Redistribution of P-selectin glycoprotein ligand-1 (PSGL-1) in chemokine-treated neutrophils: a role of lipid microdomains

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Abstract: P-selectin glycoprotein ligand-1 (PSGL-1) is a mucin-like cell adhesion molecule expressed on leukocyte plasma membranes and involved in platelet-leukocyte and endothelium-leukocyte interactions. The treatment of neutrophils with a low concentration of IL-8 induced the redistribution of PSGL-1 to one end of the cell to form a cap-like structure. We investigated the role of lipid microdomains in the redistribution of PSGL-1 and its effect on the adhesive characteristics of IL-8-treated neutrophils. The redistribution of PSGL-1 induced by IL-8 was inhibited by cholesterol-perturbing agents such as methyl-β-cyclodextrin and filipin. Sucrose density gradient centrifugation analysis revealed that PSGL-1 was enriched in a low-density fraction together with the GM1 ganglioside after solubilization of the cell membranes with a nonionic detergent, Brij 58. However, when Triton X-100 was used for the solubilization, PSGL-1 was no longer recovered in the low-density fraction, although GM1 ganglioside remained in the low-density fraction. Furthermore, immunofluorescence microscopic observation demonstrated that the localization of PSGL-1 differed from that of GM1 ganglioside, suggesting that PSGL-1 is associated with a microdomain distinct from that containing the GM1 ganglioside. Treatment of neutrophils with IL-8 increased the formation of microaggregates composed of neutrophils and activated platelets, and this treatment also enhanced reactive oxygen species production in neutrophils induced by the cross-linking of PSGL-1 with antibodies. These results suggest that the association of PSGL-1 with lipid microdomains is essential for its redistribution induced by IL-8 stimulation and that the redistribution modulates neutrophil functions mediated by interactions with P-selectin. J. Leukoc. Biol. 81: 1414–1421; 2007.

Key Words: adhesion molecule · platelet-neutrophil microaggregates · cell polarization · reactive oxygen species

INTRODUCTION

P-selectin is a member of the selectin family of adhesion molecules, and it is expressed on activated endothelial cells and platelets [1, 2]. This adhesion molecule is involved in the recruitment of leukocytes to inflammatory tissues and hemorrhagic sites via its interaction with a counter-ligand on the leukocyte cell surface [3]. A major ligand for P-selectin has been identified as P-selectin glycoprotein ligand-1 (PSGL-1), a mucin-like adhesion molecule containing sialyl Lewis X (sLeX) carbohydrate structures, which are recognized by P-selectin [4]. Several studies have suggested that the ligation of P-selectin to its ligands induces the activation of leukocytes, including the production of reactive oxygen species (ROS) in neutrophils [5, 6] and the secretion of cytokines from monocytes [7, 8]. The signals leading to the activation of leukocytes have been reported to be transmitted through PSGL-1 [9–12]. It has also been reported that PSGL-1 exhibited polarized redistribution on neutrophils upon treatment with chemotactic agents [6, 13–15]. The redistribution of PSGL-1 has been suggested to modulate leukocyte function by modifying cell adhesion mediated by an interaction between P-selectin and PSGL-1. This process is thought to be a transition state from the adhesion via P-selectin-PSGL-1 interaction to integrin-mediated adhesion, which in turn leads to transmigration through the vascular endothelium [13, 14]. We also reported that the redistribution of the P-selectin ligand was found to increase the susceptibility of neutrophils to P-selectin, thus facilitating the production of superoxide anions [6]. This redistribution was inhibited by treatment of the cells with cytochalasin [6, 13], suggesting that the translocation of PSGL-1 is driven by an actin-dependent mechanism.

