Effect of Recombinant Soluble P-Selectin Glycoprotein Ligand-1 on Leukocyte-Endothelium Interaction In Vivo
Role in Rat Traumatic Shock

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Abstract—Traumatic shock induces profound pathophysiological alterations and initiates inflammatory reactions in many tissues, thus resulting in acute multiple organ damage (eg, intestine, pancreas, and liver). In the rat, Noble-Collip drum trauma increases P-selectin expression on the vascular endothelium as a result of loss of endothelium-derived NO. Here we postulated that blockade of the earliest steps in leukocyte adhesion (ie, leukocyte rolling) via administration of a recombinant soluble form of P-selectin glycoprotein ligand-1 (PSGL-1; the recombinant soluble form is rsPSGL.Ig) would attenuate selectin-mediated events observed in the rat during traumatic shock. Using intravital microscopy of the rat mesenteric microvasculature, we found that intravenous infusion of rsPSGL.Ig significantly inhibited leukocyte-endothelium interaction (ie, leukocyte rolling, adherence, and transmigration) induced by traumatic shock as well as by activation of the microvascular endothelium with 50 μmol/L N\textsuperscript{G}-nitro-L-arginine methyl ester. Immunohistochemical detection of P-selectin on the mesenteric venular endothelial surface demonstrated that rsPSGL.Ig functionally neutralizes effects of P-selectin on the endothelial cell surface rather than attenuating P-selectin expression. Systemic administration of rsPSGL.Ig to traumatized rats prolonged survival time and survival rate, significantly attenuating ileal myeloperoxidase activity and significantly preserving mesenteric endothelial function. Furthermore, PSGL-1 mRNA levels were significantly increased in the blood of traumatized rats and were reduced after systemic administration of rsPSGL.Ig. Thus, soluble recombinant forms of PSGL-1 are able to ameliorate acute shock states by suppressing selectin-mediated leukocyte-endothelium interaction at both the functional and molecular levels. (Circ Res. 1999;84:93-102.)

Key Words: endothelial dysfunction ■ myeloperoxidase activity ■ mRNA ■ P-selectin ■ intravital microscopy

The recruitment of leukocytes into inflamed tissues plays a pivotal role in the pathogenesis of several organ injuries, including posttraumatic syndromes. With the use of biochemical techniques, we have previously demonstrated massive infiltration of leukocytes into the visceral tissues of rats subjected to Noble-Collip drum shock, a severe form of traumatic shock characterized by marked hypotension and a high mortality rate.1–3 Consistent with the concept that leukocytes initially interact with the vascular endothelium before activation,4 we have also demonstrated that this posttraumatic inflammatory response is triggered by early endothelial dysfunction.5 Under these conditions, loss of endothelium-derived NO occurs within 15 minutes following traumatic shock and is associated with increased endothelial expression of the cell adhesion molecule P-selectin.6 P-selectin is able to initiate the cascade of events that increases cell adherence and leukocyte infiltration into injured tissues by first promoting leukocyte rolling along the vascular endothelium.7 This contributes to leukocyte-mediated alterations leading to circulatory shock. Therefore, it appears that successful blockade of the earliest steps in leukocyte adhesion (ie, rolling) is effective in diminishing traumatic injury.

In 1992, Moore et al8 first described a new selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1), which is located on the microvilli of leukocytes, where it is positioned to interact favorably with its counterligands under flow conditions.9–11 Since then, other investigators have cloned soluble forms of PSGL-112 and have demonstrated that in vivo administration of these forms of soluble PSGL-1 results in amelioration of ischemia/reperfusion injury.13 Nonetheless, we are unaware of any report describing effects of in vivo administration of soluble recombinant forms of PSGL-1 (rsPSGL.Ig) on leukocyte-endothelium interaction under physiological and pathophysiological conditions in vivo. Furthermore, rsPSGL.Ig has not been studied in more complex cardiovascular disease states, such as shock or trauma, in which multiple organ dysfunction occurs.

Therefore, we hypothesized that administration of rsPSGL.Ig would attenuate the selectin-mediated endothelial...
dysfunction, tissue injury, and hemodynamic alterations typically associated with a severe form of traumatic shock. Thus, the purposes of this study were to investigate the cellular and molecular mechanisms of leukocyte-endothelium interactions in the rat mesentery following administration of rsPSGL-Ig and to determine the overall effects of rsPSGL-Ig in a well-established model of murine traumatic shock.

Materials and Methods
This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals, and all animal protocols have been approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

