Plasminogen Activator Inhibitor-1 Supports IL-8-Mediated Neutrophil Transendothelial Migration by Inhibition of the Constitutive Shedding of Endothelial IL-8/Heparan Sulfate/Syndecan-1 Complexes

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Plasminogen Activator Inhibitor-1 Supports IL-8-Mediated Neutrophil Transendothelial Migration by Inhibition of the Constitutive Shedding of Endothelial IL-8/Heparan Sulfate/Syndecan-1 Complexes

Lindsay J. Marshall,* Lara S. P. Ramdin,† Teresa Brooks,‡ Peter Charlton DPhil,§ and Janis K. Shute**

The endothelium is the primary barrier to leukocyte recruitment to sites of inflammation. Neutrophil recruitment is directed by transendothelial gradients of IL-8 that, in vivo, are bound to the endothelial cell surface. We have investigated the identity and function of the binding site(s) in an in vitro model of neutrophil transendothelial migration. In endothelial culture supernatants, IL-8 was detected in a trimolecular complex with heparan sulfate and syndecan-1. Constitutive shedding of IL-8 in this form was increased in the presence of a neutralizing Ab to plasminogen activator inhibitor-1 (PAI-1), indicating a role for endothelial plasminogen activator in the shedding of IL-8. Increased shedding of IL-8/heparan sulfate/syndecan-1 complexes was accompanied by inhibition of neutrophil transendothelial migration, and aprotinin, a potent plasmin inhibitor, reversed this inhibition. Platelets, added as an exogenous source of PAI-1, had no effect on shedding of the complexes or neutrophil migration. Our results indicate that IL-8 is immobilized on the endothelial cell surface through binding to syndecan-1 ectodomains, and that plasmin, generated by endothelial plasminogen activator, induces the shedding of this form of IL-8. PAI-1 appears to stabilize the chemoattractant form of IL-8 at the cell surface and may represent a therapeutic target for novel anti-inflammatory strategies. The Journal of Immunology, 2003, 171: 2057–2065.

Inflammatory cell migration across the endothelium, the first barrier to cellular recruitment to tissue sites of inflammation, occurs via a multistep process that is mediated via the interaction of inflammatory cells with endothelial cell adhesion molecules and chemoattractants (1). Neutrophil recruitment across the endothelium is directed by concentration gradients of the chemokine IL-8, synthesized by the endothelium in response to inflammatory signals such as TNF-α and IL-1β (2) or by cells resident in tissues and diffusing to the endothelium (3). Conversely, Gimbrone et al. (4) demonstrated that soluble IL-8 inhibits neutrophil adhesion to endothelial cells in culture. It was therefore proposed that binding of chemoattractants, including IL-8, to the endothelial cell surface promotes neutrophil transendothelial migration, while the exposure of circulating neutrophils to soluble blood-borne IL-8 inhibits their binding to the endothelium (2, 5).

All chemokines bind to heparin (6), and it was suggested that binding to heparan sulfate (HS) proteoglycans (PG) generates the transendothelial concentration gradients of these chemoattractants that are a prerequisite for successful transendothelial migration (7). It has subsequently been shown that the endothelial cell binding sites for a number of chemokines are likely to be the glycosaminoglycan (GAG) side chains of endothelial PG (8), and that GAGs interact selectively with chemokines to modulate binding to specific receptors on target cells and cellular responses (9). HS had a greater affinity for IL-8 than chondroitin or dermatan sulfate, indicating that HS may be the GAG involved in presentation of IL-8 by endothelial cells, as previously suggested (7). It was also suggested that the Duffy Ag/receptor for chemokines might play a role in chemokine presentation by endothelial cells (3). However, there is little evidence that endothelial cells express specific chemokine receptors or the Duffy Ag in vitro (8, 10), and it appears that HS is the most abundant IL-8 binding sites on these cells.

HS is synthesized on a number of cell surface proteins, but is found mainly on two major PG families, the syndecans and the glypicans (11). The syndecan family of core proteins are products of four distinct genes, and are transmembrane proteins with extended extracellular domains that carry HS chains distal from the cell membrane. Conversely, the glypican core proteins are a family of at least six gene products, and are compact molecules linked to the cell membrane via GPI linkages and carry HS chains close to the cell surface. Endothelial cells express syndecans-1, -2, and -4 and glypican-1 (12), with the syndecans carrying the bulk of the HS PG.

All cultured cells shed syndecan ectodomains as part of normal turnover, and this shedding is accelerated by agonists that enhance the activity of a tissue inhibitor of matrix metalloproteinase (TIMP)-3-sensitive metalloproteinase (14). For example, the shedding of syndecan-1 and -4 ectodomains from an endothelial cell line is enhanced by ligand activation of the thrombin (G protein-coupled) receptor and epidermal growth factor (protein tyrosine kinase)...

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Abbreviations used in this paper: HS, heparan sulfate; GAG, glycosaminoglycan; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; PG, proteoglycan; iPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; TIMP, tissue inhibitor of matrix metalloproteinase.
kinase) receptor (15). Thus, activation of multiple signaling pathways leads to increased activity of a membrane-bound metalloproteinase and cleavage of the syndecan core protein on the cell surface, close to the plasma membrane (14, 15). The HS chains of the shed ectodomains bind and regulate the activity of proteases, including neutrophil elastase and cathepsin G, and are speculated to play an important role in the regulation of wound repair (13). The proteolytic system for constitutive shedding of syndecan ectodomains has not been identified, but appears to be different from the protease involved in accelerated shedding (14).

