Raised Plasma Soluble P-Selectin in Peripheral Arterial Occlusive Disease Enhances Leukocyte Adhesion


Abstract—Raised levels of soluble P-selectin (sP-selectin) have been reported in the plasma of patients with vascular diseases; however, the functional importance of this ligand remains unclear. In this study we have examined a potential role for plasma sP-selectin in regulating neutrophil adhesion in patients with peripheral arterial occlusive disease (PAOD). Patients with PAOD had significantly higher levels of sP-selectin (mean±SD: 73.3±13.0 versus 16.7±6.4 ng/mL) and enhanced whole blood leukocyte adhesion to platelets under shear. To examine whether the raised sP-selectin levels can directly influence leukocyte adhesion, isolated neutrophils were incubated with plasma from PAOD patients before and after immunodepletion of sP-selectin. Neutrophil adhesion to fibrinogen increased 2-fold following incubation with PAOD plasma, which was abrogated on sP-selectin immunodepletion. We subsequently demonstrated that recombinant sP-selectin dose-dependently (75 to 250 ng/mL) increased leukocyte adhesion to fibrinogen and platelet monolayers. This increase was PSGL-1 and Src kinase-dependent and correlated with an increase in sP-selectin-mediated Mac-1 activation. sP-selectin–stimulated neutrophil adhesion to platelet monolayers was inversely correlated with shear, such that at low shear (50 s⁻¹) a 92.7%±15.7 increase in adhesion was observed decreasing to 38.5%±11.9 at 150 s⁻¹ and 10.1%±7.4 at 300 s⁻¹. These studies suggest a potentially important role for sP-selectin in modulating neutrophil adhesion in patients with PAOD, particularly at sites of low shear, where it raises the possibility that raised plasma sP-selectin levels may enhance leukocyte recruitment to vascular injury and promote disease progression. (Circ Res. 2006;98:90-0.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ leukocytes ■ peripheral vascular disease

Diseases of the vascular system remain the leading cause of mortality and morbidity in developed countries despite considerable therapeutic progress in recent years. Atherosclerosis is the predominant pathology underlying clinical vascular disease and consists of an intima-media plaque in conduit arteries containing cholesterol and inflammatory cells. The implications of inflammation for the development and progression of atherosclerosis have become increasingly evident, and thus the mechanisms leading to inflammatory leukocyte recruitment are of central importance. P-selectin is a member of the selectin family and is localized in the membranes of the α-granules of platelets and the Weibel-Palade bodies of endothelial cells. It is expressed on the surface of activated platelets and endothelial cells and is essential for leukocyte recruitment to sites of vascular injury and inflammation by engaging its ligand PSGL-1. This adhesive interaction supports leukocyte rolling and also transduces intracellular signals. These signals involve activation of non-receptor tyrosine kinases, eg, Src/Syk, which have previously been shown to associate with tyrosine residues in the cytoplasmic tail of PSGL-1. These kinases play an important role in initiating signals that promote the inside-out activation of surface integrins, necessary for the development of stable leukocyte adhesion contacts with the vessel wall. Along with chemokines, P-selectin is a potentially important trigger for leukocyte activation, and as such represents a potentially attractive therapeutic target in vascular disease. Consistent with this, deficiency of P-selectin or antibody-mediated inhibition of its adhesive function reduces early atherogenesis in animal models. The use of therapeutic interventions targeting P-selectin is complicated by the presence of a soluble form of the protein (sP-selectin). Low levels of sP-selectin are detected in human plasma, where it is thought to be derived from the secretion of an alternatively spliced protein lacking the transmembrane domain and/or from proteolytic cleavage of the membrane-bound form. In patients with vascular disorders such as unstable angina, postangioplasty restenosis and diabetes, levels of sP-selectin ranging from 75 to 200 ng/mL, with extreme values >1000 ng/mL having been reported.
sP-selectin has thus been suggested as a potentially valuable clinical biomarker of vascular disease risk. Although pathophysiologically relevant concentrations of sP-selectin have been reported to induce b2-integrin activation and enhance neutrophil adhesion. However, the potential pathophysiologic significance of such findings is likely to occur in vivo. Nonetheless, the demonstration that transgenic mice expressing a soluble form of P-selectin can compete with the membrane-bound form has been demonstrated to induce phosphatidylserine exposure on the surface of monocytes and sP-selectin dimers have been reported to induce b2-integrin function of leukocytes under static and shear flow conditions. Moreover, the potential pathophysiologic significance of such findings is likely to occur in vivo. Nonetheless, the demonstration that transgenic mice expressing a soluble form of P-selectin can enhance procoagulant function raises the possibility that this protein may have a functional role in vivo.

