Supporting Information

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SI Methods

Animals. Experimental and control mice (all $Apoe^{-/-}$) were generated using several approaches. $Apoe^{-/-}$ mice with macrophage-targeted overexpression of uPA (SR-uPA$^{+/0}$ mice) were generated in our laboratory (1). $Apoe^{+/+}$ mice deficient in plasminogen ($Plg^{-/-}$) or PAI-1 ($Serpine1^{-/-}$) were purchased (The Jackson Laboratory) and bred with $Apoe^{-/-}$ mice (either SR-uPA$^{+/0}$ or nontransgenic) to yield both SR-uPA$^{+/0}$ and nontransgenic mice that were either $Plg^{-/-}$ $Apoe^{-/-}$ or $Serpine1^{-/-}$ $Apoe^{-/-}$. $Apoe^{+/+}$ mice deficient in uPA ($Plau^{-/-}$) were purchased and bred with $Apoe^{-/-}$ mice to generate $Plau^{-/-}$ $Apoe^{-/-}$ mice. $Plau^{-/-}$ mice were used only as a source of $Plau^{-/-}$ macrophages and aortae for PA activity studies; all atherosclerosis studies were performed with $Plau^{+/+}$ mice (SR-uPA$^{+/0}$ or nontransgenic). SR-uPA$^{+/0}$ and nontransgenic $Plg^{+/-}$ $Apoe^{-/-}$ mice were bred to produce littermates with four experimental genotypes: SR-uPA$^{0/0}$ $Plg^{+/+}$, SR-uPA$^{+/0}$ $Plg^{+/+}$, SR-uPA$^{0/0}$ $Plg^{-/-}$, and SR-uPA$^{+/0}$ $Plg^{-/-}$. The SR-uPA transgene was never bred to homozygosity. All experimental mice were progeny of C57BL/6 backcrosses for at least 11 generations. Mice were genotyped by Southern blotting (for the SR-uPA allele) or PCR (for the *Serpine1*, *Plg*, *Plau*, *Apoe*, and SR-uPA alleles). All animal protocols were approved by the University of Washington Office of Animal Welfare.

Experimental Design. Mice were fed a diet containing 21% fat and 0.15% cholesterol by weight (Harlan Teklad: TD88137) starting at 5 wks of age and were killed at 15 wks of age. Only female mice were used for atherosclerosis studies, plasma lipid analyses, and serum chemistries. Both genders were used for uPA expression studies and for peripheral blood cell counts.

Tissue Harvest and Processing. Mice were deeply anesthetized and perfused first with 0.9% saline, and then with 10% phosphate-buffered formalin pH 7.4 containing 220 mM sucrose, 2 mM EDTA, 0.02 mM BHT (Sigma). Aortic roots were embedded in OCT medium, and cut into 8 μm-thick serial sections. The aortae were removed, stored in formalin, then pinned and stained with Sudan IV (1).

Histology. Aortic root sections were stained with H&E, Movat pentachrome, oil red O, picrosirius red (to detect fibrillar collagen), and the macrophage-specific antibodies MOMA-2 (Biosource) and Mac-2 (Cedarlane Labs) (1). A section through the midpoint of the most proximal coronary artery ostium was used as a reference point. For each of the stains, the section closest to the reference point was analyzed, as were 2–3 step sections proximally and 2–3 step sections distally (a total of 5–7 sections taken at 56 μm steps were analyzed for each stain).

Lesion Characterization. Digital images of sections and pinned aortae were analyzed by blinded observers with either NIH Image 1.62 or ImagePro software. Atherosclerosis in aortic root cross-sections was quantified by measuring the total intimal area. Oil red O-, MOMA-2, and Mac-2-positive areas were measured using color thresholding and planimetry. Picrosirius-red stained area was obtained similarly, using images of sections viewed with polarized light. Thresholds were set on each image by clicking on stained points, observing the superimposed false color, and choosing a point at which the false color maximally covered stained areas and minimally covered unstained areas. The stained area was then measured, and the percentage of lesions stained with oil red O-, MOMA-2, Mac-2, or pirosirius red was calculated by dividing the stained area by the total intimal area measured either on the same slide (for oil red O) or on a H&E-stained slide 8 μm away (for the other stains). Percentage of Sudan IV-positive area on the aortic surface was calculated by dividing Sudan IV-stained area by total aortic surface area. Aortic root circumference was measured at the level of the internal elastic lamina (IEL) on 5–7 H&E-stained step sections per aorta.

