Reduction of mouse atherosclerosis by urokinase inhibition or with a limited-spectrum matrix metalloproteinase inhibitor

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Aims
Elevated activity of urokinase plasminogen activator (uPA) and MMPs in human arteries is associated with accelerated atherosclerosis, aneurysms, and plaque rupture. We used Apoe-null mice with macrophage-specific uPA overexpression (SR-uPA mice; a well-characterized model of protease-accelerated atherosclerosis) to investigate whether systemic inhibition of proteolytic activity of uPA or a subset of MMPs can reduce protease-induced atherosclerosis and aortic dilation.

Methods and results
SR-uPA mice were fed a high-fat diet for 10 weeks and treated either with an antibody inhibiting mouse uPA (mU1) or a control antibody. mU1-treated mice were also compared with PBS-treated non-uPA-overexpressing Apoe-null mice. Other SR-uPA mice were treated with one of three doses of a limited-spectrum synthetic MMP inhibitor (XL784) or vehicle. mU1 reduced aortic root intimal lesion area (20%; \( P = 0.05 \)) and aortic root circumference (12%; \( P = 0.01 \)). All XL784 doses reduced aortic root intimal lesion area (22–29%) and oil-red-O-positive lesion area (36–42%; \( P < 0.05 \) for all doses and both end points), with trends towards reduced aortic root circumference (6–10%). Neither mU1 nor XL784 significantly altered percent aortic surface lesion coverage. Several lines of evidence identified MMP-13 as a mediator of uPA-induced aortic MMP activity.

Conclusions
Pharmacological inhibition of either uPA or selected MMPs decreased atherosclerosis in SR-uPA mice. uPA inhibition decreased aortic dilation. Differential effects of both agents on aortic root vs. distal aortic atherosclerosis suggest prevention of atherosclerosis progression vs. initiation. Systemic inhibition of uPA or a subset of MMPs shows promise for treating atherosclerosis.

Keywords
Atherosclerosis • Urokinase • Matrix metalloproteinases • Protease inhibition • Mouse models

1. Introduction
Extracellular proteases (including matrix metalloproteinases (MMPs), serine proteases, and cysteine proteases) are expressed in diseased arteries and play critical roles in several biological processes that contribute to vascular disease: cell migration, extracellular matrix metabolism, vasoconstriction, vascular wall permeability, thrombosis, apoptosis, and cell proliferation.1–3 These processes contribute—in turn—to atherosclerotic lesion growth, aneurysm formation, and plaque rupture. Proteases are among the most common drug targets.4 However, considering the diverse pathways through which proteases contribute to vascular disease—and the large number of proteases that appear to accelerate vascular disease5—protease inhibition appears underused in cardiovascular therapeutics.

Urokinase-type plasminogen activator (uPA)5,6 and certain MMPs7 are among the proteases that contribute to atherosclerosis and its complications. Accordingly, the primary goal of the present study was to begin to investigate the potential of two novel protease inhibitors—an antibody that inhibits uPA8 and a small-molecule selective MMP inhibitor (XL784)9—to prevent atherosclerosis. To begin to test these inhibitors, we administered them to atherosclerosis-prone (Apoe-/-) mice that have macrophage-specific overexpression of uPA.9 These ‘SR-uPA’
mice have accelerated atherosclerosis and aortic dilation that are mediated by activation of the uPA substrate plasminogen, itself a broad-spectrum serine protease. SR-uPA mice also have elevated aortic MMP activity and develop histologic correlates of atherosclerotic plaque instability, including intraplaque haemorrhage and fibrous cap disruption. Because—as is likely the case with humans—SR-uPA mice have protease-driven atherosclerosis, aortic dilation, and plaque rupture, they are an appropriate experimental setting for beginning to test whether pharmacological inhibition of uPA or select MMP activities can retard or prevent atherosclerosis. If ineffective in SR-uPA mice, these inhibitors would be unlikely to prevent atherosclerosis in other mouse models or humans. If effective, further preclinical testing (for example in atherosclerosis-prone mice that do not overexpress proteases or in older SR-uPA mice susceptible to plaque rupture) as well as clinical testing of these inhibitors would be warranted.

Here we report that both antibody-based uPA inhibition and selective MMP inhibition with XL784 decrease atherosclerosis in SR-uPA mice. Antibody-based uPA inhibition decreases aortic root dilation. These results encourage further development and testing of these or other uPA and MMP inhibitors as human cardiovascular therapeutics. Our data also tentatively identify MMP-13 as a downstream mediator of uPA-induced vascular pathology.

2. Methods

2.1 Animals

All mice were ApoE<sup>−/−</sup> and were progeny of at least 10 generations of C57BL/6 backcrosses. ApoE<sup>−/−</sup> mice with macrophage-specific overexpression of urorokine (‘SR-uPA’ mice, hemizygous for a transgene that includes the murine uPA gene driven by the human scavenger receptor promoter) were generated in our laboratory. SR-uPA mice were bred with non-transgenic (SR-uPA<sup>0/0</sup>) ApoE<sup>−/−</sup> mice to yield SR-uPA and non-transgenic littermate controls. Mice were housed in a specific-pathogen-free facility and transgenic mice. All animal protocols were approved by the University of Michigan Institutional Animal Care and Use Committee.