cDNA Construction of rsPSGL-Ig
rsPSGL-47 mutFc (Genetics Institute, Inc) is derived from pED0.47.Fc, a recombinant soluble form of PSGL-1 fused to human IgG1. Polymerase chain reaction (PCR) was performed on the Fc portion of this plasmid using the 5′ primer TAAATAAGCCGGCC-CACACATGCCCACGTCATCTTCCTC and the 3′ primer GCATGTGCAC-GAGGCCGACGATCA. The PCR product was digested with the restriction enzymes Not I and Kpn I and ligated to the large fragment of pED0.47.Fc restricted with the same digest. The resulting vector, pED0.47 mutFc, was confirmed by DNA sequencing and then stably transfected and amplified in a dihydrofolate reductase-negative Chinese hamster ovary cell line that was previously stably transfected with vector pMT4neo expressing both a cDNA encoding an α(1,3,4)-fucosyltransferase and a cDNA encoding core 2,1,6-N-acetylgalacaminyltransferase. Analysis of several resulting clonal cell lines revealed a cell line having readily detectable core 2, fucosyltransferase activities, and SLε-modified 47mutFc ("high-affinity rsPSGL") and another cell line lacking detectable fucosyltransferase activity and SLε-modified glycans on 47mutFc ("low-affinity rsPSGL"). Each type of these secreted rsPSGL-47mutFc molecules was separately purified from serum-free Chinese hamster ovary cell—conditioned medium essentially as described. The final purified material was formulated into 10 mmol/L histidine, 1% (wt/vol) sucrose, 260 mmol/L glycine, and 0.005% Tween 80, pH 6.6 at room temperature at a concentration of at least 3 mg/mL. Endotoxin was measured to be <10 EU/mg. Selectin binding activity was assessed via in vitro binding assays as described.

Demonstration of rsPSGL-Ig Binding to Rat Blood Cells by Flow Cytometry
Flow cytometric analysis of rsPSGL-Ig binding to freshly isolated rat platelets and neutrophils was performed according to standard procedures. Platelet-rich plasma was obtained by centrifuging rat blood, anticoagulated with sodium citrate phosphate buffer (Sigma Chemical Co), at 300g for 20 minutes. The platelet-rich plasma was then centrifuged at 2000g for 10 minutes to form a platelet-rich pellet. This pellet was washed twice in calcium-free Tyrode’s solution containing 0.2% BSA. The final cell pellet was resuspended in RPMI 1630 (Gibco) containing 0.1% sodium azide, 0.1% BSA, and 1 mmol/L CaCl2. Similarly, rat neutrophils were freshly isolated from whole blood according to the method of Williams et al. Isolated neutrophils were washed twice in calcium-free Tyrode’s solution containing 0.2% BSA and suspended in RPMI 1630. Platelet or neutrophil (5×106 cells per tube) were incubated with either rsPSGL-Ig (20 μg/mL) or a low-activity mutant rsPSGL-Ig (20 μg/mL) and maintained at 4°C for 30 minutes. Excess of primary antibody was then removed by washing the platelets or neutrophils in RPMI 1630. A goat anti-human IgG F(ab’)2, FITC conjugate, was used as the secondary antibody at a 1:100 dilution (4°C for 30 minutes). The stained platelets or neutrophils were washed twice with RPMI 1630, finally fixed in 1% paraformaldehyde, and then analyzed by flow cytometry (FACScan, Becton-Dickinson).

Intravital Microscopy
Male Sprague-Dawley rats, weighing 250 to 275 g, were anesthetized with sodium pentobarbital (60 mg/kg) injected intraperitoneally. A tracheotomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in the left carotid artery to monitor mean arterial blood pressure (MAP) as previously described. The jugular venous was cannulated for infusion of anesthetic and injection of either low-affinity mutant rsPSGL-Ig or high-affinity mutant rsPSGL-Ig. The abdominal cavity was opened via a midline laparotomy, as described earlier. A loop of ileal mesentery was exteriorized through the midline incision, placed in a temperature-controlled fluid-filled Plexiglas chamber, and transilluminated for observation of the mesenteric microcirculation via intravital microscopy. The ileum and mesentery were superfused throughout the experiment with a modified Krebs-Henseleit (K-H) solution (containing the following in mmol/L: 118 NaCl, 4.74 KCl, 2.45 CaCl2, 1.19 KH2PO4, 1.19 MgSO4, and 12.5 NaHCO3), warmed to 37°C, and bubbled with 95% N2 and 5% CO2. A Microphot microscope and a 40× water immersion lens (Nikon Corp, Tokyo, Japan) were used to visualize the mesenteric microcirculation and the mesenteric tissue. The image was projected by a high-resolution color video camera (DC-330, DAGE-MTI Inc) onto a color Sony high-resolution video monitor (Multiscan 200-sf), and the image was recorded with a videocassette recorder. All images were then analyzed using computerized imaging software (Phase 3 Image System, Media Cybernetics) on a Pentium-based IBM-compatible computer (Micron Millenia Mxe, Micron Electronics Inc). Blood red cell velocity was determined on-line using an optical Doppler velocimeter obtained from the Microcirculation Research Institute (College Station, Tex). This method gives an average red blood cell velocity, which is digitally displayed on a meter, and allows for the calculation of shear rates.