In the current study, we examined the hypothesis that pathophysiologically relevant concentrations of plasma sP-selectin have biological activity and can influence the adhesion of leukocytes under static and shear flow conditions. Moreover, the role of raised levels of sP-selectin in the plasma of patients with peripheral arterial occlusive disease (PAOD) on adhesion of leukocytes was investigated in this patient population with high atherosclerotic burden.

Materials and Methods

Materials
Histopaque-1077 and histopaque-1119 were purchased from Sigma Chemical Co. Enzyme-linked immunosorbent assays (ELISAs) for the quantification of human soluble P-selectin, recombinant monomeric human soluble P-selectin (sP-selectin), soluble E-selectin, and CHO131, a blocking mouse monoclonal antibody against human PSGL-1, were from R&D Systems. CBRM1/5, a monoclonal anti-PSGL-1 antibody, were from R&D Systems. CBRM1/5, a monoclonal antibody against human P-selectin (WAPS 12.2) over- night at 4°C. Immunoprecipitated beads were washed three times by centrifugation. Equal amounts of protein were loaded into each lane and separated on a 5% native gel, then transferred to PVDF membranes. Blots were then blocked, washed and probed with a monoclonal antibody against human P-selectin (WAPS 12.2) overnight at 4°C. The antibody bound to P-selectin was detected with a horseradish peroxidase-conjugated goat anti-mouse IgG and an enhanced chemiluminescence kit. Bands were developed on Kodak sensitive photographic film.

Plasma Soluble P-Selectin Measurement
Whole blood was taken from patients or matched controls and collected into citrate (12.9 mmol/L) for measurement of sP-selectin. For sP-selectin measurement, plasma samples were isolated by ultracentrifugation of isolated plasma for 1.5 hours at 43 000 rpm at 4°C and resuspended in Tyrode’s buffer before analysis samples were centrifuged at 1000g for 5 minutes to remove cell contaminants. Plasma (from healthy controls and PAOD patients), microparticles, or recombinant monomeric sP-selectin (from R&D systems) was incubated with protein A-sepharose beads conjugated to WAPS 12.2 overnight at 4°C. Immunoprecipitated beads were washed three times by centrifugation. Equal amounts of protein were loaded into each lane and separated on a 5% native gel, then transferred to PVDF membranes. Blots were then blocked, washed and probed with a monoclonal antibody against human P-selectin (WAPS 12.2) overnight at 4°C. The antibody bound to P-selectin was detected with a horseradish peroxidase-conjugated goat anti-mouse IgG and an enhanced chemiluminescence kit. Bands were developed on Kodak sensitive photographic film.

Western Blot Analysis for Determination of Human Plasma S-P-Selectin Conformation
Plasma was collected by centrifugation at 10,000g for 15 minutes. Microparticles from healthy controls or PAOD patients were isolated by ultracentrifugation and centrifuged plasma for 1.5 hours at 4°C, and resuspended in Tyrode’s buffer before analysis. Samples were centrifuged at 1000g for 5 minutes to remove cell contaminants. Plasma (from healthy controls and PAOD patients), microparticles, or recombinant monomeric sP-selectin (from R&D systems) was incubated with protein A-sepharose beads conjugated to WAPS 12.2 overnight at 4°C. Immunoprecipitated beads were washed three times by centrifugation. Equal amounts of protein were loaded into each lane and separated on a 5% native gel, then transferred to PVDF membranes. Blots were then blocked, washed and probed with a monoclonal antibody against human P-selectin (WAPS 12.2) overnight at 4°C. The antibody bound to P-selectin was detected with a horseradish peroxidase-conjugated goat anti-mouse IgG and an enhanced chemiluminescence kit. Bands were developed on Kodak sensitive photographic film.

Blood Cell Preparation
For platelet isolation, blood was drawn into either citrate or acid-citrate-dextrose containing theophylline, standardized phlebotomy techniques were used, and the first 10 mL of blood were discarded to reduce platelet activation. Platelets were isolated from whole blood as previously described and finally resuspended in modified Tyrode’s buffer (10 mmol/L NaHCO3, pH 7.4, 137 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L glucose). Neutrophils were prepared as follows: first, citrated whole blood (24 mL) was layered over Histopaque-1077 (12 mL) that in turn had been layered over Histopaque-1119 (12 mL), and then centrifuged at 600g for 30 minutes at room temperature. The histopaque layer containing neutrophils (directly above packed erythrocytes) was carefully removed and centrifuged at 1000g to pellet neutrophils, which were then washed twice in Hank’s balanced salt solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 0.7 mmol/L NaHPO4, 1.2 mmol/L NaHCO3, 27.5 mmol/L Tris, pH 7.4, 12.2 mmol/L dextrose), and finally resuspended to a final volume of 5 mL. Monocytes were isolated from whole blood using the commercially available monocyte negative isolation kit as previously described. The resting isolated monocyte and neutrophil fractions contained between 92% and 99% monocytes/neutrophils that were >95% viable (by trypan blue exclusion) with minimal red blood cell and platelet contamination as

