Mechanisms of Cardiac Fibrosis Induced by Urokinase Plasminogen Activator*

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Human hearts with end-stage failure and fibrosis have macrophage accumulation and elevated plasminogen activator activity. However, the mechanisms that link macrophage accumulation and plasminogen activator activity with cardiac fibrosis are unclear. We previously reported that mice with macrophage-targeted overexpression of urokinase plasminogen activator (SR-uPA+/o mice) develop cardiac macrophage accumulation by 5 weeks of age and cardiac fibrosis by 15 weeks. We used SR-uPA+/o mice to investigate mechanisms through which macrophage-expressed uPA causes cardiac macrophage accumulation and fibrosis. We hypothesized that: 1) macrophage accumulation and cardiac fibrosis in SR-uPA+/o mice are dependent on localization of uPA by the uPA receptor (uPAR); 2) activation of plasminogen by uPA and subsequent activation of transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase (MMP)-2 and -9 by plasmin are critical pathways through which uPA-expressing macrophages accumulate in the heart and cause fibrosis; and 3) uPA-induced cardiac fibrosis can be attenuated by treatment with verapamil. To test these hypotheses, we bred the SR-uPA+/o transgene into mice deficient in either uPAR or plasminogen and measured cardiac macrophage accumulation and fibrosis. We also measured cardiac TGF-β1 protein (total and active), Smad2 phosphorylation, and MMP activity after the onset of macrophage accumulation but before the onset of cardiac fibrosis. Finally, we treated mice with verapamil. Our studies revealed that plasminogen is necessary for uPA-induced cardiac fibrosis and macrophage accumulation but uPAR is not. We did not detect plasmin-mediated activation of TGF-β1, MMP-2, or MMP-9 in hearts of SR-uPA+/o mice. However, verapamil treatment significantly attenuated both cardiac fibrosis and macrophage accumulation.

Cardiac fibrosis, the accumulation of excess extracellular matrix in the heart, is a common feature of end-stage heart disease independent of etiology. Cardiac fibrosis may contribute to impaired systolic and diastolic function and is associated with both atrial and ventricular arrhythmias (1, 2). Fibrotic cardiac tissue is relatively avascular (3), and cardiac fibroblasts are unable to propagate cardiac action potentials (for review see Ref. 4). For these reasons, cardiac fibrosis will likely interfere with implementation of cell-based therapies for heart disease (5). Despite the importance of cardiac fibrosis, the mechanisms through which it develops are incompletely understood.

*This work was supported by Grants HL70941 (to A. S.-O.) and HL080597 (to D. A. D.) from the National Institutes of Health and a grant from the Locke Family Foundation (to A. S.-O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: PA, plasminogen activator; uPA, urokinase plasminogen activator; uPAR, uPA receptor; TGF-β1, transforming growth factor β1; MMP, matrix metalloproteinase.
Mechanisms of Cardiac Fibrosis

(The Jackson Laboratory). Mice were housed under specific pathogen-free conditions. Pups were weaned at 4 weeks of age and genotyped for the SR-uPA transgene by Southern blot or PCR of tail DNA. Genotyping of the Plg and Plaur alleles was performed by PCR of tail DNA using published primers (18, 19). Some of the SR-uPA+/+ mice (n = 9) were treated with verapamil (1 mg/ml in 10% dextrose as drinking water) beginning at 5 weeks of age. Littermate controls (n = 8) received 10% dextrose alone. After 60 days, the mice were killed and their hearts processed for histologic analysis of macrophage and collagen content as described below. All animal protocols were approved by the University of Washington Office of Animal Welfare.

To obtain hearts for histologic and biochemical analysis, deeply anesthetized mice were exsanguinated, and hearts were excised, placed in phosphate-buffered saline, transferred to phosphate-buffered saline with 5% dextrose and 25 mM KCl to produce cardiac arrest, and then placed in sucrose formalin fixative. Hearts were sectioned into three pieces (base, midventricle, and apex) and processed into a single paraffin block.

Histologic Analyses—Macrophages were detected with a rat monoclonal antibody (anti-Mac-3, clone M3/84, 2.5 μg/ml; Pharmingen) (10). Bound antibody was detected with peroxidase-conjugated goat anti-rat IgG (Kirkegaard and Perry) and diamobenzidine substrate. Control slides were incubated with isotype-matched primary antibodies (Pharmingen).