In the last decade, lipid microdomains have received increasing attention as a result of their importance in various biological processes [16]. The lipid microdomain [also referred to as the lipid raft, glycolipid-enriched membrane (GEM) domain, or detergent-resistant microdomain] is the submicroscopic domain in plasma membranes, where glycosphingolipid, sphingomyelin, and cholesterol are enriched, and this domain has been shown to be resistant to solubilization with nonionic detergents [17]. Immunologically important receptors (e.g., T cell and B cell antigen receptors, the FcεR) and various signaling molecules have been reported to be associated with...
lipid microdomains [18–25], and these membrane components form the functional complex for a variety of immunological responses. Handa and coworkers [26] also reported that PSGL-1 and certain signaling molecules, including CD45 and the Src family kinases, are associated with the low-density GEM fraction in T lymphocytes.

In this study, we investigated the possible association of PSGL-1 with a low-density lipid microdomain and examined the relevance of this putative association to the redistribution of PSGL-1 in IL-8-treated neutrophils. We also present evidence showing that the microdomain containing PSGL-1 differs from the GM1 ganglioside-enriched GEM prototype and that the redistribution of PSGL-1 modulates PSGL-1-mediated adhesion and the functional activation of neutrophils.

MATERIALS AND METHODS

Reagents and antibodies

Heparin from porcine intestine was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Dextran 200,000 was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Ficol-Paque was from Amersham Biosciences (Uppsala, Sweden). Anti-PSGL-1 antibody (PL1; IgG) was purchased from Serotec Ltd. (Oxford, UK). Anti-Lex antibody (KM93; IgM) [27] was a generous donation from Dr. Nobuo Hanai (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). Alexa Fluor 488-conjugated and Alexa Fluor 647-conjugated goat anti-mouse IgG antibodies were from Invitrogen (San Diego, CA, USA). HRP-conjugated goat ant-mouse IgG antibody and biotinylated goat anti-mouse IgM antibody were purchased from KPL (Gaithersburg, MD, USA) and Organon Teknika (West Chester, PA, USA), respectively. Normal murine IgG1 was purchased from Bay Bioscience (Kobe, Japan). HRP-conjugated streptavidin was from Gibco-BRL (Rockville, MD, USA). Alexa Fluor 488- or Alexa Fluor 647-conjugated goat anti-mouse IgG antibodies and streptavidin were from Molecular Probes (Eugene, OR, USA). Dextran 200,000 and Dextran 70,000 were purchased from Wako Pure Chemicals (Osaka, Japan). Ficoll-Paque was from Amersham Biosciences (Uppsala, Sweden). Anti-human PSGL-1 antibody (PL1; IgG) was from Chemicon, Temecula, CA, USA; 1/1000 dilution) for 1 h and then with HRP-conjugated secondary antibody for 30 min. Proteins were visualized with the ECL system. After the primary/secondary antibodies were removed by washing the membranes with PBS-T for three times after each reaction step. Proteins were detected with the ECL detection system (Amersham Biosciences). The dimeric form of PSGL-1 with a molecular weight of ~250 kDa was effectively detected by these procedures [29]. To detect GM1 ganglioside, an aliquot (2 μl) of each fraction was blotted onto a nitrocellulose membrane. The membranes were then blocked for 1 h in 4% BSA/Tris and incubated with HRP-conjugated cholera toxin B subunit (1:5000) for 1 h. After the membranes were washed with PBS-T three times, GM1 ganglioside was visualized with the ECL system. The phosphorylated form of p38 MAPK was analyzed by immunoblotting. Neutrophils (2 × 10⁶ cells/ml) were cultured with IL-8 (50 ng/ml) in the presence or absence of GM1 (10 μM) at 37°C for 2 h. The cell mixture was fixed with PBS containing 0.05% Triton X-100 and 0.1% sodium orthovanadate, and the lysate was subjected to SDS-PAGE (10%) and sucrose density gradient centrifugation to recover the low-density lipid microdomain.