Plasm in has been identified as a protease capable of directly inducing shedding of cell-associated syndecan from endothelial cells (15). The generation of plasm in has been implicated in the liberation of active matrix metalloproteinases (MMPs) from their latent propeptides both in vitro and in vivo (16, 17), and a number of MMPs have the potential to degrade PG (16). However, the use of specific inhibitors of members of the MMP family has indicated that these proteases may not play a role in the constitutive shedding of cell surface PG such as syndecan-1 (14). Therefore, we investigated the contribution of the plasm in/plasminogen activation system and, specifically, the effects of plasminogen activator inhibitor-1 (PAI-1) on the constitutive release of soluble ectodomains of endothelial cell surface HSPG, and the functional consequences of this on neutrophil transendothelial migration.

In this study, we speculated that endothelial IL-8 is bound, at least in part, to syndecan-1 and that shedding of this HS PG would lead to solubilization of cell surface-bound IL-8 and inhibition of IL-8-mediated transendothelial neutrophil migration. We hypothesized that proteases of the plasminogen activator/plasmin cascade are involved in the constitutive shedding of endothelial syndecan-1 and regulation of IL-8 function. To test this hypothesis, we used primary HUVECs cultured on Transwells to study the response of neutrophils when IL-8 was added as chemottractant beneath an endothelial cell layer. In these cultures, we used a neutralizing Ab to PAI-1 to unmask the role of endogenous plasminogen activator in the shedding of HS, syndecan-1, and IL-8, and investigated the effects on neutrophil transendothelial migration. We also investigated the effect of coculture with platelets, as a rich source of PAI-1, on these responses. Our results point to an important role for plasminogen activation on the endothelial cell surface in the regulation of chemokine presentation by HSPG, including syndecan-1, and subsequent neutrophil transendothelial migration.

Materials and Methods

Endothelial cell culture

Primary cultures of single donor HUVEC (Clonetics, San Diego, CA) were grown to confluence in 1% (v/v) gelatin-coated flasks in endothelial growth medium (Clonetics), endothelial basal medium supplemented with (final concentrations in brackets) FBS (2%), bovine brain extract (12 μg/ml), hydrocortisone (1 μg/ml), human epidermal growth factor (10 ng/ml), gentamicin (50 μg/ml), and amphotericin-B (50 μg/ml).

Measurement of IL-8 distribution

Endothelial cells were grown to confluence in gelatin-coated 24-well plates (Costar, Cambridge, MA) for 2 days. Mutant PAI-1 (Calbiochem, Nottingham, U.K.), an altered form of human PAI-1 containing 4 mutated aa that is virtually unable to go latent (t 1/2 = 145 h at 37°C (18)), at 1, 10, and 100 μg/ml and aprotinin at 5, 25, and 50 μM were added for 5 min. After this time, supernatants were collected, centrifuged at 200 × g for 10 min, and either assayed immediately or stored at −80°C. For determination of cell-associated IL-8, cells were lysed using 1% (v/v) Triton X-100 in PBS before analysis. The concentration of IL-8 was assayed using a commercially available ELISA kit (PelKine kit; CLB, Amsterdam, The Netherlands), as per the manufacturer’s instructions.

Measurement of tissue plasminogen activator (tPA) activity

Two mAbs to PAI-1 were used, one that neutralizes PAI-1 activity (MAI-12; Biopool, Umeå, Sweden), and one that does not (mc 3785; American Diagnostica, Greenwich, CT). The effect of these Abs on the activity of human rPAI-1 was assayed by measuring restoration of tPA activity in the presence of a concentration of PAI-1 that just inhibited tPA activity. Initially, human rPAI-1 was titrated with tPA to establish an inhibitory concentration of PAI-1 (5.2 nM) for use in the assays assessing the effects of the Abs on PAI-1 activity. A modification of the COASET tPA method (Chromogenix, Molndal, Sweden) (Nilsson et al.) was used (19). Various concentrations of the Abs (in 5 μl) were incubated with 25 μl PAI-1 and 20 μl assay buffer (50 mM Tris-HCl, pH 8.3) for 10 min at 25°C. A 20-μl aliquot of this mixture was incubated for a further 20 min at 25°C in the presence of 20 μl tPA (final concentration 100 IU/ml). Following this incubation period, 8 μl each of plasminogen and a chromogenic tPA substrate (final concentrations 0.33 μM and 0.34 mM, respectively) were added, along with 20 μl tPA stimulator (final concentration 0.24 mg/ml). The generation of plasmin was monitored at 405 nm for 10 min at 25°C. PAI-1 inhibition was expressed as the percentage restoration of tPA activity.

Platelet isolation

Platelets were isolated from EDTA-anticoagulated normal venous blood. Platelet-rich supernatant was prepared by adding 0.15 M NaCl with 77 mM EDTA, pH 7.4, and centrifuging at 200 × g for 15 min at room temperature. Platelets were pelleted at 1000 × g for 15 min at room temperature, and the pellet was washed twice with PBS containing 10 mM EDTA. Cells were resuspended at 1 × 10⁶ per ml in HBSS (Life Technologies, Paisley, U.K.), buffered to pH 7.4 with 20 mM HEPES (BDH, Poole, U.K.).

Neutrophil isolation

Neutrophils were isolated from EDTA-anticoagulated normal venous blood. RBCs were removed by sedimentation for 45 min at room temperature with 6% (v/v) Dextran 70 (Macrodex; Pharmacia, Uppsal, Sweden). Leukocyte-rich supernatants were underlayered with an equal volume of Lymphoprep (Nycomed Pharma, Oslo, Norway) and centrifuged at 300 × g for 30 min at room temperature. The upper layers were discarded, and RBCs remaining in the granulocyte pellet were subjected to hypotonic lysis. Cells were washed and resuspended at 1 × 10⁶ per ml in HBSS with 20 mM HEPES. Neutrophils isolated in this manner were >97% pure, with eosinophils as the contaminating cells.

