Affinity and kinetics of P-selectin binding to heparin

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Summary
P-selectin (CD62P), expressed on stimulated endothelial cells and activated platelets, reacts with P-selectin glycoprotein ligand-1 (PSGL-1, CD162) for leukocyte rolling. It also binds to heparin and heparan sulfate proteoglycans (HSPGs), which attenuates P-selectin mediated adhesions of leukocytes and cancer cells. Here we report that P-selectin mediated adhesion, but not rolling, of the HSPGs bearing human malignant melanoma A375 cells under shear stress. To understand its underlying molecular mechanism, we measured the biophysical properties of this interaction. Heparin inhibited the adhesion of A375 cells to immobilized P-selectin under flow (IC_{50} = 3 µM heparin) and neutralized the binding of P-selectin to A375 cells (IC_{50} = 4 µM heparin). Using surface plasmon resonance technique, we found that P-selectin bound to heparin with a dissociation constant (K_{d}) of 115 ± 6 nM. The measured off rate (k_{off}) was 3.15 ± 0.34 × 10^{-3} s^{-1} and the calculated on rate (k_{on}) was 2.75 ± 10^{4} M^{-1} s^{-1}. Taken together, our data suggest that the very slow k_{off} and the reduced k_{on}, but apparently not the K_{d}, are responsible for adhesion, but not rolling of A375 cells, to P-selectin under flow.

Keywords
P-selectin, heparin, heparan sulfate proteoglycans, affinity, kinetics

Introduction
In inflammatory responses, leukocytes are recruited to the site of infection or tissue injury, where they function as “a double-edged sword”, either fighting against invading pathogens or causing own tissue damage. The recruitment of leukocytes involves a cascade of cellular events, including rolling, weak and firm adhesion, diapedesis, transendothelial migration and chemotaxis. The binding of selectins (CD62) to their cognate glycoprotein ligands mediates rolling and weak adhesion of leukocytes. Consequently, the interaction of integrins with the immunoglobulin superfamily of cell adhesion molecules mediates firm adhesion and signal transduction, which eventually triggers diapedesis and transendothelial migration of leukocytes. The emigrated leukocytes are then guided by several large families of soluble chemoattractants to migrate to their destinations where the insult occurs. It is generally believed that the selectin family of cell adhesion molecules mediates the first step of cell-cell interactions during the recruitment of leukocytes (1-5).

P-selectin is a member of the selectin family of cell adhesion molecules. It is a pre-synthesized protein stored in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets. Upon inflammatory and thrombogenic challenges, P-selectin rapidly translocates from these intracellular granules to the cell surfaces of endothelial cells and platelets by exocytosis (in seconds). Further, P-selectin can be up-regulated by de novo synthesis in the cytokine stimulated endothelial cells (in hours; 1-5). L-selectin (CD62L) and β-2 leukocyte integrin, Mac-1 (α_{M}β_{2}, CD11b/CD18, CR3), are constitutively expressed...
receptors on most leukocytes. After leukocyte activation, L-selectin is shed from the cell surface while Mac-1 transforms from the resting state (low affinity) to the activated state (high affinity) for its ligands, such as ICAM-1 (Intercellular Cell Adhesion Molecule-1, CD54) and fibrinogen.

Heparin and its analog, HSPGs, have been shown to bind to cell adhesion molecules, such as P-selectin, L-selectin and Mac-1. These bindings can inhibit adhesion of leukocytes mediated specifically by these cell adhesion molecules in vitro and in vivo (6-12). Recently, we have shown that the cell surface HSPGs could directly mediate adhesion of human malignant melanoma A375 cells and human tongue squamous cancer Tca-8113 cells to P-selectin under flow (13). However, we observed that although human promyeloid HL-60 cells rolled on and adhered to P-selectin (a P-selectin/PSGL-1 pair), A375 cells and Tca-8113 cells only adhered to, but not rolled on, P-selectin (a P-selectin/heparin pair) under flow. Similar manifestations were also reported previously (14, 15). To understand the mechanisms governing for these distinctive cellular behaviors on the same substrate of P-selectin, we sought to measure the binding affinity and kinetics of the interaction of P-selectin with heparin, which might provide a biophysical explanation for these divergent cellular phenotypes.

