**Thrombosis**

**Effect of P-Selectin on Phosphatidylserine Exposure and Surface-Dependent Thrombin Generation on Monocytes**

Ian del Conde, Faisal Nabi, Raúl Tonda, Perumal Thiagarajan, José A. López, Neal S. Kleiman

**Objective**—Stimulation of monocytes with P-selectin induces the synthesis of an array of mediators of inflammation, as well as the expression of tissue factor (TF), the main initiator of coagulation. Because the membrane-bound reactions of coagulation are profoundly influenced by the presence of phosphatidylserine on the membranes of cells, factors that increase its expression may have an impact on coagulation.

**Methods and Results**—Using flow cytometry, we studied the effect of P-selectin on phosphatidylserine expression in blood monocytes and in the monocytic cells, THP-1. Soluble P-selectin at biologically relevant concentrations (0.31 to 2.5 μg/mL) induced a time-dependent increase in phosphatidylserine expression, an effect that could be inhibited with an anti–PSGL-1 blocking antibody, and by genistein, a tyrosine kinase inhibitor. Binding of activated platelets to THP-1 cells also resulted in a significant increase in phosphatidylserine expression that was dependent on PSGL-1. Consistent with the role of phosphatidylserine on surface-dependent reactions of coagulation, treatment of monocytic cells with soluble P-selectin led to increased thrombin generation. We excluded P-selectin induced apoptosis of monocyte as a mechanism for the increased phosphatidylserine exposure.

**Conclusion**—In summary, we show that P-selectin, either soluble or in its membrane-bound form, induces phosphatidylserine exposure in monocytes through a mechanism dependent on PSGL-1. (Arterioscler Thromb Vasc Biol. 2005; 25:1065-1070.)

**Key Words:** monocyte ■ platelet ■ phosphatidylserine ■ phospholipid ■ P-selectin

P-selectin is a transmembrane protein synthesized by platelets and endothelial cells, where it is stored in intracellular granules and is expressed on the plasma membrane only upon cell activation.1 Numerous studies show that P-selectin exerts a prothrombotic effect through its interaction with monocytes.2–4 Within the intravascular space, monocytes may be exposed to P-selectin in different forms, such as that on the surface of activated platelets, which bind to monocytes forming platelet-monocyte complexes;5 to P-selectin on inflamed endothelium;6 to P-selectin present on microparticles shed by activated platelets7 and also possibly by endothelial cells. Lastly, monocytes may also be exposed to a soluble form of P-selectin present in normal plasma, but which has been shown to be increased in various thrombotic diseases.8

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Binding of P-selectin to its main counter-receptor on monocytes, P-selectin glycoprotein ligand-1 (PSGL-1), leads to monocyte activation marked by the conformational activation of the β2 integrin, MAC-1,9 the synthesis and release of various cytokines,10 chemokines,11 and of reactive oxygen species.12 Importantly, binding of P-selectin to PSGL-1 on monocytes also induces the expression of tissue factor (TF),2 the main initiator of coagulation in vivo.

TF expressed on the surface of monocytes initiates coagulation when it binds to the serine protease factor (F) VIIa, forming the extrinsic tenase. This tenase in turn catalyzes the activation of FX, which together with its nonenzymatic cofactor, FVa, forms the prothrombinase complex, leading to thrombin generation. The reaction rates of both the tenase and the prothrombinase complexes are critically dependent on the presence of phosphatidylserine on the surface of cells.13 It is on this anionic phospholipid where the components of the enzymatic complexes of coagulation assemble. Kinetic studies reveal that the interaction between the enzyme and substrate with the membrane significantly affects the reaction rate of the tenase and prothrombinase complexes.14,15 Abrogation of the catalytic component provided by the phosphatidylserine-rich surface decreases the reaction rate of both the extrinsic tenase and prothrombinase complex by ~200-fold and 1000-fold, respectively.14,15 Likewise, increased phosphatidylserine exposure is likely to be a major contributor to the increased prothrombinase activity in endotoxin-stimulated monocytes.16 It is thus conceivable that factors affecting the extent of phosphatidylserine exposure on cells...
result in substantial modifications in the rate of thrombin generation. In the current study, we show that P-selectin binding to PSGL-1 induces phosphatidylserine exposure and increased surface-dependent thrombin generation on monocytes.