We quantified cardiac macrophages by counting Mac-3-stained cells in each of two or three sections spaced at least 1 mm apart. Cells were counted in 10 random high power (×400) microscopic fields per section (20–30 fields per heart), and the average macrophage density in each heart was calculated. Collagen accumulation was quantified by picrosirius red staining of a single section from the midventricle of each heart. Computer-assisted image analysis (Image Pro 3.0 software, Media Cybernetics) was used to quantify the red-stained area of each section. Quantification of cardiac macrophages and collagen was done by observers blinded to genotype.

Measurement of Plasminogen Activator Activity of Bone Marrow-derived Macrophages—Cultured macrophages were used for this assay, which was performed not as a direct measurement of in vivo uPA activity but to determine whether the SR-uPA transgene was still expressed by macrophages of Plg−/− mice. Bone marrow was harvested from the femurs of 8–10-week-old mice by flushing with RPMI 1640 with 2% fetal bovine serum and 5 IU/ml heparin. The marrow cells were washed in Hanks’ balanced salt solution and resuspended in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum, 1% each fetal bovine serum and 5 IU/ml heparin. The marrow cells were plated in 10% heat-inactivated fetal calf serum, 1% each fetal bovine serum and 5 IU/ml heparin. The marrow cells were washed in Hanks’ balanced salt solution and resuspended in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum, 1% each penicillin/streptomycin and l-glutamine, and 10% L-cell conditioned medium as a source of granulocyte-macrophage colony-stimulating factor (20). Culture media and nonadherent cells were removed, and new medium added at days 4 and 8. This protocol yields 10⁴-10⁶ macrophages/femur. On day 10, the medium was changed to M199. Conditioned medium was collected after 20 h and stored at −80 °C. Cells were counted and lysed, and total lysate protein was measured using the DC protein assay kit (Bio-Rad), and plasminogen activator activity was detected by incubating aliquots of macrophage-conditioned medium with Glu-plasminogen (0.4 μM; American Diagnostica) and the plasmin substrate S-2251 (0.9 mM; Chromogenix) and measuring the change in absorbance at 405 nm. PA activity was calculated with reference to a standard curve constructed with human single-chain uPA (American Diagnostica).

Measurement of TGF-β1 Secreted by Explanted Hearts—The apical half of a heart was minced and placed in M199. After two 30-min incubations in fresh M199, the pieces were transferred to fresh M199 and incubated overnight at 37 °C. Conditioned media were collected and stored at −80 °C. Active TGF-β1 (no acid activation) and total TGF-β1 (acid activated) were measured by enzyme-linked immunosorbent assay of conditioned media (Promega).

Immunoblot for Phosphorylated Smad2—The basal half of a heart was snap-frozen in liquid nitrogen, ground over ice, homogenized with a Polytron, and extracted in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, Halt protease inhibitor mixture, 10 μl/ml (Pierce)). Negative and positive controls for the presence of phospho-Smad2 consisted of lysates of AML12 cells treated with vehicle or TGF-β1 (21). Samples were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels and transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine, 5% methanol at 85 V for 3 h at 4 °C. Filters were blocked overnight with TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk. Immunoblots were analyzed for phospho-Smad-2 (Cell Signaling, catalog no. 3101) using the antibody at a 1:1,000 dilution or a β-actin antibody (Abcam, catalog no. 8227-50) at a 1:5,000 dilution. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham Biosciences) using 1:10,000 horseradish peroxidase-linked anti-rabbit secondary antisera (Abcam, catalog no. 6721-1). Blots were exposed to film for 1–10 min.

MMP Detection—The basal half of a heart was snap-frozen in liquid nitrogen, ground with a mortar and pestle over liquid nitrogen, homogenized with a Polytron, and placed in lysis buffer (10 mmol/liter cacoedetic acid, 0.15 mol/liter NaCl, 20 mmol/liter ZnCl, 1.5 mmol/liter NaNP, and 0.01% Triton X-100, pH 5.0) on ice for 30 min (22). Extracted protein was measured using the DC protein assay kit (Bio-Rad), and equivalent amounts of protein were loaded with nonreducing sample buffer into precast PA polyclonal gels containing gelatin or casein (Bio-Rad). Culture medium from HT-1080 cells stimulated with PMA was loaded as a positive control for gelatinolytic and caseinolytic activity. Gels were run at 4 °C, renatured in renaturation buffer (Bio-Rad) for 90 min at room temperature with shaking, rinsed, and placed in development buffer (Bio-Rad) at 37 °C overnight. The next day gels were stained with Coomassie Blue for 8 h, washed, and placed on a gel-drying rack.