Inclusion/Exclusion Criteria for Patient Recruitment
Volunteer patients (<75 years old) with a high atherosclerotic burden were identified by the presence of extensive PAOD. The control group consisted of age- and sex-matched volunteers with no known atherosclerosis (ie, no evidence of PAOD or other vascular disease). Patients were recruited before vascularization and inclusion criteria for PAOD patients included: symptomatic with intermittent claudication (stage II-III fontaine classification); at least 70% stenosis in major leg arteries (≥1 confirmed/measured by digital subtraction angiogram; and ankle/brachial systolic blood pressure ratio of <0.9. All inclusion criteria had to be met for recruitment. The major exclusion criteria was age (>75 years old). All patients were on either nonsteroidal antiinflammatory drugs (aspirin) and/or lipid-lowering therapy. Patient treatment was not altered within the course of the study. Control volunteers were recruited from a volunteer database, part of the Alfred Baker Medical Unit, Baker Heart Research Institute. Ethical approval for the recruitment and collection of blood from patients and controls was obtained from the Alfred Hospital Institutional Ethics Committee. Unless otherwise stated, studies of neutrophil adhesion to fibrinogen or platelet monolayers was performed on blood obtained from a separate pool of healthy consenting volunteers who had not taken either antiplatelet or antiinflammatory medication in the 2 weeks before venesection.

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assessed by an automated cell counter. Cells were stored at room temperature for a maximum of 2 hours and resuspended in Tyrode’s buffer supplemented with 1 mmol/L CaCl₂/MgCl₂ (1 × 10⁷/mL) before experimentation.

**PAOD Patient Adhesion Studies**

**Whole Blood Perfusion Studies**

Whole blood was collected from PAOD patients or matched healthy controls as described in hairdin. In some experiments whole blood from healthy matched controls were analyzed for sP-selectin levels by ELISA and then spiked with recombinant sP-selectin to increase levels up to 150 ng/mL immediately before perfusion. Whole blood was allowed to perfuse over spread platelet monolayers at 150 s⁻¹ in micro-capillary tubes for 5 minutes at 37°C (described in detail in Immobilized Fibrinogen Studies). A further 5 minutes of flow with Tyrode’s buffer was continued to quantify the number of leucocytes adhered to the platelet monolayers in 5 fields, as analyzed by phase contrast microscopy.

**Immunodepleted sP-Selectin Plasma Adhesion Studies**

To further characterize the effects of released plasma sP-selectin on neutrophil adhesion, plasma from PAOD patients or matched controls was immunodepleted of sP-selectin using protein A-sepharose beads conjugated to WAPS 12.2 or isotype matched conjugated antibody as a control, according to the same protocol used for the western blots. Isolated neutrophils from healthy volunteers (1 × 10⁶ mL) were resuspended in PAOD or matched healthy control plasma or P-selectin immunodepleted plasma and examined for neutrophil adhesion to immobilized fibrinogen in 24 well plates (described in detail in Immobilized Fibrinogen Studies).

**Healthy Volunteer Leukocyte Adhesion Studies:**

**Static Adhesion Studies**

**Immovilized Fibrinogen Studies**

To confirm the role of sP-selectin on adhesion, immobilized fibrinogen was used to exclude the interaction with membrane bound P-selectin and include the use of PSGL-1 antagonists without affecting platelet interactions. Fibrinogen (100 µg/mL) was coated onto 24-well plates overnight at 4°C. Isolated neutrophils (1 × 10⁶ mL; 500 µL) added for 15 minutes at 37°C with or without sP-selectin (10 to 250 ng/mL) or soluble E-selectin (150 ng/mL). Optimal adhesion of neutrophils was predetermined within our laboratory to be 15 minutes. Cells were fixed with 3% formaldehyde for 30 minutes and adhered cells were stained with Tyrode’s buffer to remove nonadhered neutrophils. In some experiments neutrophils were pre-incubated with α-PSGL-1 (50 µg/mL; 20 minutes), PP1, PP2, or the pharmacologically inactive inhibitor PP3 (20 µm/L; 20 minutes) before exposure to sP-selectin. Neutrophil adhesion was analyzed using phase microscopy (×40), where 5 random fields of view for each well were analyzed.

**Platelet Studies**

In some experiments, static neutrophil adhesion assays to platelet monolayers were performed to examine the effects of shear on sP-selectin mediated adhesion. Isolated platelets (2 × 10⁶/mL; 300 µL) were seeded onto coverslips within 24-well culture plates over 15 minutes at 37°C. Spread platelets monolayers were blocked with 3% human serum albumin for 10 minutes at 37°C. Platelets were washed (Tyrode’s buffer) and isolated neutrophils (1 × 10⁶/mL; 500 µL) added for 15 minutes at 37°C with or without sP-selectin (150 ng/mL). Neutrophil adhesion was quantified and analyzed as described.