Materials and methods

Materials

The same lot of heparin (sodium salt grade I-A from porcine intestinal mucosa, Cat. No. H3393, Lot No.98H0713, Sigma) was used throughout the entire experimental study. Its molecular mass was 19.5 kDa and its sulfate contents (the main constituent for its charges) were 11.75% absolute content of total sulfate and 3.25% absolute content of N-sulfate (each heparin anion). Charge densities for these divergent cellular phenotypes.

Proteins and antibodies

P-selectin receptor-globulin (P-selectin Rg; constructed by fusing the lectin domain, the epidermal growth factor-like domain and the first two complement protein-like repeats of P-selectin with the F domain and the first two complement protein-like repeats of P-selectin with heparin, which might provide a biophysical explanation for these divergent cellular phenotypes.

Cell culture

Human cell lines of promyeloid HL-60 cells (CCL 240) and malignant melanoma A375 cells (CRL 1619) were purchased from American Tissue Culture Collection (Rockville, MD). They were cultured in RPMI 1640 medium (GIBCO BRL, Shanghai, China) supplemented with 10% heat inactivated newborn bovine calf serum (BCS), 4 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in the presence of 5% CO₂.

SDS-PAGE and gel filtration chromatography

The purified P-selectin Rg was resuspended in the SDS sample buffer with or without 2% β-mercaptoethanol and boiled for 5 min. Samples were loaded on 7% SDS-polyacrylamide gel (10 µg/lane) for electrophoresis. They were stained with Coomassie brilliant blue. To determine the apparent molecular mass of P-selectin Rg in a non-denatured condition, P-selectin Rg (50 µg) was resuspended in 10 µl PBS (phosphate buffered saline, pH 7.4) and injected to a Superdex 200 column™ (Smart System®; Amersharm Pharmacia Biotech, Uppsala, Sweden) that had been pre-equilibrated with PBS. Samples were run at 50 µl/min at 22°C. The gel-filtration chromatography was calibrated by blue dextran (~2,000 kDa), horse spleen apoferritin (~443 kDa), sweet potato β-amylase (~200 kDa), yeast alcohol dehydrogenase (~150 kDa), and phenol red (0.354 kDa) (all protein markers were purchased from Sigma, St. Louis, MO).

Laminar flow assay

Polystyrene slides were coated with 2 ml human IgG (Sigma) or P-selectin Rg (both at 0.1 µg/ml) in 20 mM Tris-HCl, pH 9.5, 140 mM NaCl and 0.02% NaN₃ at 4°C overnight and blocked with 3% bovine serum albumin (BSA) in PBS at 22°C for 2 h. Slides were mounted on the stage of an inverted phase contrast Olympus microscope (Olympus Optical Co. Ltd., Tokyo, Japan). It was connected to a time-lapse videocassette recorder STLV-24P (Samsung Electronics, Suwon, Korea) using a Panasonic color CCTV camera vv-GP410/G (Matsushita Communication Industrial Co. Ltd., Okasa, Japan). HL-60 cells and A375 cells were washed and resuspended at 0.5 x 10⁶/ml in PBS supplemented with 10 mM HEPES, pH 7.4, 2 mM CaCl₂ and 2 mM MgCl₂. They were injected through the flow chamber at 0.5 dyne/cm² using a syringe pump at 22°C. The potential interactions between the cellular Fc receptors and the Fc domain of P-selectin Rg were eliminated by preincubation of the cells with 10 µg/ml of human IgG at 22°C for 20 min. The adhesive cells were quantified from videotape recordings of 10-20 fields of view obtained (3 min after flowing cells through the chamber) while scanning the lower plate of the flow chamber using a 10x objective lens. For analysis of rolling velocity, the critical velocity was defined as the velocity of a non-interacting cell in a shear flow near the wall of the flow chamber, cells with a translational velocity lower than the critical velocity were defined as rolling (19). For antibody inhibition experiments, the immobilized P-selectin Rg was preincubated with 0.3 µg/ml G1 F(ab')₂ or PS1 F(ab')₂ at 22°C for 20 min (13). For EDTA inhibition experiment, 2 mM EDTA, instead of 2 mM CaCl₂ and 2 mM MgCl₂, was used in the running buffer.
Site density determinations
The density of the immobilized P-selectin Rg was determined using $^{125}$I-labeled PS1 (20). Briefly, 96-well culture plates were coated with P-selectin Rg (40 µl/well, 0.1 µg/ml) and blocked as described above. The $^{125}$I-labeled PS1 (40 µl/well, 2 µg/ml) in PBS containing 0.1% BSA was incubated for 1 h at 22°C. The wells were washed five times and then counted in a γ counter. Specific bindings were obtained after subtraction of the bindings to the BSA-coated wells. Site densities were calculated assuming the monovalent binding of the antibody at saturation (20). All assays were performed in quadruplicate in three separate experiments.