After a 20- to 30-minute stabilization period, a 30 to 50-μm diameter postcapillary venule was chosen for observation. Rats were randomly divided into 1 of the following 5 groups: (1) control rats superfused with K-H solution (n=6), (2) K-H solution—superfused rats receiving 0.5 mg/kg IV bolus rsPSGL-Ig (n=6), (3) 30 μmol/L Nω-nitro-L-arginine methyl ester (L-NAME)—superfused rats, (4) 50 μmol/L L-NAME—superfused rats receiving 0.5 mg/kg IV bolus low-affinity mutant rsPSGL-Ig (n=6), and (5) 50 μmol/L L-NAME—superfused rats receiving 0.5 mg/kg IV bolus high-affinity mutant rsPSGL-Ig (n=6). To ascertain whether the degree of leukocyte-endothelium interaction induced by L-NAME was comparable to that produced by traumatic shock, an additional series of intravital microscopy observations was extended to traumatized control rats (n=5) and traumatized rats receiving rsPSGL-Ig (n=5). Both the low-affinity mutant rsPSGL-Ig and the high-affinity mutant rsPSGL-Ig were administered to the rats as an intravenous bolus immediately before recording leukocyte-endothelium interaction. Intravital microscopy recording was made to establish the basal values for leukocyte rolling, adherence, and transmigration (time 0). Immediately thereafter, L-NAME superfusion of the mesentery was started. Video recordings were made at 30, 60, 90, and 120 minutes after initiation of superfusion for quantification of leukocyte rolling, adherence, and transmigration. The number of rolling, adhered, and transmigrated leukocytes was determined off-line by playback analysis of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity significantly slower than that of red blood cells. Leukocyte rolling is expressed as the number of cells moving past a designated point per minute (ie, leukocyte flux). A leukocyte was judged to be adherent if it remained stationary for >30 s. Adherence is expressed as the number of leukocytes adhering to the endothelium per 100 μm of vessel length. Transmigrated leukocytes were determined in an area covering a distance of 20 μm in either direction from the vessel wall. The number of extravasated leukocytes was counted and normalized with respect to area (20×100 μm).

Immunohistochemistry
Immunohistochemical localization of P-selectin was determined in ileal samples after intravital microscopy was completed according to previously described methods.
Quantification of P-selectin was accomplished using the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent, Vector Laboratories) as previously described by Weyrich et al. Fifty venules were analyzed per tissue section, 20 sections were examined per group, and the percentage of positive-staining venules was tallied.

**Traumatic Shock Protocol**

Following anesthesia with 60 mg/kg IP sodium pentobarbital, male Sprague-Dawley rats (175 to 225 g body weight) were subjected to traumatic shock using a Noble-Collip drum apparatus.1 Traumatized rats were subjected to a total of 500 revolutions at 60 rpm. Immediately following the induction of trauma, the trachea was cannulated with PE-240 polyethylene tubing to maintain a patent airway. Polyethylene catheters (PE-50) filled with heparinized 0.9% NaCl solution were inserted into the right common carotid artery for recording MABP and into the left external jugular vein for administration of either rsPSGL.Ig or low-affinity mutant rsPSGL.Ig. The total time required to complete all surgical procedures was <10 minutes. The MABP was continuously recorded and tabulated every 30 minutes over the entire 5-hour observation period using a Grass model 7 oscillographic recorder (Grass Instruments) and Statham P23 pressure transducers. Rats were randomly assigned to 1 of 5 experimental groups: (1) sham-traumatized rats receiving K-H solution (n = 5), (2) sham-traumatized rats receiving 0.5 mg/kg IV bolus rsPSGL.Ig (n = 6), (3) traumatized rats receiving K-H solution (n = 10), (4) traumatized rats receiving 0.5 mg/kg IV bolus low-affinity mutant rsPSGL.Ig (n = 6), and (5) traumatized rats receiving 0.5 mg/kg IV bolus rsPSGL.Ig (n = 6).

Low-affinity mutant rsPSGL.Ig or rsPSGL.Ig were administered 10 minutes after trauma as a 0.25-mL IV bolus. Sham-traumatized rats were anesthetized and subjected to all of the same surgical procedures as the traumatized rats, except that they did not undergo drum trauma. Additional pentobarbital was given intraperitoneally throughout the observation period to maintain a surgical plane of anesthesia.

Five hours after trauma, or when MABP fell below 45 mm Hg, the experiments were terminated. Samples of rat small intestine were also obtained for measurement of myeloperoxidase (MPO) activity. Survival time was defined as that interval between removal from the drum to the end of the experiment (ie, MABP < 45 mm Hg). All rats were autopsyed to confirm the presence of gross evidence of traumatic injury to the splanchnic viscera (ie, bowel ischemia, serosanguinous ascites, and splanchic vascular engorgement). Rats were excluded from the study if these findings were not observed to a significant degree or if the rat died sooner than 30 minutes after trauma. Fewer than 10% of rats (ie, 1 of 13 rats) did not meet these criteria.

**Determination of Tissue MPO**

Small intestinal activity of MPO, an enzyme occurring virtually exclusively in polymorphonuclear leukocytes (PMN), was determined using the method of Bradley et al24 as modified by Mullane et al.24 One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per minute at 25°C.