2.2 Susceptibility to a uPA-activated toxin

Both SR-uPA and non-transgenic mice were injected intraperitoneally with PrAg-U2 [a mutated version of anthrax protective antigen (PrAg), in which the furin cleavage site of PrAg is replaced by a uPA substrate sequence] and FP59 (a recombinant effector protein that kills cells in a PrAg-dependent manner). When combined, PrAg-U2 and FP59 comprise a toxin that lacks the broad cellular toxicity of native PrAg and instead has a highly specific toxicity for cells with surface uPA activity. After injection, mice were monitored closely for signs of toxicity including weight loss, inactivity, and inappetence and were euthanized by carbon dioxide (CO<sub>2</sub>) inhalation when toxicity appeared. Toxicity was also considered present when an injected mouse was found dead. To identify a highly toxic dose of PrAg-U2/FP59 in C57BL/6 ApoE<sup>−/−</sup> mice, we first used a fixed dose of FP59 (10 μg per mouse; ~0.5 mg/kg body weight) along with escalating doses of PrAg-U2 (0.4–2.4 mg/kg body weight). Because we found low toxicity with this regimen we injected higher doses: 3.2 mg/kg FP59 and 9.6 mg/kg PrAg-U2. Our goal was to achieve a high level of toxicity, thereby permitting investigation of whether injection of the murine mAb mU1 (which inhibits mouse uPA activity) would block PrAg-U2/FP59 toxicity in SR-uPA (i.e. uPA-overexpressing) as well as non-transgenic mice. mU1 was generated by immunizing uPA knockout mice with recombinant mouse pro-uPA and subsequent hybridoma generation. Use of a murine mAb in these experiments eliminates concerns about immune responses to antibody therapy and ensures specificity of the antibody target. To determine whether mU1 inhibits PrAg-U2/FP59 toxicity, we first injected mice intraperitoneally with either mU1, an isotype-control mAb directed at trinitrophenol (TNP; both at 60 mg/kg), or PBS (200 μL). Preparations of both antibodies were endotoxin-free. These same injections were repeated the next day. Immediately after this second injection of antibody or PBS, mice were injected with 9.6 mg/kg PrAg-U2 and 3.2 mg/kg FP59. Mice were observed twice per day for 7 days for signs of toxicity.

2.3 Atherosclerosis studies

Mice were fed a high-fat diet (21% fat, 0.15% cholesterol by weight; Harlan-Teklad, Kent, WA #TD.88137) beginning at ~5–6 weeks of age and continuing for 10 weeks. To test for therapeutic effects of mU1 (the ‘mU1 study’; Supplementary material online, Figure S1), mice were injected with a loading dose of antibody (mU1 or anti-TNP; 100 mg/kg) or a similar volume of PBS at the time the high-fat diet was begun. The primary hypothesis tested in the mU1 study was that injection of mU1 would reduce atherosclerosis and aortic dilation compared with the anti-TNP control group. A group of PBS-injected non-transgenic mice was added while this experiment was in progress. We added this third group of mice to test a secondary hypothesis: that mU1 treatment would completely eliminate the effect of the SR-uPA transgene on atherosclerosis and aortic dilation. The half-life of mU1 is 3 days and therefore injections of antibody (50 mg/kg) or a similar volume of PBS were repeated twice weekly for the study duration. To test therapeutic effects of the MMP inhibitor XL784 (the ‘XL784 study’; Supplementary material online, Figure S1) mice were gavaged daily with a single dose of XL784 (125, 250, or 500 mg/kg; prepared freshly in vehicle) or with vehicle alone (by volume: 12.5% Cremophor EL; Sigma-Aldrich Corp, St Louis, MO, USA; 12.5% ethanol; and 75% Hank’s Balanced Salt Solution). We chose this range of XL784 doses based on unpublished data (now published) showing that 500 mg/kg/day of XL784 was highly effective in limiting experimental aneurysm formation. We also tested whether lower doses might be similarly effective. After 10 weeks on high-fat diet, mice were deeply anesthetized by i.p. injection of ketamine (225 mg/kg) and xylazine (23 mg/kg), their blood and plasma collected, then exsanguinated by saline perfusion. Aortas were removed, trimmed, rinsed, and incubated overnight in M199 (GIBCO, Carlsbad, CA, USA) at 37 °C with 5% CO<sub>2</sub>. Culture medium conditioned by the explanted aortas (CM) was then stored at ~80 °C and the aortas were fixed by incubation in formalin. Hearts (with attached aortic roots) were placed in fixative (10% phosphate-buffered formalin pH 7.4, containing 220 mM sucrose, 2 mM EDTA, 0.02 mM butylated hydroxytoluene (Sigma)) for at least 48 h. The hearts were then divided transversely. The base and attached aortic root were embedded in optimal cutting temperature medium and frozen.

2.4 Measurement of plasma total and HDL-cholesterol, triglycerides, blood counts, and aortic MMP activity

Total plasma cholesterol and triglycerides were measured with blood obtained from the retro-orbital plexus (Spectrum cholesterol assay, Abbott Diagnostics, Abbott Park, IL). L-Type TG M, Wako Diagnostics, Mountain View, CA, USA). HDL-cholesterol in the same samples was measured (Sekisui Diagnostics, Lexington, MA, USA) after PEG precipitation of apoB-containing lipoproteins, and non-HDL-cholesterol was calculated. Complete blood counts (blood collected from the same location) were performed by an outside laboratory (Phoenix Central Laboratory, Mukilteo, WA, USA). Total MMP activity in aortic CM was measured with the Omni MMP substrate (Enzo Life Sciences, Farmingdale, NY, USA). Activities of MMP-8 and MMP-13 in aortic CM were measured using fluorogenic
substrates [MMP-8 substrate: DNP-Pro-Leu-Ala-Tyr-Trp-Ala-Arg, (Sigma); MMP13 substrate: MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2; (EMD Millipore, Billerica, MA, USA)]. The MMP-13 substrate is also cleaved by MMP-8. However, according to the manufacturer, MMP-8 cleaves this substrate at lower efficiency. A separate cohort of male mice, used to measure MMP-13 activity in aortic CM, was fed the high-fat diet for 15 weeks beginning at age 6–8 weeks.