Transendothelial migration of neutrophils

Endothelial cells, between passages 4 and 8, were seeded at 30,000 cells/well on 10 μg/cm² collagen IV-coated Transwells (BD Biosciences; Oxford, U.K.). Cells were grown for 1 wk, and confluence was established at this time point by staining wells with 1% dimethyl methylene blue. One hour before neutrophils were added, the HUVEC were washed once with PBS, and, at this point, IL-8, mAbs to PAI-1, and/or aprotinin, diluted in HBSS with 20 mM HEPES, were added to the HUVEC cultures. Human tPA (the gift of I. Lindley, Novartis, Vienna), at a final concentration of 10⁻⁴ M, was added in 900 μl as neutrophil chemotactant to the lower compartment of the Transwells. Both Abs (MAI-12 and mc 3785) were added to the lower and upper Transwell compartments, at final concentrations of 1, 10, and 50 μg/ml. Aprotinin, an inhibitor of surface-bound plasmin (20), was added to both compartments at 5, 25, and 50 μM.

For platelet coculture, 250,000 platelets/well were added to the endothelial cell layers for 15 min to allow adherence, which was confirmed by light microscopy. The supernatant was removed, and neutrophils were added at 200,000 in a total final volume of 300 μl/well. Endothelial cells were cultured with neutrophils, in the absence and presence of platelets, for 3 h at 37°C. Migrated cells in the lower compartment were harvested together with transmigrated neutrophils adhering to the lower surface of the Transwell, by adding 25 μl EDTA (final concentration 77 mM) to the lower compartment and gently agitating for 1 min. The medium in the lower well was removed and centrifuged at 200 × g for 10 min at 4°C, and the supernatant was stored at −80°C. The number of neutrophils in the cell pellets was counted, and results were expressed as the percentage of cells migrated, relative to control (no Ab) conditions.

SDS-PAGE and Western blotting

Polyacrylamide mini-gels were cast using the Bio-Rad Protein II system. Proteins were separated by electrophoresis on polyacrylamide gradient gels (Fowglen, Ashby de-la Zouch, U.K.) in Tris-tricine running buffer (100 mM each Tris and tricine with 0.1% (v/v) SDS). Samples were run in parallel with prestained m.w. markers (Bio-Rad, Hercules, CA). Proteins were electrophoretically transferred onto 45-μm nitrocellulose membrane (Bio-Rad)
using a semidry transfer method. Transfer was conducted in 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, with 20% (v/v) methanol for 80 min at a constant 250 mA.

**Staining Western blots with Abs**

Nitrocellulose membranes were blocked in PBS with 2% (v/v) Tween 20 at 4°C overnight. Blots were incubated with primary Abs for 90 min at room temperature. Mouse monoclonals to HS (clone 10E4; Seikagaku Kogyo, Tokyo, Japan) and syndecan-1 (clone CD138; ImmunonContact) were used at final concentrations of 100 and 10 μg/ml, respectively, in PBS/2% (v/v) Tween 20 containing 1% (v/v) rabbit serum. For detection of bound Abs, biotinylated secondary Abs (Dako, Glostrup, Denmark), followed by a complex of streptavidin and biotinylated HRP (Dako Strept-ABCComplex), were used. Results were visualized using a chemiluminescent substrate (SuperSignal; Pierce, Warriner, U.K.) and subsequent exposure of blots to x-ray film (Kodak XLS).

No cross-reactivity between Abs to HS, syndecan-1, or IL-8 could be detected on immunoblots.

**Immunoprecipitation of IL-8**

Supernatants were diluted 1/5 in immunoprecipitation buffer, comprised of 150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.1% (w/v) SDS, 0.5% (v/v) Tergitol Nonidet P-40, and a protease inhibitor cocktail, used at one tablet in 50 ml, as per the manufacturer’s instructions (Boehringer Mannheim, Mannheim, Germany), pH 7.6. To this were added 1 μg/ml of affinity-purified goat polyclonal anti-IL-8 (the gift of I. Lindley) and 4 μl of protein A agarose beads (Bio-Rad). Control immunoprecipitations, using an irrelevant Ab, were conducted using a goat polyclonal Ab to the P2/7 receptor (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml, a concentration that precipitates the P2/7 receptor from mouse brain homogenates. Samples were incubated with shaking at 4°C overnight, followed by centrifugation at 3000 × g and 4°C for 5 min. The supernatant was removed and the pellet was washed three times with ice-cold immunoprecipitation buffer. To release the immunoprecipitated protein from the Ab/protein A agarose complex, samples were incubated with 5× Laemmli sample buffer containing 100 mM DTT at room temperature for 30 min. Samples were then centrifuged for 5 min at 3000 × g before analysis by Western blot, as described above.

**Measurement of plasmin**

Plasmin activity in culture supernatants was assayed using a modification of the Sigma Accucolor Plasminogen procedure, which employs the chromogenic substrate D-BUT-CHT-Lys-pNa. Samples were assayed neat and incubated at 37°C until a color change was apparent. The x-ray film was developed and analyzed using Scion Image software. The optical density of the color at 405 nm was determined for each sample, and the results were expressed as the percentage inhibition of plasmin activity and are representative of mean ± SD of two separate experiments. Results are expressed as the percentage inhibition of plasmin activity and are representative of mean ± SD of two separate experiments.

**Results**

**The distribution of IL-8 in endothelial cultures**

Endothelial cells are known to synthesize IL-8 constitutively. However, under baseline conditions, only 2% of the total IL-8 detected in our endothelial cell cultures was detected in association with the cell layer, with 98% in a soluble form. Because cell- or matrix-bound IL-8 is likely to be an important, proinflammatory, chemoattractant form of this chemokine (5), mechanisms leading to the shedding and solubilization of IL-8 are potentially anti-inflammatory. We therefore investigated the possibility that proteases of the plasminogen activator/plasmin cascade contributed to the release of IL-8 from pericellular binding sites. In the presence of a mutant form of plasminogen activator inhibitor-1 that only slowly reverts to latent in culture (18), the distribution of IL-8 changed significantly to 60:40 distribution between soluble and cell-associated forms (Fig. 1). The same redistribution was observed when the plasmin inhibitor aprotinin was present. The effect of both stable PAI-1 and aprotinin could only be demonstrated at the highest concentrations tested. For both inhibitors, the summed amount of IL-8 in the supernatant and cell lysate did not change, and therefore there was no evidence that the protease inhibitors were inhibiting IL-8 degradation at the cell surface. However, this was the first evidence that plasminogen activation may be directly or indirectly responsible for the shedding of IL-8 from the endothelial cell surface.