**Isolated Mesenteric Artery Ring Studies**

At the end of the experiment, the superior mesenteric artery (SMA) was rapidly removed from rats and placed in chambers containing K-H solution, and vasorelaxation responses were studied to an endothelium-dependent and endothelium-independent vasodilator according to previously described studies.6 Once a stable contraction to 100 mmol/L 9,11-epoxyethanoprostaglandin H₂ was obtained, acetylcholine (ACh), an endothelium-dependent vasodilator, was added to the bath in cumulative concentrations of 0.1, 1, 10, and 100 mmol/L. After the cumulative response stabilized, the rings were washed and allowed to equilibrate to baseline once more. The procedure was repeated with an endothelium-independent vasodilator, nitric oxide (NO), and for the control samples. Similar rat neutrophils stained 67 ± 8% positive for rsPSGL.Ig in comparison with only 7 ± 2% positive

**pH 2.0. Equal volumes of pH 2.0 solution had no vasoactive effect on rat SMA rings. We defined 100% relaxation as the return to precontraction force following U-46619–induced contraction.**

**Determination of PSGL-1 Gene Expression by Ribonuclease Protection Assays**

Total RNA was extracted from the lungs using the acid guanidinium-phenol-chloroform extraction method described by Chomczynski and Sacchi and previously modified for tissue in traumatic shock rats.26

Mouse PSGL-1 cDNA was synthesized by reverse transcriptase–PCR using mouse lung total RNA and oligo(dT) and amplified using forward primer (5′-CCTGGGAATTCACCTGCCCC-3′) and reverse primer (5′-GAAGTGAGGACGTACCAAAGG-3′). These oligonucleotides correspond to amino acid sequences 267 to 273 and 394 to 388 of mouse PSGL-1, respectively.27 A 384 bp PCR fragment was cloned using the PCR 2.1-TOPO Cloning Kit (a gift from Invitrogen Corp). The XbaI–XbaI fragment of this plasmid was recloned in pTRIExpress vector (pTRI-amp-18, Ambion). This plasmid was digested with XbaI to make with T7 polymerase a 570-base radiolabeled antisense probe containing a 384-base-protected fragment. CD45 probes were used to evaluate total RNA used for PSGL-1 gene expression analysis. Rat leukocyte-common antigen CD45 cDNA was synthesized by reverse transcriptase–PCR using rat blood RNA and oligo(dT) and amplified using forward primer (5′-ctagacTCTTTGTCCAGGCAAGGCT-3′) and reverse primer (5′-cattcTAGATGGAACCGACGAGTAC-3′). An EcoRI–EcoRV fragment of CD45 cDNA (GenBank accession No. M25823) was cloned in pBluescript II KS+ (Strategene). These constructs were verified by sequencing the insert in the plasmid. For the ribonuclease protection assay, we used 20 and 40 µg of total RNA for detection of CD45 and PSGL-1 mRNA, respectively. Water solutions of total RNA were dried under vacuum and dissolved in 25 µL of 80% formamide hybridization buffer containing 1.5×10⁶ cpm of labeled PSGL-1 probe and 1×10⁶ cpm of labeled CD45 probe. Samples were preincubated for 5 minutes at 85°C and then incubated for 16 hours at 45°C as described previously.26 The extracted, protected probe fragments were run on a 6% polyacrylamide sequencing gel in 1× Tris-borate-EDTA buffer for 2 hours at 50 mA. The gel was then dried and exposed to x-ray film (Hyperfilm MP, Amersham) at –70°C. Expression of mRNA was quantified using storage phosphor technology (Molecular Dynamics). Intensity of each PSGL-1 mRNA band was normalized for CD45 mRNA level.

**Statistical Analyses**

All values for data listed in the text and figures are presented as mean±SEM of n independent experiments. Data were compared by ANOVA using post hoc analysis with Fisher’s correct t test. Survival times were compared using Gehan’s generalized Wilcoxon test as described by Knapp and Wise.28 The survival rates were assessed by χ² analysis. Probabilities of 0.05 or less were considered significant in all cases.

**Results**

**Verification of rsPSGL.Ig Binding to Rat Platelets and Rat Neutrophils**

To confirm that rsPSGL.Ig was able to bind to its high-affinity ligand in the rat, we performed flow cytometry on freshly isolated rat platelets and neutrophils. Rat platelets stained 82 ± 12% positive for rsPSGL.Ig in comparison with only 15 ± 3% positive staining for the low-affinity mutant (P < 0.01) and 7 ± 1.8% for the control lacking the primary antibody. Additionally, the mean channel fluorescence was 99 ± 22 and 28 ± 7 for rsPSGL.Ig and the rsPSGL.Ig low-affinity mutant, respectively, and only 5 ± 0.5 for the control samples. Similarly, rat neutrophils stained 67 ± 8% positive for rsPSGL.Ig in comparison with only 7 ± 2% positive...
staining for the low-affinity mutant ($P < 0.01$) and $5 \pm 0.9\%$ for the control lacking the primary antibody. This clearly demonstrates that rsPSGL.Ig selectively binds to rat blood cells.

**Effect of rsPSGL.Ig on Leukocyte Rolling, Leukocyte Adherence, and Leukocyte Transmigration in the Mesenteric Vasculature**

In rats superfused with K-H solution, a low number of rolling and adherent leukocytes was observed in the mesenteric microvasculature (Figures 1 and 2). Superfusion of the rat mesentery with L-NAME for 120 minutes resulted in significant increases in leukocyte rolling (Figure 1) and adherence (Figure 2), starting 30 minutes after L-NAME superfusion and progressively increasing over the 120-minute observation period when it plateaued. Similarly, the number of transmigrated leukocytes increased from $1.5 \pm 0.3$ to $15 \pm 3$ cells/100x20-μm area. Likewise, traumatic shock caused a sustained increase in leukocyte rolling and leukocyte adherence (Figure 3), which was comparable to that observed following superfusion of the rat mesentery with L-NAME. Interestingly, leukocyte rolling maximally increased immediately after trauma (Figure 3). Subsequently, the number of rolling leukocytes progressively decreased, whereas the number of leukocytes undergoing firm adherence continued to increase (Figure 3). This clearly demonstrates that leukocyte rolling represents an early and crucial event in traumatic shock, which leads to severe pathophysiological alterations.