**Flow Cytometry Analysis of Mac-1 Activation**

Isolated primary neutrophils (1 × 10⁶/mL; Tyrode’s buffer) were pre-stained with CBRM1/5-FITC conjugated (5 µL) for 30 minutes at room temperature. Neutrophils were then incubated with vehicle control (Tyrode’s) or PMA (1 µmol/L) or recombinant sP-selectin (150 ng/mL) with CaCl₂ (1 mmol/L) and MgCl₂ (1 mmol/L) for 5 minutes at 37°C. Samples were then fixed (2% formaldehyde) and analyzed for neutrophil eM activation according to forward and side scatter characteristics (FL1 fluorescence) by FACS. All samples were compensated for using appropriate isotype matched negative control (anti-IgG2a-FITC).

**Perfusion Studies**

**Platelet Monolayer Studies**

Perfusion studies were performed within glass micro-capillary tubes (2×0.2×100 mm) at 37°C as previously described, and briefly washed platelets (2×10⁶/mL; 100 µL) were allowed to spread within glass micro-capillary tubes for 15 minutes at 37°C. Isolated neutrophils or monocytes (1 × 10⁶/mL) in Tyrode’s buffer supplemented with 1 mmol/L CaCl₂/MgCl₂ were perfused over spread platelet monolayers that had been blocked with 3% human serum albumin (10 minutes, 37°C) at 150 s⁻¹ (1.1 dyn/cm²) for 5 minutes, with a further wash-out with Tyrode’s buffer for 5 minutes. The 150 s⁻¹ shear rate was used as previous work within our laboratory and others has shown this rate to be optimal for studying leucocyte adhesion to platelet monolayers under shear. To examine the effects of shear on sP-selectin mediated leucocyte adhesion, further perfusion experiments were performed at differing shear rates (50 to 300 s⁻¹). Isolated leucocytes were pretreated with either vehicle control (Tyrode’s) or sP-selectin (50 or 150 ng/mL) before perfusion over spread monolayers. Leucocyte–platelet interactions were visualized using phase video microscopy (10× lens; Leica DMIRB, Leica, Heidelberg, Germany) and recorded at various times throughout perfusion for off-line analysis.

**Statistical Analysis**

All data are shown as the mean ± SD. Groups of data were evaluated statistically by ANOVA, followed by Tukey post test for multiple comparisons at each time point, Mann-Whitney test for grouped data, and Spearman rank test for identifying correlations. All significance tests were two-tailed, in which P<0.05 was considered significantly different from control treated samples. All analyses were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software). The mean intra-assay and inter-assay precision for the ELISA assays were 4.98% (CV) and 8.78% (CV), respectively. The mean intra-assay and inter-assay precision for the flow cytometry assays were 2.32% (CV) and 5.56% (CV), respectively.

**Results**

**Circulating Plasma sP-Selectin**

Patients with peripheral arterial occlusive disease show significantly elevated plasma levels of sP-selectin, with a mean ± SD of 73.3 ± 13.0 ng/mL compared with 16.7 ± 16.4 ng/mL in matched controls (Figure 1). Removing microparticles from plasma had no significant effect of sP-selectin levels in either controls (14.7 ± 12.4 ng/mL; n=4) or PAOD patients (68.3 ± 15.1 ng/mL; n=4). The mean ages of recruited patient and matched control subjects were 68.4 ± 69.4 ± 4, respectively. The mean percentage stenosis within the disease group was 78.1 ± 5.9%. There was neither significant correlation between age and sP-selectin levels (data not shown) nor any difference in sP-selectin levels between male or female patients (data not shown). Some patients had type II diabetes (n=9); however, there were no significant trends to altered sP-selectin levels within this group. Although there were no outliers or grouped data demonstrating specific effects of drug treatment on sP-selectin levels (Figure 1), the effects of drug therapy were not specifically studied in the current study. Membrane-bound P-selectin can be expressed...
as monomers or dimers, where the dimeric form has been reported to confer increased affinity to its ligand, PSGL-1.32 However, both P-selectin monomers and dimers can sustain PSGL-1 binding and therefore possible functionality.22 To determine the circulating plasma forms of sP-selectin, fresh human plasma was run on a native gel and immunoblotted for the presence of sP-selectin. sP-selectin was found to circulate as both monomers and dimers (Figure 1B). Dimer levels were not affected by disease (PAOD) (Figure 1B). Western blot analysis confirmed there was no sP-selectin associated with isolated microparticles (Figure 1B).

