not shown). After banding, numerous leukocytes infiltrated the LV in WT mice, while fewer leukocytes infiltrated the myocardium in *u-PA*^{-/-} mice (CD45⁺ leukocytes/mm² at 4 and 7 days after TAB: 120 ± 20 and 90 ± 8 in WT mice *versus* 46 ± 15 and 35 ± 7 in *u-PA*^{-/-} mice; *n* = 6; *P* < 0.05; Figure 3, a and b). Leukocyte infiltration was especially prominent at sites of focal myocyte degeneration and matrix fragmentation in the subendocardium and septal/anterior wall border (Figure 3c).

Aortic banding in WT mice also caused diffuse patchy interstitial fibrosis throughout the entire LV and septum, especially in the subendocardium and at the septal and anterior wall border (Figure 3, d and e). Quantification of Sirius red-stained area revealed that the myocardial col-

lagen content significantly increased (by ~50%) in WT, *t-PA*^{-/-}, and *MMP-9*^{-/-} mice within 2 weeks after TAB (Table 2). Hypertrophic LV from WT mice contained focal accumulation of numerous spindle-shaped cells, with ultrastructural characteristics of collagen-producing myofibroblasts, entrapped in abundant amounts of interstitial collagen and other matrix components (Figure 3, f and g). In contrast, the increase in collagen-rich ECM and spindle-shaped myofibroblasts was much smaller in the LV of hypertensive *u-PA*^{-/-} mice (Figure 2d; Table 2). Reduced interstitial fibrosis in *u-PA*^{-/-} mice was also shown by RT-PCR data for 1α pro-collagen type I (*Col 1a1*) at 4 days after TAB. Indeed, 1α pro-collagen type I (*Col 1a1*) transcript levels, while comparable in sham-operated WT and *uPA*^{-/-} mice (1α pro-collagen type I (*Col 1a1*)/1000 *HPRT* mRNA copies: 125 ± 9 in WT mice *versus* 104 ± 14 in *uPA*^{-/-} mice; *n* = 5, *P* = NS), were up-regulated in *u-PA*^{-/-} hearts to only 40% of the levels in WT hearts at 4 days after TAB (pro-Coll-1/1000 *HPRT* mRNA copies: 2340 ± 406 in WT mice *versus* 940 ± 129 in *uPA*^{-/-} mice; *n* = 5, *P* < 0.05).

Attenuated Increase of Cardiac Levels of TGF-β1 and ACE in *u-PA*^{-/-} Mice

Additional evidence that the program of cardiac fibrosis was only minimally activated in hypertensive *u-PA*^{-/-} mice was provided by measuring the myocardial levels of the angiotensin-converting enzyme (ACE) and transforming growth factor-β1 (TGF-β1), both implicated in cardiac fibrosis.^{2,82} At 14 days after TAB, myocardial ACE activity levels were increased 3.6-fold in WT mice (U/ml/mg protein: 3.3 ± 0.5 after sham *versus* 12 ± 2.2 after TAB; *n* = 7 to 10; *P* < 0.05), but only 1.9-fold in *u-PA*^{-/-} mice

Figure 1. LV hypertrophy is attenuated in the absence of u-PA or MMP-9. **a–c:** H&E staining of transverse sections through the heart. LV hypertrophy at 2 weeks after transverse aortic banding was pronounced in WT mice (**a**), attenuated in *MMP-9*^{−/−} mice (**c**) and clearly reduced in *u-PA*^{−/−} mice (**b**), (LV, left ventricular wall). **d–f:** Detail of the subendocardial area of the LV septal wall, showing marked hypertrophic growth of cardiomyocytes in WT mice (**d**) as compared to *u-PA*^{−/−} (**e**) and *MMP-9*^{−/−} mice (**f**). Bars, 500 μ m (**a–c**); 25 μ m (**d–f**).

