Serp-1, a Viral Anti-inflammatory Serpin, Regulates Cellular Serine Proteinase and Serpin Responses to Vascular Injury*

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Complex DNA viruses have tapped into cellular serpin responses that act as key regulatory steps in coagulation and inflammatory cascades. Serp-1 is one such viral serpin that effectively protects virus-infected tissues from host inflammatory responses. When given as purified protein, Serp-1 markedly inhibits vascular monocyte invasion and plaque growth in animal models. We have investigated mechanisms of viral serpin inhibition of vascular inflammatory responses. In vascular injury models, Serp-1 altered early cellular plasminogen activator (tissue plasminogen activator), inhibitor (PAI-1), and receptor (urokinase-type plasminogen activator) expression (p < 0.01). Serp-1, but not a reactive center loop mutant, up-regulated PAI-1 serpin expression in human endothelial cells. Treatment of endothelial cells with antibody to urokinase-type plasminogen activator and vitronectin blocked Serp-1-induced changes. Significantly, Serp-1 blocked intimal hyperplasia (p < 0.0001) after aortic allograft transplant and after wire-induced injury (p < 0.05) in PAI-1-deficient mice. Serp-1 also blocked plaque growth after aortic isograft transplant and after wire-induced injury (p < 0.05) in PAI-1-deficient mice indicating that increase in PAI-1 expression is not required for Serp-1 to block vasculopathy development. Serp-1 did not inhibit plaque growth in uPAR-deficient mice after aortic allograft transplant. We conclude that the poxviral serpin, Serp-1, attenuates vascular inflammatory responses to injury through a pathway mediated by native uPA receptors and vitronectin.

An integrated balance between the thrombotic and thrombo-lytic cascades, both of which are regulated by serine proteinases, activates arterial clot formation and also mediates inflammatory cell responses at sites of vascular injury (1–7). Serine proteinase inhibitors, termed serpins, in turn regulate these cascades (7). Larger DNA containing viruses have captured host serpins during millions of years of evolution, and adapted them into highly effective shields against host inflammatory responses (8). Serp-1, a secreted anti-inflammatory protein encoded by myxoma virus, is one such poxviral serpin that binds and inhibits, in vitro, the thrombotic serine proteinases tissue-type and urokinase-type plasminogen activators (tPA¹ and uPA, respectively) and plasmin (9, 10). In vivo, Serp-1 reduces inflammatory leukocyte responses to myxoma viral infection (9, 10). Furthermore, infusion of picogram to nanogram doses of purified Serp-1 protein also profoundly inhibits monocyctic cell invasion and subsequent atherosclerotic plaque growth following vascular injury induced by angioplasty (11) or allograft transplant (12, 13)² in animal models, thus providing a new class of anti-inflammatory drugs.

The precise targets and/or receptors, through which viral serpins, specifically Serp-1, inhibit inflammatory cell responses are not yet defined (10–17). uPA, when bound to the uPA receptor (uPAR), enhances inflammatory cell migration (3, 4, 7, 17, 18), cell adhesion mediated by vitronectin (3, 19, 21), cell invasion through activation of matrix metalloproteinase enzymes (20, 22), and the release and activation of growth factors (23). Serpins, inhibit individual steps in these cascades through directed one-to-one stoichiometric inhibition of many of these enzymes (3–7, 23–25). PAI-1 is a naturally occurring vascular serpin that, like Serp-1, binds to uPA and tPA in the circulating blood, inhibiting plasminogen activator activity, but, in contrast to Serp-1, does not inhibit plasmin activity (8, 21–27). Plasminogen activator inhibitor-1 (PAI-1)-deficient mice have significantly increased intimal hyperplasia after arterial injury (26–28). This injury induced intimal hyperplasia is reduced by administration of adenosinovector expressing PAI-1, suggesting that PAI-1 together with the plasminogen system acts as a central regulator of vascular wound repair responses (26–28). Recent work has demonstrated accelerated lesion growth in carotid arteries of cholesterol fed rabbits with overexpression of uPA (29) and reduced plaque after arterial injury in uPA and plasminogen-deficient mice (30, 31) confirming a pro-atherogenic role for uPA in these animal models. Other studies have been less conclusive, demonstrating either pro-atherogenic or thrombotic effects, for plasminogen and plasmin inhibitors in animal models (32, 33). Injury to the arteries of uPAR-deficient mice has not, however, been found to alter plaque development (26).

PAI-1 forms ternary complexes together with uPA and its receptor, uPAR (3–6, 34, 35). This ternary complex is rapidly internalized, effectively blocking the pro-chemotactic, adhesive, and proteolytic activity of the uPA-uPAR complex. The uPA-uPAR complex interacts with the α2-macroglobulin (low

¹The abbreviations used are: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; CHO, Chinese hamster ovary; SAA, Serp-1 reactive center loop mutant; RT, reverse transcriptase; HUVBC, human umbilical vein endothelial.