**Figure 1.** sP-selectin levels in healthy controls and patients with PAOD and plasma levels of P-selectin monomers and dimers. A, Study groups were recruited as described in Methods. Whole blood was removed and centrifuged to isolate plasma and stored at −70°C. Plasma sP-selectin levels were determined by ELISA. n=20 in duplicate (**P**<0.001 as analyzed by Mann-Whitney t test). B, Fresh human plasma was isolated by centrifugation, and microparticles were isolated from plasma by ultracentrifugation from both healthy controls and recruited PAOD patients. Recombinant monomeric and dimeric soluble P-selectin were immunoprecipitated from human plasma and microparticles. As a control, a commercially available recombinant monomeric sP-selectin was used. Equal amounts of protein were loaded into each lane and samples were immunoprecipitated overnight and run on a 5% native gel. sP-selectin products were blotted with a monoclonal antibody against P-selectin and detected using advanced chemiluminescence and developed on Kodak photographic film. A representation of 3 experiments is shown, showing both the monomeric (120 kDa) and dimeric forms of sP-selectin (240 kDa).

Effects of PAOD Plasma on Leukocyte Adhesion

To examine whether the elevated sP-selectin levels can influence neutrophil adhesion, blood from either PAOD patients or matched controls was allowed to perfuse over spread platelet monolayers for 5 minutes. Figure 2 shows that there was a significant increase in the number of leukocytes that had adhered to the platelet monolayers. Further experiments were performed to show that spiking healthy matched control whole blood with recombinant sP-selectin up to levels of 150 ng/mL also significantly increased leukocyte adhesion to platelet monolayers under shear (Figure 2).

To further examine the role of elevated plasma sP-selectin levels on neutrophil adhesion and to exclude the role of platelet membrane bound P-selectin, plasma isolated from both patients and matched controls was incubated with isolated neutrophils from healthy volunteers. Figure 3 shows that incubating isolated neutrophils with plasma from PAOD patients significantly increased neutrophil adhesion to FGN from a mean SD of 26.5 ± 9.7 in matched plasma to 48.9 ± 15.5. This increase in adhesion was reduced to 26.9 ± 11.5 after immunodepleting sP-selectin from PAOD plasma with WAPS 12.2-conjugated sepharose beads (Figure 3). There was a nonsignificant reduction in adhesion in sP-selectin immunodepleted matched control plasma (Figure 3) and in PAOD plasma incubated with an isotype matched control antibody. The mean level of sP-selectin in immunodepleted plasma was 7.71 ± 3.01 ng/mL in control and 7.89 ± 3.89 ng/mL in PAOD isolated plasma.

**Effects of Recombinant sP-Selectin on Neutrophil Adhesion to Fibrinogen**

To investigate whether the monomeric form of sP-selectin was sufficient to stimulate neutrophil adhesion on fibrinogen,
static adhesion assays were performed. Figure 4A shows that recombinant monomeric sP-selectin dose-dependently increased neutrophil adhesion to fibrinogen, with maximal effect at 150 ng/mL (from 8.1±5.6 to 25.2±6.3 cells/field; n=4; P<0.05). This increase in adhesion was PSGL-1-dependent as a blocking anti-PSGL-1 antibody completely abrogated the increase in neutrophil adhesion. In further control studies, soluble recombinant E-selectin (sE-selectin) had no effect on neutrophil adhesion even at concentrations as high as 150 ng/mL, demonstrating that the effects were specific to P-selectin (Figure 4B). The sP-selectin–induced increase in neutrophil adhesion was likely to involve direct neutrophil stimulation as it was completely blocked by the Src family kinase inhibitors PP2 or PP1, but not by the pharmacologically inactive control PP3 (Figure 4C).

PSGL-1 engagement of membrane-bound P-selectin stimulates integrin activation (CD11b) through a Src kinase-dependent manner.11 To investigate whether sP-selectin stimulates CD11b activation, isolated neutrophils were incubated with sP-selectin (150 ng/mL) in the presence of the activation-specific antibody CRBM1/5. As demonstrated in Figure 5, sP-selectin stimulated a 21.35% increase in the binding of CRBM1/5 to CD11b on neutrophils (19.9±2.0 to 25.3±3.6; n=3; P<0.05; Figure 5). This increase in CRBM1/5 binding was modest in comparison to that induced by PMA (55.5±0.1; n=4; Figure 5) but is consistent with previous findings using high concentrations of dimeric sP-selectin.26 In control studies, sE-selectin demonstrated no significant increase in CD11b activation (Figure 5) and an anti–PSGL-1 MAb completely inhibited the increase in CRBM1/5 binding induced by sP-selectin. These findings suggest that pathophysiologically relevant concentrations of soluble monomeric P-selectin can stimulate CD11b activation in a PSGL-1-dependent manner.

