Identification of Myxomaviral Serpin Reactive Site Loop Sequences That Regulate Innate Immune Responses*

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The thrombolytic serine protease cascade is intricately involved in activation of innate immune responses. The urokinase-type plasminogen activator and receptor form complexes that aid inflammatory cell invasion at sites of arterial injury. Plasminogen activator inhibitor-1 is a mammalian serpin that binds and regulates the urokinase receptor complex. Serp-1, a myxomaviral serpin, also targets the urokinase receptor, displaying profound anti-inflammatory and anti-atherogenic activity in a wide range of animal models. Serp-1 reactive site mutations, mimicking known mammalian and viral serpins, were constructed in order to define sequences responsible for regulation of inflammation. Thrombosis, inflammation, and plaque growth were assessed after treatment with Serp-1, Serp-1 chimeras, plasminogen activator inhibitor-1, or unrelated viral serpins in plasminogen activator inhibitor or urokinase receptor-deficient mouse aortic transplants. Altering the P1–P1’ Arg–Asn sequence compromised Serp-1 protease-inhibitory activity and anti-inflammatory activity in animal models; P1–P1’ Ala-Ala mutants were inactive, P1 Met increased remodeling, and P1’ Thr increased thrombosis. Substitution of Serp-1 P2–P7 with Ala allowed for inhibition of urokinase but lost plasmin inhibition, unexpectedly inducing a diametrically opposed, proinflammatory response with mononuclear cell activation, thrombosis, and aneurysm formation (p < 0.03). Other serpins did not reproduce Serp-1 activity; plasminogen activator inhibitor-1 increased thrombosis (p < 0.0001), and unrelated viral serpin, CrmA, increased inflammation. Deficiency of urokinase receptor in mouse transplants blocked Serp-1 and chimera activity, in some cases increasing inflammation. In summary, 1) Serp-1 anti-inflammatory activity is highly dependent upon the reactive center loop sequence, and 2) plasmin inhibition is central to anti-inflammatory activity.

Serine proteinase inhibitors, designated serpins, represent the majority of proteinase inhibitors in the circulating blood, functioning to balance thrombotic and thrombolytic cascades (1–3). The reactive center loop (RCL) of the prototypical serpin molecule consists of 20–27 amino acids near the C terminus, approximately P17–P10’, dependent upon the individual serpin, and defines serine proteinase target(s). The specificity of serpin recognition of protease targets is thought to principally reside in the P1–P1’ scissile bond, which acts as a pseudosubstrate for target proteases, forming a suicidal inhibitory complex (1–3). The RCL exists in a strained configuration that is required for full inhibitory function, during which the target protease cleaves the P1–P1’ and is then bound and dragged across the serpin face (10–14), rendering the enzyme inoperative. This RCL loop structure thus also has a significant role in serpin to target protease specificity (4, 5).

Activation of serine protease cascades, both in the thrombotic and thrombolytic cascades, is associated with activation of the innate immune response. Excessive or prolonged up-regulation of innate immune responses, also termed inflammation, accelerates plaque growth and even rupture, resulting in increased local thrombosis and arterial occlusion (heart attacks and strokes) or, conversely, outward remodeling and aneurysm formation. Innate immunity is an integral part of vascular healing (6–10). The urokinase-type plasminogen activator (uPA) and the uPA receptor (uPAR) form complexes that initiate thrombolysis, reducing clot formation, and act to up-regulate inflammatory cell responses (6–8). Increased uPA and uPAR are detectable after acute arterial injury in a wide range of cells (endothelial cells, monocytes, smooth muscle cells, and T lymphocytes) (11–13), where the uPA/uPAR complex activates matrix metalloproteinases and growth factors, initiating collagen and elastin breakdown and allowing cell invasion and proliferation. The uPA/uPAR complex also alters cellular activation and intracellular signaling (11–13). In mouse models, uPA deficiency reduces intimal hyperplasia, and in rabbits excess uPA increased plaque growth after vascular injury, whereas uPAR expression increased macrophage invasion in ApoE null mice (14), and uPA deficiency did not alter plaque growth (15).

The principal physiological regulator of thrombolysis is plasminogen activator inhibitor-1 (PAI-1), which binds to and internalizes the uPA/uPAR complex (2, 16). Other key serpins that bind and regulate plasminogen activators include PAI-2, which binds uPA, protease nexin, which binds both tPA and uPA (2), and neuroserpin, which binds tPA, uPA and plasmin preferentially (17, 18). PAI-1 has been reported both to increase thrombosis and plaque growth (19) and, conversely, to reduce inflammatory responses and plaque growth in various animal models (2, 15, 16, 20). Neuroserpin has been reported to reduce inflammation and stroke size in animal models (17, 18). Aneurismal remodel-
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**TABLE 1**

**Mouse aortic transplants**

| Transplant       | Treatment | Dose (µg ng/g) | Follow-up time | Number of mice at transplant | Number of mice at 4-week follow-up |
|------------------|-----------|---------------|----------------|-------------------------------|-----------------------------------|
| Isografts        |           |               |                |                               |                                   |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Saline    | 0             | 4 weeks        | 10                            | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | PAI-1 (mouse) | 1.5 (50)       | <24 h          | 6                             | 4                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 (CHO) | 1.5 (50)       | 4 weeks        | 6                             | 5                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 (mouse) | 1.5 (50)       | <24 h          | 6                             | 0                                 |
| UPAR<sup>−/−</sup> to uPAR<sup>−/−</sup> | Serp-1 (CHO) | 1.5 (50)       | 4 weeks        | 7                             | 5                                 |

| Total isografts  |           |               |                | 35                            | 20                                |