Statistical Analysis—Because much of the data were not normally distributed, data are presented as median (25–75% range) and group medians are compared with the Mann-Whitney rank-sum test.

RESULTS

uPAR Is Not Required for uPA-induced Cardiac Fibrosis or Macrophage Accumulation—To test whether uPAR is a critical mediator of macrophage accumulation and cardiac fibrosis in SR-uPA+/+ mice, we began by breeding SR-uPA+/+ mice with nontransgenic mice deficient in uPAR (Plaur−/− mice). At 15 weeks of age, SR-uPA+/+ Plaur−/− hearts had significantly more fibrosis than hearts of SR-uPA+/+ Plaur+/− littermates (8.5 (3.6–13%) versus 4.5 (0.25–1.5%) picrosirius red-positive area; p = 0.001 (Fig. 1A)). To test whether uPAR is a critical mediator of macrophage accumulation in hearts of SR-uPA+/+ mice, we counted macrophages in hearts of 15-week-old SR-uPA+/+ Plaur−/− and SR-uPA+/+ Plaur+/− littermates. SR-uPA+/+ Plaur−/− mice had significantly more cardiac macrophages than SR-uPA+/+ Plaur−/− littermates (51 (33–85) versus 1.8 (1.2–2.1) Mac-3-positive cells/mm²; p = 0.002 (Fig. 1B)). SR-uPA+/+ Plaur−/− mice had a similar degree of cardiac fibrosis and macrophage accumulation as our previously reported SR-uPA+/+ Plaur+/+ mice (10).

2 W. T. Parks (University of Washington), personal communication.
Mechanisms of Cardiac Fibrosis

Plasminogen Is Required for uPA-induced Cardiac Fibrosis—To test whether plasminogen is a critical mediator of uPA-induced cardiac fibrosis, we began by breeding SR-uPA+/o Plg+/- mice with nontransgenic Plg-/- mice. F1 mice were then intercrossed to generate SR-uPA+/+ Plg+, SR-uPA+/o Plg+, SR-uPA+/o Plg+/-, and SR-uPA+/o Plg-/- littermates. Hearts of 15-week-old SR-uPA+/+ Plg-/- mice had significantly less fibrosis than littermate SR-uPA+/+ Plg+/- mice (0.56 (0.45–0.63%) versus 5.9 (2.4–14%) picrosirius red positive area; p = 0.01 (Fig. 2)). In addition, SR-uPA+/+ Plg-/- mice had the same amount of cardiac fibrosis as nontransgenic Plg-/- littermates (0.56 (0.45–0.63%) versus 0.60 (0.46–0.79%) p = 0.68 (Fig. 2)).

Plasminogen Is Required for Macrophage Accumulation in SR-uPA+/+ Hearts—We measured macrophage accumulation in hearts of 15-week-old SR-uPA+/+ Plg+, SR-uPA+/+ Plg-/-, SR-uPA+/o Plg+/-, and SR-uPA+/o Plg-/- littermates. In Plg-/- mice, the SR-uPA+/+ transgene did not increase cardiac macrophage accumulation (0.69 (0–0.9) Mac-3-positive cells/mm² for SR-uPA+/+ Plg-/- mice versus 0.6 (0.34–0.79) for nontransgenic Plg-/- littermates; p = 1.0 (Fig. 3)). Moreover, there were significantly more macrophages in hearts of SR-uPA+/+ Plg+/- mice (21 (13–38); p = 0.008 (Fig. 3)) than in hearts of SR-uPA+/+ Plg-/- mice.