Quantification of Coronary Artery Stenosis. Percent luminal stenoses of proximal coronary artery segments were calculated using measurements made on oil red O-stained sections (1).

Macrophage Plg Activator Activity. Because Plg deficiency impairs recruitment of peritoneal macrophages, precluding their harvest from $Plg^{-/-}$ mice (ref. 2 and data not shown), we used bone marrow-derived macrophages to measure SR-uPA transgene expression. Fat-fed mice (15 wks old) were killed by cervical dislocation and perfused with saline. Bone marrow was harvested from femurs by flushing with RPMI 1640 with 2% FCS and 5 IU/ml heparin. Marrow cells were washed in Hanks Balanced Salt Solution and resuspended in DMEM with 10% heat-inactivated FCS, 1% penicillin/streptomycin and L-glutamine, and 10% L-cell conditioned media as a source of GM-CSF (3). This protocol yields approximately 10^6 macrophages per femur. Culture medium and non-adherent cells were removed and fresh medium added at days 4 and 8. On day 10, the medium was changed to M199, collected after 20 h and stored at −80 °C. Cells were counted and cell number used to calculate normalized macrophage PA activity.

Plg activator activity was detected by incubating aliquots of macrophage-conditioned medium with Glu-Plg (0.8 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 as $\Delta OD_{405}/min^2$ with reference to a standard curve constructed with human sc-uPA (American Diagnostica) (1).

Aortic Plg Activator Activity. Thoracic aortae from the same mice used for the macrophage PA activity assay were removed, trimmed, rinsed, and incubated for 6 h at 37 °C in M199 (GIBCO). Conditioned media were stored at −80 °C and PA activity was measured in the presence of Plg and S-2251, as described above. Total protein in the media was measured with the BCA assay (Pierce).

Western Analysis of Aortic uPA. SR-uPA$^{+/0}$ mice (either $Plg^{+/+}$ or $Plg^{-/-}$; $n = 5$ each) were perfused with saline and whole aortae excised. Protein was extracted (4) and 30 μg analyzed by SDS/PAGE under both nonreducing conditions and after reduction with 1.25% β-mercaptoethanol. Gels were blotted onto PVDF membranes. After blocking for 2 h in Tris-buffered saline with 0.5% Triton X-100 and 5% fat-free milk powder, blots were incubated with rabbit polyclonal antibodies against mouse uPA (1:2,000; GeneTex) at 4 °C overnight. Blots were washed and incubated with peroxidase-conjugated anti-rabbit IgG (1:5,000; GE Healthcare) for 1 h at room temperature. Bound antibody was detected using the ECL Plus system (GE Healthcare). Blots were stripped with Restore™ Western blot stripping buffer (Pierce), and re-probed with a mouse monoclonal antibody to GAPDH (1:5,000; Ambion). Bound antibody was detected using

a peroxidase-linked anti-mouse antibody (1:10,000; GE Healthcare) and the ECL Plus system.

Plasma Lipids. Retro-orbital blood was drawn after a 4-hr fast for measurement of total cholesterol and triglycerides (Spectrum cholesterol assay, Abbott, and TG triglyceride kit, Roche Molecular Biochemicals; ref. 1).

Peripheral Blood Counts and Serum Chemistries. Blood was obtained from the retro-orbital sinus of 15-wk-old (blood counts) or 8-wk-old (serum chemistries) fat-fed mice. Analyses were performed by an outside laboratory (Phoenix Central Laboratory).

Statistical Methods. Data are presented as mean ± SEM or median (25–75% range). Groups that were the focus of *a priori* hypotheses were compared by unpaired *t* test or by Mann-Whitney rank-sum test for non-normally distributed data. Two-way ANOVA on ranks was also used to discern separate as well as interdependent effects of the SR-uPA transgene and Plg on atherosclerosis. The Holm-Sidak method was used to correct for multiple comparisons. Accuracy of genotyping based on assessment of medial destruction was evaluated by Fisher Exact test. Analyses were performed with the SigmaStat program.

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