This effect of exogenous stable PAI-1 was lost in longer (1-, 3-, 24-h) cultures (data not shown). Therefore, in subsequent studies of neutrophil transendothelial migration, a PAI-1-neutralizing Ab was used to unmask the regulatory effects of endothelial PAI-1 on plasminogen activation and the form and function of IL-8 in endothelial cell cultures. In addition, because platelets are a rich source of PAI-1, experiments were conducted to investigate whether an exogenous cellular source of wild-type PAI-1 could also regulate endothelial IL-8 function.

**Inhibition of PAI-1 activity by Abs**

Two mAbs to PAI-1, MAI-12 and mc 3785, were assessed for inhibition of PAI-1 activity, measured as restoration of tPA activity and plasmin generation (Fig. 2). MAI-12 inhibited PAI-1 activity with an IC50 of 1.53 ± 0.12 nM. The second Ab tested, mc 3785, had no effects on PAI-1 activity at any of the concentrations used, and was therefore a nonneutralizing Ab.

**Neutrophil transendothelial migration**

Human rIL-8 was used as a neutrophil chemoattractant in studies of transendothelial migration. When IL-8 was added to the lower compartment at a final concentration of 10−8 M, 22.5 ± 10% of neutrophils (n = 4) had migrated to the lower chamber after 3-h incubation.
at 37°C. In the presence of platelets, the number of neutrophils that migrated was not significantly different (21.7 ± 9%).

**Analysis of soluble PG in culture supernatants**

HSPG on endothelial cell surfaces were proposed to bind and present chemokines to circulating inflammatory cells (7). We therefore investigated the effect of endogenous PAI-1 activity on the constitutive shedding of HS from endothelial cells grown on Transwells. Analysis of culture supernatants from the basal compartment by Western blot showed that inhibition of endothelial PAI-1 activity with increasing concentrations of MAI-12 induced the shedding of HS from endothelial cell layers in a concentration-dependent manner (Fig. 3). An Ab (10-E4) was used that recognizes HS chains both when they are bound to the HSPG core protein and when the core protein has been degraded (20). However, the estimated molecular mass of 140 kDa for HS indicated that the shed HS was bound to a core protein.

We investigated whether platelets, as a source of exogenous PAI-1, could induce similar changes in the distribution of IL-8, as illustrated in Fig. 1, or inhibit the shedding of HS by endothelial cells in culture, as shown in Fig. 3. A lack of effect of platelets on the distribution of IL-8 in endothelial cell platelet cocultures was observed (Table I). Despite a significant increase in the amount of IL-8 detected when endothelial cells were cultured in the presence of platelets, the same high proportion of IL-8 was present in a soluble form (Table I). Similarly, the presence of platelets had no effect on the shedding of HS (Fig. 3). Low levels of HS, detected by prolonged exposure of x-ray films, in the absence of the PAI-1 Ab were not altered in the presence of platelets. Because cell-cell interaction often excludes soluble high m.w. Ab inhibitors, we also investigated the effect of adding platelets in the presence of the Ab to PAI-1. There was no evidence that platelet-derived PAI-1 reversed the shedding of HS induced by inhibition of endothelial PAI-1 (Fig. 3).

We then proceeded to investigate the effect of PAI-1 on the shedding of HSPG during neutrophil transendothelial migration and functional effects of PAI-1 inhibition on the magnitude of this response. Supernatants harvested from the basal compartment following neutrophil transendothelial migration were analyzed by Western blot and stained for HS chains (Fig. 4). HS could be detected in the absence of MAI-12, but prolonged exposure of the blot was required, as the signal was very weak, suggesting that

![Image](http://www.jimmunol.org/)

**FIGURE 3.** PAI-1 activity prevents constitutive shedding of PG. Supernatants from the basal compartments of endothelial cells cultured alone on Transwell inserts and in the presence of platelets, in the presence of increasing concentrations of MAI-12, were analyzed by Western blotting for the presence of HS. Odd-numbered lanes show supernatants from endothelial cells alone, and even-numbered lanes endothelial cells after coculture for 3 h with platelets. Lanes 1 and 2, Show control, in the absence of MAI-12, while lanes 3 and 4, 5 and 6, and 7 and 8 are in the presence of 1, 10, and 50 μg/ml MAI-12, respectively. HS was detected at an apparent molecular mass of 140 kDa. Constitutive release was low (lanes 1 and 2), platelets had no effect on HS shedding (compare even- and odd-numbered lanes), but MAI-12 enhanced shedding in a concentration-dependent manner. The scanned image is representative of four independent experiments, and positions of m.w. markers are shown on the left of the figure.

<table>
<thead>
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<th>Supernatant</th>
<th>Lysate</th>
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<tr>
<td>% Total</td>
<td>% Total</td>
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<tr>
<td>Endothelial cells alone</td>
<td>5.59 ± 0.07</td>
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<tr>
<td>Endothelial cell platelet coculture</td>
<td>9.81 ± 0.5</td>
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*Endothelial cells were cultured for 15 min in the absence and presence of platelets. Endothelial cells produced IL-8, as measured by ELISA, that was detected predominantly in the culture supernatant with a small percentage remaining cell associated. Although the amount of IL-8 present in the cultures was significantly (p < 0.05) increased in the presence of platelets, there was no change in the relative distribution between soluble and cell-associated forms.