Intravenous infusion of 0.5 mg/kg IV bolus rsPSGL.Ig significantly attenuated L-NAME–induced leukocyte rolling (Figure 1) and leukocyte adherence (Figure 2) in the absence of any significant change in the number of circulating leukocytes or platelets (Table). In particular, at 120 minutes, leukocyte rolling was significantly ($P < 0.01$) reduced $\approx 80\%$ in rats given rsPSGL.Ig (Figure 1). Similarly, rsPSGL.Ig significantly attenuated leukocyte adherence and leukocyte transmigration to the microvascular endothelium. Following a 120-minute L-NAME (50 μmol/L) superfusion, 17±3 cells/100 μm².
100 μm were adherent to the microvasculature and 15±2 cells/100×20-μm area had emigrated into the mesenteric tissue. In contrast, intravenous infusion of rsPSGL.Ig resulted in only 5±1.5 adherent cells/100 μm (Figure 2) and 4±1 extravasated cells/100×20-μm area (P<0.01). Moreover, infusion of low-affinity rsPSGL.Ig did not result in any significant attenuation of leukocyte rolling (Figure 1), adherence (Figure 2), or transmigration in L-NAME–superfused rats. In addition, systemic administration of rsPSGL.Ig to traumatized rats significantly attenuated the number of rolling and adherent leukocytes (Figure 3). Thus, rsPSGL.Ig, but not its low-affinity mutant, markedly curtails leukocyte-endothelium interaction in the mesenteric microvasculature.

Mean values for MABP ranged from 120 to 135 mm Hg for all 5 groups of rats studied, over the entire experimental time (data not shown). In addition, the venular shear rates were calculated in the 5 experimental groups. Initial shear rate values were 679±33, 706±55, 701±47, 695±50, and 709±54 seconds for the 5 experimental groups. These values are not significantly different from each other and did not change significantly over the 120-minute observation period for any group. These findings indicate that the adhesive interactions observed between leukocytes and endothelial cells were not due to changes in physical hydrodynamic forces or to spontaneous hemodynamic alterations brought about by the infusion of rsPSGL.Ig.

**Effect of rsPSGL.Ig on P-Selectin Expression**

Immunolocalization of P-selectin was studied in the venular endothelium of the rat ileum immediately after intravitral microscopy was completed. The percentage of venules staining positively for P-selectin in ileal sections from control rats superfused with K-H and given rsPSGL.Ig was consistently low (13±2% positive venules). In contrast, superfusion with 50 μmol/L L-NAME for 120 minutes resulted in a significant increase in P-selectin expression as quantified by the percentage of venules staining positively for P-selectin (67±4% positive venules; P<0.01 versus control). Intravenous infusion of rsPSGL.Ig (0.5 mg/kg IV bolus) did not attenuate the number of venules staining positively for P-selectin (64±4% positive venules; NS versus L-NAME superfused mesentery). This clearly suggests that inhibition of leukocyte-endothelium interaction induced by rsPSGL.Ig is due to functional neutralization of P-selectin on the endothelial cell surface rather than to significant attenuation of P-selectin expressed on the microvascular endothelium.

**Effect of rsPSGL.Ig on MABP of Rats Subjected to Noble-Collip Drum Trauma**

Administration of rsPSGL.Ig (0.5 mg/kg IV bolus) did not significantly influence MABP over the entire observation period in sham-operated control rats (Figure 4). Thus, the MABP in the sham trauma group remained at a relatively constant value between 120 and 125 mm Hg over the entire observation period. However, Noble-Collip drum trauma initially led to a sustained hypotension, with MABP falling to ~75 mm Hg in all trauma groups. These initial MABP values were not significantly different among the 3 trauma groups, suggesting a comparable degree of trauma experienced by all trauma groups. Moreover, treatment with rsPSGL.Ig resulted in a gradual and sustained recovery of MABP, which became significant 150 minutes after

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**Numbers of Circulating Leukocytes and Platelets in Peripheral Blood of Control Rats and Control Rats Given High-Affinity rsPSGL-1 (0.5 mg/kg IV Bolus)**