| Allografts       |           |               |                |                               |                                   |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Saline    | 0             | 4 weeks        | 16                            | 11                                |
| PAI-1<sup>+/+</sup> to PAI-1 | PAI-1 (rat) | 1.5 (50)       | <24 h          | 10                            | 0                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | PAI-1 (mouse) | 1.5 (50)       | <24 h          | 6                             | 0                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Serp-1 (CHO) | 1.5 (50)       | 4 weeks        | 4                             | 4                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 CHO | 1.5 (500)      | 4 weeks        | 6                             | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 VV | 1.5 (50)       | 4 weeks        | 7                             | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Serp-1 (SAA) | 1.5 (50)       | 4 weeks        | 7                             | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 (a1-PI) | 1.5 (50)       | 4 weeks        | 7                             | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Serp-1 (PAI-2) | 1.5 (50)       | 4 weeks        | 6                             | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 (CrmA) | 1.5 (50)       | 4 weeks        | 8                             | 5                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-1 (Ala<sub>α</sub>) | 1.5 (50)       | 4 weeks        | 8                             | 6                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | Serp-2 | 1.5 (50)       | 4 weeks        | 6                             | 5                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | CrmA | 1.5 (50)       | 4 weeks        | 9                             | 5                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | D294A | 1.5 (50)       | 4 weeks        | 5                             | 5                                 |
| PAI-1<sup>+/+</sup> to PAI-1 | D294E | 1.5 (50)       | 4 weeks        | 5                             | 5                                 |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Saline | 0             | 4 weeks        | 11                            | 10                                |
| PAI-1<sup>+/+</sup> to PAI-1<sup>+/+</sup> | Serp-1 (CHO) | 1.5 (50)       | 4 weeks        | 12                            | 12                                |
| uPAR<sup>−/−</sup> to uPAR<sup>−/−</sup> | Saline | 0             | 4 weeks        | 6                             | 6                                 |
| uPAR<sup>−/−</sup> to uPAR | Serp-1 (VV) | 1.5 (50)       | 4 weeks        | 6                             | 6                                 |
| uPAR<sup>−/−</sup> to uPAR<sup>−/−</sup> | Serp-1 (SAA) | 1.5 (50)       | 4 weeks        | 6                             | 6                                 |
| uPAR<sup>−/−</sup> to uPAR<sup>−/−</sup> | Serp-1 (PAI-2) | 1.5 (50)       | 4 weeks        | 9                             | 6                                 |
| uPAR<sup>−/−</sup> to uPAR<sup>−/−</sup> | Serp-1 (Ala<sub>α</sub>) | 1.5 (50)       | 4 weeks        | 8                             | 6                                 |

| Total allografts |           |               |                | 169                           | 128                               |
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The mutated RCL sequence is underlined, P1–P1' sequence is in boldface type; I, inhibitor; S, substrate/cleaved; αPC, activated protein C; Pls, plasmin; Thr, thrombin; Try, trypsin; hNE, human neutrophil elastase; Cat, cathepsin; Chy, chymotrypsin; Cas, caspase; α1-PI, α 1-proteinase inhibitor; CrmA, cytokine response modifier; NA, not applicable.

| Native serpins | Source | RCL sequence (amino acids) | Primer sequences | P1–P1' | Target proteinases | \(K_{i}\) uPA/plasm | \(u^{-1} s^{-1}\) |
|----------------|--------|-----------------------------|-----------------|--------|-------------------|----------------------|----------------------|
| PAI-1          | Human  | TAVIVSARMAPE                | NA              | RM     | I: tPA, uPA, Thr, αPC | 1.1 × 10¹⁴          | inactive            |
| PAI-2          | Human  | TGGVMTGRTHG                  | NA              | RT     | I: uPA, Pls        | NA                   | RT                   |
| α1-PI          | Human  | TVLQVMPSMSP                  | NA              | MS     | I: Try, hNE, Chy, CatG | 2.59 × 10⁸          | 4.8 × 10⁸            |
| Serp-1/Spi4    | Myxoma | TAILTRPRNALT                 | NA              | RN     | I: tPA, uPA, Pls, Xa | 2.05 × 10⁴          | Enhancing           |
| Serp-2/Spi     | Myxoma | CTGVCTDFGGT                  | NA              | DF     | I: Cas 1, Grz B    | NA                   | NA                   |
| CrmA/Serp2     | Cowpox | ATCALVADCAST                 | NA              | DC     | I: Cas 1, 8, Grz B | NA                   | NA                   |
| Serp-1 (SAA)   | Myxoma | TAILTPAAALT                  | 5'-ATC GCC GTG AGG GCG GCC GCG GGG ATG AGG GTG AT-3' | AA     | I: none | Inactive | NA       |
| Serp-1 (α1-PI) | Myxoma | TAILTRPNALT                  | 5'-CGTGGATCCTACCTCTCATCCCCCATGAACGACTCA-3' | MN     | I: Thr, hNE, CatG, Chy | Inactive | NA       |
| Serp-1 (PA-2)  | Myxoma | TGGVMTGRTHG                  | 5'-CGTGGTGATGTTATGCAAGGCCAAGCGGCTTA-3' | RT     | I: Thr             | NA                   | NA                   |
| Serp-1 (Ala5)  | Myxoma | TAAAAAAARNALT                | 5'-CTGCCCATCACCTCTCATCCCCCATGAACGACTCA-3' | RN     | I: none | 2.05 × 10⁴ | Enhancing |
| Serp-1 (CrmA)  | Myxoma | TAILTRPLCALT                 | 5'-CTGCCATACCTCCATCCCCCGACTGACACTCA-3' | DC     | I: tPA             | NA                   | NA                   |
| Serp-2 (D299A) | Myxoma | CTGVCTDFGGT                  | NA              | AF     | I: none             | NA                   | NA                   |
| Serp-2 (D294E) | Myxoma | CTGVCTEFGGT                  | NA              | EF     | I: none             | NA                   | NA                   |