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Neutrophil transendothelial migration does not alter MAI-12-dependent HS shedding. Supernatants from the basal compartment following neutrophil transendothelial migration were analyzed by Western blotting for the presence of HS (A). Odd-numbered lanes show supernatants from neutrophil-endothelial cell cocultures, and even-numbered lanes cocultures in the additional presence of platelets. Lanes 1 and 2, Show control, and lanes 3 and 4, 5 and 6, and 7 and 8 are in the presence of 1, 10, and 50 μg/ml MAI-12, respectively. The transmigration of neutrophils, either in the absence or presence of platelets, did not alter the pattern or extent of HS shedding in response to increasing MAI-12, compared with the effect of MAI-12 on the constitutive shedding of HS (Fig. 3). Positions of m.w. markers are shown on the left of the figure. The results are representative of four independent experiments. B, Shows the quantitative analysis of the intensity of HS staining; data are represented as mean ± SEM (n = 4). HS release following neutrophil transmigration in the absence (filled bars) and presence (open bars) of platelets is shown. Levels of soluble HS were significantly enhanced at all concentrations of MAI-12 studied compared with control (p < 0.001) under all culture conditions.
during the 3-h incubation, constitutive turnover was relatively low. In the presence of MAI-12, at 1, 10, and 50 μg/ml, supernatants contained increasing amounts of HS. HS was most abundant as an immunopositive band with an apparent molecular mass of ~140 kDa, and this was true for supernatants from both the lower and upper compartments (data not shown). The larger volume of the lower compartment allowed multiple analyses, and this compartment was therefore used for all subsequent analyses. Fig. 4 demonstrates the marked increase in the amount of HSPG released into the basal compartment on addition of increasing MAI-12. Quantification of these data by image analysis (Fig. 4B) revealed that, even at 1 μg/ml, MAI-12 significantly \((p < 0.001)\) increased the release of soluble HS into the basal compartment. Further increases in the amount of soluble HS were detected with increasing concentrations of MAI-12. Additionally, aliquots of the basal supernatants were separated on SDS-PAGE, and the gels were silver stained to confirm that the increase in HS observed was not the result of an increase in the total protein content of the supernatant (data not shown).

Most (~95%) of the HS on endothelial cells is present in the syndecan PG (13). We therefore investigated the presence of the syndecan-1 core protein in the endothelial culture supernatants. The same supernatants were analyzed in this experiment as those stained for HS (Fig. 4). Western blots stained using a mAb to syndecan-1 showed a single major band with an apparent molecular mass of ~140 kDa, and the intensity of staining increased with increasing concentrations of MAI-12. Additionally, aliquots of the basal supernatants were separated on SDS-PAGE, and the gels were silver stained to confirm that the increase in HS observed was not the result of an increase in the total protein content of the supernatant (data not shown).

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**IL-8 is associated with syndecan-1**

To determine whether IL-8 was associated with the ectodomain of syndecan-1, a polyclonal Ab to IL-8 was used to immunoprecipitate all forms of IL-8 present in the culture supernatants. Analysis (Fig. 6) of the immunoprecipitate for the presence of HS (top) and syndecan-1 (bottom) indicated that IL-8 in culture supernatants was present in a complex with both HS and syndecan-1. The estimated molecular mass of 140 kDa for both of these forms of IL-8 indicated that IL-8 was present as a complex with the HS-containing syndecan-1 ectodomains. This was confirmed by immunoprecipitation of syndecan-1 from culture supernatants with Western blot analysis to confirm the presence of both HS and IL-8 in the immunoprecipitate (data not shown). Immunoprecipitates prepared from culture supernatants using the irrelevant P2×7 receptor polyclonal Ab control contained no detectable IL-8 or HS (not shown).

The association of IL-8 with the syndecan ectodomain was resistant to high concentrations of DTT present in the buffer used to recover immunoprecipitated proteins, suggesting that disulfide

**FIGURE 5.** Syndecan-1 is associated with the shed HS. Supernatants were analyzed by Western blotting for the presence of the HSPG core protein, syndecan-1. Lanes are labeled as in Fig. 4. The pattern of syndecan-1 staining was identical with that of HS (Fig. 4). Syndecan-1 was detected at an apparent molecular mass of 140 kDa, and release was low in the absence of MAI-12 (lanes 1 and 2) and was dose dependently increased in the presence of this Ab. Positions of m.w. markers are shown on the left of the figure. The results are representative of four independent experiments. B. Shows the quantitative analysis of staining intensity; data are represented as mean ± SEM \((n = 4)\). Syndecan-1 release following neutrophil transmigration in the absence (filled bars) and presence (open bars) of platelets is shown. Soluble syndecan-1 increased significantly with increasing MAI-12 compared with control \((p < 0.001)\).

**FIGURE 6.** IL-8 is associated with the HS-syndecan-1 ectodomain. Using a polyclonal Ab, IL-8 was immunoprecipitated from supernatants following neutrophil transendothelial migration, in the presence and absence of platelets. The immunoprecipitate was analyzed by Western blotting for HS (A) and syndecan-1 (B), with lanes labeled as in Fig. 4. HS and syndecan-1 were detected as a major band at ~140 kDa for both, indicating that IL-8 forms part of the shed PG complex. In addition, the intensity of HS and syndecan-1 associated with IL-8 increased dose dependently with increasing concentration of MAI-12.
bonds were not responsible for the association. In addition, the increased intensity of staining for soluble HS and syndecan-1 in the presence of increasing concentrations of MAI-12 was apparent in the IL-8 immunoprecipitates, indicating that shedding of this form of IL-8 was regulated by PAI-1. This was confirmed by staining for IL-8 in the immunoprecipitates, which showed that increasing amounts of IL-8 were released in the presence of MAI-12 (results not shown).