PA Activity of SR-uPA+/+ Macrophages Is Not Affected by the Absence of Plasminogen—To exclude the possibility that SR-uPA transgene expression in SR-uPA+/+ mice might be altered in the Plg-/- background or that SR-uPA transgene expression might have been lost over time, we collected conditioned media from bone marrow-derived macrophages harvested from SR-uPA+/+ Plg+/-, SR-uPA+/+ Plg-/-, and SR-uPA+/+ Plg-/- mice. Media from SR-uPA+/+ Plg-/- macrophages had PA activity equal to the PA activity of media from SR-uPA+/+ Plg+/- macrophages (1.5 (1.1–2.0) versus 0.9 (0.7–1.5) IU/10⁶ cells/20 h; p = 0.29 (Fig. 4)). Media from SR-uPA+/+ Plg-/- macrophages had significantly greater PA activity than media conditioned by macrophages from nontransgenic Plg-/- mice, in which PA activity was uniformly below the limit of detection (p < 0.02; Fig. 4).

FIGURE 1. uPAR is not required for uPA-induced cardiac fibrosis and macrophage accumulation. A, percent cardiac fibrillar collagen measured by picrosirius red stain of hearts from 15-week-old mice. Data points represent individual hearts; bars are group medians. B, quantification of macrophages (Mac-3 immunostain) in hearts of 15-week-old mice. F1 mice were then intercrossed to generate SR-uPA+/+ Plg+, SR-uPA+/o Plg+, SR-uPA+/o Plg+/-, and SR-uPA+/o Plg-/- mice, respectively. Fibrillar collagen stains red. Size bar = 50 μm.

FIGURE 2. Plasminogen is required for uPA-induced cardiac fibrosis. A, percent cardiac fibrillar collagen content measured by picrosirius red stain in hearts from 15-week-old mice. Data points represent individual hearts; bars are group medians. B and C, picrosirius red stain of hearts from 15-week-old SR-uPA+/+ Plg+/- and SR-uPA+/+ Plg-/- mice, respectively. Fibriellar collagen stains red. Size bar = 50 μm.
Mechanisms of Cardiac Fibrosis

Cardiac Fibrosis in SR-uPA<sup>+/o</sup> Mice Is Not Associated with Increased TGF-β1 Activity—Because plasmin can convert TGF-β1 to active TGF-β1 (23), and active TGF-β1 can cause cardiac fibrosis (24), we tested the hypothesis that uPA-induced cardiac fibrosis is associated with increased cardiac TGF-β1 protein and activity. Total and active TGF-β1 protein were measured in media conditioned by hearts explanted from 5-week-old SR-uPA<sup>+/o</sup> Plg<sup>+/o</sup>, SR-uPA<sup>+/o</sup> Plg<sup>−/−</sup>, SR-uPA<sup>o/o</sup> Plg<sup>+/+</sup>, and SR-uPA<sup>o/o</sup> Plg<sup>−/−</sup> littermates. We chose the 5-week time point because it is after the onset of macrophage accumulation but before the onset of fibrosis in SR-uPA<sup>+/o</sup> mice (10). Neither total nor active TGF-β1 protein was increased in explant cultures of hearts of 5-week-old SR-uPA<sup>+/o</sup> mice (Fig. 5, A and B). To gain confidence that we had not missed an increase in cardiac TGF-β1, we repeated this assay with hearts from 7–8-week-old SR-uPA<sup>+/o</sup> Plg<sup>−/−</sup> and SR-uPA<sup>o/o</sup> Plg<sup>−/−</sup> mice. Again, neither total nor active TGF-β1 was increased in media conditioned by SR-uPA<sup>+/o</sup> hearts (Fig. 5, C and D). Finally, to determine whether cardiac TGF-β1 signaling was increased even in the absence of detectable differences in TGF-β1 protein in conditioned media, we assayed extracts of hearts of 7–8-week-old SR-uPA<sup>+/o</sup> Plg<sup>−/−</sup> and SR-uPA<sup>o/o</sup> Plg<sup>−/−</sup> mice for phosphorylated Smad2 (phospho-Smad2). Phospho-Smad2 was detected in all heart extracts; however, there was no increase in phospho-Smad2 in hearts of SR-uPA<sup>+/o</sup> mice (Fig. 6).