Flow cytometric assay
The adherent A375 cells were detached by PBS containing 0.02% EDTA (Verene®; GIBCO BRL), washed once with PBS, and resuspended in PBS/BSA (PBS supplemented with 2 mM CaCl₂, 2 mM MgCl₂ and 1% BSA; 1 × 10⁶ cells/ml). Each aliquot (0.5 ml) of cells was incubated with 1 µg human IgG or P-selectin Rg followed by 1 µg of an FITC-conjugated Ab against human IgG (Pierce) at 22°C for 1 h with end-to-end rotation. Cells were centrifuged at 1,500 rpm for 5 min and supernatants were discarded. For antibody inhibition experiments, 1 µg P-selectin Rg was preincubated with 3 µg of G1 F(ab')₂ or PS1 F(ab')₂ in 50 µl PBS/BSA at 22°C for 30 min. For heparin inhibition experiments, 1 µg P-selectin Rg was preincubated with the indicated amounts of heparin in 50 µl PBS/BSA at 22°C for 30 min. Each aliquot was then resuspended in 0.5 ml PBS/BSA for immediate flow cytometric analysis (FACScan®; Becton Dickinson & Co., Mountain View, CA).

Heparin conjugation
Unfractionated heparin was end-conjugated to BSA by a method described for the synthesis of celllobiose conjugates (21, 22). Briefly, 9.12 mg heparin and 34 mg BSA were dissolved in 5 ml of 200 mM potassium phosphate buffer, pH 8.0. Sodium cyanoborohydride (25 mg) was then added and the mixture was incubated at 37°C for 2 days. The mixture was dialyzed thoroughly against H₂O, lyophilized to the dryness and reconstituted in 250 mM bicarbonate buffer, pH 8.0. The resulting high molecular weight complex was separated from unconjugated BSA and free heparin, by gel-filtration chromatography, on a Sepharose 4B column (1.6 × 100 cm; Amersham Pharmacia Biotech). As a mock control, BSA (without heparin) was treated with sodium cyanoborohydride under identical conditions, as described above.

Surface plasmon resonance measurements
The mock BSA and the heparin-conjugated BSA were biotinylated with N-hydroxysuccinimidobiotin (Sigma), as previously described (23). Surface plasmon resonance experiments were conducted at 22°C with a BIACore™ biosensor (Pharmacia Biosensor, Uppsala, Sweden) that was upgraded to model 1000 specifications. The running buffer was 10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.005% detergent-P20, which had been passed through 0.2 µm filters and degassed. The biotinylated mock BSA and the biotinylated heparin-conjugated BSA were captured on SA5 sensor chips containing immobilized streptavidin on their surfaces at a flow rate of 10 µl/min for 1 min. A typical immobilization resulted in the coupling of the heparin-conjugated BSA at a
density of 0.4 ng/mm² (400 RU). For measurements of binding affinity and kinetics, P-selectin Rg, at various concentrations, were injected at a flow rate of 20 µl/min for 30 sec, followed by a 1-min dissociation phase, when only running buffer was passed over the surface. Identical results were obtained when different amounts of P-selectin Rg were injected at 10 µl/min or when different lots of P-selectin Rg preparations were used (data not shown). The bound P-selectin Rg was removed by injection of 5 µl of 10 mM EDTA when less than 50 nM P-selectin Rg was used or 5 µl of 20 mM SDS when more than 50 nM P-selectin Rg was used. For antibody inhibition experiments, 3 µg/ml P-selectin Rg was preincubated with 9 µg/ml G1 F(ab')2 or PS1 F(ab')2 at 22°C for 20 min. For EDTA inhibition experiment, 2 mM EDTA, instead of 2 mM CaCl₂ and 2 mM MgCl₂, was used in the running buffer. Data on equilibrium binding were analyzed by the nonlinear curve fitting of Langmuir binding isotherm to the primary data using BIAevaluation software (Pharmacia Biosensor) and by the linear regression analysis of Scatchard plots. Recombinant P-selectin Rg used here was calculated as a dimer (24, 25), according to the determination data of gel-filtration chromatography (Fig. 1B).