2.5 Measurement of MMP-13 mRNA and protein

MMP-13 mRNA was measured by quantitative RT-PCR using RNA extracted from aortas of non-transgenic Apoe−/− recipients of either SR-uPA Apoe−/− or non-transgenic Apoe−/− bone marrow. These aortic mRNA samples were available from past experiments.11,14 PCR primers for MMP-13 were: 5′-AAAGATTATCCCGCCTCAT-3′ and 5′-TGGGC CCATTGGAAAGTAG-3′. MMP-13 mRNA levels were normalized to GAPDH mRNA measured in the same samples.11 MMP-13 protein was detected by western blot analysis of aortic CM obtained from mice in the present study. For each sample, 24 μL of CM were separated by SDS–PAGE under reducing conditions, both activities inhibited by EDTA (data not shown). Fragments of APMA-activated MMP-13 cleaved both the OMNI-MMP and MMP-13 substrates, with activity showing far lower levels of uPA than SR-uPA mice)5,14 were included in experiments that primarily compare mU1 with anti-TNP treatment and secondarily compare mU1-treated SR-uPA mice to PBS-treated non-transgenic mice. This approach does not correct for multiple comparisons; however, we believe that it is justified by our experimental design, which prioritizes the first comparison as the primary end point. In experiments testing multiple doses of XL784, we used one-way ANOVA (followed by Dunnett’s method for post hoc correction for multiple comparisons) to test whether each of the treatment groups differed significantly from the control group. When conditions of normality and equal variance were not met, we used Kruskal–Wallis ANOVA, also with post hoc correction. To identify trends that might guide future research, we also performed t-tests between the XL784-treated groups and the control group. Results of these t-tests are not corrected post hoc for multiple comparisons and accordingly should be viewed as hypothesis-generating. Toxicity-free survival curves were generated with the Kaplan–Meier method and compared with the log-rank test. Categorical data (e.g. presence or absence of PrAg-U2/FPS9 toxicity) were analysed by chi square or Fisher exact test. Correlations were analysed using the Pearson Product Moment test. All tests were carried out with the SigmaStat program (Systat Software, Chicago, IL, USA).

3. Results

3.1 Activity of a uPA-Activated toxin in SR-uPA mice

Identification of a highly toxic dose of the uPA-activated toxin PrAg-U2/FPS915 was necessary in order to enable us to test whether the uPA-inhibitory antibody mU113 could inhibit uPA activity in uPA-overexpressing SR-uPA Apoe−/− mice. Experiments with low PrAg-U2/FPS9 doses (used in published studies)16,22 revealed equivalent but modest toxicity in both SR-uPA and non-transgenic mice (data not shown). A larger experiment with a higher dose of PrAg-U2 (2.4 mg/kg with ~0.5 mg/kg FPS9) also showed equivalent—but still submaximal—toxicity (10 of 20 SR-uPA mice and 14 of 20 non-transgenic mice; P = 0.3). We therefore increased the doses of both agents (to 9.6 mg/kg PrAg-U2 and 3.2 mg/kg FPS9), achieving 91% toxicity (10 of 11 mice). We anticipated that this highly toxic dose of PrAg-U2/FPS9 would allow detection of protective effects of mU1 using a relatively small number of mice.

3.2 MU1 delays toxicity of a uPA-activated toxin in SR-uPA mice

We next tested whether mU1 could protect SR-uPA mice from uPA-dependent PrAg-U2/FPS9 toxicity. Non-transgenic mice (which express far lower levels of uPA than SR-uPA mice)5,14 were included as a positive control, potentially useful in the event mU1 was ineffective in SR-uPA mice. To our knowledge, susceptibility of mice to PrAg-U2/FPS9 is the most sensitive and specific measure of in vivo uPA activity.15,16 Therefore, protection from PrAg-U2/FPS9 toxicity would be the best indication that mU1 decreases in vivo uPA activity in SR-uPA mice. Only 3 of 22 (14%) of PrAg-U2/FPS9-injected mice that received either anti-TNP or PBS were still healthy by the end of day 2. These included 2 of 14 (14%) SR-uPA mice and 1 of 8 (12%) non-transgenic mice (see Supplementary material online, Table S1). In contrast, mU1 delayed toxicity beyond day 2 in 6 of 6 (100%) PrAg-U2/FPS9-injected SR-uPA mice and 3 of 4 (75%) PrAg-U2/FPS9-injected non-transgenic mice (P = 0.4 for comparison of SR-uPA and non-transgenic mice, indicating equivalent efficacy of mU1 in both lines). Combining the data from SR-uPA and non-transgenic mice, mU1 significantly extended survival of PrAg-U2/FPS9-injected
mice (see Supplementary material online, Figure S2; \( P = 0.02 \)). Therefore, mU1 is equally (although incompletely) protective in both uPA-overexpressing SR-uPA and non-transgenic mice. Because mU1 significantly decreases in vivo uPA activity (required for PrAg-U2/FP59 toxicity)\(^\text{15}\) in \( \text{Apo}^{-/-} \) mice, mU1 is a useful reagent for testing the hypothesis that in vivo pharmacological suppression of uPA activity in SR-uPA \( \text{Apo}^{-/-} \) mice will prevent uPA-induced atherosclerosis and its complications.

### 3.3 MU1 partially prevents accelerated atherosclerosis and aortic dilation in SR-uPA mice

Compared with mice treated with the control anti-TNP antibody, SR-uPA mice treated with mU1 had less aortic root intimal lesion area (20% decrease; \( P = 0.05 \); Figure 1A, C and D) and smaller aortic root circumferences (12% decrease; \( P = 0.01 \); Figure 1B–D). mU1 decreased total intimal macrophage-positive and ORO-positive areas by similar amounts (20–30%); however, these differences were not statistically significant (\( P = 0.3 \) and \( P = 0.4 \), respectively; Table 1). Accordingly, neither the percentage of intimal lesion area occupied by macrophages nor the percentage occupied by lipid was decreased by mU1 (\( P = 1.0 \) and 0.4, respectively; Table 1). Analysis of Sudan IV-stained, pinned aortas revealed non-significant trends towards less total aortic surface area (5% decrease) and lower percent coverage of the aortic lumen surface with lesions (20% decrease) in mU1-treated vs. anti-TNP-treated mice (\( P = 0.1 \) for both; Table 1). The number of individual lesions on pinned aortic surfaces did not differ between the two groups (\( P = 0.3 \); Table 1).