Gelatinase Activity Is Not Increased in Hearts of SR-uPA<sup>+/o</sup> Mice—To test whether activity of the gelatinases MMP-2 and -9 was increased in hearts of SR-uPA<sup>+/o</sup> mice at the onset of macrophage accumulation and before the onset of cardiac fibrosis, we performed gelatin zymography of extracts of hearts from 5-week-old mice. Extracts of hearts of SR-uPA<sup>+/o</sup> Plg<sup>−/−</sup>, SR-uPA<sup>+/o</sup> Plg<sup>−/−</sup>, and SR-uPA<sup>o/o</sup> Plg<sup>−/−</sup> mice had equivalent activity of pro-MMP-2 and no detectable active MMP-2 or MMP-9 (Fig. 7). To measure activity of MMP-3, -7, and -13, aliquots of the same samples were electrophoresed into casein-containing gels. Culture medium from HT-1080 cells was used as a positive control. No caseloinyotic activity was detected in any of the samples (data not shown).

Treatment with Verapamil Attenuates uPA-induced Cardiac Fibrosis and Macrophage Accumulation—To test whether verapamil could prevent cardiac fibrosis, we treated SR-uPA<sup>+/o</sup> mice with verapamil from 5 to 13.5 weeks of age. We chose the 5-week time point because it is after the onset of macrophage accumulation but before the onset of fibrosis in SR-uPA<sup>+/o</sup> mice (10). Hearts of 13.5-week-old SR-uPA<sup>+/o</sup> mice treated with placebo (3.0 (2.0–3.4%) versus 10 (8.8–15%) picrosirius red-positive area; p = 0.008 (Fig. 8A)). Verapamil-treated mice also had significantly less cardiac macrophage accumulation (37 (24–48) versus 132 (88–181) Mac-3-positive cells/mm<sup>2</sup>; p = 0.005 (Fig. 8B)).

DISCUSSION

We used a mouse model of macrophage-targeted uPA overexpression (SR-uPA<sup>+/o</sup> mice) to identify downstream mediators of uPA-in-
duced macrophage accumulation and cardiac fibrosis. Our major findings were as follows. 1) uPAR is not required for uPA/plasmin-induced cardiac macrophage accumulation and fibrosis. 2) Plasminogen is necessary for macrophage accumulation and for the development of cardiac fibrosis. 3) TGF-β1 is not a critical mediator of uPA/plasmin-induced cardiac fibrosis. 4) The activity of gelatinolytic or caseinolytic cardiac MMPs is not increased before the onset of uPA/plasmin-induced cardiac fibrosis. 5) Treatment with verapamil after the onset of macrophage accumulation substantially limits further cardiac macrophage accumulation and fibrosis. Thus, although plasmin is a crucial mediator of uPA-induced cardiac fibrosis, plasmin substrates previously associated with cardiac fibrosis (TGF-β1 and MMPs) do not appear to contribute to either cardiac macrophage accumulation or fibrosis in SR-uPA+/−/mice. However, uPA-induced cardiac macrophage accumulation and fibrosis depend substantially on verapamil-sensitive pathways.

Increased cardiac macrophage accumulation and PA activity are associated with cardiac fibrosis in humans and in animal models of human cardiac disease (7, 9, 25–27). Our previous work, showing that mice with macrophage-targeted overexpression of uPA develop cardiac macrophage accumulation and fibrosis, suggests a causal link between uPA-expressing macrophages and cardiac fibrosis (10). Moreover, PAI-1-null mice, in which uPA activity is increased, are more susceptible than wild-type mice to cardiac fibrosis (10, 28) and uPA-null mice are less susceptible to cardiac fibrosis than wild-type mice (11, 12). Therefore, several studies link cardiac macrophage accumulation and increased cardiac uPA activity with cardiac fibrosis; however, none of these studies identifies the downstream mediators and pathways through which uPA acts.

Because plasminogen is the major physiologic substrate of uPA, we hypothesized that plasminogen is a critical mediator of both macrophage accumulation and fibrosis in the hearts of SR-uPA+/−/mice. A role for plasminogen in cardiac macrophage accumulation and fibrosis is supported by the observation that, compared with wild-type mice, Plg−/− mice have less cardiac fibrosis after myocardial infarction (29) and decreased macrophage migration in response to an inflammatory stimulus (30). However, uPA could also cause macrophage accumulation and cardiac fibrosis through plasminogen-independent pathways. Moreover, uPA has non-plasminogen substrates (31, 32) including MMP-2 (33), and MMP-2 activity is elevated in fibrotic human hearts (34). An overexpression model such as the SR-uPA+/−/mouse is a setting in which uPA activity on non-plasminogen substrates might be manifest. Nevertheless, complete loss of the cardiac phenotype in Plg−/− mice argues strongly that plasminogen is the only important uPA substrate in SR-uPA+/−/mice.