Materials and Methods
Soluble recombinant human P-selectin (sP-selectin) was a generous gift from Dr Andrew Weyrich (University of Utah). This P-selectin consists of the ectodomain of the mature protein (it lacks the transmembrane domain and cytoplasmic tail) and is monomeric, confirmed by subjecting the protein to electrophoresis in a nondenaturing, nonreducing polyacrylamide gel, and visualizing a single band with a molecular weight of 120 kDa. We also tested this P-selectin for endotoxin contamination using the limulus amebocyte lysate test (Associates of Cape Cod, East Falmouth, Mass) and found it to contain very low levels (<0.03 endotoxin U/mL) of endotoxin. The well-characterized monocytic cell line, THP-1, was from American Type Culture Collection (ATCC, Manassas, Mass). RPMI 1640 medium was from Invitrogen (Carlsbad, Calif). The PSGL-1–blocking mouse monoclonal antibody, KPL-1, was a generous gift from Dr Karen R. Snapp (University of Illinois at Chicago). Nonspecific mouse IgG was purchased from Pierce (Rockford, Ill). Annexin-V was produced as previously described. Some of the annexin-V was conjugated with the fluorophore fluorescein isothiocyanate (FITC) using the FluoroReproter protein labeling kit (Molecular probes, Eugene, Ore), following the manufacturer’s instructions. All other reagents were purchased from Sigma (St. Louis, Mo).

Isolation of Platelets and Monocytes
Blood was drawn from healthy individuals into a sodium citrate (0.38%, final concentration)-anticoagulated syringe. All donors gave their informed consent under a protocol approved by the institutional review board of Baylor College of Medicine. Platelets were isolated by centrifuging blood at 700 g for 15 minutes and were then resuspended in Tyrode buffer, pH 7.4, with 2 mmol/L CaCl2. By light microscopy and Wright staining, the monocyte purity was 80%; lymphocytes were the most common contaminating cells.

Annexin V Binding in Monocytes Stimulated With P-selectin
Annexin-V is a 34-Kd protein that binds phosphatidylserine with high affinity and specificity in a Ca2+-dependent manner. Annexin V binding is therefore a well-established method for measuring phosphatidylserine exposure. We incubated 107 fixed “phosphatidylserine-exposed” platelets with a density of 1.068 g/mL Optiprep (Accurate, Westbury, NY) and carefully overlaid with 2 mmol/L CaCl2. The sample was centrifuged at 2000 g for 30 minutes, and the sample was taken, cells were sedimented at 2000 g for 5 minutes, removing all unbound platelets in the supernatant. Cells were resuspended in TBS with 2 mmol/L CaCl2 and then analyzed by flow cytometry, as described.

Thrombin Generation
THP-1 cells (1×106) were treated with either 2.5 μg/mL sP-selectin or buffer control, at 37°C. At the indicated time points, an aliquot of the sample was taken, cells were sedimented at 2000g for 5 minutes, and gently resuspended in equal volumes of Tris–human serum albumin buffer with calcium (0.05 mol/L Tris-HCl, 0.12 mol/L NaCl, 3 mmol/L CaCl2, 0.5 mg/mL human serum albumin). Suspended cells were transferred onto a glass cuvette with a Teflon-coated stir bar, stirring at 300 rpm, at 37°C. A mixture of 6 nM Xa and 3 nM Va (both final concentrations) in Tris–human serum albumin buffer was added, and 1 minute later, 4 μmol/L purified prothrombin was added. The reaction was allowed to continue for 5 minutes. Aliquots (25 μL) of the reaction sample were then taken and quenched by adding them to 1 mL of TBS with 2 mmol/L EDTA. The amount of thrombin generated was measured by adding 25 μL of thrombin-specific chromogenic substrate (S-2338; Chromogenix). The changes in color were recorded in a microplate reader at an absorbance of 405 nm.