Cell Adhesion and Migration Assays—For the adhesion assay, platelets, isolated from normal volunteers, were labeled with 5 μM calcein acetoxyethyl ester (Molecular Probes, Inc., Eugene, OR) for 1 h. Excess fluorescent probe was removed by washing with phosphate-buffered saline, and cells were then resuspended in buffer and treated with Serp-1 (500 ng/ml), PAI-1 (500 ng/ml), or saline, and adherence to fibronectin (5 μg)-coated 96-well, black plates (Greiner Bio-one, Inc., Longwood, FL) was measured after 1 h. Adherent cells were quantified by calcein fluorescence (excitation 485 nm; emission, 527 nm) using a spectrofluorometer (Thermolab Systems) (36, 37).

Fluorescence-activated Cell Sorting Analysis—Peritoneal exudates from mice injected with MCP-1 were washed with phosphate-buffered saline containing 2% fetal bovine serum and treated with RBC lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.4). RBC-free cells were isolated by layering with 0.5 ml of fetal bovine serum and centrifuging at 500 × g for 5 min. Cells were identified using fluorescein isothiocyanate-labeled CD4 and phycoerythrin-labeled CD11b (FACS-Calibur; BD Biosciences) (38).
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for Serp-1 RCL cassette mutagenesis (PNAIwRC3 [GGGGAACGAGCGGGTGCTGACAGTCAACCTCCCATCC]), which maintains a Ser codon synonym with codon TCA and Thr with codon ACT, and pNAIwO2 [GACGCCCCATAGAGATTAGGGGTGGACGC], which maintains Leu with codon TTA). Mutagenic mismatch positions are indicated in bold. The resulting construct, termed pAS-Alw, was subcloned into pUC9 to generate pUC-Salw, allowing construction of RCL cassette mutants of Serp-1 by cloning in synthetic oligonucleotide primers using BamHI and HindIII endonucleases. (Table 2). The resulting RCL chimeric mutants were subcloned into the pMI-601 vector using BamHI and HindIII endonucleases. Vectors expressing Serp-1 or Serp-1 RCL mutants were adsorbed to vaccinia-infected Buffalo green monkey kidney cells (2 × 108 cells) at 5 plaque-forming units/cell. Medium containing secreted viral proteins was purified by sequential column chromatographic separations as follows: 1) Hi-trap Q (Amersham Biosciences) washed with 20 mM Tris, pH 8.0, and eluted with 75 mM NaH2PO4, pH 7.0; 2) copper-cooperated chelating column, washed with 0.1 M NH4Cl and eluted with 1 M NH4Cl; 3) Superdex 75 gel filtration column, buffer-exchanged to 150 mM NaCl, 25 mM Tris, pH 8.0. Eluates were analyzed by Western blot. Serpin concentration was measured by enzyme-linked immunosorbent assay (24–32, 39), and protein was deemed >95% pure (silver-stained gel) (24–32, 39).

Analysis of Serp-1 and Chimera Protease Inhibition—Serp-1 or individual Serp-1 RCL chimeras (180 nl) were incubated with a slight molar excess of target proteinases, tPA, tPA, plasmin, thrombin, trypsin, cathepsin G, bovine chymotrypsin, or human neutrophil elastase, obtained from Sigma, in 100 mM NaCl, 2 mM CaCl2, 0.005% Triton X-100, 100 mM Tris-HCl, pH 7.5. Samples were separated on a 4–20% linear gradient or 10% Tris-glycine SDS-polyacrylamide gels using the Laemmli buffer system (23, 27, 28, 39) and transferred to Hybond C-extra (Amersham Biosciences) nitrocellulose by electroblotting, blocked in TBS (150 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween 20. Bands containing Serp-1 were identified by 0.05% (v/v) monoclonal anti-Serp-1 antibodies AQ.H9 and AG.F11 (kindly provided by Leona Ling, Biogen, Inc.) and secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad). Labeled bands were visualized using ECL detection (Amersham Biosciences) on Eastman Kodak Co. x-ray detection type film. Reactions with complete protease cleavage of Serp-1 were repeated at shorter incubation times to test for transient, high molecular weight complexes. Reactions in which the serpin appeared unchanged at the end of the incubation period were repeated at higher enzyme concentrations for longer incubation periods. Serp-1 and the Serp-1 (SAA) and (Ala6) constructs were also assessed for thermal stability. All individual Serp-1 RCL chimeras (180 nM) were incubated with a slight molar excess of target proteinases, uPA and plasmin (chromogenic pefachrome substrate) (Sigma). 0.19 μg/ml plasmin was added to reactions containing 0.75 mM S-2251 substrate and varying concentrations of Serp-1 (at 0–6 μg/ml) in the reaction buffer (100 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2, 0.005% Triton X-100, pH 7.5). The pseudo-first-order rate constant (kcat) at each inhibitor concentration was determined from a nonlinear regression fit, and data were collected every 5 min (300 s).

Statistics—Mean cell count from three high power fields and mean plaque area from individual animals were assessed for significant change by unpaired Student’s t test and analysis of variance with Fisher’s protected least significance difference post-test analysis (41). Regression analysis was also used to assess correlations for histologic parameters and for serpin protease inhibition kinetics.