<table>
<thead>
<tr>
<th>Time, Minutes</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes, 10³/mm³</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control (n=6)</td>
<td>9.5±0.1</td>
<td>9.1±0.3</td>
<td>8.6±0.7</td>
<td>9.1±0.11</td>
<td>9.2±0.8</td>
<td>10.0±0.7</td>
</tr>
<tr>
<td>High-affinity rsPSGL-1 (n=6)</td>
<td>9.3±0.1</td>
<td>8.7±0.1</td>
<td>8.2±0.4</td>
<td>8.3±0.1</td>
<td>9.0±0.1</td>
<td>9.9±0.2</td>
</tr>
<tr>
<td>Platelets, 10³/mm³</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control (n=6)</td>
<td>288±2.6</td>
<td>265±4.6</td>
<td>261±53.1</td>
<td>286±5.1</td>
<td>281±6.2</td>
<td>284±5.9</td>
</tr>
<tr>
<td>High-affinity rsPSGL-1 (n=6)</td>
<td>278±0.9</td>
<td>290±2.0</td>
<td>287±3.2</td>
<td>291±4.8</td>
<td>300±4.7</td>
<td>297±4.9</td>
</tr>
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</table>

All values are mean±SEM.
rsPSGL.Ig Inhibits Leukocyte Injury in Shock

Figure 4. Time course of MABP over the experimental time period for the 5 experimental groups of rats: sham-operated control rats given K-H buffer, sham-operated control rats given rsPSGL.Ig, traumatized rats given K-H buffer, traumatized rats given the low-affinity mutant rsPSGL.Ig, and traumatized rats given high-affinity mutant rsPSGL.Ig. Each point represents a value that is mean±SEM; numbers in parentheses indicate surviving rats at each interval. *P<0.05 vs traumatized rats given low-affinity mutant rsPSGL.Ig.

Figure 5. Mean survival time (A) and survival rate (B) for the 5 experimental groups of rats: sham-operated control rats given K-H buffer, sham-operated control rats given rsPSGL.Ig, traumatized rats given K-H buffer, traumatized rats given low-affinity mutant rsPSGL.Ig, and traumatized rats given high-affinity mutant rsPSGL.Ig. Data are mean±SEM. Numbers at the bottom of each bar represent the number of rats in each group (A) or number of survivors/total number in each group (B). Statistical significance for survival rate was assessed by χ² analysis.

Effect of rsPSGL.Ig on Survival Time and Survival Rate Following Traumatic Shock

Survival times for each experimental group are presented in Figure 5A. All 6 sham-traumatized rats survived the entire 300-minute observation period. In contrast, traumatized rats receiving the low-affinity mutant rsPSGL.Ig demonstrated a survival time of only 120±24 minutes, a value significantly (P<0.001) lower than that of sham-traumatized rats. A significant prolongation of survival time was observed in traumatized rats treated with 0.5 mg/kg IV bolus rsPSGL.Ig (P<0.001), with survival time doubling (Figure 5A). Survival rate was also significantly (P<0.01) increased in traumatized rats treated with rsPSGL.Ig compared with traumatized rats treated with the low-affinity mutant rsPSGL.Ig (Figure 5B). These results suggest that systemic administration of rsPSGL.Ig significantly increases both survival time and survival rate in this model of traumatic shock.

Effect of rsPSGL.Ig on Ileal MPO Activity of Traumatized Rats

The accumulation of neutrophils in intestinal tissue was determined by measuring ileal MPO activity. The MPO enzyme activity (Figure 6) was low in sham-operated control rats (1.3±0.4 U/mg wet tissue). However, Noble-Collip drum trauma led to a significant 4-fold increase in the intestinal accumulation of neutrophils in the low-affinity mutant rsPSGL.Ig-treated rats (Figure 6). In contrast, the MPO activity of traumatized rats given rsPSGL.Ig was significantly attenuated to 2±0.5 U/mg wet tissue (P<0.05), a value not significantly different from that of sham-operated controls (Figure 6). Thus, these data provide evidence that rsPSGL.Ig treatment significantly retarded the accumulation of neutrophils in intestinal tissue following traumatic shock.

Effect of rsPSGL.Ig on SMA Endothelial Function of Traumatized Rats

Endothelial function was measured by comparing vasoactivity of isolated SMA rings in response to the endothelium-dependent vasodilator ACh and endothelium-independent vasodilator NaNO₂ (Figure 7). Isolated SMA rings from sham-traumatized rats, receiving the low-affinity mutant rsPSGL.Ig, exhibited full (>90%) relaxation to both vasodilators. In contrast, the endothelium-dependent vasorelaxant responses to ACh in SMA rings obtained from traumatized rats given the low-affinity mutant rsPSGL.Ig were significantly reduced (Figure 7). Nevertheless, these rings relaxed...
fully in response to \( \text{NaNO}_2 \) (>95%). However, the degree of relaxation to \( \text{ACh} \) in rings isolated from traumatized rats treated with rsPSGL.Ig was significantly preserved (\( P<0.05 \)), thus indicating that rsPSGL.Ig significantly protected against endothelial dysfunction occurring during traumatic shock (Figure 7).

**Effect of rsPSGL.Ig on Endogenous PSGL-1 mRNA Expression in Traumatized Rats**

Rat blood cell levels of mRNA codifying for endogenous PSGL-1 were assessed by ribonuclease protection assay. As shown in Figure 8, the intensity of each PSGL-1 mRNA band was normalized to that of CD45. No significant change in the level of PSGL-1 mRNA was found in control rat blood cells or in blood cells obtained from control rats given rsPSGL.Ig (Figure 8). In contrast, the PSGL-1 transcript was significantly (\( P<0.05 \)) increased in the blood of rats subjected to Noble-Collip drum trauma and given the low-affinity mutant rsPSGL.Ig (Figure 8). Systemic administration of rsPSGL.Ig to the rat significantly (\( P<0.05 \)) attenuated this increase in mRNA following traumatic shock (Figure 8). Therefore, in vivo administration of rsPSGL.Ig in rats results in attenuation of de novo synthesis of counterligands for adhesion molecules during inflammatory disease states such as trauma.