²R. Zhong, personal communication.
density lipoprotein related protein) receptor at the cellular membrane, an interaction that is believed to regulate intracellular tyrosine kinase activity (3, 4, 20, 34, 35). This inhibition of the plasminogen activators reduces activation of the pro-form of matrix metalloproteinase enzymes to active forms (3–7, 17, 18, 20, 22, 35), thus potentially halting cellular invasion at sites of vessel trauma. Vitronectin is a multifunctional adhesion molecule that binds to uPAR forming more stable PAI-1-uPAR complexes and has also been reported to also enhance inhibition of thrombin (34, 36).

We have postulated that Serp-1 interacts with the uPA-uPAR pathway, to inhibit inflammatory responses to arterial injury (11–14). We initially examined uPAR-linked regulation of vascular and endothelial cell serpin expression following Serp-1 treatment after arterial injury in rat models. Based on those studies, we subsequently examined the effect of Serp-1 infusion on plaque growth after aortic transplant in mouse models. The capacity of Serp-1 to inhibit plaque growth differed dramatically when the PAI-1 (PAI-1<sup>−/−</sup>) and uPAR (uPAR<sup>−/−</sup>)-deficient mouse strains were compared, indicating that Serp-1 inhibits inflammatory cell responses through native vascular uPA receptors. Delineation of the mechanisms through which viral serpins inhibit arterial inflammatory responses provides a new approach to the investigation of inflammatory cell responses and their regulation, both innate and virally mediated.

**EXPERIMENTAL PROCEDURES**

**Animal Models of Arterial Surgery**

**General Surgical Method**—All research protocols and animal care conformed to the Guiding Principles for Animal Experimentation of the Canadian Council on Animal Care. Surgeries were performed under general anesthesia (6.5 mg per 100 g of body weight intra-muscular injection of Somnotrol, MTC Pharmaceuticals, Cambridge, Canada). Serp-1 or controls (1.0 ml volume/rats, 0.2 ml/mice) were given by intra-arterial injection through the central balloon lumen immediately after balloon or wire injury and by venous injection (penile vein) for aortic allograft transplant surgery. Animals were sacrificed with euthanized general anesthetic (6.5 mg per 100 g of body weight intra-muscular injection of Somnotrol, MTC Pharmaceuticals, Cambridge, Canada).

**Table I**

| Study 1 | 30 SD rats |
|---------|------------|
| Iliofemoral angioplasty |
| 30 rats |
| Saline (12 R) |
| S-1 CHO (6 R) |
| S-1 VV (6 R) |
| SAA (6 R) |
| 4 weeks |
| 0, 4, 12, 24 hrs or 10 ds |
| Histological analysis |
| Morphometric analysis |
| RT PCR |
| SQ |

| Study 2 | 207 SD rats |
|---------|-------------|
| Iliofemoral angioplasty |
| 120 rats |
| Saline (60 R) |
| S-1 CHO (60 R) |
| (12 R/time/treatment) |
| 4 weeks |
| 0, 4, or 24 hrs |
| RT-PCR |
| Q-Real time |
| SQ |

| Study 3 | No injury |
|---------|-----------|
| 36 rats |
| Saline (18 R) |
| S-1 CHO (18 R) |
| (6 R/time/treatment) |
| 0, 12, 24 hrs |
| RT-PCR |
| SQ |

Abbreviations: SD – Sprague-Dawley; SQ – semi-quantitative; RT-PCR, Q – quantitative real-time RT-PCR; R, rats; S-I, Serp-1, nyl (Bimeda-MTC Animal Health Ltd., Cambridge, Ontario, Canada), 0.05 ml for mice and 0.25 ml for rats given intramuscularly. No increase in mortality was detected for any of the rats with angioplasty injury or mice after aortic transplant using either PAI-1- or uPAR-deficient mouse strains (p = 0.49). Serp-1 infusion did not result in any increase in adverse events or mortality.