Annexin V Binding in Monocytes Stimulated With Activated Platelets
Washed platelets were prepared as described. Platelets (2×107) suspended in 100 μL TBS with 2 mmol/L CaCl2 were either left resting or activated with 10 μmol/L thrombin receptor-activating peptide (TRAP-SFLNLRC) for 15 minutes at 37°C. Phosphatidylserine on the platelets was “cloaked” by incubating the platelets with 100 μg/mL unlabeled annexin V for 30 minutes at room temperature. Samples were then fixed with paraformaldehyde 1% for 45 minutes at 4°C, and washed 3 times in 15 mL TBS with 2 mmol/L CaCl2. P-selectin expression in the fixed, activated platelets was confirmed by flow cytometry using a FITC-conjugated anti–P-selectin antibody (Becton Dickinson, Franklin Lakes, NJ). Also, complete blockade of annexin-V binding sites was demonstrated by showing no additional binding of FITC-annexin-V to these platelets above background levels set with a sample treated with 20 mmol/L EDTA. THP-1 cells (5×105) were incubated with 2×107 fixed “phosphatidylserine-cloaked” washed platelets for 8 hours at 37°C, and then sedimented at 2000g for 5 minutes, removing all unbound platelets in the supernatant. The data were analyzed using ANOVA to assess the effects of either KPL-1 (5 μg/mL) or mouse IgG (5 μg/mL) for the indicated time points, at 37°C. The same protocol was used for propidium iodide representing apoptosis. Cells treated for 8 hours at 37°C with 500 ng/mL of actinomycin D served as a positive control for apoptosis.

Statistical Analysis
The data were analyzed using ANOVA to assess the effects of various treatments of THP-cells and monocytes on their expression size particles (15 to 20 μm), and not cell fragments. To set the background fluorescence, we treated samples with 20 mmol/L EDTA, because in the absence of calcium, annexin-V does not bind phosphatidylserine. In some experiments, cells were treated for 5 minutes with either 100 μmol/L genistein, a tyrosine kinase inhibitor, or with an equal volume of DMSO as a vehicle control, before the addition of P-selectin. Results are presented as fold increases over control and expressed numerically as arbitrary units of mean fluorescence intensity (MFI).
Soluble P-Selectin Induces Phosphatidylserine Exposure in Monocytes Via PSGL-1

We next investigated the identity of the molecule on the monocytic cell surface involved in P-selectin–induced phosphatidylserine exposure. The obvious candidate was P-selectin glycoprotein ligand-1, or PSGL-1, because this receptor is known to mediate the earliest adhesive interactions between activated endothelial cells or platelets and monocytes, and also because it has been shown in the past to mediate other P-selectin–induced responses in monocytes. We therefore tested the effect of the PSGL-1 blocking monoclonal antibody, KPL-1, on P-selectin–induced phosphatidylserine exposure on monocytes. Pretreatment of THP-1 cells with 10 μg/mL KPL-1 prevented almost all of the phosphatidylserine exposure induced by sP-selectin. In contrast, phosphatidylserine expression in cells treated with nonspecific mouse IgG was not significantly different than that of sP-selectin alone (Figure 1d).

P-Selectin on the Surface of Activated Platelets Induces Phosphatidylserine Exposure