**Discussion**

This study was undertaken to determine the molecular and cellular mechanisms involved in the inhibition of leukocyte-endothelium interaction observed following in vivo administration of a soluble recombinant form of PSGL, rsPSGL.Ig. We also evaluated the effects of rsPSGL.Ig on the inflammatory response and subsequent end-organ injury produced by a severe form of traumatic shock. Our data clearly indicate that rsPSGL.Ig is a potent inhibitor of leukocyte-endothelium interaction in vivo and that rsPSGL.Ig exerts significant effect of trauma on blood PSGL-1 mRNA expression is summarized in the right panel. Data are mean±SEM. Numbers at the bottom of each bar represent the number of rats studied.
rsPSGL.Ig Inhibits Leukocyte Injury in Shock

100 protective effects in a murine model of traumatic shock. Specifically, intravenous administration of rsPSGL.Ig resulted in a significant prolongation of survival time and an attenuation of leukocyte infiltration as measured by lower intestinal MPO activity. rsPSGL.Ig also preserved endothelial function as evidenced by the maintained relaxation responses of SMA rings to the endothelium-dependent vasodilator ACh. These findings are consistent with recent work demonstrating that rsPSGL.Ig plays a significant role in limiting organ injury following ischemia/reperfusion of the kidney or the liver.13,29

Acute endothelial dysfunction associated with enhanced leukocyte-endothelium interaction is a common and critical early pathophysiological event resulting from either inhibition of NO synthesis in the rat mesentery30 or traumatic shock.6,31 In this regard, we have previously established a functional relationship between loss of endothelium-derived NO and the upregulation of P-selectin on the venular endothelium of the rat mesenteric microcirculation in shock-like states.31,32 Thus, blocking NO synthesis via NAME results in increased leukocyte adherence in the mesenteric microvasculature,33 coincident with increases in microvascular permeability.34 Accordingly, organic nitrates, which release NO and act as NO donors, can also protect the mesenteric microcirculation during acute inflammatory states.35 In this regard, a progressive reduction in endothelial NO release in the rat mesenteric microvasculature has been observed in traumatic shock.6 Moreover, we have shown that endothelial dysfunction occurs 15 to 20 minutes after trauma and that the resulting loss of functional release of NO is associated with an increased PMN adherence to the mesenteric endothelium 30 minutes after trauma.6 In the present model of trauma, total P-selectin mRNA increased in several vital organs of traumatized rats, including the intestine.26 Therefore an early, critical event common to all acute models of inflammation is the increased expression of adhesion molecules subsequent to impaired release of endothelium-derived NO.

The recruitment of leukocytes from the circulation to sites of tissue injury is mediated by cell adhesion molecules, which orchestrate the initial contact between leukocytes and the endothelium, followed by the firm adherence of leukocytes to the endothelium and the subsequent extravasation of leukocytes into the affected tissue.4 In particular, 3 groups of cell adhesion molecules are implicated in the recruitment of leukocytes into inflamed tissue, and they are the selectin family (ie, P-, L-, and E-selectin), the β2-integrin family (ie, CD11/CD18), and the immunoglobulin superfamily (ie, intercellular adhesion molecule-1 and platelet endothelial cell adhesion molecule-1). Leukocyte rolling is the first step and is a prerequisite for firm adherence, since integrin-mediated adherence is relatively ineffective at physiological shear rates.36 In this regard, several investigators have demonstrated that inhibition of the rolling phase of leukocytes plays a key role in attenuating the inflammatory response.37,38 Consistent with such findings, we now demonstrate that the administration of a soluble form of PSGL-1, which has selectin binding properties, significantly attenuates extravasation of leukocytes in the rat mesenteric microvasculature following inhibition of NO synthesis in vivo. This inhibitory effect on leukocyte-endothelium interaction exerted by rsPSGL.Ig results in normalization of these pathophysiological events in traumatic shock. Inhibition of leukocyte extravasation exerts a key role during inflammation, because activated neutrophils, which have adhered to the endothelium, release cytotoxic mediators including proteases, eicosanoids, cytokines, and oxygen-derived free radicals,37,38 each of which can promote tissue injury and exacerbate endothelial dysfunction. Therefore, our data strongly suggest that the beneficial effects exerted by systemic administration of rsPSGL.Ig are due significantly to an inhibition of leukocyte-endothelial cell interaction. This reduced leukocyte-endothelium interaction may also explain the mechanism by which systemic administration of rsPSGL.Ig preserves endothelium-dependent vasodilation in our traumatic shock model.