(U/ml/mg protein: 2.8 ± 0.6 after sham versus 5.4 ± 0.7 after TAB; $n = 10$; $P < 0.05$). Cardiac levels of active TGF- β 1 (pg TGF- β 1/ μ g protein) were 16 ± 2 in WT mice versus 4 ± 1 in *u-PA*^{−/−} mice ($n = 8$; $P < 0.05$) at 7 days after TAB. The lower myocardial levels of ACE and TGF β -1 in hypertensive *u-PA*^{−/−} mice may be attributable to the reduced accumulation or activation of cardiac fibroblasts and leukocytes, which produce these factors.^{2,82,83}

Preserved Myocardial Vascularization in *u-PA*^{−/−} Mice after Pressure Overload

To evaluate the consequences of the increased pressure loading on the coronary vasculature, vessels in WT and *u-PA*^{−/−} mice were immunostained for the endothelial cell marker thrombomodulin (TM) at 2 weeks after TAB. A comparable capillary density was present in the LV of both genotypes after sham-surgery (capillaries per mm²:

6100 ± 260 in WT versus 6000 ± 310 in *u-PA*^{−/−} mice; $n = 6$; $P = \text{NS}$). Capillary density was more extensively reduced in WT than in *u-PA*^{−/−} mice after TAB (capillaries per mm²: 3600 ± 380 in WT versus 4800 ± 190 in *u-PA*^{−/−} mice; $n = 6$; $P < 0.05$). The decrease in capillary density was strongest in the subendocardial area (capillaries per mm²: 3200 ± 230 in WT versus 4100 ± 210 in *u-PA*^{−/−} mice; $n = 6$; $P < 0.05$). In addition, intercapillary distances were larger in WT than in *u-PA*^{−/−} mice after banding (9.4 ± 0.5 μ m in WT versus 9.6 ± 0.6 μ m in *u-PA*^{−/−} mice after sham-surgery; $n = 6$; $P = \text{NS}$; 13.4 ± 0.8 μ m in WT versus 10.4 ± 0.8 μ m in *u-PA*^{−/−} mice after TAB; $n = 6$; $P < 0.05$). The capillary-to-cardiomyocyte ratio, however, remained constant (1.2 ± 0.2 in WT versus 1.1 ± 0.2 in *u-PA*^{−/−} mice after sham-surgery; $n = 6$; $P = \text{NS}$; 1.0 ± 0.3 in WT versus 1.2 ± 0.1 in *u-PA*^{−/−} mice at 14 days after TAB; $n = 6$; $P = \text{NS}$), indicating that the reduced capillary density was due to the cardiomyocyte hypertrophy and not to capillary loss. Fibrosis around arterioles and larger coronary arteries was comparable in WT and *u-PA*^{−/−} mice (not shown). These findings suggest that WT cardiomyocytes, because of their increased hypertrophy, are at greater risk than *u-PA*^{−/−} cardiomyocytes to suffer insufficient oxygen delivery (ischemia).

Loss of u-PA Attenuates LV Dysfunction and Remodeling after Pressure Overload

When stressed by pressure overload, the heart initially improves its function compensatorily, but subsequently, the heart cannot sustain the overload and cardiac function progressively deteriorates, ultimately resulting in pump failure.^{5,6} In healthy WT mice, the initial improvement of cardiac function occurs rapidly, ie, within the first days, and is evidenced by an increased LV contractility (measured as dP/dt_{max}) which is capable of maintaining normal levels of fractional shortening.^{84,85} In the subsequent weeks, the initial gain of LV contractility is lost and LV contractility deteriorates below normal levels, resulting in progressively lower levels of fractional shortening.^{84,85} Consistent with these previous findings, we observed that LV contractility (dP/dt_{max}) was preserved at 2 weeks but subsequently impaired at 7 weeks after TAB, and was accompanied with a progressive loss of fractional shortening, as measured by M-mode echocardiography (Tables 1 and 3). In contrast, in *u-PA*^{−/−} mice, LV contractility was improved and fractional shortening levels were maintained at 2 weeks and even at 7 weeks after TAB (Tables 1 and 3), thereby resembling the compensatorily improved cardiac response, as it initially occurs in normal WT mice.^{84,85} Cardiac function was also better preserved in *MMP-9*^{−/−} mice, though the response was intermediate (Tables 1 and 3).