Next, because several important differences may exist between membrane-bound P-selectin and its soluble, monomeric form, for example, in their ability to cluster PSGL-1, we investigated the effect of P-selectin on the surface of activated platelets on phosphatidylserine exposure on monocytic cells. For this, we prepared fixed, activated, washed platelets expressing P-selectin on their surfaces. Because activated platelets also express phosphatidylserine, we cloaked all annexin-V binding sites on platelets by incubating them with saturating concentrations of unlabeled annexin-V. TRAP-activated, but not resting, platelets prepared in this manner expressed a considerable amount of P-selectin (Figure 2a) but did not bind fluorescently labeled annexin-V (Figure 2b). Incubation of THP-1 cells with resting platelets for 8 hours led to a small but consistent increase in phosphatidylserine exposure relative to THP-1 cells treated with buffer alone (MFI, 70.2 versus 38.4). In contrast, THP-1 cells that were incubated with activated platelets had a significant 3.4-fold increase in their expression of phosphatidylserine relative to the control sample (MFI, 132.1 versus 38.4; P=0.04) (Figure 2c). Similar to the experiments using soluble P-selectin, KPL-1 prevented the expression of phosphatidylserine on the outer membrane leaflet of the THP-1 cells, indicating that PSGL-1 is also required for the P-selectin–induced effects (Figure 2c).

Tyrosine Phosphorylation Induced by P-selectin Binding to PSGL-1 Is Required for Phosphatidylserine Exposure

It is well-established that P-selectin binding to PSGL-1 induces intracellular signaling in neutrophils and monocytes, with concomitant protein tyrosine phosphorylation. We investigated whether transmembrane signaling through ligated PSGL-1 was required for phosphatidylserine exposure on THP-1 cells. Treatment of cells with 100 μmol/L

Results

Soluble P-Selectin Induces Phosphatidylserine Exposure in Monocytes in a Concentration-Dependent Manner

We tested whether soluble P-selectin (sP-selectin) induces phosphatidylserine exposure in the monocytic cells, THP-1. Cells were incubated for 8 hours with either a buffer control or increasing concentrations of sP-selectin that ranged from 0.15 μg/mL to 2.5 μg/mL. Under baseline conditions, THP-1 cells had a relatively small amount of phosphatidylserine on their surfaces (MFI, 21±11). Phosphatidylserine exposure increased with increasing sP-selectin concentrations, becoming discernible at concentrations >0.31 μg/mL (MFI, 39±13), and reaching a plateau at concentrations >1.25 μg/mL (MFI, 84±16) (Figure 1a).

Soluble P-Selectin–Induced Phosphatidylserine Exposure in Monocytes Is Time-Dependent

To determine the time course of the P-selectin–induced phosphatidylserine exposure on monocytes, we treated THP-1 cells with either a buffer control or 2.5 μg/mL of sP-selectin for up to 24 hours. Within the 1 hour incubation period, cells treated with sP-selectin exhibited a 1.2-fold increase in phosphatidylserine expression relative to untreated cells (MFI, 40.7 versus 18.3; n=3; P=0.02). This difference increased in a time-dependent manner, until it leveled-off at 8 hours with sP-selectin–treated cells having, on average, a phosphatidylserine expression that was 4.4-fold greater than that in control cells, and that persisted for at least 24 hours (Figure 1b). Similar findings were obtained with freshly isolated blood monocytes, recapitulating our observations in THP-1 cells (Figure 1c).

Figure 1. Soluble P-selectin induces phosphatidylserine exposure in monocytes. Expression of phosphatidylserine was determined by flow cytometry using FITC-annexin V. A, THP-1 cells were incubated for 8 hours with either a buffer control or with increasing concentrations of sP-selectin that ranged from 0.15 μg/mL to 2.5 μg/mL. B, THP-1 cells with either a buffer control or 2.5 μg/mL of sP-selectin for up to 24 hours. C, Treatment of freshly isolated monocytes with 2.5 μg/mL sP-selectin led to a marked increased phosphatidylserine exposure. D, THP-1 cells were treated with 10 μg/mL of the anti-PSGL-1 monoclonal antibody, KPL-1, or nonimmune mouse IgG, before being treated with 2.5 μg/mL of sP-selectin for 8 hours (n=4; * P=0.02 vs baseline; ** P=0.01 vs sP-selectin).
genistein, a tyrosine kinase inhibitor, completely prevented the increased phosphatidylserine expression elicited by sP-selectin (Figure 3).