Isolation of lipid microdomain

The lipid microdomain was isolated by sucrose density gradient centrifugation as described by Rodgers and Rose [23]. Human neutrophils (5 × 10⁶ cells) were treated with or without IL-8 (50 ng/ml) at 37°C for 20 min. Cells were then lysed in 1 ml lysis buffer [25 mM Tris-HCl (pH 7.5) containing Brij 58 (0.5%), aprotinin (10 μg/ml), and PMSF (1 mM)], and the lysates were homogenized with 20 strokes in a glass Dounce homogenizer at 0°C. In some experiments, Triton X-100 (1%) was used instead of Brij 58. After the homogenates were centrifuged at 14,000 rpm for 5 min, the supernatants were mixed with an equal volume of 80% sucrose. Mixtures were placed in a centrifugation tube, overlaid with discontinuous sucrose density layers (5 ml 34% sucrose, then 6 ml 5% sucrose), and centrifuged at 200,000 g for 20 h. Fractions (1.5 ml) were collected from the top to the bottom of the gradient. The detergent-insoluble, low-density lipid microdomain was recovered mainly in Fraction 4 (5%/34% interface). The protein concentration of each fraction was determined with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Immunoblot analysis

PSGL-1 and GM1 ganglioside in the fractions obtained by sucrose density gradient centrifugation were analyzed by immunoblotting. For the detection of PSGL-1, an aliquot (24 μl) of each fraction was subjected to SDS-PAGE on 7.5% polyacrylamide gel under nonreducing conditions, and the proteins were transferred electrophoretically to a nitrocellulose membrane (HyBond ECL™, Amersham Biosciences). After being treated for 1 h in 4% BSA/Tris, the membranes were treated sequentially with KM93 (5 μg/ml), biotinylated goat anti-mouse IgM antibody (1:500), and HRP-conjugated streptavidin (1:5000). The membranes were washed with PBS containing 0.05% Tween 20 (PBS-T) three times after each reaction step. Proteins were detected with the ECL detection system (Amersham Biosciences). The dimeric form of PSGL-1 with a molecular weight of ~250 kDa was effectively detected by these procedures [29]. To detect GM1 ganglioside, an aliquot (2 μl) of each fraction was blotted onto a nitrocellulose membrane. The membranes were then blocked for 1 h in 4% BSA/Tris and incubated with HRP-conjugated cholera toxin B subunit (1:5000) for 1 h. After the membranes were washed with PBS-T three times, GM1 ganglioside was visualized with the ECL system.

Flow cytometric analysis of neutrophil-platelet microaggregate formation

The formation of platelet-neutrophil microaggregates was measured by flow cytometry, essentially as described previously [29, 30]. Briefly, platelets in PRP were fluorescently labeled with BICEF-AM (0.05 mM) for 30 min at 37°C. BICEF-labeled platelets were suspended in an original volume of PBS and stimulated with 0.125 U/ml thrombin for 10 min. An aliquot (0.05 ml) of activated platelet suspension was mixed with neutrophil suspension (4 × 10⁶ cells/ml, 0.05 μl) and incubated for 20 min at 37°C in the presence or absence of IL-8 (50 ng/ml). The cell mixture was diluted with 3 ml PBS and analyzed by a flow cytometer (FACScanlibur, BD Biosciences, San Diego, CA, USA). Neutrophils were gated on a forward-scatter versus side-scatter plot. The platelet-neutrophil microaggregates were quantified by counting neutrophils positive for fluorescence associated with BICEF-labeled platelets. Data for 2000 cells gated for neutrophils were collected.
RESULTS

Redistribution of PSGL-1 on the neutrophil cell surface was induced by IL-8 treatment and inhibited by cholesterol-perturbing agents