To determine whether treatment with mU1 eliminated the contribution of uPA overexpression to murine atherosclerosis, we compared atherosclerosis and aortic root circumference between mU1-treated SR-uPA mice and PBS-treated non-transgenic mice. PBS-treated non-transgenic mice had significantly smaller aortic root lesion area and aortic root circumference than mU1-treated SR-uPA mice (\( \sim 30 \) and 15% smaller, respectively; \( P = 0.01 \) for both; Figure 1A, B, and E). PBS-treated non-transgenic mice also had significantly less aortic root lesion oil red O area and tended to have less aortic root lesion macrophage area than mU1-treated SR-uPA mice (\( \sim 30 \) decrease for both; \( P = 0.03 \) and 0.07, respectively; Table 1).

### 3.4 XL784 significantly reduces atherosclerosis in SR-uPA mice

Treatment with all three doses of XL784 significantly reduced aortic root intimal lesion area (20–30%; \( P < 0.05 \) for all doses; Figure 2A). All three doses of XL784 also significantly reduced total aortic root lesion oil red O-positive area (36–42%; \( P < 0.05 \) for all three doses; Figure 2B). All XL784 doses also nominally reduced total aortic root lesion macrophage-positive area (15–33%). This result was of only borderline statistical significance (\( P = 0.1 \) by one-way ANOVA); however, the highest XL784 dose had a significant effect by \( t \)-test (\( P = 0.02 \); Figure 2C). Neither the percentage of lesion oil red O-positivity nor the percentage of macrophage positivity in aortic root lesions were significantly affected by XL784 (\( P \geq 0.2 \); Table 2). All doses of XL784 also yielded non-significant trends towards reduced aortic root circumference (6–10% for all three; \( P = 0.17 \) by one-way ANOVA; by \( t \)-test the lowest dose reduced circumference significantly; \( P < 0.05 \); Figure 2D).

**Figure 1** Antibody-mediated inhibition of uPA decreases aortic root atherosclerosis and aortic root dilation in uPA-overexpressing (SR-uPA) mice. SR-uPA mice were treated for 10 weeks with either the control anti-TNP antibody (\( n = 9 \)) or the uPA-inhibitory antibody mU1 (\( n = 10 \)). Littermate non-transgenic (Ntg) mice were treated with PBS (\( n = 11 \)), also for 10 weeks. (A) Mean intimal area of aortic root lesions. (B) The circumference of the aortic root at the level of the internal elastic lamina (IEL). Data points are individual mice and bars are group means. (C–E) Aortic root lesions in uPA-overexpressing (SR-uPA) mice treated with (C) the control anti-TNP antibody or (D) the uPA-inhibitory antibody mU1, and (E) in non-transgenic mice treated with PBS. Haematoxylin and eosin stain; size bar (C) = 500 \( \mu \)m. (C–E) are representative images of 9–11 aortas per group.
Table 1 Atherosclerosis in mU1- and anti-TNP-treated SR-uPA mice

<table>
<thead>
<tr>
<th></th>
<th>SR-uPA&lt;sup&gt;+/0&lt;/sup&gt; + α-TNP (n)</th>
<th>SR-uPA&lt;sup&gt;+/0&lt;/sup&gt; + mU1 (n)</th>
<th>P (α-TNP vs mU1)</th>
<th>SR-uPA&lt;sup&gt;+/0&lt;/sup&gt; + PBS (n)</th>
<th>P (mU1 vs SR-uPA&lt;sup&gt;+/0&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>Aortic root Mac-2 area (µm&lt;sup&gt;2&lt;/sup&gt; × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>1.50 ± 0.81 (9)</td>
<td>1.20 ± 0.58 (10)</td>
<td>0.3</td>
<td>0.79 ± 0.23 (11)</td>
<td>0.07</td>
</tr>
<tr>
<td>Aortic root Mac-2 area (% of intimal area)</td>
<td>16 ± 7.8 (9)</td>
<td>17 ± 9.0 (10)</td>
<td>1.0</td>
<td>16 ± 5.0 (11)</td>
<td>0.8</td>
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<tr>
<td>Aortic root oil red O area (µm&lt;sup&gt;2&lt;/sup&gt; × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>1.3 ± 0.30 (9)</td>
<td>1.1 ± 0.42 (10)</td>
<td>0.4</td>
<td>0.77 ± 0.22 (11)</td>
<td>0.03</td>
</tr>
<tr>
<td>Aortic root oil red O area (% of intimal area)</td>
<td>15 ± 3.9 (9)</td>
<td>17 ± 6.6 (10)</td>
<td>0.4</td>
<td>16 ± 3.1 (11)</td>
<td>0.5</td>
</tr>
<tr>
<td>Total aortic lumen surface area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>59 (56–60) (9)</td>
<td>56 (53–58) (10)</td>
<td>0.1</td>
<td>53 (51–55) (11)</td>
<td>0.2</td>
</tr>
<tr>
<td>Sudanophilic lesions (% of total aortic lumen surface area)</td>
<td>2.2 (1.8–3.0) (9)</td>
<td>1.7 (1.5–2.0) (10)</td>
<td>0.1</td>
<td>0.45 (0.27–2.1) (11)</td>
<td>0.3</td>
</tr>
<tr>
<td>Number of lesions on pinned aortic surface</td>
<td>14 (12–17) (9)</td>
<td>13 (11–16) (10)</td>
<td>0.3</td>
<td>11 (8–14) (11)</td>
<td>0.2</td>
</tr>
<tr>
<td>Peripheral blood monocytes (per µL)</td>
<td>140 ± 100 (5)</td>
<td>265 ± 160 (5)</td>
<td>0.2</td>
<td>180 ± 160 (5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Peripheral blood monocytes (% total leukocytes)</td>
<td>3.0 (2.0–4.0) (5)</td>
<td>3.0 (2.5–7.0) (5)</td>
<td>0.7</td>
<td>2.0 (2.0–4.5) (5)</td>
<td>0.6</td>
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<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>1140 ± 197 (8)</td>
<td>1140 ± 178 (8)</td>
<td>0.9</td>
<td>1260 ± 101 (11)</td>
<td>0.08</td>
</tr>
<tr>
<td>Plasma TG (mg/dL)</td>
<td>51 ± 23 (8)</td>
<td>37 ± 13 (10)</td>
<td>0.1</td>
<td>34 ± 16 (11)</td>
<td>0.6</td>
</tr>
<tr>
<td>Plasma HDL (mg/dL)</td>
<td>27 ± 6.3 (8)</td>
<td>27 ± 5.0 (10)</td>
<td>0.9</td>
<td>18 ± 5.2 (11)</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma non-HDL-cholesterol (mg/dL)</td>
<td>1110 ± 199 (8)</td>
<td>1120 ± 177 (10)</td>
<td>0.9</td>
<td>1240 ± 102 (11)</td>
<td>0.06</td>
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Values are mean ± SD or median (25–75%) range. P values are from unpaired t test or Mann–Whitney rank-sum test. P values in right column compare SR-uPA<sup>+/0</sup> + mU1 and SR-uPA<sup>+/0</sup> + PBS.

n = number of mice in each group.