P-Selectin Increases Surface-Dependent Thrombin Generation on Monocytes

Our findings indicated that P-selectin engages PSGL-1 and induces phosphatidylserine exposure on the surface of monocytes. Because there is ample evidence that this anionic phospholipid plays a critical role in surface-bound reactions of coagulation, we studied the effect of sP-selectin on surface-dependent thrombin generation. For this, we used a reconstituted system in which all of the soluble components of the prothrombinase complex (factors Xa, Va, and prothrombin) are in high concentrations and in the presence of calcium ions. This leaves the surface on which these coagulation factors assemble as the only variable determining thrombin generation during the course of our experiments. THP-1 cells were treated for 0, 1, 6, and 12 hours with 2.5 μg/mL sP-selectin or a buffer control. Cells treated with sP-selectin generated more thrombin than untreated cells, with the difference becoming discernible at 1 hour incubation and increasing in a time-dependent manner (Figure 4). By 12 hours of incubation, sP-selectin–treated cells generated 2.5-fold more thrombin than did control cells (OD 405 nm: 0.646 versus 0.254; n = 3; *P = 0.01) (Figure 4).

P-Selectin Does Not Induce Apoptosis in Monocytes

One possible explanation for the P-selectin–induced phosphatidylserine exposure we found is that prolonged stimulation of monocytes with P-selectin stimulates them to undergo apoptosis. We addressed this possibility by treating monocytes for 8 hours with either a buffer control or 2.5 μg/mL of sP-selectin. Cells treated with actinomycin D, a chemotherapeutic agent known to induce apoptosis, served as a positive control for inducing apoptosis. The number of apoptotic cells in the control and sP-selectin–treated samples was comparable (2.28% versus 3.0%, respectively; *P = 0.75; n = 3). In contrast, 12.59% of cells treated with actinomycin D underwent apoptosis (Figure 5).
Discussion

Phosphatidylserine on the outer membranes of cells serves many important biological roles, such as supporting the assembly of the macromolecular complexes of coagulation, and as a signal for the removal of apoptotic bodies or cells from the circulation. In the current studies, we investigated whether P-selectin induces phosphatidylserine expression in monocytes. Within the circulation, monocytes are frequently exposed for prolonged periods of time to P-selectin, either in its soluble form or associated with cell membranes, such as those on activated platelets, activated endothelial cells, or on microparticles shed by either of these 2 types of cells. We have found that P-selectin induces phosphatidylserine exposure on monocytes. Monocytes stimulated with biologically relevant concentrations of soluble P-selectin had a significant increase in their phosphatidylserine expression. This effect became evident at s-selectin concentrations >0.31 µg/mL, and reached a plateau at concentrations >1.25 µg/mL. Because the normal plasma concentrations of s-selectin are generally <0.200 µg/mL, it is unlikely that this phenomenon occurs in a significant degree in healthy individuals. However, in diseases characterized by elevated levels of s-selectin in plasma, such as in acute coronary syndromes or in acute lung injury in which levels of s-selectin were reported to reach up to 0.842 µg/mL, it is likely that the s-selectin–induced phosphatidylserine exposure on monocytes becomes significant. Importantly, the recombinant soluble P-selectin we used consists exclusively of the extracellular domain of the protein and is monomeric, thus resembling the soluble P-selectin that is present in plasma and which was characterized by Dunlop et al.

Monocytic cells stimulated with s-selectin expressed phosphatidylserine within 1 hour of stimulation, reached a plateau at 8 hours, and persisted for up to 24 hours. Taking this time course in the context of the findings of Celi et al, who showed that s-selectin–induced TF expression in monocytes peaks at 6 hours, it is likely that s-selectin–induced TF expression coincides with increased phosphatidylserine exposure. Because the efficiency by which TF initiates coagulation is greatly influenced by phosphatidylserine on the cell surface, increased phosphatidylserine expression induced by P-selectin may have a significant impact on the initial phases of coagulation occurring on the surface of TF-bearing monocytes. However, given these long time courses, it is unlikely that such a mechanism plays an important role in the acute thrombosis in response to vessel injury. Instead, P-selectin–induced phosphatidylserine exposure in monocytes may contribute to a more systemic hypercoagulable state. Such an effect may be advantageous in settings such as autoimmune thrombocytopenia, in which a bleeding tendency coexists with elevated levels of plasma sP-selectin.