**Rat Angioplasty Injury Model**—In Study 1 (Table I) we analyzed the effects of either 30 ng (per animal) of Serp-1 purified from vaccinia vector (Serp-1 VV) (11) or isolated from Chinese hamster ovary (CHO) cells (Serp-1 CHO) (13, 14), the Serp-1 reactive loop mutant (SAA) (11, 14), or saline (Fig. 1) infusion on intimal hyperplasia after ilio-femoral angioplasty balloon injury in 30 Sprague-Dawley rats (250–350 g, Charles River Laboratories, Wilmington, MA) at 28 days (11, 14). In Study 2 (Table I), 120 rats were treated with either Serp-1 CHO (60 rats) or saline (60 rats) and mRNA levels of tPA, uPA, PAI-1, and uPAR were analyzed by semi-quantitative RT-PCR analysis in rat arteries at 0, 4, 12, 24 h and 10 days after angioplasty injury (12 rats/treatment group/per time point). A subset of 21 rats had arterial injury with Serp-1 CHO (12 rats) or saline (9 rats) treatment for real time PCR analysis. A set of 30 rats treated with saline, rat PAI-1, SAA, or Serp-1 CHO infusion were sacrificed at 12 h (Table I). Thirty-six rats were sacrificed at 0, 12, and 24 h after Serp-1 or saline infusion without angioplasty injury (6 rats/treatment group). In Study 3, 48 rats treated with either 30 ng of Serp-1 CHO (6 rats) or saline (6 rats) were sacrificed at 0, 12, and 24 h and 28 days after angioplasty for analysis of tPA and PAI-1 protein expression and enzyme activity. All rats were maintained on a normal rat diet.

**Mouse Aortic Allograft Transplant Model**—We performed segmental 0.3-cm aortic isograft and allograft transplantation (12, 37) using all combinations of Balb/c (PAI-1<sup>−/−</sup>), C57Bl/6 (PAI-1<sup>−/−</sup>), and C57Bl/6J (PAI-1<sup>−/−</sup>) mice and similarly C57Bl/6 (uPAR<sup>−/−</sup>) to Balb/c (uPAR<sup>−/−</sup>) (Table II). Mice were purchased directly from Jackson Laboratories (Bar Harbor, ME). Sharpoin 11/0 nylon sutures (Surgical Specialties Corporation, Reiding, MA) were used for end to end aortic anastomosis (12, 14). Mice were sacrificed at 28 days follow up.

**Mouse Femoral Arterial Wire Injury Model**—We performed wire injury in the femoral artery of 12 C57Bl/6J PAI-1<sup>−/−</sup> mice and 6 C57Bl/6 PAI-1<sup>−/−</sup> mice although under general anesthesia. A 0.014-inch angioplasty guide wire (Medtronic Inc., Mississauga, Ontario, Canada) was introduced through a femoral arteriotomy, advanced to the level of the abdominal aorta and then withdrawn 3 times and removed. The site was then sealed with surgical glue, n-butyl cyanocrylate monomer (Nexaband Veterinary Products Laboratories, Phoenix, AZ), as previ-
Table II

| Isograft | Recipient mouse | Treatment | Number mice |
|----------|-----------------|-----------|-------------|
| Total, 22 mice | Balb/c (PAI-1−/−) | saline | 6 |
| C57bl/6 (PAI-1−/−) | C57bl/6 (PAI-1−/−) | Serp-1 CHO, 500 ng/g | 6 |
| C57bl/6J (PAI-1−/−) | C57bl/6J (PAI-1−/−) | Serp-1 CHO, 500 ng/g | 6 |

Table III

| Target gene | Primers | Length |
|-------------|---------|--------|
| Rat PAI-1 | 5'-AGTCTTTCGCCAAAAGAGAC, 3'-CCAGTTTTCTGCCCACAAGGA, 5'-ATAGACCCAGGAGAGAATG | 273 bp |
| Rat tPA | 5'-GGCCCTAGGCACATACAAACA, 3'-GGCCTGAGGCAATACAAAC, 5'-GGCCTGAGGCAATACAAAC | 230 bp |
| Rat uPAR | 5'-GCACAGGAGCCATTTGGATGT, 3'-CAGGGGAGCAAAGTGGATGATA, 5'-CAGGGGAGCAAAGTGGATGATA | 296 bp |
| Mouse β-actin | 5'-CTGTCCTGCGGACACCCCTCCCATC, 3'-TTGTCATCACTTCTGAATCCCCATA | 212 bp |
| Human PAI-1 | 5'-CTGCTCTGCTGCGGACACCCCTCCCATC, 3'-TTGTCATCACTTCTGAATCCCCATA | 212 bp |
| Human PAR-1 | 5'-CTGCTCTGCTGCGGACACCCCTCCCATC, 3'-TTGTCATCACTTCTGAATCCCCATA | 212 bp |
| Human PAR-2 | 5'-CTGCTCTGCTGCGGACACCCCTCCCATC, 3'-TTGTCATCACTTCTGAATCCCCATA | 212 bp |
| Human PAR-3 | 5'-AGTCTTTCGCCAAAAGAGAC, 3'-CCAGTTTTCTGCCCACAAGGA, 3'-AGTCTTTCGCCAAAAGAGAC | 271 bp |
| Human PAR-4 | 5'-AGTCTTTCGCCAAAAGAGAC, 3'-CCAGTTTTCTGCCCACAAGGA, 3'-AGTCTTTCGCCAAAAGAGAC | 271 bp |