Effects of sP-Selectin on Neutrophil Adhesion to Platelets Under Shear

To investigate whether sP-selectin had any effect on neutrophil adhesion to spread platelet monolayers under physiological shear conditions (150 s⁻¹) and a leukocyte purified system, flow-based adhesion assays were performed as described in Methods. As demonstrated in Figure 6, sP-selectin increased total neutrophil adhesion to spread platelet mono-

Figure 4. Neutrophil static adhesion to fibrinogen. The 24-well plates were coated in fibrinogen (100 µg/mL) overnight at 4°C. Isolated neutrophils (1×10⁶/mL) were added (30 minutes) to wells and incubated with (A) sP-selectin (0 to 250 ng/mL) or pre-incubated with (B) PP1, PP2, or PP3 (1 µmol/L; 20 minutes) or (C) anti–PSGL-1 (50 µg/mL; 20 minutes) and analyzed for total cell adhesion (×40); n=4 in triplicate. *P<0.05 from vehicle control treated neutrophils (0), as analyzed by ANOVA followed by Tukey post-test.

Figure 3. Effect of raised plasma sP-selectin levels on neutrophil adhesion to fibrinogen. The 24-well plates were coated with fibrinogen (100 µg/mL) overnight at 4°C. Isolated neutrophils (1×10⁶/mL) were added (30 minutes) to wells and incubated with plasma isolated from healthy matched control whole blood, or blood from patients with PAOD. In some experiments, both matched control plasma and plasma from patients was immunodepleted (ID) with a MAb against P-selectin or isotype matched control (anti-mouse-IgG; Ctrl Post-ID). Total cell adhesion was analyzed by phase contrast microscopy (×40); n=4 in triplicate, and probability values are given below the figure as analyzed by ANOVA followed by Tukey post-test. *P<0.05 from matched controls.
layers in a time-dependent manner. This increase in adhesion was most significant at 150 ng/mL, with mean \( \pm \) SD cells/field of 72.2 \( \pm \) 31.1 compared with control treated neutrophils of 57.3 \( \pm \) 30.3 (\( n = 9 \); \( P < 0.05 \)). At sP-selectin levels comparable to the highest values seen in healthy individuals (50 ng/mL), there was no significant difference in adhesion from vehicle controls at 51.3 \( \pm \) 13.8 cells/field from 57.3 \( \pm \) 30.3 (\( n = 9 \)). Further analysis revealed that sP-selectin significantly decreased the rolling kinetics to stationary adhesion of neutrophils (Figure 6B) from a mean \( \pm \) SD time to reach stationary adhesion from tethering of 12.2 \( \pm \) 4.6 to 8.1 \( \pm \) 6.1 seconds after sP-selectin treatment (\( n = 6 \); \( P < 0.05 \)). Similar results were observed for monocyte adhesion to platelets, where sP-selectin dose-dependently increased monocyte adhesion to spread platelet monolayers under shear (data not shown) with maximal levels of adhesion observed at a sP-selectin concentration of 150 ng/mL (data not shown).

Effects of Different Shear Rates on sP-Selectin–Mediated Neutrophil Adhesion to Platelets

An unexpected finding was the observation that increases in neutrophil adhesion mediated by sP-selectin (150 ng/mL) under static conditions (Figure 4A; 333.3%) was significantly greater than under flow (Figure 6A; 38.5%). We therefore proceeded to explore the effects of different shear rates on sP-selectin stimulated neutrophil adhesion to platelets. Figure 7 shows that increasing shear leads to a decline in neutrophil adhesion; however, it showed a disproportionate decrease in the ability of sP-selectin to enhance neutrophil adhesion to platelet monolayers, whereby at a shear rate of 50 s\(^{-1}\) sP-selectin increased neutrophil adhesion by 92.7% compared to controls.

Figure 5. sP-selectin stimulates neutrophil Mac-1 activation. Isolated neutrophils were stimulated with PMA (1 \( \mu \)mol/L), sP-selectin (150 ng/mL), sE-selectin (150 ng/mL), or pre-incubated with anti–PSGL-1 (50 \( \mu \)g/mL; 20 minutes) and analyzed for CD11b activation (\( \alpha \)-CD11b:FITC) via FACS. Average FL1 GeoMn florescence from 3 experiments in triplicate is shown. \( ** P < 0.01 \) and \( * P < 0.05 \) from controls as analyzed by ANOVA followed by Tukey post-test.

Figure 6. Neutrophil adhesion to spread platelet monolayers under shear. A, Neutrophils were isolated from whole blood. Platelets were isolated according to standardized methods and allowed to spread in 0.2-mm glass capillaries at 37°C for 15 minutes and exposed glass blocked by human serum albumin. Neutrophils were treated with sP-selectin (50 & 150 ng/mL) and allowed to flow (150 s\(^{-1}\)) over spread platelet monolayers at 37°C. The total number of cells adhered over 10 minutes in 5 fields were analyzed; \( n = 9 \) in triplicate. \( ** P < 0.01 \) from controls as analyzed by ANOVA followed by Tukey post-test. B, sP-selectin and control treated neutrophils were analyzed from Figure 5A for their rolling kinetics before stationary adhesion; \( n = 20 \). \( * P < 0.05 \) from controls as analyzed by Mann-Whitney \( t \) test.

