Overexpression of Urokinase by Macrophages or Deficiency of Plasminogen Activator Inhibitor Type 1 Causes Cardiac Fibrosis in Mice

Hideaki Moriwaki, April Stempien-Otero, Michal Kremen, Aaron E. Cozen, David A. Dichek

Abstract—Several studies implicate elevated matrix metalloproteinase activity as a cause of cardiac fibrosis. However, it is unknown whether other proteases can also initiate cardiac fibrosis. Because absence of urokinase plasminogen activator (uPA) prevents development of cardiac fibrosis after experimental myocardial infarction in mice, we hypothesized that elevated activity of uPA or deficiency of the uPA inhibitor plasminogen activator inhibitor-1 (PAI-1) might cause cardiac fibrosis. We used mice with scavenger-receptor (SR)-directed, macrophage-targeted uPA overexpression (SR-uPA<sup>+/−</sup> mice) and PAI-1 null mice to test these hypotheses. Our studies revealed that SR-uPA<sup>+/−</sup> mice developed cardiac fibrosis beginning between 5 and 10 weeks of age. Fibrosis was preceded by cardiac macrophage accumulation, implicating uPA-secreting macrophages as important contributors to development of fibrosis. A key role for uPA-secreting macrophages in development of cardiac fibrosis was supported by experiments in which recipients of bone marrow transplants from SR-uPA<sup>+/−</sup> donors but not nontransgenic donors developed cardiac macrophage accumulation and fibrosis. SR-uPA<sup>+/−</sup> mice and recipients of SR-uPA<sup>+/−</sup> bone marrow had neither macrophage accumulation nor fibrosis in other major organs despite the presence of higher levels of uPA in these organs than in hearts. PAI-1 null mice but not congenic, age-matched controls also developed macrophage accumulation and fibrosis in hearts but not in other organs. We conclude: (1) either elevated macrophage uPA expression or PAI-1 deficiency is sufficient to cause cardiac macrophage accumulation and fibrosis; (2) macrophages are important contributors to the development of cardiac fibrosis; and (3) the heart is particularly sensitive to the effects of excess uPA activity. (Circ Res. 2004;95:637-644.)

Key Words: cardiac disease □ collagen □ inflammation □ metalloproteinases

Cardiac fibrosis, the abnormal accumulation of extracellular matrix (ECM) in the heart, is a common feature of ischemic and nonischemic end-stage heart disease. Fibrotic hearts have impaired systolic as well as diastolic function and are prone to ventricular arrhythmias. Cardiac fibrosis is found in hearts in which myocytes have been lost because of toxic or ischemic injury. In this setting, the fibrosis is termed “replacement” and is viewed as an adaptive process that preserves the structural integrity of the heart. Cardiac fibrosis also occurs independently of myocyte loss, in which case it is termed “interstitial” fibrosis and is less clearly adaptive. The mechanisms that drive cardiac fibrosis are poorly understood.

Efforts to elucidate mechanisms causing fibrosis in other organs have focused on identifying either increased ECM synthesis (ie, increased expression of genes encoding matrix proteins) or decreased ECM degradation (ie, deficient ECM proteolysis). However, several studies suggest that increased protease activity in the heart may paradoxically contribute to fibrosis. For example, matrix metalloproteinase (MMP) activity is increased in failing human hearts. Moreover, mouse hearts overexpressing human MMP-1 have increased collagen accumulation. After experimental myocardial infarction, mice deficient in MMP-9 have less cardiac collagen accumulation than wild-type mice, and administration of MMP inhibitors decreases cardiac collagen accumulation in animal models of cardiomyopathy and myocardial infarction. These data support a role for MMP activity in the pathogenesis of cardiac fibrosis.

The serine protease urokinase plasminogen activator (uPA) and its substrate plasminogen might also play a role in cardiac fibrosis. A role for the uPA/plasminogen system in cardiac fibrosis is suggested by experiments in which mice deficient in uPA or plasminogen were less able to form fibrotic scars after myocardial infarction. Absence of uPA or plasminogen in these mice appears to impair the ability of fibroblasts to migrate into infarcted tissue and synthesize collagen. According to this model, excess uPA activity or, alternatively, deficiency of a uPA inhibitor might each be sufficient to cause cardiac fibrosis.
We recently generated transgenic mice with macrophage-specific overexpression of uPA (SR-uPA+/0 mice) and showed that when bred into the apolipoprotein E (apoE) null background, the SR-uPA transgene accelerated atherosclerosis. Here we report use of SR-uPA+/0 mice and mice deficient in PA inhibitor-1 (PAI-1, the physiologic inhibitor of uPA) to test the hypothesis that elevated uPA activity or lack of a uPA inhibitor can cause cardiac fibrosis.