When unfracionated culture supernatants were analyzed for IL-8, some samples contained small amounts of the free 8-kDa form, but all samples contained the 140-kDa form, and the latter was the most abundant (results not shown). These results indicate that IL-8 was released from endothelial cells bound to the HS side chains of syndecan-1.

**PAI-1 activity is required for neutrophil transendothelial migration**

The shedding of soluble complexes of IL-8 with syndecan ectodomains was predicted to inhibit IL-8-induced transendothelial migration; therefore, cell migration was quantified in the absence and presence of the PAI-1-neutralizing Ab (Fig. 7). Under control conditions, in the absence of the PAI-1-neutralizing Ab, neutrophils (45,075 ± 18,107) migrated through the endothelial cell layer over 3 h. We considered the possibility that platelet PAI-1 may prevent shedding of IL-8/HS/syndecan-1 and promote cell migration. However, in line with the results indicating that platelets did not prevent shedding of IL-8/HS/syndecan-1, neither did platelets promote cell migration (Fig. 7). The average number of neutrophils harvested from the lower compartment was not altered (43,413 ± 18,372) in the presence of platelets. However, incubation with the PAI-1 inhibitor, MAI-12, significantly decreased the number of transmigrating neutrophils in a dose-dependent manner (Fig. 7).

With the addition of MAI-12 at a concentration of 1 μg/ml, the number of neutrophils recovered from the basal compartment of the Transwells was only 50% of control values (p < 0.05). An increase in the concentration of MAI-12 to 50 μg/ml produced a further, significant decrease in neutrophil transmigration, to just 25% of control levels (p < 0.01).

**PAI-1 prevents PG shedding and maintains the chemoattractant gradient**

We hypothesized that inhibition of PAI-1 activity in the presence of MAI-12 increased tPA activity, and that the associated plasmin activity increased IL-8/PG shedding and reduced neutrophil transendothelial migration. Therefore, in the transmigration assays, we used a second Ab, mc 3785, which did not inhibit PAI-1 activity (Fig. 2). Analysis of supernatants from 3-h neutrophil-endothelial cell cocultures in the presence of mc 3785 (Fig. 8) supports this theory. To visualize the soluble PG, Western blots had to undergo prolonged exposure, as levels were low. Fig. 8A shows that the main immunopositive band had an apparent molecular mass of ~140 kDa for both HSPG and syndecan-1, indicating that the form of PG released was the same as that observed previously (Figs. 3–5). Quantitative analysis of these data (Fig. 8B) revealed that increasing concentrations of mc 3785 did not alter the levels of

**FIGURE 7.** PAI-1 activity is permissive for neutrophil transendothelial migration. The number of neutrophils in the basal compartment of endothelial cell cultures, in the absence (filled bars) and presence (open bars) of platelets, was assessed after 3-h culture in the presence of increasing concentrations of the PAI-1 inhibitory Ab, MAI-12. The data (mean ± SEM, n = 4) are expressed as a percentage of control, in which control was the number of neutrophils migrating in the absence of MAI-12 and was given the value of 100%. MAI-12 significantly and dose dependently reduced the number of neutrophils migrating through the endothelial cell layer compared with control (+, p < 0.005), and this effect was apparent both in the absence and presence of platelets (compare filled and open bars, respectively).

**FIGURE 8.** PAI-1 activity inhibits HSPG shedding and supports neutrophil transendothelial migration. In the presence of increasing concentrations of a nonneutralizing Ab to PAI-1, mc 3785, analysis of culture supernatants by Western blot showed no increase in HS (A, left panel) or syndecan-1 (A, right panel). Constitutive release of HSPG and syndecan-1 was low, and these blots had to be overexposed to visualize the proteins and allow the semiquantitative analysis shown in B. HS and syndecan were both detected with apparent molecular mass of 140 kDa, in agreement with the data shown in Figs. 4 and 5. The concentration of mc 3785 is indicated above the lane, and positions of m.w. markers are shown on the left of the image. The results are representative of three independent experiments. B, Quantitative analysis of these data confirmed that there was no change in the level of soluble HS (open bars) or syndecan-1 (shaded bars) with increasing concentrations of mc 3785. C, Indicates that, when transendothelial migration was assayed in the presence of uninhibited PAI-1 activity, there was no effect on the percentage of neutrophils migrating through the endothelial cell layer. Data are represented as mean ± SEM, n = 3.
transmigration. Data are represented as mean ± SEM. To date, the evidence that IL-8 binds to endothelial cell HSPG with effects on target cell function has been indirect only. Studies in vivo have demonstrated a site for IL-8 binding on the lumenal surface of endothelial cells in postcapillary venules, although its identity was not elucidated (3, 10). Hoogewerf et al. (8) demonstrated binding and oligomerization of IL-8 on HUVEC monolayers, and the dependency of binding on the presence of intact GAGs was demonstrated by digestion of GAGs with a mixture of glycosidases that reduced IL-8 binding by 50%. These authors also demonstrated that cell surface GAGs increased the affinity of specific chemokine binding to cells transfected with seven transmembrane-spanning domain chemokine receptors (8). Subsequently, it was shown that the binding of IL-8 to HUVECs was competed with GAGs in the order of efficacy, heparin > HS > dermatan sulfate > chondroitin sulfate, indicating the relative affinity of IL-8 for heparin and HS (9). Functionally, soluble GAGs had an inhibitory effect on target cell responses, heparin and HS inhibiting IL-8-induced neutrophil calcium fluxes (9), in line with our previous observation that heparin inhibits IL-8-induced neutrophil chemotaxis (21).