Figure 7. Effects of shear on sP-selectin and fMLP stimulated neutrophil adhesion. Isolated neutrophils were incubated with 150 ng/mL of sP-selectin or fMLP (1 \( \mu \)mol/L; 10 minutes before perfusion) and perfused over spread platelet monolayers (isolated from whole blood) at various shear rates (50 to 300 s\(^{-1}\)) over 5 minutes. For static experiments (0) isolated platelets were allowed to spread on in 24-well plates before addition of control, sP-selectin (150 ng/mL), or fMLP (1 \( \mu \)mol/L) treated neutrophils. The total number of cells adhering to platelet monolayers was assessed after 5 minutes of flow or 15 minutes in static assays (defined as optimal conditions for stationary adhesion; see Methods). The total number of cells adhered at each shear at 5 minutes in 5 fields were analyzed; \( n = 3 \) in triplicate.
increased levels of leukocyte b2-integrin activation and the percentage increase in cell adhesion after treatment with sP-selectin was observed in patients with vascular disease; however, to our knowledge this is the first report demonstrating a direct role for other circulating proinflammatory molecules, such as C-reactive protein or sCD40L in regulating leukocyte adhesion function. Whether sP-selectin dimers or oligomers can further increase neutrophil adhesion due to their reported increased avidity to PSGL-1 remains unknown and will require further investigation. In this context, it was of interest that although the increase in mean sP-selectin levels from control to PAOD patients was ≈60 μg/mL, the enhancing effect of patient plasma on neutrophils was larger than that using isolated neutrophils spiked with recombinant monomeric sP-selectin, raising the possibility that sP-selectin dimers/oligomers and or other proinflammatory cytokines may contribute to the leukocyte adhesion process.

It is well-known that leukocyte adhesion at higher shear rates decreases overall adhesion, which is confirmed within our results; however, a further novel finding in our flow studies was the demonstration that the effects of sP-selectin on neutrophil adhesion were dependent on the prevailing blood flow conditions, such that at very low shear rates (0 to 50 s⁻¹) the enhancing effects on neutrophil adhesion were up to 4-fold greater than at 150 s⁻¹. This observation is potentially important when considering the rheological conditions operating at sites of atherosclerosis. Typically atheromatous plaques develop at sites of arterial bifurcations where disturbances of blood flow are common and shear rates significantly lower than in nonbranching arteries. Such shear rates can also be lower than those encountered in post-capillary venules, the preferred sites where leukocytes adhere and transmigrate into inflamed tissue, raising the interesting possibility that high levels of sP-selectin may preferentially influence leukocyte adhesive function in atherosclerotic-prone regions.

Our studies showing increases in leukocyte adhesion conflict with previous studies describing decreases in adhesion of activated leukocytes to endothelial cells. However, the previous studies were performed under static conditions and the potential pathophysiological significance of such findings under flow conditions remains unclear. Similarly the concentrations of sP-selectin used in some of these assays are unlikely to occur in vivo. Further, whether similar effects of sP-selectin will be observed on endothelial adhesion under the conditions described here requires further investigation. Previous studies show that drug therapies in vascular disease can alter sP-selectin levels. The current study was not designed to explore the effects of therapy on levels or function, but our data support the hypothesis that lowering plasma sP-selectin through treatment would be beneficial in reducing sP-selectin mediated leukocyte adhesion. Further limitations of this study include the use of in vitro models of leukocyte adhesion and require further investigation of sP-selectin’s role in vivo models of human leukocyte adhesion to both endothelium and platelet thrombi.

### Discussion

Our studies demonstrate for the first time to our knowledge a functionally important role for sP-selectin in regulating leukocyte adhesion in patients with PAOD. Furthermore, we demonstrate that pathophysiologically relevant concentrations of monomeric sP-selectin are biologically active and induce neutrophil activation through engagement of PSGL-1 independent of contributing effects of membrane-bound platelet P-selectin. This activating mechanism is Src kinase-dependent and leads to an upregulation in MAC-1 adhesive function. We have demonstrated that the effects of sP-selectin on neutrophil adhesion to platelets are shear-dependent, with maximal neutrophil adhesion at low shear rates. Overall, our studies raise the possibility that sP-selectin may promote leukocyte recruitment to sites of vessel wall injury in patients with vascular disease.

The demonstration of raised levels of sP-selectin in patients with PAOD is consistent with previous findings suggesting that this protein represents a useful biomarker for advanced cardiovascular disease. It is also well-known that increased levels of leukocyte b1-integrin activation and the presence of leukocyte–platelet aggregates is a common finding in patients with vascular diseases;33,34 however, to our knowledge this is the first report demonstrating a direct functional relationship between raised sP-selectin levels in an atherogenic cohort and increased leukocyte adhesion. Whereas our studies do not exclude a functionally important role for other circulating proinflammatory molecules, such as C-reactive protein or sCD40L in regulating leukocyte adhesion,30,35 they nonetheless point to a potentially important role for sP-selectin in this process. Future studies will be required to more fully delineate the role of these various inflammatory markers in regulating leukocyte adhesive function.