Materials and Methods

Experimental Animals

Transgenic mice with macrophage-targeted overexpression of uPA (SR-uPA+/0 mice) were described previously. Nontransgenic C57BL/6 Apoe−/− mice (used as bone marrow transplant recipients), C57BL/6 mice deficient in PAI-1 (Serpine1−/−), and C57BL/6 Serpine1−/− controls were purchased (The Jackson Laboratory). Some mice were fed a Western-type diet containing 21% fat and 0.15% cholesterol (TD88137; Harlan-Tekland) beginning at 5 weeks of age. Bone marrow recipients were fed this diet beginning after transplantation (8 weeks of age). Serpine1−/− mice and their controls were not fed the Western-type diet. All mice except for the bone marrow donors were female.

Lipid Analysis

Plasma cholesterol was measured with a colorimetric assay (Serpine1−/−, Abbott Laboratories).

Immunostaining and Histochemistry

In initial experiments, mice were perfused via cardiac puncture with 0.9% saline followed by buffered formalin. Tissues were stored in formalin and embedded in paraffin. In later experiments, mice were exsanguinated, hearts were excised, placed in PBS with 5% dextrose, 25 mM KCl, then fixed and processed into paraffin. Serial sections were cut at 4 levels: the aortic root and the base, midventricle, and apex of the heart. Sections of lung, liver, and kidney were also processed into paraffin. Macrophages and leukocytes were detected with rat anti-mouse monoclonal antibodies: anti–Mac-3 clone M3/84 and anti–mouse CD45 clone 30-F11, respectively (Pharmingen). Bound antibody was detected with peroxidase-conjugated goat anti-rat IgG (Kirkegaard & Perry Laboratories). Control slides were incubated with isotype-matched primary antibodies (Pharmingen). Other sections were stained with hematoxylin and eosin (H&E), Masson trichrome, or picrosirius red.

Quantification of Leukocytes, Macrophages, and Collagen in Tissue Sections

Leukocyte (CD45) and macrophage (Mac-3) infiltration were quantified by counting stained cells in 10 high-power (×400) microscopic fields in each of 3 step sections per heart (30 fields per heart). In some mice, Mac-3-positive and CD45-positive cells were also counted in 10 high-power fields of a section of lung. Sections of heart, kidney, liver, lung, and spleen were also stained with H&E and Masson trichrome to assess qualitatively whether fibrosis was present. In mice in which cardiac fibrosis was evident (and in their matched controls), collagen accumulation was quantified by picrosirius red staining of a section from the midventricle and computer-assisted planimetry (Image Pro 3.0 software; Media Cybernetics).

Measurement of Plasminogen Activator Activity

Mice were exsanguinated by saline perfusion. Hearts, lungs, and kidneys were sliced into 1-mm-thick sections, which were incubated overnight at 37°C in M199 medium. Plasminogen activator (PA) activity was detected by incubating aliquots of explant culture media with glu-plasminogen (0.4 μmol/L; American Diagnostica) and the plasmin substrate S-2251 (0.9 mM; Chromogenix) and measuring optical density at 405 nm.

Bone Marrow Transplantation

Eight-week-old female nontransgenic Apoe−/− mice were lethally irradiated. Eight-week-old, male Apoe−/− mice (either SR-uPA+/0 or nontransgenic) were used as donors of 6×106 bone marrow cells per recipient.

Statistical Analysis

Data are presented as mean±SD for normally distributed data or median (25% to 75% range) for data not normally distributed. Group means were compared with the t test, and medians were compared with the Mann–Whitney rank-sum test. Additional details of the materials and methods used can be found in the online data supplement available at http://circres.ahajournals.org.