There was no significant effect of any of the XL784 doses on total aortic surface area, percent aortic surface Sudan IV-positivity, or number of individual lesions on pinned aortas (P ≥ 0.4; Table 2).

3.5 Neither mU1 nor XL784 alters peripheral blood monocyte counts or plasma cholesterol levels in SR-uPA mice; XL784 decreases plasma triglycerides

Compared with SR-uPA mice injected with anti-TNP, injection of mU1 did not affect either total peripheral blood monocyte counts or percentages of circulating monocytes (P ≥ 0.2; Table 1). Similarly, mU1 treatment did not significantly affect plasma cholesterol, HDL-cholesterol, non-HDL-cholesterol, or triglycerides (P = 0.9 vs. anti-TNP-treated mice for all cholesterol measurements; P = 0.1 for triglycerides; Table 1). HDL-cholesterol was increased in both groups of SR-uPA mice vs. non-transgenic PBS-injected mice. Compared with vehicle-treated mice, none of the three doses of XL784 significantly affected either total peripheral blood monocyte counts, percentages of circulating monocytes, total plasma cholesterol, HDL-cholesterol, or non-HDL-cholesterol (P ≥ 0.2; Table 2). All three doses of XL784 significantly decreased plasma triglycerides (50–60%; P = 0.001 by ANOVA).

3.6 MMP-13 activity is significantly increased in aortas of SR-uPA mice and correlates with total MMP activity

Decreased atherosclerosis in SR-uPA mice treated with XL784 suggests that uPA might accelerate atherosclerosis—at least in part—via MMP activation. A role for MMPs in uPA-accelerated atherosclerosis was suggested by our finding of elevated total MMP activity in aortic-conditioned media (CM) of SR-uPA vs. non-transgenic Apoe<sup>−/−</sup> mice.11 We used the inhibitory profile of XL784<sup>8</sup> to guide experiments aimed at identifying specific MMP(s) that are activated in aortas of SR-uPA mice. Four MMPs are inhibited by XL784 with IC<sub>50</sub> values in the low nanomolar range: MMP-2 (0.81 nM), MMP-8 (10.8 nM), MMP-9 (18 nM) and MMP-13 (0.56 nM).<sup>8</sup> Of these, MMP-9 and MMP-13 are activated ex novo by Apoe<sup>−/−</sup> macrophages,<sup>23</sup> making them more likely candidates. However, our previous data provided no support for activation of MMP-9 (or MMP-2) in aortas of SR-uPA mice. Therefore, we focused our attention on MMP-8 and MMP-13. We found no increase in MMP-8 activity in aortic CM from SR-uPA mice (Supplementary material online, Figure S3). In contrast, MMP-13 activity was easily detected in aortic CM from all groups of mice in the mU1 study and was significantly higher in both groups of SR-uPA mice [anti-TNP and mU1-treated; 31 ± 5.4 and 32 ± 3.4 RFU/min, respectively] than in PBS-treated non-transgenic mice [25 ± 5.3 RFU/min, P < 0.05 vs. both groups of SR-uPA mice]. Moreover, MMP-13 activity in aortic CM from all three groups of mice correlated highly and significantly with total MMP activity in the same individual samples (r<sup>2</sup> = 0.81; P < 10<sup>−10</sup>, Figure 3A). We repeated these measurements using aortic CM from the four groups of mice in the XL784 study and again found that MMP-13 activity was highly and significantly correlated with total MMP activity in the same samples (r<sup>2</sup> = 0.81; P < 10<sup>−10</sup>, Figure 3B). To prospectively test the hypothesis that overexpression of uPA increases MMP-13 activity in aortas of SR-uPA mice (and avoid potentially confounding effects of the antibody treatments), we enrolled new groups of SR-uPA (<sup>n</sup> = 12) and non-transgenic (<sup>n</sup> = 16) Apoe<sup>−/−</sup> mice and measured MMP-13 activity in aortic CM generated after 15 weeks of high-fat diet. MMP-13 activity was significantly increased in aortic CM from SR-uPA mice [37 ± 3.5 vs. 29 ± 5.3 RFU/min, P < 0.001, Figure 3C].

3.7 Increased MMP-13 mRNA in aortas of recipients of SR-uPA BM

To test whether elevated aortic MMP-13 activity was associated with elevated expression of MMP-13 in aortas of SR-uPA mice, we measured MMP-13 mRNA in aortic mRNA available from a previous study in which non-transgenic Apoe<sup>−/−</sup> mice received bone marrow transplants from either SR-uPA Apoe<sup>−/−</sup> or non-transgenic Apoe<sup>−/−</sup> donors.11,14
Figure 2 The selective MMP inhibitor XL784 decreases aortic root atherosclerosis in uPA-overexpressing (SR-uPA) mice. SR-uPA mice were treated for 10 weeks with either vehicle (V) \((n=9)\) or with XL784 at one of three doses \((125, 250, \text{ or } 500 \text{ mg/kg/day}; n=10, 11, \text{ or } 12, \text{ respectively})\). (A) Mean intimal area of aortic root lesions. (B) Mean aortic root intimal lesion area staining positively for oil red O (ORO). (C) Mean aortic root intimal lesion area staining for the macrophage marker Mac-2. (D) The circumference of the aortic root at the level of the internal elastic lamina (IEL). Data points are individual mice; bars are group means. Aortic root lesions in uPA-overexpressing (SR-uPA) mice treated with vehicle \((E \text{ and } I)\) or three different doses of XL784 \((F \text{ and } J)\) \((125 \text{ mg/kg/day}); G \text{ and } K \((250 \text{ mg/kg/day}); \text{ and } H \text{ and } L \((500 \text{ mg/kg/day}))\). Haematoxylin and eosin stain \((E \text{–} H)\); oil red O stain \((I \text{–} L)\); size bar \((E) = 500 \mu\text{m}\). \((A, B) P\) values are from one-way ANOVA with post hoc correction; \((C, D) P\) values are from unpaired t-test. \((E \text{–} L)\) are representative images of 9–12 aortas per group.