After 7 weeks of high blood pressure, WT mice exhibited obvious signs of LV systolic dysfunction and LV dilatation (Tables 1 and 3), with 3 of 10 WT mice even exhibiting signs of severe respiratory distress and pulmonary congestion (lung/body weight: 8.8 ± 1.1 mg/g at 7 weeks after TAB versus 5.3 ± 0.3 mg/g in sham-operated mice; $n = 11$; $P < 0.05$). In contrast, at 7 weeks after TAB,

Table 2. Morphological Characteristics after Aortic Banding (TAB)

	LV/body weight (mg/g)	LV myocyte area (μm^2)	LV collagen content (% of total area)
Sham			
WT (u/t-PA)	3.8 ± 0.2	139 ± 11	7 ± 1
WT (MMP-9)	3.6 ± 0.1	138 ± 9	7 ± 1
<i>t-PA</i> ^{-/-}	3.4 ± 0.3	142 ± 6	7 ± 1
<i>u-PA</i> ^{-/-}	3.5 ± 0.2	138 ± 8	6 ± 1
<i>MMP-9</i> ^{-/-}	3.6 ± 0.4	144 ± 9	7 ± 0.5
TAB 2 weeks			
WT (t/u-PA)	$5.2 \pm 0.3^*$	$228 \pm 12^*$	$14 \pm 2^*$
WT (MMP-9)	$5.4 \pm 0.4^*$	$233 \pm 10^*$	$14 \pm 3^*$
<i>t-PA</i> ^{-/-}	$5.5 \pm 0.3^*$	$253 \pm 15^*$	$15 \pm 2^*$
<i>u-PA</i> ^{-/-}	$4.4 \pm 0.4^{*†}$	$158 \pm 9^{*†}$	$8 \pm 1^{*†}$
<i>MMP-9</i> ^{-/-}	$5.0 \pm 0.3^*$	$197 \pm 15^*$	$12 \pm 1^*$
TAB 7 weeks			
WT (u-PA)	$5.9 \pm 0.3^*$	$241 \pm 7^*$	$12 \pm 3^*$
WT (MMP-9)	$5.7 \pm 0.2^*$	$243 \pm 8^*$	$11 \pm 1^*$
<i>u-PA</i> ^{-/-}	$4.3 \pm 0.2^{*†}$	$176 \pm 12^{*†}$	$7 \pm 1^{*†}$
<i>MMP-9</i> ^{-/-}	$5.1 \pm 0.3^{*†}$	$210 \pm 11^*$	$11 \pm 2^*$
TAB 2 weeks after gene transfer (WT mice)			
AdRR5	5.1 ± 0.1	220 ± 8	11 ± 0.5
AdPAI-1	$3.9 \pm 0.3^\ddagger$	$160 \pm 11^\ddagger$	$9 \pm 1^\ddagger$
AdTIMP-1	$3.8 \pm 0.2^\ddagger$	$150 \pm 6^\ddagger$	$7 \pm 1^\ddagger$

Sham, sham-operated normal mice; TAB, transverse aortic banding studied 2 and 7 weeks after surgery; LV, left ventricular; WT, wild-type mice. $n = 6$ in sham-operated mice; $n = 6$ to 10 in banded mice. *, $P < 0.05$ in TAB as compared to sham mice; †, $P < 0.05$ in gene-inactivated as compared to wild-type mice after TAB; ‡, $P < 0.05$ in PAI-1 and TIMP-1-treated mice as compared to control RR5-treated mice after TAB.

LV systolic dysfunction and dilatation were only marginally detectable in *u-PA*^{-/-} mice (Tables 1 and 3) without signs of pulmonary edema (lung/body weight: 5.7 ± 0.2 mg/g in *u-PA*^{-/-} mice after TAB versus 5.4 ± 0.3 mg/g in *u-PA*^{-/-} mice after sham-operation; $n = 10$; $P = \text{NS}$). The cardiac response of *MMP-9*^{-/-} mice to pressure overload was intermediate to that of WT and *u-PA*^{-/-} mice: LV dilatation was minimal and fractional shortening was preserved at 7 weeks after TAB (Table 3), with no signs of

pulmonary edema (lung/body weight ratio in mg/g: 5.4 ± 0.3 in *MMP-9*^{-/-} mice after TAB versus 5.5 ± 0.4 in *MMP-9*^{-/-} mice after sham-operation; $n = 10$; $P = \text{NS}$).