Results

Macrophage and Collagen Accumulation in Hearts of SR-uPA+/0 Apoe−/− Mice

In a previous study, we reported that macrophage-targeted overexpression of uPA in Apoe−/− mice (SR-uPA+/0 Apoe−/− mice) caused accelerated aortic atherosclerosis, severe proximal coronary artery stenoses, complete coronary occlusions, myocardial infarcts, and early mortality. As expected, on the basis of our previous examination of H&E-stained sections, hearts of 15-week-old SR-uPA+/0 Apoe−/− mice had significantly more collagenous scar formation than hearts of nontransgenic Apoe−/− mice (10% [8.8% to 13%] versus 0.015% [0.0% to 0.075%]; picrosirius red positive area; P<0.001; Figure 1A). SR-uPA+/0 Apoe−/− hearts were also larger (Table) and had far more inflammatory cells than nontransgenic Apoe−/− hearts. Inflammatory cells were essentially all macrophages: 110 (62 to 130) Mac-3–positive cells/mm2 and 110 (98 to 160) CD45-positive cells/mm2 in transgenic hearts compared with 3.8 (1.3 to 4.4) Mac-3–positive cells/mm2 and 3.0 (2.0 to 5.2) CD45-positive cells/mm2 in nontransgenic hearts (P<0.001; Figure 1A). Compared with organs of nontransgenic mice, other organs of SR-uPA+/0 Apoe−/− mice did not have increased inflammation or fibrosis. For example, lungs (an organ that is relatively rich in macrophages) of 15-week-old SR-uPA+/0 Apoe−/− mice had 114 (60 to 250) Mac-3–positive cells/mm2 compared with 140 (70 to 190) for nontransgenic Apoe−/− controls (P=0.96; χ 2=8 to 9).

The presence of collagenous scars and inflammation in hearts but not in other organs of mice with occlusive coronary disease was not surprising. However, the patterns of inflammation and collagen accumulation in SR-uPA+/0 Apoe−/− hearts differed from what one would expect if these histologic findings were attributable only to ischemic necrosis. Specifically, inflammation and collagen accumulation were not always circumscribed (ie, confined to territories supplied by individual epicardial coronary arteries). Rather, they were often diffuse and patchy (Figure 1D, 1F, and 1H). We hypothesized that uPA-secreting macrophages, migrating through the heart toward areas of infarction or attracted to the heart independently of ischemic necrosis, might cause cardiac fibrosis.

Macrophage Accumulation in Hearts of SR-uPA+/0 Apoe−/− Mice Aged 3 to 5 Weeks

To begin to test the hypothesis that SR-uPA+/0 macrophages accumulate in the heart and cause cardiac fibrosis indepen-
dently of ischemic heart disease, we examined hearts of
SR-uPA<sup>−/−</sup> mice aged 3 and 5 weeks (ie, before development of significant atherosclerosis). Sections of hearts of 3- and 5-week-old 
Apoe<sup>−/−</sup> mice (either SR-uPA<sup>−/−</sup> or nontransgenic) did not show abnormal collagen accumulation (H&E and Masson’s trichrome stains; data not shown). The 3-week-old hearts were not enlarged (Table) and contained few leukocytes, with no increase in SR-uPA<sup>−/−</sup> mice (2.5 [0.8 to 4.0] versus 1.2 [0.8 to 2.5] Mac-3–positive cells/mm<sup>2</sup>; 2.0 [1.0 to 4.8] versus 3.9 [3.1 to 5.2] CD45-positive cells/mm<sup>2</sup> [P=0.02; Figure 2A]). However, 5-week-old SR-uPA<sup>−/−</sup> hearts had more inflammatory cells (almost exclusively mononuclear cells) than nontransgenic controls: 3.6 (0.30 to 12) versus 0.30 (0.00 to 0.52) Mac-3–positive cells/mm<sup>2</sup> (P=0.04) and 40 (16 to 55) versus 1.6 (0.40 to 17) CD45-positive cells/mm<sup>2</sup> (P=0.007; Figure 2B). Thus, leukocytes, including macrophages, accumulate in SR-uPA<sup>−/−</sup> hearts before

**Figure 1.** Fibrosis and macrophage accumulation in hearts of 15-week-old Apoe<sup>−/−</sup> mice. A, Fibrillar collagen measured by picrosirius red stain of sections from hearts of nontransgenic (non-tg; n=8) and transgenic mice (SR-uPA<sup>−/−</sup>; n=6). B, Density of macrophages (using Mac-3 immunostain) and total leukocytes (using CD45 immunostain) in hearts of non-tg (n=6 to 9) and SR-uPA<sup>−/−</sup> (n=10) mice. C and D, Masson trichrome stain. E and F, Picrosirius red stain. G–I, Mac-3 immunostain with hematoxylin counterstain. Arrows (D, F, and H) indicate areas in which collagen and macrophages colocalize. Scale bars: C–H, 200 μm; I, 50 μm. Points in A and B represent individual mice; horizontal lines are group medians. *P<0.001 vs non-tg.

### Mouse Heart and Body Weights

<table>
<thead>
<tr>
<th>Age</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt; Background</th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt; Background</th>
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<tr>
<td>Age</td>
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<td>5 weeks</td>
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<tr>
<td>Genotype</td>
<td>non-tg</td>
<td>SR-uPA&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Body weight, g (n)</td>
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<td>6.9±1.4 (9)</td>
</tr>
<tr>
<td>Heart weight, mg (n)</td>
<td>46±8 (7)</td>
<td>45±9 (8)</td>
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<tr>
<td>Heart/body weight ratio (n)</td>
<td>6.8±0.9 (7)</td>
<td>6.6±0.7 (8)</td>
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Values represent mean±SD. n indicates the number of mice in each group.