RESULTS

Effects of Serp-1 and Chimeras on Plaque Growth after PAI-1-deficient Aortic Transplant—The effect of altering Serp-1 RCL sequences to sequences mimicking known mammalian and viral serpins (Table 2) was assessed after PAI-1−/−→PAI-1+/+ aortic allograft transplant. As previously reported (29), Serp-1, markedly inhibited mononuclear cell invasion (p < 0.001) and plaque growth (p < 0.01) at 4 weeks follow-up (Fig. 1A). The Serp-1 RCL chimeras tested did not inhibit mononuclear cell invasion or intimal plaque formation (Fig. 1, B–F). Aortic allograft segments treated with Serp-1 (SAA) (Fig. 1B), Serp-1 (α1-PI), Serp-1 (PAI-2) (Fig. 1D), and Serp-1 (CrmA) RCL chimeras demonstrated marked inflammatory cell invasion (Fig. 1E) and plaque growth (Fig. 1F), comparable with that seen after saline treatment.

Despite the preservation of an intact Serp-1 RN sequence at the P1-P1’ site, the Serp-1 (Ala6) chimera did not retain anti-inflammatory or anti-atherogenic activity, producing instead an accelerated inflammatory cell response (p < 0.03) (Fig. 1, C and E). Mononuclear cell invasion into the intimal and adventitial layers (p < 0.0001) (Fig. 1, C and E) and mean plaque area (p < 0.0002) (Fig. 1, C and F) increased 2–3-fold after treatment with the Serp-1 (Ala6) chimera when compared with all other treatments (Fig. 1, E and F). Areas of chronic, organized thrombosis (Fig. 1, C and G) were also profoundly increased after Serp-1 (Ala6) treatment (p < 0.028). Associated with increased plaque area, inflammatory cell invasion, and thrombosis in the Serp-1 (Ala6)-treated mice was marked positive (outward) remodeling with focal aneurysm formation (p < 0.01) (Fig. 1, C–H).

Infusions of Serp-1 (α1-PI) also increased outward remodeling or aneurysm formation (p < 0.023 compared with saline, p = 0.118 compared with Serp-1 (SAA) chimera), comparable with that seen after treatment with Serp-1 (Ala6) (Fig. 1, C and H). However, Serp-1 (α1-PI) did not increase associated inflammatory cell invasion (p = 0.104) (Fig. 1E), thrombosis (p = 0.939) (Fig. 1G), or plaque growth (p = 0.70) (Fig. 1F) to the same extent as Serp-1 (Ala6) (Fig. 1, A–H). The infusion of the inactive serpin, Serp-1 (SAA), did not significantly alter plaque growth, cell invasion, thrombus formation, or aneurysm formation (Fig. 1, B and E–H).

Treatment with the S-1 (PAI-2) chimera (Fig. 1, D–H) induced a moderate, nonsignificant increase in thrombosis (p = 0.57) (Fig. 1, D and G) and aneurysm formation (p = 0.14) (Fig. 1H) with no increase in plaque growth (p = 0.74) (Fig. 1E) when compared with saline treatment. Serp-1 (PAI-2) produced, conversely, a decrease in mononuclear cell invasion (p < 0.0003 upon comparison with Serp-1 (Ala6), p = 0.004 upon comparison with saline or Serp-1 (SAA) treatment) (Fig. 1E). Despite increased cell invasion, plaque area, thrombosis, and outward remodeling induced by treatment with the Serp-1 (Ala6) (Fig. 1, C–H) mutant and Serp-1 (PAI-2) and Serp-1 (α1-PI) (Fig. 1, E–H) chimeras, there was no increase in early or late mortality after PAI-1−/−→aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model apparently activates aortic allograft transplants (Table 1). The marked arterial remodeling seen with the Serp-1 (Ala6) chimera in the PAI-1−/−→aortic allograft model appears...
Serp-1 P1-P1’s scissile bond results in loss of anti-inflammatory activity, and mutation of P2–P7 exacerbates inflammation, thrombosis, and plaque growth with attendant aneurismal dilation.

Effects of Viral Cross-class Serpins on Plaque Growth in PAI-1-deficient Aortic Transplants—Two unrelated intracellular viral serpins, Serp-2 and CrmA, that inhibit granzyme B and caspases 1 and 8 (30, 31), but not thrombolytic proteases, were tested in the PAI-1–/– aortic allograft transplant model. CrmA (Fig. 2A) increased mononuclear cell invasion (p < 0.001), plaque growth, and arterial dilation after aortic transplant when compared with saline infusion. Serp-2 as well as two RCL mutants of Serp-2 (Table 2), Serp-2 (D294A; Fig. 2B) and Serp-2 (D294E) with P1 Asp substitutions of Ala and Glu, respectively, were also tested. The Serp-2 (D294A) mutant also increased cell invasion (p < 0.001), plaque area (Fig. 2C), and aneurysm formation (Fig. 2D). Serp-2 (D294E) had no effect (Fig. 2, C and D), whereas wild type Serp-2 reduced mononuclear cell invasion into the adventitia (but not the intima) when compared with saline, CrmA, and the Serp-2 (D294A and D294E) mutants (p < 0.0001; not shown). CrmA and Serp-2 (D294A) also markedly increased thrombus formation.
(p < 0.0001 for CrmA, p < 0.0006 for Serp-2 (D294A); data not shown) to a greater degree than the Serp-1 (Ala6) mutant (p < 0.045). CrmA treatment reduced survival (p < 0.05) when compared with Serp-1 or Serp-2 (D294A and D294E) mutants (Table 1) but not significantly when compared with saline (p = 0.391) or Serp-2 (p = 0.155).