Analysis of Gene Expression—Reverse Transcriptase and Northern Blot Analysis

Total RNA was isolated from tissue and cells for (RT-PCR) analysis using TRizol reagent (Invitrogen) (40). Preliminary experiments demonstrated that the amount of tPA, uPA, PAI-1, uPAR, PARs 1, and β-actin cDNA (PCR products) reached plateau levels over 36–38 cycles of reaction and were therefore co-amplified using 32 cycles with β-actin as an internal standard (PTC-100 Programmable Thermal Controller; MJ Research, Watertown, MA) (40, 41). Primers are shown in Table III. Real time PCR was performed as described using SYBR green dye and AmpliTaq Gold DNA polymerase in an ABI Prism 7900HT sequence detection system (AB Applied Biosystems, Warrington, UK) (39). RT-PCR products were verified by sequencing cDNA from the gel band (Gem Extraction Kit, Qiagen) with an ABI 377 automated sequencer (PE Applied Biosystems Inc., Mississauga, ON, Canada) using a Sony Power HAD63CC color video camera attached to the microscope and calibrated to the microscope objective (11, 12, 14). The mean total cross-sectional area of the intima was calculated for each arterial specimen.

Expression and Purification of Serp-1 and Serp-1 Chimeras

Serp-1 CHO was purified from the supernatant of a recombinant CHO cell line (Biogen, Inc., Boston, MA) and Serp-1 VV and SAA were harvested and purified from Buffalo green monkey kidney cell supernatants as previously described (10–14). SAA was prepared by mutating the Serp-1 P1-P1 reactive center loop site (R-N) to an A-A sequence as previously reported (10–12, 14). Serp-1 or SAA proteins were more than 95% pure as judged by overloaded Coomassie-stained SDS-PAGE gels and reverse-phase HPLC (11–14). Serp-1 was tested and found to be free of endotoxin.3

Analysis of Gene Expression—Reverse Transcriptase and Northern Blot Analysis

Total RNA was isolated from tissue and cells for (RT-PCR) analysis using Ambion reagent (Invitrogen) (40). Preliminary experiments demonstrated that the amount of tPA, uPA, PAI-1, uPAR, PARs 1, and β-actin cDNA (PCR products) reached plateau levels over 36–38 cycles of reaction and were therefore co-amplified using 32 cycles with β-actin as an internal standard (PTC-100 Programmable Thermal Controller; MJ Research, Watertown, MA) (40, 41). Primers are shown in Table III. Real time PCR was performed as described using SYBR green dye and AmpliTaq Gold DNA polymerase in an ABI Prism 7900HT sequence detection system (AB Applied Biosystems, Warrington, UK) (39). RT-PCR products were verified by sequencing cDNA from the gel band (Gem Extraction Kit, Qiagen) with an ABI 377 automated sequencer (PE Applied Biosystems Inc., Mississauga, ON, Canada). Northern blot analysis was carried out by the chemiluminescence method (42). RNA was detected (30 μg from HUVEC cultures treated with control protein or Serp-1) in Northern blots by chemiluminescence as previously described (43, 44) using disodium 3-(4-methoxyphenyl)-2,4-dioxetane-3,2'-5'-chloro-tricyclo-[3.3.1.1decan]-2,4-yl phenyl phosphate substrate (Roche Diagnostics; 0.25 mM final concentration), and exposed to Kodak XAR-5 film (Sigma)

Cell Culture and Preparation

Human umbilical vein endothelial (HUVEC, CC-2519 Clonetics, Walkersville, MD, passages 2–5), rat aortic smooth muscle (passages 3–5) (39), or THP-1 cells (ATCC TIB 202) were incubated with saline, 4 ng/ml Serp-1, or SAA. Cells were also incubated with 20 μg/ml antihuman antibodies to uPAR, α5-macroglubulin, or vitronectin, or combinations of proteins and antibodies.

HUVEC were cultured in EGM™ bullet kit CC-3124 (Clonetics) medium and isolated at passages 2–5 for all experiments. Rat aortic smooth muscle cells, collected at passages 3–5, were isolated and grown as previously described (39) in Medium 199 (Sigma) with HEPES (25 mM), and α-glutamine (2 mM) (Invitrogen). THP-1 cells (ATCC TIB 202) provided by Dr. M. Sandig (Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada) were cultured in RPMI 1640 medium (Invitrogen) with mercaptoethanol (2 × 10−5 M). Cells were cultured with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen).