*P<0.05; **P=0.058 vs non-tg.
the onset of occlusive coronary artery disease or cardiac fibrosis.

uPA Accumulation in SR-uPA<sup>+/0</sup> Apoe<sup>−/−</sup> Hearts

Because only the hearts of SR-uPA<sup>+/0</sup> mice appeared abnormal, we hypothesized that uPA protein, secreted by transgenic macrophages, might accumulate selectively in SR-uPA<sup>+/0</sup> hearts, reaching levels that are higher than in other organs, and initiating tissue damage. To test this hypothesis, we explanted hearts, lungs, and kidneys from 5-week-old mice (ie, before onset of fibrosis) and measured uPA released from these organs (Figure 2C). We chose lungs because they are relatively rich in macrophages and kidneys because they are the predominant site of uPA production in mice. As expected, in nontransgenic mice, uPA levels were higher in kidney explant cultures than in lung or heart cultures. Cultures of SR-uPA<sup>+/0</sup> organs contained higher levels of uPA than corresponding cultures of nontransgenic organs. However, SR-uPA<sup>+/0</sup> heart cultures contained far less uPA than SR-uPA<sup>+/0</sup> kidney or lung cultures. Moreover, SR-uPA<sup>+/0</sup> heart cultures contained approximately the same amount of uPA as nontransgenic kidney cultures. Thus, nontransgenic kidneys and SR-uPA<sup>+/0</sup> kidneys and lungs contain as much or more uPA as SR-uPA<sup>+/0</sup> hearts, yet SR-uPA<sup>+/0</sup> hearts become inflamed and fibrotic, whereas kidneys and lungs remain normal in appearance. Therefore, the pathology observed in SR-uPA<sup>+/0</sup> hearts cannot be attributed solely to selective accumulation of high levels of uPA in hearts versus other organs.

Transplantation of SR-uPA<sup>+/0</sup> Bone Marrow to Nontransgenic Mice

Because cardiac macrophage accumulation in SR-uPA<sup>+/0</sup> mice preceded cardiac fibrosis, it seemed likely that SR-uPA<sup>+/0</sup> macrophages caused cardiac fibrosis. However, we could not exclude the possibility that low-level SR-uPA transgene expression in nonhematogenous cells of the heart caused subtle cardiac damage, provoking macrophage accumulation as a secondary event. We therefore tested whether presence of the SR-uPA<sup>+/0</sup> transgene in hematogenous cells is sufficient to cause macrophage and collagen accumulation in the heart. Bone marrow was transplanted from male Apoe<sup>−/−</sup> donors (either SR-uPA<sup>+/0</sup> or nontransgenic) to female Apoe<sup>−/−</sup> recipients (all nontransgenic). Hearts harvested 10 weeks later revealed higher macrophage and collagen accumulation in recipients of SR-uPA<sup>+/0</sup> marrow (16 [14 to 28] versus 5.6 [3.6 to 9.6] Mac-3–positive cells/mm<sup>2</sup> [P<0.05]; 4.7% [0.54% to 6.1%] versus 0.0% [0.0% to 0.0%] picrosirius red-positive area [P=0.01; see supplemental figure, available online at http://www.circresaha.org]). Macrophage and collagen accumulation were not found in kidneys, livers, or lungs of recipients of SR-uPA<sup>+/0</sup> bone marrow. For example, we found no difference in density of Mac-3–positive cells in lungs of recipients of SR-uPA<sup>+/0</sup> versus nontransgenic bone marrow (89 [77 to 110] versus 76 [59 to 110] cells/mm<sup>2</sup> [P=0.44; see supplemental figure]). Thus, SR-uPA<sup>+/0</sup> macrophages accumulate specifically in the heart and cause cardiac fibrosis independent of the presence of the SR-uPA<sup>+/0</sup> transgene in nonhematogenous cells.