We first examined the effects of IL-8 treatment on the distribution of PSGL-1. In unstimulated neutrophils, PSGL-1 was distributed uniformly on the plasma membrane, whereas it was translocated to one end of the cell to form a cap-like structure upon treatment with IL-8 (Fig. 1, A and B). The redistribution of PSGL-1 was observed within 1 min after IL-8 treatment and was maintained for at least 60 min (data not shown). To evaluate the role played by the lipid microdomain in the redistribution of PSGL-1, we examined the effects of two cholesterol-perturbing agents, MβCD and filipin [32]. The cap formation of PSGL-1 in IL-8-treated cells was inhibited by treatment with MβCD or filipin (Fig. 1, C and D), and almost complete inhibition was achieved at 10 mM MβCD or at 10 μg/ml filipin (Table 1). We then examined the effects of MβCD treatment on p38 MAPK activation induced by IL-8 to assess possible inhibition of the IL-8 signaling processes by cholesterol depletion. However, the MβCD treatment did not affect IL-8-induced activation of p38 MAPK, as analyzed by immunoblot analysis (Fig. 1E). Treatment of neutrophils with PMA (100 pg/ml), an activator of protein kinase C, also induced a moderate level of cap formation of PSGL-1, and the redistribution was inhibited in the presence of MβCD (10 mM; Table 1). These results suggest that the perturbation of lipid microdomains by these reagents results in the abrogation of the redistribution of PSGL-1. The total expression of PSGL-1 on neutrophils was not changed after the treatment with MβCD or filipin, as assessed by flow cytometric analysis (data not shown).

Localization of PSGL-1 in low-density lipid microdomains

The postnuclear fraction obtained by the treatment of neutrophils with Brij 58 was separated by sucrose density gradient centrifugation to isolate the low-density lipid microdomain (Fig. 2A). The fractions obtained after the centrifugation were assayed for PSGL-1 and GM1 ganglioside by immunoblot analysis using anti-sLeX antibody (KM93; Fig. 2B) and cholera toxin B subunit (Fig. 2C), respectively. PSGL-1 was studied with a molecular weight of 250 KDa (dimeric form) was recovered in Fraction 4 (Fig. 2B), which corresponded to the interface of 5%/34% sucrose layers. GM1 ganglioside, a marker of low-density lipid microdomains, was also enriched in the same fraction (Fig. 2C). These results strongly suggest that PSGL-1 is a component of the low-density lipid microdomains of neutrophil membranes. To explore the possible disengagement of PSGL-1 from a lipid microdomain during the course of redistribution, IL-8-treated neutrophils were subjected to sucrose density gradient centrifugation after Brij 58 extraction. However, treatment with IL-8 did not alter the localization of PSGL-1 or GM1 ganglioside in the low-density fraction (Fig. 2, B and C). When the neutrophils were pretreated with MβCD (10 mM), PSGL-1 was distributed in a wider range of the sucrose density gradient (Fractions 4–9 in Fig. 2B). In contrast, the distribution of GM1 ganglioside in the low-density fraction remained unaffected by treatment with 10 mM MβCD (Fig. 2C), thus indicating the possibility that PSGL-1 is located in lipid microdomains, which are distinct from the GM1 ganglioside-containing mi-
crodomains. When we used Triton X-100 instead of Brij 58 for the extraction of the cells, PSGL-1 was recovered in the Triton X-100-soluble, higher-density fractions (Fractions 8 and 9, Fig. 3). However, most of the GM1 ganglioside was recovered in the lower-density fraction, as was also the case when Brij 58 was used for extraction.

Differences in the intracellular distribution of PSGL-1 and GM1 ganglioside

The intracellular distribution of PSGL-1 and GM1 ganglioside was analyzed by immunofluorescence microscopy. In IL-8-treated neutrophils, GM1 ganglioside was distributed uniformly throughout whole-cell membranes, and it was not colocalized with the PSGL-1, which had accumulated at the cappling site (Fig. 4A). Moreover, PSGL-1 formed clusters on the cell surface of unstimulated neutrophils after treatment with a combination of anti-PSGL-1 antibody and the antimouse IgG F(ab')2 fragment, whereas the distribution of GM1 ganglioside clearly differed from that of the PSGL-1 clusters (Fig. 4B). When cell-surface GM1 ganglioside was induced to form patches by treatment with the cholera toxin B subunit plus anticholera toxin antibody, PSGL-1 and GM1 ganglioside exhibited no colocalization with each other (Fig. 4C). These observations suggest that PSGL-1 and GM1 ganglioside are distributed independently on cell membranes, although these molecules were recovered in the low-density lipid microdomains.