Results

Characterization of recombinant P-selectin Rg

In this study, we investigated the biophysical properties for the interaction of P-selectin with heparin. Figure 1A shows the Coomassie brilliant blue staining of recombinant human P-selectin Rg following electrophoresis on a polyacrylamide gel. It had apparent molecular masses of ~85 kDa under reducing conditions and ~170 kDa under non-reducing conditions. Figure 1B illustrates a single peak of the gel-filtrated P-selectin Rg on a Superdex 200 column™ (Smart® System). This peak was larger than β-amylase (~200 kDa), but smaller than apoferritin (~443 kDa), indicating that P-selectin Rg was a dimeric molecule. No earlier elution peaks were observed, suggesting that there had no detectable oligomers of P-selectin Rg existed. To further make sure that P-selectin Rg used in this study was not oligomeric, all binding experiments were
performed using the peak fractions of P-selectin Rg within 48 h of the gel filtration chromatography.

**Adhesion, but not rolling, of A375 cells to P-selectin**
We then compared the cellular behaviors of human promyeloid HL-60 cells and human malignant melanoma A375 cells on immobilized P-selectin Rg (the calculated density was 24.7 ± 0.9 molecules/μm²) under flow conditions. As shown in Figures 2A and B, HL-60 cells and A375 cells adhered avidly to immobilized P-selectin Rg under shear stress. These adhesions of HL-60 cells and A375 cells were abrogated by G1 F(ab')2 (a leukocyte adhesion blocking mAb to P-selectin) and EDTA (a chelator for divalent cations), but not by PS1 F(ab')2 (a leukocyte adhesion non-blocking mAb to P-selectin). Interestingly, only HL-60 cells, but not A375 cells, could roll avidly on immobilized P-selectin Rg under flow (Figs. 2C and D). Again, the rolling of HL-60 cells on immobilized P-selectin Rg was abolished by G1 F(ab')2 and EDTA, but not by PS1 F(ab')2. Figures 3A and B show that, in this laminar flow assay, heparin inhibited adhesions of HL-60 cells and A375 cells to P-selectin Rg in dose-dependent manners (IC₅₀ = 10 μM heparin for HL-60 cells and IC₅₀ = 4 μM heparin for A375 cells).

**Figure 3:** Heparin inhibits adhesion of A375 cells to P-selectin under flow. The immobilized P-selectin Rg was preincubated with various amounts of heparin as indicated. HL-60 cells (A) and A375 cells (B) in the presence of the same amounts of heparin were perfused through the slides pretreated with the same amount of heparin, as described above. All results were expressed as the mean ± S.D. values determined in ten to twenty measurements of four to five separate experiments.