Macrophage and Collagen Accumulation in Hearts of SR-uPA<sup>+/0</sup> Apoe<sup>+/−</sup> mice

Our experiments suggested that elevated macrophage uPA expression is sufficient to cause cardiac macrophage accumulation and fibrosis. However, these experiments were all performed in Apoe<sup>−/−</sup> mice, leaving the possibility that absence of apoE, severe hyperlipidemia, atherosclerosis, or associated myocardial damage might be required for the effects of elevated macrophage uPA expression to be manifest. To exclude these possibilities, we bred the SR-uPA<sup>+/0</sup> transgene into the Apoe<sup>+/−</sup> background and examined hearts of SR-uPA<sup>+/0</sup> Apoe<sup>+/−</sup> mice. To avoid introducing diet as an uncontrolled variable, Apoe<sup>+/−</sup> mice (SR-uPA<sup>+/0</sup> and nontransgenic littermate controls) were fed the Western-type diet beginning at 5 weeks of age. The SR-uPA transgene does not cause early mortality in Apoe<sup>+/−</sup> mice. Mice were killed 10 weeks later. As expected, plasma cholesterol in Apoe<sup>+/−</sup> mice fed the atherogenic diet was only minimally elevated and did not differ between SR-uPA<sup>+/0</sup> mice (132±18 mg/dL; n=7) and nontransgenic controls (155±36 mg/dL; n=7; P=0.13). Examination of aortic roots of several Apoe<sup>+/−</sup> mice (4 SR-uPA<sup>+/0</sup> and 2 nontransgenic) revealed no atherosclerosis. However, SR-uPA<sup>+/0</sup> Apoe<sup>+/−</sup> mice had more massive hearts.
(Table) and greater cardiac macrophage and collagen accumulation than nontransgenic Apoe<sup>−/−</sup> mice (73 [58 to 83] versus 1.3 [0.0 to 2.5] Mac-3–positive cells/mm<sup>2</sup>; 100 [71 to 140] versus 3.0 [3.0 to 3.0] CD45-positive cells/mm<sup>2</sup>; 6.6 [5.5 to 9.2] versus 0.0050% [0.0% to 0.035%] picrosirius red-positive area [P<0.001 for all; Figure 3]). SR-uPA<sup>+</sup>/0 Apoe<sup>−/−</sup> mice also developed cardiac macrophage accumulation and fibrosis when fed normal chow (data not shown).

**PAI-1–Deficient Mice Develop Cardiac Fibrosis**

Cardiac fibrosis in SR-uPA<sup>−/0</sup> (transgenic) mice and recipients of SR-uPA<sup>−/0</sup> bone marrow appeared to be caused by elevated cardiac uPA activity attributable to the presence of SR-uPA<sup>−/0</sup> macrophages. Alternatively, fibrosis could be attributable to enhanced cardiac macrophage accumulation in SR-uPA<sup>−/0</sup> mice, with uPA-independent macrophage-related cardiotoxicity. To begin to discriminate these possibilities, we tested whether Serpine1<sup>−/−</sup> mice (deficient in PAI-1, the major physiologic inhibitor of uPA) would develop cardiac fibrosis. To maximize our ability to detect fibrosis, we examined hearts of older C57BL/6 Serpine1<sup>−/−</sup> and C57BL/6 Serpine1<sup>−/+</sup> mice (aged 366 [310 to 470] and 404 [366 to 441] days, respectively; P=0.9). Hearts of C57BL/6 Serpine1<sup>−/−</sup> mice had significantly more macrophages (8.1 [0.90 to 12] versus 0.25 [0.16 to 0.47] Mac-3–positive cells/mm<sup>2</sup> [P<0.005]); and collagen (16 [9.9 to 17] versus 3.9% [1.6% to 5.8%] picrosirius red-positive area [P=0.006]) than hearts from age-matched C57BL/6 Serpine1<sup>−/+</sup> controls (Figure 4). Fibrosis was not present in liver, spleen, lungs, or kidneys of Serpine1<sup>−/−</sup> mice or Serpine1<sup>−/+</sup> mice (data not shown).

Because diffuse fibrosis could be caused by microvascular disease, we examined sections of hearts for evidence of small vessel occlusion. Small vessel disease was not present in hearts of SR-uPA<sup>+</sup>/0 or Serpine1<sup>−/−</sup> mice.

**Discussion**

We investigated whether mice with macrophage-targeted overexpression of uPA (SR-uPA<sup>−/0</sup> mice) or deficiency of PAI-1 develop cardiac fibrosis independently of atherosclerosis. Our major findings were as follows: (1) Macrophages accumulate in hearts of SR-uPA<sup>−/0</sup> Apoe<sup>−/−</sup> mice at 3 to 5 weeks of age, before the onset of occlusive coronary disease or cardiac fibrosis; (2) Other major organs of SR-uPA<sup>−/0</sup> Apoe<sup>−/−</sup> mice develop neither macrophage accumulation nor fibrosis despite the presence of higher levels of uPA in these organs than in SR-uPA<sup>−/0</sup> Apoe<sup>−/−</sup> hearts; (3) Apoe<sup>−/−</sup> recipients of SR-uPA<sup>−/0</sup> Apoe<sup>−/−</sup> bone marrow develop macrophage accumulation and fibrosis in their hearts but not in other major organs; (4) The SR-uPA transgene causes cardiac macrophage accumulation and fibrosis even when introduced...
into nonatherosclerotic Apoe<sup>−/−</sup> mice; and (5) Mice deficient in PAI-1 develop cardiac macrophage accumulation and fibrosis. These data suggest that macrophage-expressed uPA plays a critical role in regulating macrophage migration to the heart and in development of cardiac fibrosis.