Table 2 Atherosclerosis in XL784- and vehicle-treated SR-uPA mice

<table>
<thead>
<tr>
<th></th>
<th>Vehicle ((n))</th>
<th>XL784 (mg/kg)</th>
<th>XL784 (mg/kg)</th>
<th>XL784 (mg/kg)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>125 ((n))</td>
<td>250 ((n))</td>
<td>500 ((n))</td>
<td></td>
</tr>
<tr>
<td>Aortic root oil red O area (% of intimal area)</td>
<td>15 ± 4.6 (9)</td>
<td>12 ± 3.7 ((10))</td>
<td>13 ± 3.1 ((11))</td>
<td>12 ± 4.1 ((12))</td>
<td>0.4</td>
</tr>
<tr>
<td>Aortic root Mac-2 area (% of intimal area)</td>
<td>22 ± 7.3 (9)</td>
<td>22 ± 8.7 ((10))</td>
<td>20 ± 6.3 ((11))</td>
<td>16 ± 5.9 ((12))</td>
<td>0.2</td>
</tr>
<tr>
<td>Thoracic aortic lumen surface area (mm²)</td>
<td>36 ± 1.8 (9)</td>
<td>37 ± 4.0 ((10))</td>
<td>37 ± 3.4 ((11))</td>
<td>35 ± 1.7 ((11))</td>
<td>0.4</td>
</tr>
<tr>
<td>Sudanophilic lesions (% of thoracic aortic luminal surface area)</td>
<td>3.5 ((2.9–3.9)) ((9))</td>
<td>3.7 ((2.0–4.1)) ((10))</td>
<td>3.3 ((2.6–3.5)) ((11))</td>
<td>3.3 ((2.0–6.7)) ((11))</td>
<td>1.0</td>
</tr>
<tr>
<td>Number of lesions on pinned thoracic aorta</td>
<td>16 ± 3.8 (9)</td>
<td>17 ± 4.7 ((10))</td>
<td>18 ± 5.1 ((11))</td>
<td>17 ± 4.0 ((11))</td>
<td>0.7</td>
</tr>
<tr>
<td>Peripheral blood monocytes (per µL)</td>
<td>100 ± 140 (5)</td>
<td>140 ± 53 (5)</td>
<td>290 ± 240 (5)</td>
<td>230 ± 150 (5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Peripheral blood monocytes (% total leukocytes)</td>
<td>2.2 ± 2.2 (5)</td>
<td>4.2 ± 3.6 (5)</td>
<td>4.6 ± 3.6 (5)</td>
<td>4.0 ± 2.6 (5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>1360 ± 213 (9)</td>
<td>1560 ± 277 (10)</td>
<td>1430 ± 337 (11)</td>
<td>1280 ± 295 (12)</td>
<td>0.2</td>
</tr>
<tr>
<td>Plasma TG (mg/dL)</td>
<td>51 ± 22 (6)</td>
<td>25 ± 8.2 (6)</td>
<td>27 ± 6.7 (6)</td>
<td>19 ± 6.7 (6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma HDL (mg/dL)</td>
<td>48 ± 19 (9)</td>
<td>53 ± 23 (9)</td>
<td>47 ± 30 (11)</td>
<td>53 ± 36 (12)</td>
<td>0.9</td>
</tr>
<tr>
<td>Plasma non-HDL-cholesterol (mg/dL)</td>
<td>1310 ± 204 (9)</td>
<td>1490 ± 276 (9)</td>
<td>1390 ± 334 (11)</td>
<td>1230 ± 318 (12)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SD or median (25–75%) range. Number of mice in each group is in parentheses \((n)\). \(P\) values are from one-way ANOVA.
Aortic MMP-13 mRNA expression was 70% higher in recipients of SR-uPA BM [1.7 \pm 0.49 vs. 1.0 \pm 0.51 arbitrary units (AU); \( P = 0.02; \text{Figure 4A} \)]

### 3.8 The level of an MMP-13 fragment correlates with MMP-13 activity in aortic CM

Western analysis of aortic CM from SR-uPA (\( n = 6 \)) and non-transgenic (\( n = 9 \)) mice detected only a trend towards increased \(~65\text{ kDa}\) pro-MMP-13 in aortic CM of SR-uPA mice (1.3 \pm 0.36 vs. 1.0 \pm 0.33 arbitrary units; \( P = 0.1; \text{Figure 4B and C and data not shown} \)). To begin to investigate whether active MMP-13 might be increased in SR-uPA aortas, we first activated recombinant human MMP-13 with APMA and visualized the resulting fragments by SDS–PAGE and silver staining (\text{Figure 4D}). A major fragment, at \(~29\text{ kDa}\), was identified—based on reference to the literature—as a potential MMP-13 catalytic fragment. We repeated western blotting of aortic CM, selecting samples with either high or low MMP-13 activity (\( n = 4 \) each; from SR-uPA and non-transgenic mice, respectively). These blots were exposed for longer times, revealing a lower molecular weight fragment (\text{Figure 4E}) that co-migrated with the \(~29\text{ kDa}\) fragment produced by APMA treatment of human MMP-13 (data not shown). The immunoreactive \(~29\text{ kDa}\) fragment in aortic CM therefore could be an MMP-13 catalytic fragment, generated either by autolysis or cleavage by a different protease. Supporting this hypothesis, the density of the \(~29\text{ kDa}\) band in these eight CM samples correlated highly and significantly with fluorogenic MMP-13 activity measured in the same samples (\( r^2 = 0.7; P = 0.006; \text{Figure 4F} \)).