**Figure 4:** Heparin inhibits the binding of P-selectin to A375 cells. A). A375 cells were incubated with human IgG or P-selectin Rg (designated as P-Rg) and a FITC-conjugated Ab against human IgG. For antibody inhibition experiments, P-selectin Rg was preincubated with G1 F(ab')2 (a leukocyte adhesion blocking mAb to P-selectin) or PS1 F(ab')2 (a leukocyte adhesion non-blocking mAb to P-selectin). The binding events were analyzed by flow cytometry (FACScan; Becton Dickinson & Co., Mountain View, CA). B). P-selectin Rg was preincubated with heparin at various concentrations, prior to addition of the washed A375 cells and a FITC-conjugated Ab against human IgG. Results were presented as histograms of the log fluorescence intensities from 10⁴ cells from the representative of three to five independent experiments.
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and IC$_{50}$ = \(3 \mu M\) heparin for A375 cells). These results confirmed the earlier reports from others (14, 15) that unlike HL-60 cells, A375 cells could adhere to, but could not roll on, P-selectin. They further suggested a reasonably high affinity for the interaction of P-selectin with heparin. It should be pointed out that although G1 F(\(ab'^\)2) and EDTA inhibited the adhesions of HL-60 cells (Fig. 2A) and A375 cells (Fig. 2B) to the base lines, heparin, at as high as 1 mM concentration, apparently did not completely abrogate their adhesions to immobilized P-selectin (Fig. 3A and B), indicating that there had the multifaceted and/or non-specific effects of heparin in this laminar flow assay system.

Heparin inhibits P-selectin binding to A375 cells

To support the above findings, we employed a cell-surface binding assay to measure the inhibitory effects of heparin on the binding of P-selectin Rg to A375 cells. In this assay, a FITC-conjugated Ab to human IgG was used to report the binding events by flow cytometry. Figure 4A shows that compared to human IgG, P-selectin Rg bound robustly to A375 cells. Preincubation of P-selectin Rg with G1 F(\(ab'^\)2) (a leukocyte adhesion blocking mAb to P-selectin), but not with PS1 F(\(ab'^\)2) (a leukocyte adhesion non-blocking mAb to P-selectin), attenuated this binding, demonstrating the specificity for this binding assay. Using this assay, we carried out the dose course of heparin inhibition experiments. Fig. 4B shows that heparin potently interfered with the binding of P-selectin Rg to A375 cells (IC$_{50}$ = \(4 \mu M\) heparin).

**Kinetics of P-selectin binding to heparin-conjugated BSA**

Using surface plasmon resonance technique, we further measured, in real-time, the affinity and kinetics of P-selectin binding to heparin-conjugated BSA. In our preliminary experiments, we found that it was difficult to covalently couple heparin-conjugated BSA in a sufficient quantity to a CM5 sensor chip through its primary amino groups (data not shown). Thus, we had to biotinylate the heparin-conjugated BSA and then to capture it on a SA5 sensor chip carrying immobilized streptavidin on its surface.

Injection of P-selectin Rg over the heparin-conjugated BSA surface, but not over the unconjugated BSA surface, caused a
binding response (Fig. 5A). In contrast, injections of human IgG over the heparin-conjugated BSA surface did not induce a significant binding response (data not shown). Preincubation of P-selectin Rg with G1 F(ab’)2 (a leukocyte adhesion blocking mAb to P-selectin), but not PS1 F(ab’)2 (a leukocyte adhesion non-blocking mAb to P-selectin), blocked the binding. Further, EDTA, a chelator for divalent cations, also abrogated this binding.

As the binding detectable to BIAcore was presented as a change in refractive index, the rapid kinetics of P-selectin binding to heparin-conjugated BSA might be difficult to distinguish from bulk refractive changes, especially when high protein concentrations were used. We therefore conducted parallel injections of P-selectin Rg on both surfaces of the heparin-conjugated BSA and the unconjugated BSA (as the experimental control). Each of them was individually captured on a SA5 sensor chip following the biotinylation. In this manner, the response units (RU) attributed to bulk refractive index on the unconjugated BSA surface could be subtracted from the RU values measured on the heparin-conjugated BSA surface for the specific bindings. It should be mentioned that the RU values measured on the unconjugated BSA were always much lower than those on the heparin-conjugated BSA (Fig. 5B).

The equilibrium affinity of P-selectin binding to heparin was plotted with the subtracted equilibrium responses (R_E) versus the concentrations of P-selectin Rg. The dissociation constant (K_d = 115 ± 6 nM) was derived from nonlinear curve fitting (Fig. 5C). Alternatively, Scatchard plot analysis (K_d = 117 ± 5 nM) was used (Fig. 5D). Both methods yielded very similar results. The measured k_off was 3.15 ± 0.34 × 10^3 s⁻¹ and the calculated k_on was 2.75 ± 0.42 × 10⁴ M⁻¹ s⁻¹.