Thus, despite the greater cardiomyocyte hypertrophy in WT than *u-PA*^{-/-} mice, cardiac function was, overall, better preserved in *u-PA*^{-/-} than in WT mice. In WT mice, the increase in cardiomyocyte size was accompanied by an increase in intercapillary distance (and thus likely an impaired delivery of oxygen to the center of the myocyte)

Table 3. *In Vivo* Echocardiographic Assessment after TAB

	Posterior wall thickness (mm)	Septal wall thickness (mm)	LV diastolic dimension (mm)	LV systolic dimension (mm)	Fractional shortening (%)
Sham					
WT (t/u-PA)	0.67 ± 0.3	0.72 ± 0.1	3.7 ± 0.2	2.1 ± 0.3	44 ± 3
WT (MMP-9)	0.68 ± 0.2	0.71 ± 0.3	3.6 ± 0.1	2.1 ± 0.2	44 ± 4
<i>u-PA</i> ^{-/-}	0.64 ± 0.1	0.78 ± 0.05	3.7 ± 0.2	1.8 ± 0.2	45 ± 2
<i>MMP-9</i> ^{-/-}	0.64 ± 0.1	0.75 ± 0.05	3.7 ± 0.1	2.0 ± 0.1	46 ± 3
TAB 2 weeks					
WT (t/u-PA)	$0.96 \pm 0.1^*$	$1.5 \pm 0.05^*$	3.7 ± 0.3	$2.7 \pm 0.4^*$	$32 \pm 5^*$
WT (MMP-9)	$0.95 \pm 0.1^*$	$1.4 \pm 0.05^*$	3.6 ± 0.2	$2.8 \pm 0.4^*$	$34 \pm 4^*$
<i>u-PA</i> ^{-/-}	$0.75 \pm 0.05^{*†}$	$0.84 \pm 0.1^\ddagger$	3.7 ± 0.1	$2.0 \pm 0.7^\ddagger$	$48 \pm 6^\ddagger$
<i>MMP-9</i> ^{-/-}	$0.86 \pm 0.05^*$	$0.99 \pm 0.05^*$	3.6 ± 0.2	1.9 ± 0.2	46 ± 2
TAB 7 weeks					
WT (t/u-PA)	$0.91 \pm 0.1^*$	$1.1 \pm 0.1^*$	$4.3 \pm 0.2^*$	$3.3 \pm 0.1^*$	$26 \pm 2^*$
WT (MMP-9)	$0.92 \pm 0.1^*$	$1.2 \pm 0.1^*$	$4.2 \pm 0.2^*$	$3.1 \pm 0.1^*$	$26 \pm 1^*$
<i>u-PA</i> ^{-/-}	$0.81 \pm 0.1^*$	$0.83 \pm 0.1^\ddagger$	3.7 ± 0.3	$2.0 \pm 0.2^\ddagger$	$46 \pm 4^\ddagger$
<i>MMP-9</i> ^{-/-}	$0.89 \pm 0.2^*$	$1.0 \pm 0.05^*$	3.8 ± 0.1	$2.1 \pm 0.1^\ddagger$	$45 \pm 3^\ddagger$
TAB 2 weeks after gene transfer (WT mice)					
AdRR5	1.0 ± 0.1	1.2 ± 0.2	3.8 ± 0.4	2.7 ± 0.2	31 ± 2
AdPAI-1	$0.81 \pm 0.1^\ddagger$	$0.84 \pm 0.1^\ddagger$	3.5 ± 0.3	$2.0 \pm 0.1^\ddagger$	$43 \pm 2^\ddagger$
AdTIMP-1	$0.75 \pm 0.05^\ddagger$	$0.81 \pm 0.1^\ddagger$	3.7 ± 0.1	$2.1 \pm 0.2^\ddagger$	$42 \pm 3^\ddagger$

Sham, sham-operated mice; TAB, transverse aortic banding studied 2 or 7 weeks after operation; LV, left ventricular; AdRR5, control replication deficient adenovirus; AdPAI-1, replication deficient adenovirus carrying the human PAI-1 gene; WT, wild-type mice. $n = 6$ in sham-operated mice; $n = 6$ to 10 in banded mice. *, $P < 0.05$ in TAB as compared to sham mice; †, $P < 0.05$ as compared to WT mice after TAB; ‡, $P < 0.05$ in PAI-1 and TIMP-1-treated mice as compared to control RR5-treated mice after TAB.