Infusion of the Serp-1 (CrmA) chimera with CrmA P1-P1 amino acid sequence DC neither decreased nor increased mortality (Table 1) (p = 0.698), inflammatory cell response, or plaque growth at 4 weeks follow-up (Fig. 1, E and F), as noted above. We have concluded that the infusion of unrelated, foreign viral serpins does not reproduce the anti-inflammatory and anti-atherogenic effects of Serp-1 infusion but can for some (CrmA and D294A) initiate a proinflammatory response. The substitution of the Serp-1 P1-P1’ sequence with CrmA P1-P1’ DC sequence, however, did not initiate a proinflammatory response, indicating that the...
combination of a negatively charged Asp residue at P1 with a polar uncharged Cys at P1* in the Serp-1 RCL did not increase inflammation, such as was seen with Serp-1 (Ala PI) and (Ala6) chimeras.

**Effects of Serp-1 and RCL Chimeras in uPAR-deficient Aortic Allograft Transplants**—As previously reported, Serp-1 anti-inflammatory and anti-atherogenic activity is blocked in uPAR-deficient (uPARΔ) allografts, indicating that uPAR is required for Serp-1 anti-inflammatory activity (29). The capacity of Serp-1 and RCL chimeras to alter inflammatory cell invasion, thrombosis, plaque growth, and vessel remodeling was therefore also tested after uPARΔ to uPAR+/+ aortic transplant. Serp-1 was again ineffective in blocking plaque growth (Fig. 2E) (p = 0.212) and mononuclear cell invasion (Fig. 2F) (p = 0.194) after uPAR−/− aortic transplant. Serp-1 also had no effect on thrombosis (Fig. 2G) and aneurysm formation (Fig. 2H) in this model, whether compared with saline or Serp-1 (SAA) treatment. The Serp-1 (SAA) mutant was similarly ineffective (p = 0.518) (Figs. 2, E–H). There was a modest, nonsignificant trend toward increased plaque area with both Serp-1 and Serp-1 (SAA) (p = 0.292) (Fig. 2E).

Conversely, the Serp-1 (Ala6) and the Serp-1 (PAI-2) mutants increased thrombosis (Fig. 2G, p < 0.037) and outward arterial remodeling (Fig. 2H, p < 0.043) in uPAR−/− allografts. The prothrombotic effect of the Serp-1 (PAI-2) chimera and the increased remodeling produced by Serp-1 (Ala6) were greater in the uPAR−/− aortic allograft model (p < 0.037 and p < 0.004, respectively, upon comparison with saline) (Fig. 2, E–H) and when compared with Serp-1 (PAI-2) and Serp-1 (Ala6) mutant treatment after PAI-1−/− aortic allograft transplant (Fig. 1). A significant increase in plaque area was also detected in the uPAR−/− model after Serp-1 (PAI-2) and Serp-1 (Ala6) treatment (Fig. 2F). Despite the increased thrombosis, remodeling and plaque growth, none of these chimeras increased mortality during the term of this study (Table 1) (p = 0.355). These findings suggest that uPAR expression in the donor aorta is not required for the increased thrombosis and aneurysmal remodeling observed after treatment with some of the Serp-1 mutants. However, uPAR is present in the recipient aorta and in circulating cells in the recipient mouse and thus may still have a role in thrombosis and aneurysm formation.

**Effects of Viral and Mammalian Serpins on Thrombosis and Mortality**—Both PAI-1 and Serp-1 target tPA and uPA but diverge in their ability to bind and inhibit plasmin, thrombin, and factor Xa. PAI-1, similar to Serp-1, has also been demonstrated to have anti-inflammatory and anti-atherogenic activity in selected animal models, whereas Serp-1 can compensate for PAI-1 deficiency in mouse PAI-1−/− aortic allografts (29). In order to assess the relative efficacy of Serp-1 and PAI-1 for inhibiting inflammatory cell responses and plaque growth, we compared PAI-1 and Serp-1 treatment of PAI-1−/− aortic allograft transplants. The mortality for Serp-1-treated mice after aortic transplant ranged from 0 up to 14.29%, less than or equal to that seen in saline-treated controls, where mortality was 0–31.25% (Table 1) (p = 0.079 for Serp-1). In stark contrast, rat PAI-1 treatment resulted in acute thrombosis in the transplanted aorta with early (less than 24-h) mortality in all mice treated (Table 1) (p < 0.0001). Mouse PAI-1 treatment was also tested to rule out a possible reaction to rat PAI-1 in the mouse models, but infusion of mouse PAI-1 also produced acute thrombosis with 100% mortality (p < 0.0001) (Table 1). PAI-1 infusion also caused a marked early thrombotic response with 100% mortality in uPAR−/− aortic allograft (Table 1) and uPAR−/− isograft transplants (Table 1). Serp-1 treatment did not alter mortality in the uPAR−/− isograft or the uPAR−/− (0–28.57%) allograft transplant models (Table 1).

Treatment with the Serp-1 RCL mutants, similar to Serp-1 treatment, did not significantly increase thrombosis or early mortality after PAI-1−/− allograft transplant (0–40% mortality) (Table 1), the greatest mortality being detected for mice treated with the Serp-1 (CrmA) chimera (37.5% mortality) in PAI-1−/− allograft transplants (p = 0.695 compared with saline; p = 0.062 compared with Serp-1). The mortality for saline treatment after wild type C57Bl/6 to Balb/c aortic transplant was also low (9.09%) (Table 1). For Serp-1 treatment, mortality in wild type mouse aortic transplant models, with normal PAI-1 and uPAR expression, was low (0%) (Table 1). For all models where early mortality occurred, death was invariably associated with graft thrombotic occlusions.