The molecular and cellular mechanisms that cause cardiac fibrosis are poorly understood. Increased ECM production, a prerequisite for the development of cardiac fibrosis, occurs when cardiac fibroblasts are “activated” to a “myofibroblast” phenotype in settings such as infarction, hypertension, and cardiomyopathy. The stimuli that promote myofibroblast activation in vivo are thought to include angiotensin II, aldosterone, transforming growth factor type β1 (TGF-β<sub>1</sub>), endothelin, catecholamines, stretch, hypoxia, and diabetes. Myofibroblasts activated by these stimuli increase production of ECM components such as collagen, fibronectin, and laminin. When cardiac ECM synthesis outpaces degradation by cardiac MMPs, cardiac fibrosis develops.

Paradoxically, despite the requirement that ECM synthesis outpace MMP-mediated ECM degradation for cardiac fibrosis to develop, extensive data implicate MMP-mediated ECM degradation in initiation of cardiac fibrosis. These data include early upregulation of MMPs during the transition from cardiac hypertrophy to dilation and the finding that MMPs facilitate myofibroblast migration and upregulate the activity of potentially fibrogenic cytokines such as TNF-α and TGF-β. To test more directly whether increased MMP activity can cause cardiac fibrosis, Kim et al overexpressed human MMP-1 in mouse hearts and found increased cardiac fibrosis at 6 months of age. This observation led to the hypothesis that collagen degradation products, generated by active MMP-1, stimulated collagen synthesis by cardiac fibroblasts. Development of cardiac fibrosis in mice with cardiac MMP-1 overexpression supports a cause-and-effect relationship between elevated cardiac protease expression and fibrosis. However, the applicability of this animal model to human cardiac fibrosis (which is a progressive condition) is limited by the fact that the predominant phenotype of the MMP-1–overexpressing mice is cardiac hypertrophy rather than fibrosis. In addition, by 12 months of age, MMP-1–overexpressing mice have less cardiac fibrosis than nontransgenic controls. Therefore, an animal model of protease-induced, progressive cardiac fibrosis has not yet been reported.

Here we report that overexpression of uPA, a protease outside the MMP family, causes cardiac fibrosis. Cardiac fibrosis in SR-uPA<sup>+/+</sup> mice is evident at 10 weeks of age and persists for ≥1 year (data not shown). Others have suggested that uPA might initiate cardiac fibrosis through activation of MMPs. Nevertheless, our report is the first to show that overexpression of uPA is sufficient to cause cardiac fibrosis. The absence of cardiac fibrosis in mice that overexpress uPA in hepatocytes suggests that macrophage-expressed uPA rather than elevated uPA per se is critical in generating the phenotypes reported here.

There are 2 hypothetical pathways through which uPA could cause cardiac fibrosis: (1) primary activation of cardiac fibroblasts (the “fibroblast activation” hypothesis); and (2) primary cardiomyocyte toxicity with secondary, reactive fibrosis (the “cardiotoxic” hypothesis). The fibroblast activation hypothesis proposes that uPA activates cardiac fibroblasts indirectly through plasmin-mediated activation of
Expression causes fibrosis, whereas PAI-1 deficiency or uPA-dependent MMP activation leading to proteolysis of cell matrix contacts. Disruption of these contacts could cause cardiomyocyte dysfunction and death. However, we did not find any differences in levels of active MMP-2 or MMP-9 in zymograms of heart extracts from 5-week-old SR-uPA+/− and nontransgenic Apoe−/− mice (n=8 from each group). There was no difference between the groups (1.9×10⁴ erythrocytes/mm² in both groups; P=0.8). Moreover, we never observed spontaneous hemorrhage in SR-uPA+/− mice, suggesting that they have normal hemostasis. In contrast, mice lacking fibrinogen have abnormal hemostasis but do not develop cardiac fibrosis. Therefore, uncontrolled fibrinolysis does not appear to be the cause of cardiac fibrosis in SR-uPA+/− mice. Alternatively, uPA could damage cardiomyocytes by plasminogen-dependent MMP activation leading to proteolysis of cell-matrix contacts. Disruption of these contacts could cause cardiomyocyte dysfunction and death. However, we did not find any differences in levels of active MMP-2 or MMP-9 in zymograms of heart extracts from 5-week-old SR-uPA+/− and nontransgenic mice (data not shown). Nevertheless, we cannot exclude that other MMPs are activated in SR-uPA+/− mice or that MMP-2, MMP-9, or other proteases might be activated at other time points. Further experimentation is required to elucidate the mechanisms of uPA-mediated cardiac fibrosis.