### 4. Discussion

We used mice with macrophage-specific overexpression of uPA and elevated aortic uPA and MMP activity (SR-uPA mice) to test whether inhibition of uPA or selected MMPs could prevent atherosclerosis. Our major findings were: (i) systemic inhibition of uPA with a murine mAb (mU1) significantly inhibited aortic root atherosclerosis and dilatation; (ii) oral administration of a selective MMP inhibitor (XL784) significantly inhibited aortic root atherosclerosis; (iii) MMP-13 mRNA and activity are up-regulated in aortas of SR-uPA mice; and (iv) increased aortic MMP-13 activity appears largely responsible for increased total aortic MMP activity in SR-uPA mice. These experiments provide further proof-of-concept for systemic protease inhibition as a treatment for atherosclerosis and its complications, identify MMP activity as a likely downstream mediator of uPA/plasminogen-induced atherosclerosis, and tentatively identify MMP-13 as a key downstream mediator of uPA/plasminogen-induced atherosclerosis.

Our first step was to determine whether mU1 could significantly inhibit uPA in (uPA-overexpressing) SR-uPA mice. To our surprise, SR-uPA mice were indistinguishable from non-transgenic mice in their susceptibility both to the uPA-activated toxin PrAg-U2/FP59 and to rescue from PrAg-U2/FP59 toxicity by mU1. These findings support the hypothesis that PrAg-U2/FP59 toxicity is mediated at a limited number of cell-surface uPA binding sites rather than systemically, and suggest that—in SR-uPA mice—cell-surface uPA is increased far less than total body uPA mRNA and protein. Accordingly, only a small percentage of uPA in PrAg-U2/FP59-injected SR-uPA mice may be biologically active with the remainder in the inactive single-chain form. This small percentage of active (cell surface) uPA appears to be inhibited by the same dose of mU1 in both SR-uPA and non-transgenic mice. A corollary of this interpretation is that—although SR-uPA mice have dramatically increased atherosclerosis that is caused by uPA catalytic activity—they appear to have only a modestly increased level of in vivo uPA activity, as measured by the bioassay of in vivo PrAg-U2/FP59 toxicity (the toxicity of which is clearly mediated by cell-surface uPA activity). ^{16}

In addition to protecting against PrAg-U2/FP59 toxicity, mU1 decreased atherosclerosis and limited aortic dilatation in SR-uPA mice. This result supports a role for cell-surface uPA activity in accelerating atherosclerosis and aneurysm formation and suggests that uPA inhibitors may be therapeutically useful. More complete inhibition of cell-surface uPA either by more potent antibodies or with small-molecule inhibitors would likely be more effective than mU1 both in blocking...
Protease inhibition to treat atherosclerosis

PrAg-U2/FP59 toxicity and in preventing vascular pathology. When these more potent inhibitors are available, they could be tested first in the present model and then in other mouse models, including atherosclerosis-prone mice that do not overexpress uPA and bone marrow-chimeric SR-uPA mice that exhibit increased plaque rupture.11

XL784 also inhibited atherosclerosis, with a trend towards less aortic dilation at all doses. Data from transgenic and knockout mice show that individual MMPs can both prevent and accelerate atherosclerosis.7,12 Accordingly, broad-spectrum MMP inhibitors have not reduced atherosclerosis in animal models.7,12,25 and the effect on atherosclerosis of a limited-spectrum MMP inhibitor such as XL784 was not predictable. Other, more specific MMP inhibitors have shown promise in mice, including an MMP-12 inhibitor that reduced plaque size24 and an MMP-13 inhibitor that increased plaque collagen content (with no

Figure 4 Expression and activity of MMP-13 mRNA and protein in aortas of SR-uPA and non-transgenic mice. (A) MMP-13 mRNA was measured by qRT-PCR of aortic RNA of mice21 that had received bone marrow transplants from either non-transgenic (n = 8 recipients) or SR-uPA donors (n = 7 recipients) (AU = arbitrary units). (B) Representative image of western blot of medium conditioned by aortas of either non-transgenic (n = 5) or SR-uPA mice (n = 4). Bands in the upper panel were identified as pro-MMP-13 by their migration distance (~65 kDa). (C) Densitometry analysis of bands in western blot shown in (B) and in a western blot of other samples (not shown). Samples are from a total of nine non-transgenic and six SR-uPA mice. (D) Detection of an MMP-13 fragment that may represent the catalytic domain (arrow) by gel electrophoresis and silver staining. Recombinant MMP-13 was analysed either without (left lane) or with (right 2 lanes) pre-treatment with amino-phenyl mercuric acetate (APMA). The MMP-13 was then loaded either without dilution (centre lane) or after 1:10 dilution. Major protein bands were identified by their migration distances, with reference to the literature.16 (E) Western blot of medium conditioned by aortas of either SR-uPA or non-transgenic mice (n = 4 for each). Pro-MMP-13 and an MMP-13 fragment that could represent the catalytic domain (arrow) were identified by their migration distances, with reference to the literature.16 Membrane was stripped and reprobed with anti-GAPDH antibody. (F) Correlation of MMP-13 activity [Δ relative fluorescence units (RFU)/min] with density of ~29 kDa band [arrow in (E)] on western blot of the same conditioned medium samples. Samples from SR-uPA and non-transgenic mice are indicated. AU = arbitrary units. (A–C, E, F) Each lane or point represents one mouse; bars (A, C) are group means. Size markers (D, E) and are in kDa.
Our study is the first to show that a semi-selective MMP inhibitor can retard atherosclerosis. Moreover, the trend towards smaller aortic diameter in XL784-treated mice is consistent with a recent study, showing that XL784 was protective in a mouse model of elastase-induced aortic aneurysm. The larger effect of XL784 in this study may be due to the more severe aortic damage in elastase-treated vs. uPA-overexpressing mice. Interestingly, in the present study XL784 was marginally more effective at the lowest dose (by t-test the decrease in aortic diameter of mice treated with XL784 at 125 mg/kg was statistically significant; \( P = 0.04 \)). Lack of dose–response effects of XL784 might be due to saturation of MMP active sites at the lowest XL784 dose or inhibition of protective MMPs at higher XL784 doses. Overall, the XL784 results suggest that MMP activity may be a downstream mediator of uPA-induced atherosclerosis and identify XL784—which is well tolerated so far in humans—as a clinically promising MMP inhibitor.