Mechanisms of Cardiac Fibrosis

**FIGURE 5.** Total and active TGF-β1 secretion from experimental and control hearts. TGF-β1 was measured by enzyme-linked immunosorbent assay of explant culture media from transgenic and nontransgenic hearts of both Plg+/− and Plg−/− mice. A and B, total and active TGF-β1 from 5-week-old hearts. C and D, total and active TGF-β1 from 7–8-week-old hearts (Plg+/−/mice only). Data points represent individual hearts; bars are group medians.

**FIGURE 6.** Immunoblot for phosphorylated Smad2. Immunoblot of protein extracts of hearts from 7–8-week-old SR-uPA+/− or nontransgenic mice. Blots were probed with antibodies to phospho-Smad2 (P-Smad2) and β-actin.

**FIGURE 7.** Overexpression of uPA does not increase cardiac gelatinase activity. Gelatin zymography of extracts from 5-week-old transgenic and nontransgenic hearts (Plg+/− and Plg−/−). Culture medium conditioned by HT1080 cells treated with PMA was a positive control.
Mechanisms of Cardiac Fibrosis

We used Plaur−/− mice to investigate whether cardiac macrophage accumulation and fibrosis in SR-uPA+/− mice were mediated through either nonproteolytic or proteolytic pathways that require binding of uPA to uPAR. Binding of uPA to uPAR can initiate cell activation in the absence of proteolysis (for review, see Ref. 14). Moreover, uPA binding to uPAR enhances the catalytic efficiency of uPA-mediated plasminogen activation (35). Although the initial characterization of Plaur−/− mice did not reveal any impressive phenotypes (19, 36), subsequent studies suggest a role for uPAR in macrophage migration in vivo (37–39). It was therefore logical to investigate a role for uPAR in cardiac macrophage accumulation in SR-uPA+/− mice. However, uPAR is not required for the SR-uPA transgene to produce cardiac macrophage accumulation and fibrosis.

We also considered that the critical role of plasminogen in uPA-induced macrophage accumulation and cardiac fibrosis might be as an activator of pro-uPA to two-chain (active) uPA rather than as a downstream effector of two-chain uPA (40). However, substantial conversion of pro-uPA to a two-chain uPA occurs in Plg−/− mice (18, 41). Therefore, the absence of macrophage accumulation and cardiac fibrosis in Plg−/− mice appears to be due to the absence of plasmin and not the absence of two-chain uPA.

Because plasmin can activate latent TGF-ß1 by proteolytic cleavage (23), and because expression of active TGF-ß1 in either hearts or livers (24, 42) of transgenic mice causes cardiac fibrosis (for a review of TGF-ß1 and cardiac fibrosis, see Ref. 15), we investigated whether TGF-ß1 protein was increased in hearts of SR-uPA+/− mice. TGF-ß1 is an attractive candidate for downstream mediator of plasmin-induced cardiac fibrosis, because active TGF-ß1 stimulates cardiac fibroblasts to produce collagen (43, 44) and cardiac TGF-ß1 mRNA and protein are increased in human and animal cardiomyopathies (45, 46).

We measured total as well as active TGF-ß1 in heart extracts because we considered total (latent + active) TGF-ß1 to be a more sensitive measure of TGF-ß1 production as compared to active TGF-ß1 alone. Active TGF-ß1 can induce TGF-ß1 gene transcription (47); therefore, a small increase in active TGF-ß1 can lead to a large increase of latent (and therefore total) TGF-ß1. We measured TGF-ß1 secreted by hearts of both 5- and 7-week-old SR-uPA+/− mice (i.e. after the onset of cardiac macrophage accumulation but before the onset of cardiac fibrosis). If plasmin-mediated activation of cardiac TGF-ß1 stimulated fibroblast collagen synthesis, we would expect that elevations of active and total cardiac TGF-ß1 would precede the appearance of fibrosis in SR-uPA+/− hearts.