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for 20 min at room temperature. Each membrane was probed first for PAI-1 and then stripped and re-probed with β-actin or tRNA.

Western Blot and Enzyme Activity Assay

Arterial sections, from balloon-injured rat ilio-femoral branches at designated time points (0, 12, 24 h and 28 days) after Serp-1 or saline control treatment, were used for the enzyme activity assays and Western blot analysis (45). Arterial sections were homogenized on ice in buffer (20 mM Tris-HCl, 125 mM NaCl, pH 7.4) containing 100 μg/ml phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin proteinase inhibitor (Sigma). Protein concentrations for each sample tested were measured by colorimetric assay (Bio-Rad). For Western analysis, after blocking nonspecific binding sites with blocking solution (5% skim milk, 3% bovine serum albumin, and 0.1% Tween 20 in phosphate-buffered saline) overnight at 4°C, blots were incubated with 1:800 dilution of rabbit anti-rat PAI-1 or anti-rat tPA (American Diagnostics, Inc.), followed by a 1:100,000 dilution of a monoclonal anti-rabbit IgG (alkaline phosphatase conjugate, Sigma). The color reaction was performed using 5-bromo-4 chloro-3-indoyl phosphate/nitro blue tetrazolium liquid (Bio-Rad).

tPA and uPA activity were measured by chromogenic assay (American Diagnostics), using des-aa-fibrinogen substrate (5 mg/ml, DESAFIB™, American Diagnostics, Inc.), incubated for 75 min at 37°C. Plasmin activity was determined by analyzing absorbance at 405 nm on a Bio-Rad automated microplate reader (Bio-Rad). For the PAI-1 assay arterial extracts were mixed with 100 μl of tPA substrate (American Diagnostics, Inc.) followed by des-aa-fibrinogen substrate. Absorbance was read at 405 nm.

Statistics

Mean plaque area for individual animals was used for statistical analyses. Plaque area, enzyme activity, and RT-PCR ratios were assessed by unpaired Student’s t test and analysis of variance. RT-PCR densitometry ratios were compared by paired t test and analysis of variance. A p value less than 0.05 was considered significant.

RESULTS

Plaque Growth Is Reduced after Vascular Angioplasty Injury by Serp-1 Infusion

To extend the generality of prior studies in rabbit angioplasty (11) and rat allograft transplant models (12–14), we tested Serp-1 efficacy for plaque inhibition in a rat angioplasty injury model (Table I). The plaque growth detected at 28 days follow up after infusion of control SAA (an inactive variant of Serp-1) (Fig. 1A) was significantly greater than that following active Serp-1 infusion (Fig. 1B) (p < 0.016). Arrows in panel A indicate limits of intimal plaque growth. A bar graph of mean plaque area ± S.E. for each treatment group demonstrates similar and significant reductions (p < 0.05) in the plaque area after treatment with Serp-1 VV or Serp-1 CHO when compared with the SAA mutant (C). Magnification ×50.

Altered Serine Proteinase and Serpin Expression following Rat Angioplasty Injury

Semi-quantitative and real time RT-PCR analysis of rat arterial sections using altered gene expression after Serp-1 treatment was analyzed in rat arteries using a balloon-induced vascular angioplasty injury model (Table I). Semi-quantitative RT-PCR analysis demonstrated significant changes in mRNA
expression in the rat arterial wall at early time points after angioplasty injury and Serp-1 treatment when compared with saline control treatment \( (p < 0.01) \). A time course of mRNA expression in representative arterial isolates after balloon angioplasty injury and treatment with either saline control or Serp-1 is shown in Fig. 2. A significant reduction in mRNA for tPA (Fig. 2A, lanes 2-9) and a relative increase in both PAI-1 (Fig. 2B, lanes 2-9) and uPAR (Fig. 2C, lanes 2-9) at 4 and 12 h after injury were detected in arterial isolates after Serp-1 treatment when compared with saline controls \( (p < 0.01) \). Real time PCR analysis confirmed similar significant changes in tPA, PAI-1, and uPAR mRNA expression after angioplasty injury at 4 and 24 h. No significant change in uPA was detected after injury plus treatment with Serp-1 (not shown).

Saline control-treated rat arteries following injury had significant increases in the levels of mRNA expression for tPA \( (p < 0.013) \) and uPAR \( (p < 0.0006) \), but not PAI-1, at selected time points after angioplasty injury when compared with the baseline \( (0 \text{ h}) \) time point (Fig. 3, A-C). SAA control treatment of rat arteries after angioplasty injury had no effect on PAI-1 mRNA levels in the rat artery compared with Serp-1 treatment (Fig. 4, lanes 1-3). Treatment of rats with PAI-1 at equivalent, or 10-fold higher, doses to Serp-1 infusion, also had no detected significant effect on PAI-1 up-regulation (Fig. 4, lanes 4 and 5). Normal, non-injured rat ilio-femoral arterial branches had no demonstrated change in expression of PAI-1, tPA, or uPAR (not shown) with Serp-1 treatment.