PSGL-1 is a highly extended homodimer of 2 disulfide-linked subunits, each of which displays N-linked glycans and sialylated O-linked glycans that present Slax.14 However, expression of PSGL-1 protein is not sufficient to allow binding to its primary high-affinity ligand, P-selectin. Under physiological conditions, functional binding of PSGL-1 to P-selectin requires a number of posttranslational modifications by α(1,3/1,4)-fucosyltransferase, which include sialylation, sulfation, and fucosylation of the primary protein structure.9,14 All 3 selectins demonstrate a high affinity for sialylated, fucosylated saccharide structures such as Slax, and although there are several leukocyte-bound structures that present Slax, it appears as though P-selectin must bind with PSGL-1 in order for leukocytes to efficiently roll along the endothelium under physiological flow conditions.39,40 Consistent with this concept, we found that in vivo administration of rsPSGL.Ig inhibits NAME-induced leukocyte rolling in the rat mesenteric microcirculation, thus also blocking firm adherence and subsequent transmigration of leukocytes. Since intravenous infusion of rsPSGL.Ig did not attenuate endothelial cell surface expression of P-selectin, this activity of rsPSGL.Ig is likely due to functional neutralization of P-selectin on the endothelial cell surface rather than to quantitative attenuation of P-selectin expression on the endothelium.

Interestingly, in this study, rsPSGL.Ig was also found to inhibit de novo synthesis of PSGL-1, as confirmed by quantification of PSGL-1 mRNA levels in the blood of traumatized rats treated with rsPSGL.Ig. This result suggests that the mode of action of rsPSGL.Ig may be extended not only to inhibition of leukocyte-endothelial interaction, but also to attenuation of leukocyte-leukocyte and leukocyte-platelet interactions. Therefore, the observed reduced PSGL-1 mRNA blood levels may contribute to inhibition of the widespread increase in cell-to-cell interaction during acute inflammatory conditions such as ischemia-reperfusion and traumatic shock. In this regard, previous observations have demonstrated binding of PSGL-1 to both L- and E-selectin.41,42 However, inhibition of E-selectin-mediated leukocyte-endothelial cell interaction by rsPSGL.Ig in our 120-minute study using intravital microscopy is highly unlikely. E-selectin expression requires de novo protein synthesis having a lag time of 4 to 6 hours for protein expression on
the endothelial surface. This suggests that, during the early stage of inflammatory responses, the protective effect of rsPSGL.Ig is primarily mediated via P-selectin–dependent mechanisms. Furthermore, functional inhibition of P-selectin can result in protective effects in longer experimental models of inflammation than our 2-hour intravital microscopy model.31 In this regard, Skurk et al31 have previously demonstrated that administration of a monoclonal antibody against P-selectin exerts a beneficial effect in traumatic shock and that the degree of this protection is comparable to that observed following systemic administration of a sialyl Lewis*-oligosaccharide, a specific ligand for P-, L-, and E-selectin. Therefore, most of the protection of sialyl Lewis*-oligosaccharide is via blockade of P-selectin. Interestingly, other authors have reported that inhibition of P-selectin by soluble PSGL-1 partially blocks the induction of E-selectin mRNA in the ischemic-reperfused kidney.13 One possible result of this finding is that by inhibiting the initial P-selectin–mediated tethering of leukocytes to endothelium, rsPSGL.Ig diminishes the localized production of proinflammatory cytokines, which induce later E-selectin expression. In contrast to E-selectin, L-selectin–mediated cell-to-cell interactions have been shown to be important in the development of tissue injury during the acute phase of inflammatory disease states.43 L-selectin is not only capable of mediating leukocyte-endothelium interaction but also of contributing to leukocyte-leukocyte interaction. Although PSGL-1 has been shown to bind with lower affinity to L-selectin than to P-selectin,42 the interaction of L-selectin and PSGL-1 could initiate leukocyte-leukocyte interactions that represent the first step in leukocyte aggregation during inflammatory states.41 Unstimulated leukocytes have been shown to roll on immobilized PSGL-1 under flow conditions, and treatment of the immobilized PSGL-1 with an L-selectin monoclonal antibody inhibits not only leukocyte rolling on immobilized PSGL-1 but also leukocyte rolling on adherent leukocytes.44 Even though activated PMNs shed their L-selectin, PSGL-1 remains intact. Thus, adherent activated PMNs that have shed L-selectin are capable of interacting with flowing PMNs if the adherent PMNs are expressing PSGL-1 and activated integrins, while flowing PMNs express L-selectin and the integrin counterreceptor.41 Therefore, reduced synthesis of PSGL-1 by circulating white blood cells represents a further mechanism by which rsPSGL.Ig attenuates tissue injury during acute inflammatory diseases and shock states.

In conclusion, we have demonstrated significant in vivo beneficial effects of a recombinant soluble human form of PSGL-1 in a severe model of traumatic shock. These effects are consistent with reduced leukocyte-endothelial interaction induced by rsPSGL.Ig in the rat mesenteric microvasculature. These impressive effects are probably due to the fact that PSGL-1 has ~4 orders of magnitude greater avidity for binding to P-selectin than previously identified selectin ligands.45 These data demonstrate the significant role that PSGL-1 plays in the development and progression of inflammation in disease states such as traumatic shock.

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References

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Effect of Recombinant Soluble P-Selectin Glycoprotein Ligand-1 on Leukocyte-Endothelium Interaction In Vivo: Role in Rat Traumatic Shock

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