PAI-1 infusion was also tested in PAI-1-deficient isografts where PAI-1 expression should be absent or minimal. In PAI-1−/− to PAI-1−/− isograft transplants, infusion of PAI-1 did not increase mortality (Table 1) (mortality 33.3%). We have concluded that the presence of the Arg-Met P1-P1’ RCL sequence in PAI-1 can produce anti-inflammatory and anti-atherogenic activity in some animal models, but in the presence of circulating PAI-1 in recipient mice, infusion of low dose PAI-1 increased thrombosis.

**Analysis of Clotting Time and Platelet Activation after Serp-1 and PAI-1 Treatment**—Histology analysis represents findings at 4 weeks follow-up in the aortic transplant segments but does not allow analysis of early changes in the arterial wall immediately after transplant. Thus, early effects of Serp-1, PAI-1, and the Serp-1 RCL chimeras on bleeding time, platelet activation, and mononuclear cell invasion were also assessed.

The bleeding time for wild type Balb/c mice was significantly shorter for PAI-1 than for Serp-1 treatment (p < 0.014) (Fig. 3A). Although both serpins reduced the bleeding time, only PAI-1 produced a significant and reproducible reduction in the bleeding time (p < 0.016). Adherence of platelets, isolated from normal human volunteers, to fibronectin was also assessed in the presence and absence of treatment with Serp-1 and PAI-1, in vitro. Serp-1 reduced human platelet adhesion to fibronectin (p < 0.05), whereas PAI-1 increased platelet adhesion (p < 0.05) (Fig. 3B).

**Effects of Serp-1, PAI-1, and Serp-1 RCL Mutant on Peritoneal Monocyte Invasion**—Serp-1 reduced migration of mononuclear cells into mouse ascsites in response to intraperitoneal injection of MCP-1 in PAI-1−/− mice. PAI-1 infusion, in contrast, had no effect on monocyte migration into ascites fluid after injection of MCP-1, indicating that Serp-1 has a greater inhibitory activity for monocyte invasion, even in mice lacking PAI-1 (p < 0.01) (Fig. 3C). Serp-1 (SAA) had no effect. Serp-1 also reduced the total number of migrating mononuclear cells in normal Balb/c mice after stimulation with MCP-1 (p < 0.02) (Fig. 3D), with associated equal reductions in CD4-positive T cells (p < 0.011) (Fig. 3E) and CD11b-positive monocytes (data not shown) as assessed by fluorescence-activated cell sorting analysis. The Serp-1 (Ala6) RCL chimera conversely increased total cell invasion (p < 0.02) (Fig. 3D) with significant and equivalent percentage increases in CD4+ T cells (p < 0.011) (Fig. 3F) and CD11b-positive mononuclear cells (not shown) into mouse peritoneal fluid when compared with Serp-1. We have concluded that Serp-1 inhibits early mononuclear cell invasion in response to chemokine activation, whereas PAI-1 does not. In contrast, the Serp-1 (Ala6) chimera produced a marked increase in mononuclear cell invasion.

**Kinetics of Protease Inhibition**—Specific P1-P1’ sequences and protease-inhibitory activity for Serp-1 and individual chimeras were also compared with the capacity for each serpin and chimera to alter cell invasion, thrombosis, plaque growth, and aneurysm formation after aortic transplant. Western blot analysis of serpin binding to selected proteases and protease cleavage as well as analysis of kinetics of uPA and
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FIGURE 3. Bleeding time in Balb/c mice was significantly reduced by intravenous injection of PAI-1 injection (p < 0.016). Serp-1 reduced the bleeding time but not significantly (A). Adhesion of platelets from normal volunteers to fibronectin was significantly reduced by Serp-1 treatment (p < 0.05) (B). Serp-1 infusion, but not PAI-1 infusion, reduced mononuclear cell invasion into mouse ascites in PAI-1-/- mice after intraperitoneal injection of MCP-1 (C). Total numbers of invading mononuclear cells were also reduced after intravenous injection of Serp-1 into normal mice with MCP-1 intraperitoneal injection (D) but markedly increased after injection of Serp-1 (Ala6) (E). ANOVA, analysis of variance.

plasmin inhibition are provided (Table 2). Serp-1, as previously reported (26, 39), bound to and inhibited tPA, uPA, plasmin, and factor Xa but was cleaved by thrombin. The Serp-1 (PAI-2) RCL chimer failed to interact with uPA, tPA, or plasmin but formed inhibitory complexes with thrombin and was cleaved by trypsin, human neutrophil elastase, and cathepsin G, exhibiting a product of ~50 kDa, consistent with cleavage at or near the RCL. Serp-1 (PAI-2) is also cleaved by chymotrypsin, without the formation of an inhibited complex. The Serp-1 (PAI-1) chimera binds tPA and Thr, forming SDS-stable complexes. However, no inhibited complex was observed with either uPA or plasmin, both of which cleaved Serp-1 (PAI-1) without forming a visible inhibitory complex. Serp-1 (PAI-1) was cleaved by trypsin, chymotrypsin, human neutrophil elastase, and cathepsin G. Serp-1 (CrmA) had properties similar to those observed for Serp-1 (PAI-1), forming a visible inhibitory complex with tPA, but similar to the Serp-1 (SAA) mutant, it failed to bind to uPA, plasmin, or thrombin and was cleaved by trypsin, human neutrophil elastase, cathepsin G, and chymotrypsin. Serp-1 (Ala6) failed to form SDS-stable complexes with any of the proteinases tested in gel shift assays, but a 50-kDa cleaved form was observed upon incubation with uPA, tPA, plasmin, thrombin, trypsin, and chymotrypsin. Incubation of Serp-1 (Ala6) chimera with human neutrophil elastase and cathepsin G failed to exhibit either substrate or inhibition activity.