Treatment with the anti–PSGL-1 blocking monoclonal antibody, KPL-1, prevented nearly all the exposure of phosphatidylserine induced by sP-selectin, indicating that PSGL-1 is the receptor on the monocyte surface mediating the effects elicited by sP-selectin. There is internal consistency between these findings and those of several studies. It has been shown both in vitro and in vivo that monocytes stimulated with P-selectin shed microvesicles. Because the outer membrane leaflet of the microvesicles is rich in phosphatidylserine, this phospholipid is likely to have also been present on the surface of the monocytes at the time of microvesicle shedding.

In addition to sP-selectin, monocytes in flowing blood may also be exposed for prolonged periods of time to membrane-bound P-selectin, either in P-selectin–bearing microparticles, or on activated platelets forming platelet-monocyte complexes. We found that similar to its soluble form, P-selectin on platelets increased phosphatidylserine expression on monocytes through a mechanism dependent on PSGL-1. These findings are particularly relevant to diseases in which circulating platelet–monocyte complexes increase, such as in acute coronary syndromes or in percutaneous coronary intervention. Increased exposure of phosphatidylserine on monocytes forming these complexes may play various roles. For example, increased expression of phosphatidylserine on platelet–monocyte complexes may promote coagulation and may also mark the complexes for their clearance by the reticuloendothelial system, similar to the way in which apoptotic bodies are removed from the circulation.

Because we used washed platelets in the experiments using activated platelets as a source of P-selectin, the effects observed are unlikely to be the result of soluble mediators released by activated platelets. Although our data point out that PSGL-1 is required for phosphatidylserine exposure induced by activated platelets, it is likely that the P-selectin–PSGL-1 adhesive interaction serves as an initial docking system allowing other platelet membrane proteins to interact with receptors on monocytes, inducing (or inhibiting) the exposure of phosphatidylserine on the monocyte. For example, binding of activated platelets to monocytes via P-selectin–PSGL-1 would allow the cross-linking of other receptor-ligand pairs (eg, CD40–CD40L) known to activate monocytes. Similarly, one possibility is that the P-selectin–induced phosphatidylserine exposure is not a direct effect of signaling through ligated PSGL-1, but rather the effect of mediators (eg, tumor necrosis factor-α) released by the monocyte when stimulated with P-selectin. The small amount of phosphatidylserine expression on THP-1 cells
incubated with unstimulated platelets very likely resulted from a slight degree of activation of the platelets that occurred during their preparation. This interpretation is supported by the observation that an anti–PSGL-1 antibody reduced phosphatidylserine exposure in the cells incubated with activated platelets to levels below those observed in THP-1 cells incubated with unstimulated platelets.

Consistent with the role of phosphatidylserine in prothrombinase activity, sP-selectin–treated cells had increased surface-dependent thrombin generation. Although the sP-selectin–induced increase in thrombin generation is most probably a direct consequence of increased phosphatidylserine exposure, other effects cannot be ruled out. Signaling through PSGL-1 may induce the synthesis of membrane proteins that facilitate the binding and assembly of the prothrombinase complex to the monocyte membrane.

In summary, we have shown that P-selectin binding to PSGL-1 induces phosphatidylserine exposure and increases surface-dependent thrombin generation on monocytes. These studies reveal an additional mechanism by which P-selectin and PSGL-1 induces phosphatidylserine exposure and increases prothrombinase complex to the monocyte membrane. Through PSGL-1 may induce the synthesis of membrane proteins probably a direct consequence of increased phosphatidylserine exposure, other effects cannot be ruled out. Signaling through PSGL-1 may induce the synthesis of membrane proteins that facilitate the binding and assembly of the prothrombinase complex to the monocyte membrane.

References


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