Surprisingly, we did not detect elevation of either active or total TGF-ß1 in hearts of 5- or 7-week-old SR-uPA+/− mice. The absence of an increase in active TGF-ß1 in hearts of SR-uPA+/− mice was not because of lack of latent (i.e. activatable) TGF-ß1 protein. In fact, the level of latent TGF-ß1 protein in explant cultures of 5- and 7-week SR-uPA+/− hearts was considerably greater than the level of latent TGF-ß1 in explant cultures of hearts of older transgenic mice that over-express TGF-ß1 in their hearts and develop cardiac fibrosis (Ref. 48 and data not shown). We conclude that TGF-ß1 does not contribute to cardiac fibrosis in SR-uPA+/− mice. The absence of increased TGF-ß1 activation in hearts of SR-uPA+/− mice (despite substantially increased cardiac PA activity (10)) is consistent with other studies that discount the role of the uPA/plasminogen system as an important in vivo activator of TGF-ß1 (49, 50).

We also attempted to identify MMPs that might be key downstream mediators of uPA-induced cardiac fibrosis. MMPs are logical candidates for this role because they are activated by plasmin, and increased MMP activity is associated with cardiac fibrosis in mice and humans (16, 22, 34). For example, fibrotic human hearts have increased plasmin activity and increased amounts of both active MMP-2 and MMP-9 (25, 51). Moreover, mice deficient in MMP-2 or MMP-9 have less cardiac macrophage accumulation and fibrosis than wild-type mice after myocardial infarction (52, 53). However, in the present study, SR-uPA+/− heart extracts obtained before the onset of fibrosis did not reveal increased activity of either MMP-2 or MMP-9. The absence of increases in either gelatinolytic or caseinolytic activity in extracts of SR-uPA+/− hearts suggests that MMP-2, -3, -7, -9, and -13 are not critical mediators of uPA-induced cardiac fibrosis. It remains possible that other MMPs, lacking gelatinolytic or caseinolytic activity, contribute to cardiac fibrosis in SR-uPA+/− hearts. However, our data argue strongly against roles for MMP-2 and MMP-9, the two MMP family members that have been most closely associated with cardiac fibrosis in other settings (52, 53).

Because verapamil attenuates cardiac fibrosis in several animal models of cardiomyopathy (54–57) and because uPA can cause arterial constriction, (17) which might be relieved by verapamil, we tested whether verapamil could limit cardiac fibrosis in SR-uPA+/− mice. Not only did verapamil treatment largely prevent cardiac fibrosis (70% decrease versus placebo-treated mice; Fig. 8A), it also significantly blunted the increase in cardiac macrophages that occurs between 5 and ~15 weeks in this model (also a 70% decrease; Fig. 8B). Because verapamil blocks both fibrosis (which follows macrophage accumulation in this model) and further macrophage accumulation, it appears that verapamil blocks a critical step through which uPA/plasmin-induced cardiac fibrosis is either initiated or perpetuated.

There are several potential mechanisms through which verapamil could block macrophage accumulation and cardiac fibrosis. Verapamil could have a direct effect on macrophages that diminished activation or...
adherence to endothelium (58–61). Verapamil could act directly on cardiomyocytes by blocking calcium currents that lead to cell death (62, 63). Increased cardiomyocyte survival would likely retard further macrophage infiltration and could prevent vasospasm that leads to further cardiomyocyte death (54). It is also possible that verapamil acts directly on vascular smooth muscle cells to block coronary artery spasm that is induced either directly by uPA or that occurs as a direct result of cardiomyocyte death, as in other mouse models of cardiomyopathy (64). Elucidation of the exact cellular targets through which verapamil mitigates uPA/plasmin-induced cardiac macrophage infiltration and fibrosis is important and will require additional experimentation that extends far beyond the present study.

In summary, plasminogen is a critical mediator of uPAR-independent, uPA-induced cardiac fibrosis. Neither TGF-β1 nor MMPs appear to be the mediators of plasmin-induced cardiac fibrosis. A process that is blocked by the calcium channel inhibitor verapamil is a critical component of uPA/plasmin-induced cardiac macrophage accumulation and fibrosis.

Acknowledgments—We thank Margo Weiss for administrative assistance and Valerie Carlb erg for technical assistance.

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