Figure 2. Ultrastructural signs of hypertrophy and myocyte degeneration. **a–b:** Normal cardiomyocytes in sham-operated WT (**a**) and *u-PA*^{−/−} mice (**b**). **c–f:** At 4 days after TAB, hypertrophic growth of cardiomyocytes is prominent in WT mice (**c**) but minimal in *u-PA*^{−/−} mice (**d**). In WT mice, cardiomyocyte degeneration is evidenced by mitochondrial vacuolization (**arrowheads**) and myofibril disorganization (**arrows**) (**e**), and myocyte necrosis can be seen by fragmentation and edema of the interstitial matrix (Ma) (**f**). **Bars**, 10 μ m (**a–f**).

and by hemodynamically undesirable structural changes (eg, myolysis, fibrosis). In contrast, in *u-PA*^{−/−} mice, the modest cardiomyocyte hypertrophy, together with the preserved oxygen delivery and the minimal myocyte degeneration and interstitial fibrosis seemed to suffice to allow cardiac function to respond to the increased loading conditions. Loss of *MMP-9* caused similar but less striking changes as deficiency of *u-PA*.

PAI-1 or *TIMP-1* Gene Transfer Preserve Cardiac Function after Pressure Overload

An adenovirus expressing human *PAI-1* (Ad*PAI-1*), human *TIMP-1* (Ad*TIMP-1*), or a control Ad*RR5* adenovirus was injected intravenously 48 hours before TAB; this procedure is known to result in markedly elevated plasma levels of *PAI-1* and *TIMP-1* within 10 to 14 days.³⁴ Ad*PAI-1* elevated plasma *PAI-1* levels to 48 ± 5 μ g/ml and Ad*TIMP-1* increased plasma *TIMP-1* levels to 32 ± 9

Figure 3. Leukocytes and myofibroblasts mediated matrix remodelling. **a–b:** CD45 staining, revealing that infiltration of leukocytes in the LV at 4 days after TAB is significant in WT mice (**a**) but only minimal in *u-PA*^{−/−} mice (**b**). **c:** Ultrastructural analysis of the LV at 4 days after TAB, showing infiltration of leukocytes (**arrow**) into the endocardium at sites of myocyte degeneration and matrix degradation. **d–e:** Sirius red staining of collagen in the subendocardial region of the septal wall at 2 weeks after TAB, showing that collagen deposition in the LV is pronounced in WT mice (**d**) but only minimal in *u-PA*^{−/−} mice (**e**). **f–g:** Detail of the interstitial matrix in the LV of WT mice during hypertension, showing spindle-shaped myofibroblasts (MyoF) entrapped in large amounts of collagen (Co) and other matrix components (Ma). **Bars**, 50 μ m (**a**, **b**, **d**, and **e**); 10 μ m (**c**); and 5 μ m (**f–g**).

μ g/ml within 5 days after gene transfer, while human *PAI-1* or *TIMP-1* were undetectable after control virus transfer.

In general, overexpression of *PAI-1* or *TIMP-1* yielded a comparable degree of protection against TAB-induced LV dysfunction. Moreover, *PAI-1* gene transfer was as efficient as *u-PA* deficiency, while *TIMP-1* gene transfer was more effective than *MMP-9* deficiency, likely because *TIMP-1* also inhibits other MMPs than *MMP-9* alone. Because *PAI-1* and *TIMP-1* levels were only elevated for 10 to 14 days after adenoviral gene transfer, we limited our analysis to the 2-week time point only. In mice expressing elevated plasma levels of *PAI-1* or *TIMP-1*, TAB induced only a modest degree of LV hypertrophy