**Discussion**

Heparin and HSPGs have been shown to bind to P-selectin and, consequently, to inhibit the adhesions of leukocytes and cancer cells to P-selectin. We previously reported that the cell surface HSPGs directly mediated adhesion of human malignant melanoma A375 cells and human tongue squamous cancer Tca-8113 cells to P-selectin (13). Here we showed that P-selectin mediated adhesion, but not rolling, of A375 cells under shear stress. Although others (14, 15) also reported the similar findings, its molecular mechanism remains to be explored. In this context, it has been shown that sialyl Lewis x mediates the rolling on P-selectin while tyrosine sulfation, although not required, enhances it (26, 27). Since both A375 cells and Tca-8113 cells have no apparent expression of sialyl Lewis x on their cell surfaces (13), our results appear to support this finding.

In the present study, we determined the potencies of heparin for inhibition of A375 cell adhesion to P-selectin under flow (IC₅₀ = 3 μM heparin) and for attenuation of P-selectin binding to the cell surface of A375 cells (IC₅₀ = 4 μM heparin). Using the technique of surface plasmon resonance, we further examined the affinity and kinetics of P-selectin binding to heparin (K_d = 115 ± 6 nM, k_off = 3.15 ± 0.34 × 10^3 s⁻¹ and k_on = 2.75 × 10⁴ M⁻¹ s⁻¹). Apparently, these very divergent approaches yielded the relatively convergent results for the binding affinity of P-selectin to heparin.

It was worth noting that the K_d value of 115 ± 6 nM measured by the technique of surface plasma resonance was ~20-fold lower than those determined by other methods, such as the laminar flow assay (3 μM heparin; Fig. 3B) and the flow cytometric assay (4 μM heparin; Fig. 4B). Although we currently did not know the exact explanation(s), we speculate that this difference could result from a number of potentially variable factors. These included the differences in the determination methods, the amounts of P-selectin Rg used, and the densities of heparin-conjugated BSA, the stoichiometry of P-selectin Rg binding to heparin and the topology of HSPGs on the cell surface of A375 cells.

In literature, P-selectin was reported to bind to sulfatides secreted by granulocytes and tumor cells (28-30). Further, using site-directed mutagenesis, the amino acids on the lectin domain of P-selectin for the bindings of myeloid cells and sulfatides were shown to be overlapping (31). Likewise, we found that G1, a leukocyte adhesion blocking mAb to the lectin domain of P-selectin, blocked the binding of P-selectin to heparin-conjugated BSA (Fig. 5A). These observations suggest that sialyl Lewis x, sulfatides and heparin may bind to the same or very similar motif(s) in the lectin domain of P-selectin.

The moderate binding affinity, the fast on rate (k_on) and the fast off rate (k_off) are known to be the three biophysical elements critically required for leukocyte rolling on selectin molecules under shear stress (23, 24, 32, 33). Compared with the parameters for the interaction of P-selectin with PSGL-1 (K_d = 320 ± 20 nM, k_off = 1.4 ± 0.1 s⁻¹ and k_on = 4.4 × 10⁶ M⁻¹ s⁻¹) (32), the interaction of P-selectin with heparin has a characteristic slow k_off (a ~1,000-fold increase) and a reduced k_on (a ~100-fold reduction). Given the similar binding affinities for the P-selectin/PSGL-1 and P-selectin/heparin interactions, these data suggest that the k_off and k_on values are apparently the key determinators for rolling (fast k_off and k_on) of leukocytes versus adhesion (slow k_off and k_on) of cancer cells on P-selectin under flow. By extrapolation, as carbohydrate ligands can dramatically alter the parameters of the binding kinetics to P-selectin, our finding also indicate that the carbohydrate ligands can determine the biophysical properties of these selectin-mediated cell-cell interactions.

**Abbreviations**

BCS, bovine calf serum; BSA, bovine serum albumin; HSPGs, heparan sulfate proteoglycans; PBS, phosphate buffered saline, pH 7.4; PSGL-1, P-selectin glycoprotein ligand-1; Rg, receptor-globulin; RU, response unit.
References


23. Wang, Geng: Characteristics of P-selectin/heparin interaction.