Perhaps the most intriguing aspect of our data is our finding that the heart is uniquely susceptible to macrophage infiltration and fibrosis caused by either overexpression of uPA or absence of PAI-1. uPA-expressing macrophages that are either autologous or were transplanted from a congenic donor accumulate specifically in the heart. This observation is reminiscent of the requirement for uPA, plasminogen, and fibrinogen in zymograms of heart extracts from 5-week-old SR-uPA+/− and nontransgenic mice. Because of these findings, we suggest that uPA may have an important role in cardiac fibrosis. The second part of a hypothesis that would explain the apparently paradoxical increase in cardiac fibrosis in Serpine1−/− mice is that the heart is particularly susceptible to damage by dysregulated proteolysis. Unlike other organs, the heart is in constant motion with alternating contraction and relaxation, and is subject to repetitive hemodynamic stresses. Resisting these stresses and performing the work of systole and diastole requires coordination of cardiomyocyte function, which is normally achieved by proteins that link cardiomyocytes and their cytoskeletons to the surrounding ECM and to each other. These proteins include the dystrophin/sarcoglycan complex and matrix proteins such as laminin, to which this complex attaches. Proteolysis of this complex causes cardiomyopathy. Fibrosis might develop in hearts but not in other organs of Serpine1−/− mice because of a unique dependence of the heart on extracellular matrix. Of interests, insights into these mechanisms will be useful in developing therapies that prevent myocarditis, enhance infarct healing, and prevent cardiac failure and fibrosis.

Finally, our data suggest a new physiologic role for PAI-1: cardioprotection. Because PAI-1 null mice have relatively unopposed uPA activity, spontaneous cardiac fibrosis in these mice is consistent with our finding of cardiac fibrosis in SR-uPA+/− mice and with the report of impaired collagenous scar formation in infarcted hearts of uPA null mice. However, these data contrast with results obtained in animal models of lung and kidney fibrosis, in which PAI-1 overexpression causes fibrosis, whereas PAI-1 deficiency or uPA overexpression protects from fibrosis. In addition, a recent report of decreased perivascular fibrosis in infarcted hearts of Serpine1−/− mice appears to conflict with our finding of increased fibrosis in hearts of aging Serpine1−/− mice.
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**Materials and Methods**

**Experimental Animals**

Transgenic mice with macrophage-targeted overexpression of uPA (SR-uPA^{+/0} mice) were described previously.\(^1\) SR-uPA^{+/0} mice in the current experiments had been backcrossed into the C57BL/6 background for at least 8 generations. All comparisons between SR-uPA^{+/0} and nontransgenic mice are between littermates. Nontransgenic C57BL/6 Apoe\(^{-/-}\) mice (used as bone marrow transplant recipients), C57BL/6 mice deficient in PAI-1 (Serpine1\(^{-/-}\)), and C57BL/6 Serpine1\(^{+/+}\) controls were purchased (Jackson Laboratory). All mice were housed under specific pathogen-free conditions. Pups were weaned at 4 weeks of age and genotyped by Southern blot or PCR analysis of tail DNA. Some mice were fed a Western-type diet containing 21% fat and 0.15% cholesterol (TD88137; Harlan-Tekland). SR-uPA\(^{+/0}\) mice and their nontransgenic controls were fed this diet beginning at 5 weeks of age. Bone marrow transplant recipients were fed this diet beginning after transplantation (8 weeks of age). Serpine1\(^{+/+}\) mice and their controls were not fed the Western-type diet. All mice except for the bone marrow donors were female. All animal protocols were approved by the Institutional Animal Care and Use Committees.