(see LV/body weight ratio, cardiomyocyte size, and LV wall thickness; Tables 2 and 3) and myocardial fibrosis (Table 2). In contrast to *AdRR5*-injected mice, where leukocytes massively infiltrated the heart after TAB, significantly fewer leukocytes accumulated in hearts of *AdPAI-1*- or *AdTIMP-1*-treated mice (CD45-stained leukocytes per mm²: 29 ± 6 in *AdPAI-1*; 27 ± 4 in *AdTIMP-1* versus 95 ± 10 in *AdRR5*-treated mice at 7 days after TAB; $n = 6$; $P < 0.05$). Moreover, because of the more modest cardiomyocyte hypertrophy, myocardial capillary densities were higher after *AdTIMP-1* and *AdPAI-1* than after *AdRR5* treatment (capillaries per mm²: 4700 ± 430 in *AdPAI-1*, 4900 ± 220 in *AdTIMP-1* versus 3700 ± 350 in *AdRR5*-treated mice at 14 days after TAB; $n = 6$; $P < 0.05$). *PAI-1* or *TIMP-1* overexpression did not, however, alter the capillary-to-cardiomyocyte ratio (not shown). Importantly, elevated *PAI-1* or *TIMP-1* plasma levels not only prevented the development of cardiac dilatation, they also allowed the stressed heart to improve its contractility ($dP/dt_{\max}$) and largely preserved the fractional shortening index (Tables 1 and 3). Taken together, gene transfer of PA or MMP proteinase inhibitors permitted the stressed heart to respond in a more beneficial way to the increased pressure loading, thereby mimicking the gene-inactivation studies.

Discussion

LV hypertrophy is a natural adaptation of the heart to pressure overload, which permits cardiomyocytes to generate the additional force required to accommodate the increased pressure load without compromising pump function. Unfortunately, the initially adequate response may become maladaptive when ischemic cardiomyocytes degenerate, fibrosis occurs, and LV remodeling develops, all contributing to a progressive decline of cardiac pump function.²⁻⁶ These structural and functional changes were observed in WT mice at 2 weeks, but predominantly at 7 weeks after transverse aortic banding. At 2 weeks, LV cardiomyocytes were hypertrophic, but the mice already exhibited initial signs of maladaptation, eg, cardiomyocyte degeneration, fibrosis, increased intercapillary distance, slight LV dilatation, and impaired fractional shortening. LV contractility was "apparently" normal at 2 weeks: since an increase in LV contractility would be expected to occur in a normally adapting heart (as observed in the *u-PA*^{-/-} mice; see below), the "apparently" normal LV contractility in WT mice at 2 weeks more likely reflects the already ongoing process of progressive LV deterioration. At 7 weeks, LV remodeling was more prominent, and LV contractility and fractional shortening were further impaired; overall, cardiac pump function was impaired to the extent that the mice suffered respiratory distress due to pulmonary congestion. In contrast, the cardiac response to pressure overload was remarkably different in *u-PA*^{-/-} mice or in WT mice after *PAI-1* gene transfer; cardiomyocytes were still capable of enlarging in size, though the degree of hypertrophy was less marked than in WT mice (see below). Unlike WT mice, *u-PA*^{-/-} mice showed only minimal signs of mal-

adaptation such as myolysis, fibrosis, increased intercapillary distance, or LV remodeling. Consequently, LV contractility was enhanced and *u-PA*^{-/-} mice were capable of preserving normal fractional shortening without signs of cardiac failure or pulmonary congestion. Thus, in the absence or on inhibition of uPA, the heart is capable of adapting in a more adequate manner to severe pressure overload, with only minimal signs of maladaptation occurring after 7 weeks.

How does u-PA affect the cardiac adaptation to pressure overload? One likely mechanism is that u-PA itself or via generation of plasmin is involved in the remodeling of the myocardial ECM.⁶⁴ MMPs are secreted as inactive pro-enzymes and first need to be activated; plasmin is a possible activator of pro-MMPs.^{34,63} u-PA may indeed affect the remodeling of the interstitial collagen matrix, which plays an important role in maintaining the interaction of cardiomyocytes with each other and their three-dimensional organization in the entire heart.^{2,20} When this interstitial collagen network is broken down, cardiomyocyte slippage may occur, resulting in LV dilatation. Our ultrastructural analysis revealed that the interstitial matrix in WT mice was broken down after pressure overload, preceding subsequent LV dilatation. In contrast, the interstitial matrix was better preserved in *u-PA*^{-/-} mice, which were protected against LV remodeling.