In a separate series, the protein levels and enzyme activity for tPA and PAI-1 in the rat ilio-femoral arterial wall were directly measured after angioplasty with Serp-1 or control treatment. A marked reduction in tPA levels, as detected both by immunohistochemical analysis and Western blot analysis (not shown), was observed following Serp-1 treatment. A significant attenuation in tPA enzyme activity increase following injury was also detected in Serp-1-treated rat arteries by 24 h after angioplasty injury (tPA enzyme activity measured by absorbance: for saline-treated arteries \( 0.814 \pm 0.03 \), for Serp-1-treated arteries \( 0.654 \pm 0.022, p < 0.01 \)).
inhibitory activity was reduced during the same time frame, and this reduction was attenuated by Serp-1 treatment (PAI-1 inhibitory activity measured by absorbance: for saline-treated arteries = 0.396 ± 0.039, for Serp-1-treated arteries = 0.526 ± 0.023, p < 0.01).

**Analysis of Plasminogen Activator and Inhibitor Expression in Cell Culture**

We next examined the effect of Serp-1 treatment on selected cell lines by RT-PCR analysis of several select genes in the host serpin pathway with potential Serp-1 regulation. PAI-1 mRNA was significantly increased (p < 0.05) in HUVEC cultures after incubation with Serp-1 when compared with saline controls (Fig. 5, lanes 2 and 3). Serp-1 treatment produced an increase in PAI-1 mRNA starting at 4 h and continuing to 24 h post-treatment (Fig. 5, lane 3 illustrates 12 h), a time frame similar to that observed for the increase in PAI-1 detected in angioplasty-injured rat arteries with Serp-1 treatment. In contrast, Serp-1 had no effect on PAI-1 expression in smooth muscle or THP-1 monocytic cell cultures (not shown). Serp-1 also had no effect on tPA, uPA, protease-activated receptors 1–4 (PARs1–4), or uPAR mRNA expression as measured by RT-PCR analysis in all cell lines tested (not shown). To further confirm this finding, PAI-1 mRNA was also assessed at 24 h after Serp-1 treatment using Northern blot analysis. Serp-1, but not saline, PAI-1, or SAA (not shown), again significantly increased levels of PAI-1 mRNA on Northern blot analysis.

**Inhibition of HUVEC Responses to Serp-1 with Antibodies to uPAR and Vitronectin**

Addition of uPAR or vitronectin blocking antibodies alone to HUVEC cultures had no effect on PAI-1 mRNA levels (Fig. 5, lanes 4 and 6). When antibody to either uPA receptor or vitronectin was given during Serp-1 treatment of HUVEC cultures, however, the up-regulation of PAI-1 mRNA produced by Serp-1 treatment was partially attenuated (Fig. 5, lanes 2, 3, 5, and 7). Treatment of HUVEC cells with both antibodies together completely prevented the Serp-1-mediated increase in PAI-1 mRNA (Fig. 5, lane 8). Treatment with antibody to α2-macroglobulin had no effect on Serp-1-induced up-regulation of PAI-1 expression (not shown). Based on these results, we examined whether PAI-1 or uPAR might play a role in mediating the anti-inflammatory properties of Serp-1 in knockout mouse models.

**Treatment of PAI-1-deficient Mouse Strains with Serp-1 after Vascular Transplant**

Based upon the studies reported above demonstrating altered expression of tPA, PAI-1, and uPAR in rat arteries after treatment with Serp-1, we tested Serp-1 for inhibition of plaque growth after aortic allograft transplant in mouse models deficient for PAI-1 or uPAR (Table II). The aortic allograft transplant model provides a model of chronic rejection with a marked vascular inflammatory response. The PAI-1-deficient mouse aortic allograft transplant provided an analysis of the capacity of Serp-1 to block plaque growth in animals with no functional PAI-1 expression. The PAI-1-deficient mouse allograft transplant provided analysis of the relative effects of PAI-1 deficiency in the local donor aortic implant (PAI-1−/− to PAI-1−/+ allografts) and in the recipient aorta and systemic blood (PAI-1+/− to PAI-1+/− allografts) and the capacity for Serp-1 to block plaque growth under both conditions. The uPAR-deficient mouse allograft transplant model provided an analysis of Serp-1 anti-atherogenic activity in donor aorta lacking uPAR.