Kinetic analysis of inhibition of uPA and plasmin was also performed for Serp-1 and the Serp-1 (SAA) and Serp-1 (Ala6) mutants to further define specific changes in protease inhibition. These studies detected specific interactions for Serp-1 (Ala6) with uPA and plasmin, interactions not detectable by simple gel shift assays (Fig. 4). Altering the P1-P1’ Arg-Asn sequence resulted in loss of uPA inhibition, as seen with the Serp-1 (SAA) mutant. The rate of reaction (Kcat) for wild type Serp-1 with uPA was 2.59 × 10^6 M^-1 s^-1; Kcat for plasmin was 4.8 × 10^5 M^-1 s^-1; Kcat for Serp-1 (SAA) was inactive or equal to 0 for uPA and plasmin; and Kcat for Serp-1 (Ala6) was 2.05 × 10^5 M^-1 s^-1 for uPA and with relative
enhancement of activity for plasmin (Fig. 4). Retention of the Serp-1 P1–P1′ RN sequence in Serp-1 (Ala6) reduced inhibitory activity for uPA (Table 2). However, mutation of the P2–P7 arm to a series of 6 Ala residues not only removed the capability to inhibit plasmin but in fact produced a modest increase, up to 1.3-fold, in plasmin activity, as detected by the increased slope of curves in the plasmin assays (Fig. 4B). Upon comparison of curves with control, there is an increase of reaction velocity along with increased Serp-1 (Ala6) concentrations (Fig. 4B).

The stoichiometry of inhibition (SI) for Serp-1 was measured for uPA and plasmin at 1.0 and 1.9, respectively (40). Both SI values are in the range deemed consistent with the inhibitory pathway rather than the substrate pathway; SI values between 1 and 2 are indicative of an inhibitory pathway. Using the SI value multiplied by the $K_a$, provides an adjusted $K_a$ as previously reported by Schechter and Plotnick (40). The calculated adjusted $K_a$ values for Serp-1 do not vary significantly from prior $K_a$ analyses ($5.9 \times 10^7$ for uPA and $9.12 \times 10^6$ for plasmin). If one makes the assumption that the SI values for the wild type Serp-1 can be used to calculate the adjusted $K_a$ for Serp-1 (Ala6) with uPA, the adjusted $K_a$ remains $2.05 \times 10^4$. No SDS stable complexes were, however, detectable for Serp-1 (SAA) and Serp-1 (Ala6) mutants when incubated together with uPA or plasmin on gel shift analysis, and thus calculation of SI for these mutants is inaccurate. Further detailed analyses will be needed to provide a final accurate assessment of the kinetics for each serpin analyzed in these studies.

In summary, when a positively charged Arg residue is at the P1 position and a polar, uncharged Asn residue is present at P1′ in the wild type Serp-1 protein with preserved RCL sequences, Serp-1 demonstrates specific and potent anti-inflammatory and anti-atherogenic activity. Altering the Serp-1 P1–P1′ sequence resulted in a reduction or loss of uPA and plasmin inhibition in the mutants tested. When the P1 Arg residue was retained, Serp-1 (PAI-2) formed thrombin-binding complexes, but Serp-1 (Ala6) did not. When Asn (N) was retained in the Serp-1 (a1-PI) and Serp-1 (Ala6) mutants, there was no consistent formation of protease-inhibitory complexes. Serp-1 (a1-PI) and Serp-1 (PAI-2) both bound thrombin, whereas Serp-1 (a1-PI) and Serp-1 (CrmA) both formed inhibitory complexes with tPA, but the P1–P1′ sequences are dissimilar: MN for Serp-1 (a1-PI), RT for Serp-1 (PAI-2), and DC for Serp-1 (CrmA). Retaining the Serp-1 P1–P1′ sequence in the Serp-1 (Ala6) with mutation of the P2–P7 arm to Ala, reduced uPA inhibition and removed plasmin inhibitory activity, also causing a moderate increase in plasmin activity.

**DISCUSSION**

The arterial wall has a highly modifiable architecture with responses to injury that range from cellular proliferation with negative or inward remodeling to cellular necrosis with outward or positive remodeling and all possible variations between. These highly plastic responses in the arterial wall are initiated by a host of cells that include activated platelets, macrophages, inflammatory T cells, and smooth muscle cells (8–10, 42, 43). The balance between synthesis and degradation of connective tissue and proliferation and necrosis of cells is regulated in part by serine proteases in the thrombotic and thrombolytic cascades and by their attendant regulatory serpins (2, 6, 7, 9, 11–13, 15, 44). This balance can determine the final outcome of vascular responses to injury, specifically vascular healing, lumen obliteration, or aneurysm formation.

We report here a viral serpin, Serp-1, that binds to and inhibits plasminogen activators yet demonstrates a remarkable selectivity for inhibition of inflammatory responses without associated thrombosis. In this series of studies, Serp-1 significantly reduced early platelet adhesion and monocye migration following aortic allograft transplant. Selective changes in the reactive center loop of Serp-1 markedly altered the balance between inflammation, thrombosis, and healing in the vascular wall. Our studies have demonstrated that 1) the Serp-1 P1–P1′ sequence with Arg (a bulky, positively charged amino acid) and Asn (a polar uncharged amino acid), both containing NH$_2$ groups, provides a unique anti-inflammatory and anti-atherogenic activity, 2) substitution of either the P1 or the P1′ amino acid results in a loss of anti-inflammatory activity, and 3) altering the P2–P7 RCL arm amino acid sequence increases plasmin activity and induces a widely divergent proinflammatory response. We have also demonstrated that, despite similarities in protease targets, the mammalian serpin, PAI-1, induces a markedly prothrombotic response, whereas Serp-1 has a neutral effect on thrombosis and can reduce platelet activation *in vitro*.