Another mechanism whereby u-PA might affect the cardiac response to pressure overload is by facilitating the infiltration of inflammatory cells and collagen-producing cardiac fibroblasts, as both cell types require u-PA to migrate into the damaged myocardium.³⁴ Fewer leukocytes and fibroblasts were present in *u-PA*^{-/-} mice, underscoring the critical role of this proteinase. We and others previously documented that u-PA may have a dual function; one related to proteolytic matrix breakdown, the other related to matrix production via proteolytic activation of growth factors, such as the fibrogenic TGF- β 1. This dual role of u-PA may explain why, at one side, ECM degradation and, at the other side, collagen deposition both were attenuated in the absence of u-PA.

Deficiency of *MMP-9* and gene transfer of *TIMP-1* also attenuated LV remodeling and dysfunction after pressure overload, with *TIMP-1* gene transfer being more efficient. This may not be surprising, since *TIMP-1* inhibits other MMPs in addition to *MMP-9*. Genetic and pharmacological studies indeed support the involvement of other MMPs in the development of chronic heart failure (see Introduction). All these findings suggest that various MMPs, each possibly having distinct functions, cooperate with each other in the cardiac response to pressure overload. Our results are also consistent with previous studies demonstrating a prominent role of *MMP-9* in myocardial ECM remodeling in cardiac rupture³⁴ and LV remodeling.³⁵ The similar phenotypes after *TIMP-1* and *PAI-1* gene transfer are also not surprising, when considering that u-PA-mediated plasmin activates the inactive pro-MMPs.⁸⁶

The role of u-PA in the cardiac response to pressure overload has not been previously reported. However, our findings about the role of u-PA are consistent with and extend previous observations on the role of MMPs,

though some differences exist. In general, inhibition or genetic deficiency of MMPs attenuates LV maladaptation to conditions such as pressure or volume overload, myocardial infarction, and pacing,^{26,35,36,42–49,51,52} while the opposite phenotype develops when MMP activities are enhanced.^{33,38–40} Our findings also indicate that proteinase inhibition promotes a favorable adaptation. But, there are also differences, such as in the hypertrophy of cardiomyocytes after proteinase inhibition, which was attenuated in our study and in others,²⁶ unaffected in response to volume overload⁴⁷ or during spontaneous hypertension,⁴⁶ and even increased in a pacing-induced model of cardiac failure in pigs.⁵² These differences are likely attributable, at least in part, to differences in animal models. For instance, the cardiomyocyte cross-sectional area is increased after TAB in the mouse model, but reduced in the pacing-induced heart failure pig model.⁵² Proteinase inhibition resulted, however, in both models in a favorable adaptive response. Some of the quantitative differences in phenotypes may also be due to additional differences in the type of LV loading (eg, acute pressure overload, volume overload,⁴⁷ chronic spontaneous hypertension,⁴⁶ rapid pacing⁵²), the species (eg, mouse, rat, pig), and the type and dose of proteinase inhibitor and its method of delivery (adenoviral gene transfer yields very high proteinase inhibitor levels; TIMP-1 is a natural efficient inhibitor of several MMPs; pharmacological MMP inhibitors differ greatly in their ability to block various MMPs).^{46,47,52}

In conclusion, this study demonstrates that inactivation or inhibition of u-PA attenuates LV hypertrophy, remodeling, and dysfunction after pressure overload. These genetic insights should encourage future analysis as to whether long-term pharmacological inhibition of u-PA may offer any therapeutic opportunities for the treatment of maladaptive LV remodeling, often leading to fatal heart failure.

Acknowledgments

We thank A. Bouché, M. De Mol, E. Gils, S. Jansen, L. Kieckens, A. Manderveld, and K. Maris, (Center for Transgene Technology and Gene Therapy, KULeuven, Leuven, Belgium) for technical assistance.

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