**Isograft Aortic Transplants in PAI-1-deficient (PAI-1−/−) Mice**—Aortic sections from isograft transplants in Balb/c and C57Bl/6 mice with normal PAI-1 expression had only small areas of intimal hyperplasia 4 weeks after transplant (Fig. 6A). In contrast, PAI-1-deficient mouse aortic isografts had larger areas of plaque development (Fig. 6A). Morphometric analysis of plaque area demonstrated a significant increase in plaque area in the PAI-1−/− isografts when compared with either mouse strains expressing PAI-1 (p < 0.011 for C57Bl/6 and p = 0.021 for Balb/c mice) (Fig. 6A). Serp-1 treatment significantly inhibited plaque growth in the PAI-1−/− isograft transplants (p < 0.025) indicating that up-regulated arterial PAI-1 expression is not needed for Serp-1-mediated blockade of inflammatory responses and plaque growth (Fig. 6A). Up-regulation of PAI-1 expression might, nevertheless, provide additive inhibitory activity.

**Allograft Aortic Transplants in PAI-1−/− Mice**—Aortic allograft transplants also demonstrated increased plaque development when compared with the isografts (Fig. 6, B–G). Transplants using PAI-1−/− mice as either the donor (Fig. 6, B and F) or recipient (Fig. 6, C and G) had larger areas of plaque development when compared with the isografts. When compared with aortic allografts from mice where both the donor and the recipient expressed PAI-1 (PAI-1−/+−) (p < 0.04 for C57Bl/6 to Balb/c when compared with C57Bl/6J to Balb/c and p < 0.06 for Balb/c to C57Bl/6 when compared with Balb/c to C57Bl/6J) plaque area was increased for the PAI-1-deficient mice (Fig. 6, F and G). Of interest was the finding that when the aorta from PAI-1−/− mice was used for the donor transplant aortic arterial segment, the plaque area was larger, although not significant (p = 0.093), than when the PAI-1−/− mouse was the recipient (Fig. 6, F and G), suggesting a greater local effect of aortic serpin expression on transplant vasculopathy development.

**Serp-1 Infusion after Aortic Allograft Transplant in PAI-1−/−-deficient Mice**—Serp-1 treatment reduced plaque...
and G/H11546 PAI-1

A area with Serp-1 treatment of the PAI-1/H11546 FIG. 6.

Bar graphs/H11001 Bar graphs/H11001), had increased plaque when compared with the PAI-1/H11001 recipients (G/H11001).

Allograft transplants, whether PAI-1/H11546/H11001 isografts and a significant decrease in plaque B and C/H11001 or PAI-1/H11001 donors to PAI-1/H11001/H11001/V18569 isograft transplants. Serp-1 treatment also showed a trend toward reducing plaque growth after aortic allograft transplant in PAI-1/H11001/H11001 mouse models (C57Bl/6 to Balb/c, p = 0.143 and Balb/c to C57Bl/6, p = 0.057), but this did not reach significance (FIG. 6, E and G). We conclude that Serp-1 retains the ability to mediate plaque reduction even in the absence of PAI-1 in either the donor or recipient mouse aorta.

Serp-1 Infusion after Femoral Arterial Wire Injury in PAI-1/H11001-deficient Mice

A significant increase in plaque area was seen in the PAI-1-deficient mice when compared with normal PAI-1 expressing C57Bl/6 mice after wire injury. Serp-1 treatment reduced the excess plaque area in the PAI-1-deficient mice as follows: plaque area – saline-treated C57 Bl/6 PAI-1/H11001/H11001 mice = 0.002 ± 0.0009 mm², saline-treated C57Bl/6J PAI-1/H11001/H11001 mice = 0.028 ± 0.022 mm², Serp-1 treated C57Bl/6J PAI-1/H11001/H11001 mice = 0.00009 ± 0.00004 mm², p < 0.05). This work indicates that Serp-1 is capable of preventing the increase in plaque seen in mice lacking PAI-1, both in models of aortic allograft transplant and in this second model of mechanical, wire-induced arterial injury.

Serp-1 Infusion after Aortic Allograft Transplant from uPAR-deficient Mice

The transplant of aortic allograft segments from uPAR-deficient (uPAR/H11001) into Balb/c mice had no effect on generalized plaque growth when compared with uPAR/H11001 C57Bl/6/H11001/H11001 aortic transplant (p = 0.121). Treatment of mice after uPAR/H11001 aortic transplant with Serp-1 at doses proven to inhibit plaque growth in the PAI-1/H11001 mouse model no longer blocked plaque growth (FIG. 7, A–C) (p = 0.85), indicating that Serp-1 inhibits plaque growth through mechanisms dependent, at least in part, on the uPAR complex. Thus we conclude that Serp-1 targets serine proteinases that utilize uPAR on cells found in the donor transplanted tissue.