Immunohistochemical analysis revealed an abundance of HS on the surface of HUVECs in culture (data not shown), yet most (98%) of the IL-8 in unstimulated HUVEC cultures was not associated with the cell surface, even in short, 5-min incubations. We considered the possibility that endogenous proteases of the plasminogen activator/plasmin cascade were responsible for shedding IL-8 binding site(s) from the endothelial cell surface. This was confirmed by the ability of PAl-1 and aprotinin to induce a change in the distribution of IL-8 in these short-term cultures, so that significantly more IL-8 was associated with the cells. This effect of exogenous PAI-1 was lost in cultures longer than 1 h (data not shown). Because PAI-1 is a suicide inhibitor, reacting only once with the target protease, this may indicate the extent of constitutive tPA activity and turnover of the putative IL-8 binding sites. The anti-inflammatory effects of soluble IL-8, such as down-regulation of functional IL-8Rs on neutrophils (22) and inhibition of neutrophil adhesion, as described by Gimbrone et al. (4), contrast with the proinflammatory effects of cell surface-bound IL-8, which appears to be an important form of the active neutrophil chemottractant (2, 5). Thus, mechanisms that change the relative distribution of IL-8 between cell-bound and soluble forms are likely to have profound effects on the net response of neutrophils to this chemokine.

To investigate the functional effects of changing the distribution of IL-8 between cell-bound and soluble phases, we studied the transendothelial migration of neutrophils in response to a gradient of IL-8 that was generated by adding IL-8 to the lower well of the Transwells. Under these conditions, Western blot analysis showed that 8-kDa IL-8 rapidly disappeared from the culture medium (data not shown), presumably by binding to the endothelial cells and forming the haptotactic gradient that is essential for successful transendothelial migration (2, 5). A number of studies have described the induction of IL-8-dependent neutrophil transendothelial migration by TNF-α and IL-1α (23, 24). However, TNF-α and IL-1 were not used in these studies because they also induce the synthesis of PAI-1 by endothelial cells (25), which, as discussed later, may contribute to their efficacy as stimulators of neutrophil migration. In the model we chose to use, it was shown that the endothelium is not activated by IL-8, and the presence of endothelial cells facilitates IL-8-induced neutrophil migration compared with bare filters (26). However, the endothelial factor(s) involved in

![FIGURE 9.](https://www.jimmunol.org/)

**FIGURE 9.** Aprotinin restores MAI-12-inhibited neutrophil transendothelial migration. A, In the presence of 50 µg/ml MAI-12, the concentration of Ab shown to inhibit neutrophil migration by 75% of control (Fig. 7), aprotinin (25 and 50 µM) significantly (p < 0.05) increased MAI-12-inhibited neutrophil transmigration, restoring the level to 105 ± 8.8% of control at the highest concentration. B, Aprotinin, alone, at 50 µM significantly increased neutrophil transmigration. Data are represented as mean ± SEM, n = 3.
the facilitation of IL-8-stimulated transcellular migration was not elucidated. Our results shed light on this phenomenon, indicating an important role for endothelial HSPG, including syndecan-1, in supporting IL-8-stimulated neutrophil transcellular migration.

The Abs to PAI-1 used in this study both recognize PAI-1 on Western blot (data from suppliers), and we confirmed that the MAI-12, but not the mc 3785, Ab neutralizes PAI-1 activity. Using these tools, we were able to demonstrate that inhibition of endogenous endothelial PAI-1 activity resulted in an increase in the shedding of HS, suggesting a role for plasminogen activation in the shedding of HS. PAI-1 accounts for ~12.5% of protein released by endothelial cells in culture (27) and is an inhibitor of both tPA and urokinase-type plasminogen activator (uPA) (27, 28). However, our immunohistochemical analysis (data not shown) has demonstrated that endothelial cells are a source of tPA, and not uPA, confirming previous reports that expression of uPA is an artifact of cell culture (28).

Platelets are a rich source of PAI-1 that is indistinguishable from endothelial PAI-1, concentrating it to levels 4 times higher than plasma (29). Significant interactions between platelets and endothelial cells in the mediation of active PAI-1 release have been reported (30). However, in the absence and presence of the neutralizing PAI-1 Ab, the presence of platelets had no effect on the extent of HS shedding. Endothelial cells have a high capacity for binding plasminogen compared with other cells, 567 times higher than platelets and 131 times higher than granulocytes (31), indicating that in our cocultures the site of plasmin formation is the endothelial cell surface. We anticipated that even in the presence of the PAI-1 Ab, release of PAI-1 from platelets adherent to endothelial cells might inhibit local plasminogen activation (31). The absence of an effect of platelets indicates either that under our culture conditions platelet-PAI-1 was released mainly in latent form, previously shown for the response of platelets to various activators (30), or that on the endothelial cell surface tPA is protected from the effects of inhibitors (31). However, the ability of exogenously added mutant PAI-1, which only slowly reverts to latent, to prevent solubilization of IL-8 would support the former explanation. Thus, it appears that the PAI-1-neutralizing Ab used in our experiments inhibits endothelial PAI-1, which is localized on the endothelial cell surface in active form (32, 33).

Our evidence indicates that IL-8 was shed from endothelial cells in culture as a complex with HS and syndecan-1. IL-8 binding to HS has been reported to occur through an interaction of IL-8 monomers with N-sulfated domains in HS chains (34), suggesting that IL-8 in endothelial culture supernatants was bound to the HS chains of the syndecan-1 ectodomain. This ectodomain was solubilized, in the presence of platelets and neutrophils, to an extent that was dependent on inhibition of endogenous endothelial PAI-1. Importantly, increased shedding of IL-8/H5/syndecan-1 was accompanied by a significant decrease in the number of neutrophils migrating across the endothelium. Our interpretation of these findings is that plasminogen activation resulted in cleavage of a chemotactrant form of IL-8 from the cell surface, and that this form of IL-8 is a complex with the syndecan-1 HS. The ability of the potent plasmin inhibitor, aprotinin, to dose dependently reverse the effects of the PAI-1-neutralizing Ab indicates that increased plasmin activity mediated shedding of IL-8/H5/syndecan-1 and inhibition of cell migration. The ability of aprotinin alone to increase neutrophil migration further suggests a role for plasmin activity in the regulation of chemotactrant function on the endothelial cell surface.