Our studies demonstrate that human plasma contains both the monomeric and dimeric forms of sP-selectin and demonstrate that concentrations of the monomeric forms found in vivo can alter leukocyte adhesive function. Monomeric sP-selectin appears to activate leukocytes in a similar manner to its membrane-bound form, through PSGL-1 dependent Src kinase activation, leading to an upregulation in αM adhesion function. Whether sP-selectin dimers or oligomers can further increase neutrophil adhesion due to their reported increased avidity to PSGL-1 remains unknown and will require further investigation. In this context, it was of interest that although the increase in mean sP-selectin levels from control to PAOD patients was ≈60 μg/mL, the enhancing effect of patient plasma on neutrophils was larger than that using isolated neutrophils spiked with recombinant monomeric sP-selectin, raising the possibility that sP-selectin dimers/oligomers and or other proinflammatory cytokines may contribute to the leukocyte adhesion process.

It is well-known that leukocyte adhesion at higher shear rates decreases overall adhesion, which is confirmed within our results; however, a further novel finding in our flow studies was the demonstration that the effects of sP-selectin on neutrophil adhesion were dependent on the prevailing blood flow conditions, such that at very low shear rates (0 to 50 s⁻¹) the enhancing effects on neutrophil adhesion were up to 4-fold greater than at 150 s⁻¹. This observation is potentially important when considering the rheological conditions operating at sites of atherosclerosis. Typically atheromatous plaques develop at sites of arterial bifurcations where disturbances of blood flow are common and shear rates significantly lower than in nonbranching arteries. Such shear rates can also be lower than those encountered in post-capillary venules, the preferred sites where leukocytes adhere and transmigrate into inflamed tissue, raising the interesting possibility that high levels of sP-selectin may preferentially influence leukocyte adhesive function in atherosclerotic-prone regions.38

Our studies showing increases in leukocyte adhesion conflict with previous studies describing decreases in adhesion of activated leukocytes to endothelial cells. However, the previous studies were performed under static conditions and the potential pathophysiological significance of such findings under flow conditions remains unclear. Similarly the concentrations of sP-selectin used in some of these assays are unlikely to occur in vivo. Further, whether similar effects of sP-selectin will be observed on endothelial adhesion under the conditions described here requires further investigation. Previous studies show that drug therapies in vascular disease can alter sP-selectin levels. The current study was not designed to explore the effects of therapy on levels or function, but our data support the hypothesis that lowering plasma sP-selectin through treatment would be beneficial in reducing sP-selectin mediated leukocyte adhesion. Further limitations of this study include the use of in vitro models of leukocyte adhesion and require further investigation of sP-selectin’s role in vivo models of human leukocyte adhesion to both endothelium and platelet thrombi.

### Table 1: Mean±SD Percentage Increase in Neutrophil Adhesion From Controls

<table>
<thead>
<tr>
<th>Shear Rate (s⁻¹)</th>
<th>sP-Selectin-Treated (150 ng/mL)</th>
<th>fMLP-Treated (1 μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>123.5±45.9</td>
<td>304.1±124.1</td>
</tr>
<tr>
<td>50</td>
<td>92.7±30.2</td>
<td>291.1±35.9</td>
</tr>
<tr>
<td>100</td>
<td>64.5±29.8</td>
<td>306.7±25.5</td>
</tr>
<tr>
<td>150</td>
<td>38.5±11.4*</td>
<td>322.3±82.6</td>
</tr>
<tr>
<td>300</td>
<td>10.1±7.4*</td>
<td>310.4±99.2</td>
</tr>
</tbody>
</table>

*P<0.05 from 0 s⁻¹ in each treatment group.

Percentage increase from vehicle control treated neutrophil adhesion to platelet monolayers. The mean percentage increase±SD in cell adhesion after sP-selectin or fMLP treatment compared against vehicle controls (correlates with Figure 7). *P<0.05 from neutrophil adhesion at 0 s⁻¹ in each treatment group, as analyzed by ANOVA followed by Tukey post-test at 5 minutes of perfusion.
In summary, our studies demonstrate a potentially important role for elevated levels of sP-selectin in regulating leukocyte adhesive function in patients with advanced atherosclerotic disease. Although it remains to be established whether such effects enhance leukocyte recruitment to vascular injury and promote disease progression in experimental models, they nonetheless raise the interesting possibility that pharmacological targeting of sP-selectin may represent a novel therapeutic approach to reduce inflammatory infiltration in diseased vessels.

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References

Raised Plasma Soluble P-Selectin in Peripheral Arterial Occlusive Disease Enhances Leukocyte Adhesion

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