DISCUSSION

We report here that Serp-1 treatment following vascular injury reduces vascular serine proteinase (tPA) expression and induces increased vascular uPAR and serpin (PAI-1) expression levels in the injured arterial wall. In addition, Serp-1 up-regulates expression of PAI-1 in human endothelial cells (HUVEC), specifically through signaling that is dependent upon the uPA receptor and vitronectin. Based upon these findings we postulated that the viral serpin, Serp-1, altered cellular responses by commandeering the mammalian cellular uPA/ uPAR serine proteinase response system and altering gene expression. The vascular serpin PAI-1 is known to bind to uPAHIV complexes that regulate cellular responses both through extracellular activity involving activation of matrix metalloproteinases and growth factors and enhancing cellular migration and adhesion and also via intracellular signaling through low density lipoprotein-related protein (3–6, 20, 34, 35). The uPAuPARPAI-1 ternary complex effectively blocks uPAuPAR activity through internalization and breakdown of controls. Serp-1 treatment reduced plaque growth after allograft transplant with more significant inhibitory effect in PAI-1/H11001 to PAI-1/H11001 allografts (B) than PAI-1/H11001 to PAI-1/H11001 allografts (C and G).
the uPA/uPAR complex. Vitronectin further enhances PAI-1-mediated inhibition. We have proposed that Serp-1, acting as a native cellular serpin mimic, supplants PAI-1 blocking the uPA/uPAR complex-directed extracellular and intracellular activity.

To test these postulates, we assessed the role of PAI-1 and the uPA receptor in Serp-1-mediated inhibition of inflammation (11–14) in mouse models of aortic transplant. Significantly, we detected inhibition of plaque growth with Serp-1 treatment in PAI-1-deficient, but not in uPAR-deficient, mouse strains. Serp-1 thus successfully exploits the mammalian cellular uPA receptor to alter expression of a key arterial regulator, PAI-1, and to regulate inflammatory cell responses and plaque growth in the arterial wall (3–7, 17, 18, 15, 26). This study provides a definitive demonstration of a viral serpin acting to alter cellular expression and regulation of a mammalian host cell proteinase/receptor system.

Serp-1-mediated up-regulation of PAI-1 was unexpected, but has the potential to amplify inhibition of uPA/uPAR complex-associated cellular invasion in accelerated inflammatory responses. The altered expression of host vascular plasminogen activators and inhibitors is observed beginning as early as 4 h after Serp-1 treatment, suggesting that this amplification of host-mediated regulatory activity plays a role in subsequent anti-inflammatory and anti-atherogenic activity of Serp-1 (Figs. 2 and 3). Our finding that Serp-1 blocked plaque growth in the PAI-1 knockout isografts and after wire injury in PAI-1-deficient mice indicated, however, that Serp-1 anti-inflammatory activity does not depend on increased expression of PAI-1 (Fig. 6A). Increased expression of PAI-1 and uPAR have been previously reported after vascular injury (46–48) and also after injury of other organ systems (48, 49) or invasive carcinoma (50, 51). Our work demonstrates similar increases in control saline-treated animals, but comparison of Serp-1-treated specimens with the saline controls at each time point detected significant alterations in tPA and uPAR gene expression that were greater than the changes in gene expression observed after injury alone.

Dysregulated expression of PAI-1 and tPA induced by Serp-1 is consistent with a reduction in inflammation and cellular invasion, as mediated by the uPA/uPAR receptor complex. The inability of the biochemically inactive Serp-1 mutant, SAA, or PAI-1 itself to up-regulate PAI-1 gene expression suggests that this was not a nonspecific reaction to local arterial injury. The finding that Serp-1 altered PAI-1 and uPAR expression, but not expression of protease-activated receptors, PARs 1 to 4 (the main thrombin receptors), suggests that Serp-1 activity is specifically mediated through the cell surface uPA/uPAR system, and not circulating thrombin or the clotting cascade. Also, the ability of Serp-1 to trigger effects in human endothelial cells is of great significance and suggests that Serp-1 initiates a non-species-specific response in the arterial wall via a conserved regulatory mechanism. The inhibition of PAI-1 increase by the combined action of blocking antibodies to uPAR and vitronectin is also consistent with mediation of Serp-1 anti-inflammatory activity through the uPA/uPAR system. Vitronectin is known to bind and stabilize the uPA/uPAR/PAI-1 complex and thereby enhance PAI-1 inhibitory activity (35–37, 50). The mechanism for the increase in uPAR expression in the arterial wall by Serp-1 remains to be explained, but is likely to be of functional
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