Stimulated neutrophils release a number of neutral proteases, including gelatinase, collagenase, elastase, cathepsin G, and uPA. Most reports on the role of proteases in neutrophil transendothelial migration have focused on the role of neutrophil-derived enzymes. An early report indicated that uPA and plasmin were not required for transendothelial migration of neutrophils (35). Subsequently, a role for gelatinase and elastase in neutrophil migration across basement membrane components was suggested (36), although later studies showed that these enzymes were not involved in the migration of neutrophils across an endothelial barrier (37). Evidence that neutrophils can migrate across the endothelium and endothelial basement membrane without the involvement of activated MMPs (37–39) also precludes a role for plasmin as an activator of MMPs in this process. Thus, it appears that neutrophil-derived proteases may not be involved in successful transendothelial migration. However, our evidence indicates that proteases of the plasminogen activator/plasmin cascade play an important role in the regulation of this process, and that activation of these proteases on the endothelial cell surface inhibits transmigration. Proliferative cytokines such as IL-1β and TNF-α not only stimulate the expression of endothelial IL-8, but also stimulate expression of PAI-1, with concomitant inhibition of tPA (26). These observations lead us to suggest that at sites of inflammation, IL-1β and TNF-α generate transendothelial gradients of IL-8 that are stabilized in the presence of PAI-1 to facilitate neutrophil recruitment.

Our evidence supports a proinflammatory role for endogenous PAI-1 at the level of IL-8-induced recruitment of neutrophils, and possibly other leukocytes (40), across the endothelium. Conversely, a recent study suggested that, in the absence of an endothelial cell layer, plasmin mediates cosinophil migration across basement membrane components (41). However, PAI-1 is a multifunctional protein, and a number of effects of PAI-1 are independent of its function as an inhibitor of plasminogen activation. Cell migration is a complex process requiring a delicate balance between adhesion and detachment, and previous reports (reviewed in Refs. 42 and 43) describe the ability of PAI-1 to inhibit the migration of smooth muscle cells, and amnion and epidermoid carcinoma cell lines in vitro. PAI-1 impairs the interaction between the vitronectin receptors αvβ3 and α5β1 and vitronectin, and the interaction between uPAR (the uPA receptor) and vitronectin, resulting in inhibition or activation of cell migration on or toward vitronectin in vitro, depending on the context. However, in vivo, high levels of PAI-1 are prognostic of both breast and renal tumor progression (43), and, in mice, absence of host PAI-1 prevents cancer invasion and vascularization (44).

The physiological role of the shed ectodomains in the regulation of neutrophil-directed IL-8 function is unknown. The evidence from Kuschert et al. (9), demonstrating that soluble GAG-chemokine complexes are unable to bind the specific receptor, resulting in a block of the biological activity, indicates that the same may be true for the IL-8/HS/syndecan-1 complex. Thus, a soluble form of IL-8 may be present in the circulation that does not down-regulate neutrophil IL-8Rs nor inhibit neutrophil adhesion, anti-inflammatory effects previously reported for soluble 8-kDa IL-8 (4, 23). However, processing of the soluble HSPG ectodomains by GAG-degrading heparanases at sites of inflammation may further change the function of this form of IL-8, as previously shown for the functional effects of the syndecan-1 ectodomain on fibroblast growth factor-2 (45). Proteolysis in the pericellular environment regulates many cellular processes, and a number of substrates for plasmid have been identified (46). Cell surface substrates for plasmid include fibronectin, fibrin/fibrinogen, tenascin, laminin, aggrecan, and other PG (reviewed in Ref. 46 list).

The proinflammatory role for PAI-1 in maintaining stable chemotactrant gradients of a chemokine indicates that inhibitors of PAI-1 may prove to be useful anti-inflammatory agents. Extensive inflammation is often linked with the development of fibrosis, for example, in bleomycin-induced pulmonary fibrosis in mice (47). In
this model, overexpression of PAI-1 did not prevent accumulation of leukocytes, as our data would predict. Furthermore, it was suggested that inhibitors of PAI-1 or administration of plasminogen activators would limit the development of fibrosis by enhancing fibrinolysis. Our data suggest that PAI-1 inhibition may prove to be a useful therapeutic strategy for attenuation of both inflammatory and profibrotic processes. This may be especially important in the treatment of asthma, an inflammatory disease characterized by subepithelial fibrosis, that has recently been associated with polymorphisms in the PAI-1 gene (48, 49). In vivo studies using the low m.w. PAI-1 inhibitor XRS118 as a thrombolytic agent showed no effect on bleeding time and, in vitro, no effect on blood coagulation tests (50). It remains to be seen whether systemic inhibition by PAI-1 inhibitors would have adverse effects in humans.

References

CORRECTIONS

To draw attention to additional relevant research by Holtappels et al., the authors wish to add the following as the fourth sentence in the third paragraph of the Discussion.

Enrichment of pp89- and m164-specific but not m3-, m04-, or M84-specific CD62Llo effector memory CD8+ T cells in the latent phase of MCMV infection has been recently observed by Holtappels et al. using a model of MCMV-induced interstitial pneumonia after immunosuppression by irradiation and syngeneic bone marrow transplantation, and a role for such cells in control of viral latency proposed (12, 54).

The authors also wish to add the following reference.


The fourth author’s degree was listed in error in the author line. The correct listing of his name is Peter Charlton.


Due to typographical errors, the first sentence of the legend for Figure 9 appeared incorrectly. The correct sentence is shown below.

GM-CSF induces types I and III CIITA, but not type IV, transcripts in monocytes.