The mammalian serpin, PAI-1, which is similar to Serp-1 in terms of substrate specificity, binds plasminogen activators but displays a very different profile of activity in this allograft model, producing local thrombosis, vascular occlusion, and inevitable early fatality. PAI-1 is known to regulate and block thrombolysis with the potential for increased thrombosis (16, 45, 46), but PAI-1 has also been reported to have both anti-inflammatory and proinflammatory actions in differing animal models (15, 16, 19–21, 29). Despite the shared capacity of Serp-1 and PAI-1 to inhibit serpins, these two serpins differ in their reaction rates for plasminogen activators (29, 39). PAI-1, but not Serp-1, displayed prothrombotic activity in the mouse tail bleeding time measurements and increased platelet adhesion, whereas Serp-1 reduced platelet adhesion to fibronectin, consistent with prior reports of increased thrombosis with PAI-1 (16, 45). Serp-1 thus either has additional functions or potentially lacks domains that cause platelet activation and thrombosis. These additional anti-thrombotic actions of Serp-1 may be the result of the much lower reaction rates for Serp-1 than PAI-1 with respect to plasminogen activators or may be due to an additional inhibition of clotting factor proteases, such as factor Xa (26, 39).

Substituting Met for P1 in the Serp-1 (a1-PI) chimera increased aneurysmal dilation and resulted in a loss of anti-inflammatory and anti-atherogenic activity. Substituting Thr for the P1′ in the Serp-1 (PAI-2) chimera also removed anti-inflammatory and anti-atherogenic activity and caused a slight increase in thrombosis in PAI-1-deficient allografts. Serp-1, Serp-1 (PAI-2), and Serp-1 (Ala6) all share an Arg at the P1 scissile bond site, but the presence of Arg in the P1 position was not enough to provide anti-inflammatory or anti-atherogenic activity. Substitution of the wild type Serp-1 P2–P7 RCL arm amino acid sequence of Ala, Ile, Thr, Leu, Ile, Pro (A1T1P1) with 6 Ala residues represents a substitution with nonpolar aliphatic Ala for the wild type Serp-1 P2–P7 sequence, which is predominantly composed of nonpolar, aliphatic amino acid R groups (5 of 6). This 6-Ala substitution in the Serp-1 (Ala6) chimera produced an excessively proinflammatory protein that induced excess plaque growth, thrombosis, and aneurysm formation.

The properties of the Serp-1 (Ala6) chimera are of particular interest in that while retaining the P1–P1′ RCL site of Serp-1 (RN), alteration of the RCL P2–P7 arm with Ala6 caused a profound acceleration of all of the functions that native Serp-1 inhibited. Unlike wild type Serp-1, the Serp-1 (Ala6) chimera induced a marked increase in thrombotic and inflammatory responses along with accelerated plaque growth and aneurysm formation. The proinflammatory activities of the Serp-1 (Ala6) chimera as demonstrated here differ from the other serpins and chimeras tested in that the Ala6 substitution of the P2–P7 RCL residues altered binding to and inhibition of uPA, reducing the $K_a$ modestly (Table 2), and also increased plasmin activation (Fig. 4) with resulting proinflammatory functions and extensive local aneurysm formation.
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(Fig. 1). A loss of inhibitory activity for uPA and plasmin and an increase in plasmin activity would be predicted to increase promatrix metallo-proteinase activation with increased inflammatory cell invasion and aneurysm formation. Certainly, the P1-P1’ and adjacent RCL amino acid residues have been previously documented to alter activity of other serpins, such as anti-thrombin (1, 47, 48), and additionally other domains of serpins are known to alter serpin-mediated inhibitory actions (1, 49, 50).

Serp-1-mediated inhibition of inflammatory cell invasion and plaque growth was dependent upon the expression of uPAR in the donor aortic transplant artery, whereas the prothrombotic activity of Serp-1 (PAI-2) and the aneurysm formation induced by Serp-1 (Ala6) are not dependent upon uPAR expression. The increase in inflammatory cell invasion, thrombosis, plaque growth, and aneurysm formation in the uPAR+/−/− allografts for some of the Serp-1 mutants may be associated with either the absence of uPAR in the donated aorta or with the presence of uPAR expression in adjacent recipient aorta or in circulating inflammatory cells in the recipient blood stream. Prior work has indicated that uPAR is associated with but not always required for cell migration, plaque growth, and aneurysm formation (46).

The aortic allograft model used here provides a model of extensive vascular inflammation and rapid plaque growth. Although inducing a highly inflamed vascular environment, it should be noted that this is an artificial model utilized to mimic the marked inflammatory vascular responses seen in transplanted organs, so-called chronic rejection. Thus, immune allograft responses in surgical trauma are both induced, providing a robust model where the inflammatory and immune responses are up-regulated.

We conclude that the highly potent viral serpin, Serp-1, has unique anti-inflammatory and anti-atherogenic actions, distinct from the mammalian serpin PAI-1. Serp-1 is also unique in displaying both platelet and monocyte inhibitory actions. Whereas the anti-inflammatory and anti-atherogenic actions require Arg-Asn at the Serp-1 P1-P1’, the mobile RCL P2-P7 arm adjacent to the P1-P1’ scissile bond also has a key role. When the P1-P1’ is altered, Serp-1 activity transforms to a dramatically more proinflammatory state and promotes thrombosis, plaque growth, and aneurysmal dilation. Mutation of the P2-P7 sequence in the RCL to Ala8 in the Serp-1 (Ala8) mutant removed tPA, uPA, and plasmin protease-inhibitory activity and enhanced plasmin activity. The altered protease-inhibitory activity and enhanced plasmin activity of Serp-1 (Ala8) promoted an excessive proinflammatory response with aneurysm formation.

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