Our experiments do not identify the mechanisms through which mU1 and XL784 retard atherosclerosis, but presumably they do so by inhibiting atherogenic proteolytic activities of uPA and MMPs. These activities could include enhancement of foam cell formation, activation of growth factors, and stimulation of pro-inflammatory pathways. The recent study, showing that XL784 was protective in a mouse model of atherosclerosis,37 promising MMP inhibitor.

We used a substrate-screening approach, guided by the inhibitory profile of XL784, to identify which MMP is responsible for increased total MMP activity in SR-uPA aortas. Several lines of evidence implicate MMP-13: (i) MMP-13 mRNA is up-regulated in aortas of SR-uPA mice; (ii) the magnitude of elevated MMP-13 activity in SR-uPA aortic CM (30%) is identical to the magnitude of elevated total MMP activity in SR-uPA aortic CM; and (iii) total MMP activity and MMP-13 activity in individual aortic CM samples are highly correlated. Moreover, others have shown that MMP-13 is present in atherosclerotic mouse aorta and is activated (in vitro) by uPA-generated plasmin.

We also found a 30% increase in pro-MMP-13 protein in SR-uPA vs. non-transgenic aortic CM. This increase—while not statistically significant—is essentially identical to the 27% increase in MMP-13 activity in aortic CM of SR-uPA mice. Lack of statistical significance of the increase in pro-MMP-13 protein could be due to the semi-quantitative nature of western blotting, compared with the more precisely quantitative assays used to measure MMP-13 mRNA (qRT-PCR) and MMP-13 activity (a fluorogenic assay). Our finding of a high correlation between the abundance of a possible MMP-13 catalytic fragment (also found by others in vivo) and the level of MMP-13 activity in the same aortic CM samples also supports a conclusion that MMP-13 is the mediator of increased total MMP activity in SR-uPA mice. Nevertheless, extensive efforts that we made to generate further support for this conclusion were unsuccessful. These included: the use of an MMP-13 antibody to remove MMP activity from aortic CM by immunoprecipitation (this was successful but so were control immunoprecipitations); elution of proteins immunoprecipitated from aortic CM by the MMP-13 antibody used for western blotting and identification of the proteins by mass spectrometry (no MMP-13 fragments were identified; possibly because the antibody recognizes denatured but not native mouse MMP-13); and treatment of aortic CM with a proprietary MMP-13 inhibitor (it did not suppress total MMP activity). The possibility that the MMP-13 substrate detects other activities must be considered. However, the most probable candidates for cleaving this type of substrate [the collagenases MMP-8 and MMP-1 (see also manufacturer’s product sheet)] are unlikely because we found no activity with an MMP-8 substrate and mice do not have MMP-1. More definitive evidence of a biological role for MMP-13 as a downstream mediator of uPA-stimulated proteolysis, total MMP activity, and vascular pathology awaits further experimentation, possibly in MMP-13-null mice. We are aware of reports that deletion of MMP-13 does not reduce mouse atherosclerosis. However, as with uPA, increased expression of MMP-13 may affect atherosclerosis more profoundly than MMP-13 deletion.

Another question for the future is why—and among all MMPs that are present in atherosclerotic aortas—uPA activates only MMP-13. Quillard et al. also found that MMP-13 is preferentially activated in mouse aorta, at least in comparison with MMP-8. We speculate that preferential activation of MMP-13 in aortas of SR-uPA mice could be because MMP-13 can be directly activated by plasmin, because MMP-13 may have a lower

effect on lesion size. Our study is the first to show that a semi-selective MMP inhibitor can retard atherosclerosis. Moreover, the trend towards smaller aortic diameter in XL784-treated mice is consistent with a recent study, showing that XL784 was protective in a mouse model of elastase-induced aortic aneurysm. The larger effect of XL784 in this other study may be due to the more severe aortic damage in elastase-treated vs. uPA-overexpressing mice. Interestingly, in the present study XL784 was marginally more effective at the lowest dose (by t-test the decrease in aortic diameter of mice treated with XL784 at 125 mg/kg was statistically significant; \( P = 0.04 \)). Lack of dose–response effects of XL784 might be due to saturation of MMP active sites at the lowest XL784 dose or inhibition of protective MMPs at higher XL784 doses. Overall, the XL784 results suggest that MMP activity may be a downstream mediator of uPA-induced atherosclerosis and identify XL784—which is well tolerated so far in humans—as a clinically promising MMP inhibitor.

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affinity for vascular MMP inhibitors, or because selective extracellular localization of MMP-13 may facilitate its association with uPA and plasmin. Interaction of the uPA receptor and MMP-13 supports the notion of selective extracellular localization of MMP-13 that could lead to preferential activation by uPA/plasminogen.

In summary, a specific antibody directed at murine uPA and a small-molecule selective MMP inhibitor both suppress atherosclerosis in SR-uPA mice. Direct uPA inhibition also suppresses aortic dilation. These results (obtained in a model in which atherosclerosis and aortic dilation are clearly protease-driven) support a role for uPA in both atherogenesis and aneurysm formation. Future studies will test these interventions in other animal models, including those without protease overexpression and in animal models of protease-driven plaque rupture. Efficacy in these settings would further support our hypothesis that mU1, XL784, or related protease inhibitors have promise for translation into human cardiovascular therapeutics.

Supplementary material is available at Cardiovascular Research online.

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References