**Lipid Analysis**

Plasma total cholesterol was measured with a colorimetric assay (Spectrum cholesterol assay; Abbott Laboratories).
Immunostaining and Histochemistry

In initial experiments, mice were deeply anesthetized, then perfused with 0.9% saline followed by 10% phosphate-buffered formalin with 7.5% sucrose, 2.0 mM EDTA, and 0.02 mM BHT via cardiac puncture. Tissues were stored in 10% phosphate-buffered formalin overnight at 4°C and embedded in paraffin. In later experiments anesthetized mice were exsanguinated, hearts were excised, placed in PBS with 5% dextrose, 25 mM KCl, then fixed and processed into paraffin as above. Serial sections (each 5 µm thick) were taken at four levels: the aortic root, and the base, mid-ventricle, and apex of the heart. The right lung, a segment of liver, and the right kidney were also processed into paraffin. Macrophages and leukocytes were detected with rat anti-mouse monoclonal antibodies: anti-Mac-3, clone M3/84 (2.5 µg/ml) and anti-mouse CD45, clone 30-F11 (62.5 ng/ml), respectively (Pharmingen). Bound antibody was detected with peroxidase-conjugated goat anti-rat IgG (Kirkegaard and Perry) and a diaminobenzidine substrate followed by hematoxylin counterstaining. Control slides were incubated with isotype-matched primary antibodies (Pharmingen). Other sections were stained with hematoxylin and eosin (H & E), Masson trichrome, or picrosirius red.

Quantification of Leukocytes, Macrophages, and Collagen in Tissue Sections

Observers blinded to genotype quantified the extent of leukocyte (CD45) or macrophage (Mac-3) infiltration by counting stained cells in three sections, one each from the base, mid-ventricle, and apex of the heart of each mouse. Cells were counted in ten random high-power (400X) microscopic fields per section (30 fields per heart) and the average
cell density calculated. In some mice, Mac-3- and CD45-positive cells were also counted in 10 high-power fields of a single section of lung. Sections of heart, kidney, liver, lung, and spleen were also stained with H & E and Masson trichrome to assess, qualitatively, whether fibrosis was present. In groups of mice in which fibrosis was evident by trichrome stain (and in hearts of mice in matched control groups) collagen accumulation was quantified by picrosirius red staining of a section from the mid-ventricle and computer-assisted color thresholding and measurement of stained and total areas (Image Pro 3.0 software, Media Cybernetics).

**Measurement of Plasminogen Activator Activity**

Deeply anesthetized mice were exsanguinated by saline perfusion. Hearts, lungs, and kidneys were sliced in pieces of approximately 1 mm thickness. The pieces were weighed then incubated overnight at 37 °C in M199 medium. Plasminogen activator (PA) activity was detected by incubating aliquots of explant culture media with gliplasminogen (0.4 µM; American Diagnostica) and the plasmin substrate S-2251 (0.9 mM; Chromogenix) and measuring OD$_{405}$. uPA activity was calculated with reference to a standard curve constructed with human single-chain uPA (American Diagnostica).

**Bone Marrow Transplantation**

8 week-old, female nontransgenic Apo$_e^{-/-}$ mice were lethally irradiated (9.5 Gy from a $^{137}$[Cs] source). 8 week-old, male Apo$_e^{-/-}$ mice (either SR-uPA$^{+/0}$ or nontransgenic) were used as donors of whole bone marrow.$^2$ Briefly, donor mice were euthanized, femurs and tibias removed and marrow flushed out with RPMI 1640, 2% FBS, 5 U/ml heparin. Bone
marrow cells were dispersed, counted, and resuspended in PBS at $2 \times 10^7$ nucleated cells/ml. Irradiated mice received $6 \times 10^6$ bone marrow cells from either a SR-uPA$^{+/0}$ Apoe$^{+/-}$ or nontransgenic Apoe$^{+/-}$ donor, by tail vein injection. In our laboratory, this protocol leads to replacement of approximately 80% of peripheral blood leukocytes with donor-derived cells by 9 weeks after transplantation (data not shown).

**Statistical Analysis**

Data are presented as mean ± SD for normally distributed data or median (25–75% range) for data not normally distributed. Group means were compared with the t test and medians were compared with the Mann-Whitney rank-sum test.

**References**


**Figure.** Transplantation of transgenic (SR-uPA$^{+/0}$) bone marrow to nontransgenic (non-tg) recipients causes cardiac macrophage accumulation and fibrosis. Density of cardiac (A) and lung (B) macrophages and cardiac collagen content (C) in non-tg mice 10 weeks
after receipt of bone marrow from a non-tg donor ($n = 8 – 9$) or a SR-uPA$^{+/0}$ donor ($n = 6$). (D – G) Mac-3 immunostain with hematoxylin counterstain; F and G are high-power views of boxes in D and E. Arrows (F, G) indicate Mac-3 positive cells. (H – I) picrosirius red stain. Size bars: D, E, H, I: 200 µm; F, G: 50 µm. * Points in A – C represent individual mice; horizontal lines are group medians. $P < 0.05$; ** $P = 0.01$. 