Formation of a complex of neutrophils and activated platelets

To examine whether the redistribution of PSGL-1 influences P-selectin-dependent adhesion, we analyzed the formation of microaggregates composed of neutrophils and thrombin-activated platelets by flow cytometry. When fluorescently labeled platelets were incubated with unstimulated neutrophils and were applied to flow cytometry, the percentage of neutrophils positive for fluorescence associated with platelets was estimated to be 38.1% (Fig. 5). The pretreatment of neutrophils with IL-8 (50 ng/ml) increased the percentage of fluorescence-positive neutrophils to 59.7%. The formation of neutrophil-platelet microaggregates was almost completely inhibited in the presence of EDTA (5 mM), anti-PSGL-1 antibody, or anti-P-selectin antibody but not in the presence of antibody against platelet GPIb (data not shown), which was reported to be involved in a platelet-leukocyte interaction [33]. The increased microaggregate formation with IL-8-treated neutrophils was abrogated by treatment of these cells with MβCD (10

### TABLE 1. Effects of Cholesterol-Perturbing Agents on the Redistribution of PSGL-1 Induced by IL-8 or PMA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cap-forming cells (%) Mean ± SD</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.7 ± 2.6</td>
</tr>
<tr>
<td>IL-8 (50 ng/ml)</td>
<td>69.9 ± 10.6</td>
</tr>
<tr>
<td>+ MβCD (5 mM)</td>
<td>28.6 ± 4.4</td>
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<tr>
<td>+ MβCD (10 mM)</td>
<td>0.9 ± 1.1</td>
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<tr>
<td>+ filipin (5 μg/ml)</td>
<td>19.3 ± 14.0</td>
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<tr>
<td>+ filipin (10 μg/ml)</td>
<td>1.2 ± 1.6</td>
</tr>
<tr>
<td>PMA (100 pg/ml)</td>
<td>37.1 ± 8.0</td>
</tr>
<tr>
<td>+ MβCD (10 mM)</td>
<td>1.4 ± 1.7</td>
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* Mean percentage (±SD) of cap-forming cells in 20 fields.

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**Fig. 2.** Localization of PSGL-1 in detergent-insoluble lipid microdomain. Neutrophils were treated with or without IL-8 (50 ng/ml, 37°C, 20 min) and lysed with a buffer containing 0.5% Brij 58 at 4°C. Postnuclear supernatants were centrifuged in a 5%/34% discontinuous sucrose density gradient (A) at 200,000 g for 20 h. Fractions (Fr.; 1.5 ml) from top to bottom were collected, and each fraction was analyzed by Western blotting (WB) with anti-sLeX IgM (KM93) plus biotin-conjugated anti-IgM antibody and HRP-conjugated streptavidin (for PSGL-1 staining; B) and by dot-blotting (DB) with HRP-conjugated cholera toxin B subunit (for GM1 ganglioside staining; C).

**Fig. 3.** Differential distribution of PSGL-1 and GM1 ganglioside in the detergent-insoluble, lipid microdomain. Neutrophils were lysed with a buffer containing 0.5% Brij 58 or 1% Triton X-100 at 4°C. Postnuclear supernatants were centrifuged in a 5%/4% discontinuous sucrose density gradient at 200,000 g for 20 h. Fractions (1.5 ml) from top to bottom were collected, and each fraction was analyzed by immunoblotting with anti-sLeX antibody (for PSGL-1) and with HRP-conjugated cholera toxin B subunit (for GM1 ganglioside).
mM) at 37°C for 20 min (Table 2). Based on these results, it is reasonable to conclude that the redistribution of PSGL-1 induced by IL-8 treatment leads to the formation of a stable complex of neutrophils and activated platelets.

ROS production in neutrophils induced by the cross-linking of PSGL-1

As it had already been demonstrated that PSGL-1 acts as a receptor to generate signals leading to cellular activation [9, 11, 34–36], we examined the inter-relationship between the redistribution of PSGL-1 and neutrophil activation induced by the cross-linking of PSGL-1 in IL-8-treated or untreated neutrophils. When intracellular ROS production was analyzed by flow cytometry, it was found that treatment of unstimulated neutrophils with a combination of anti-PSGL-1 antibody (PL1) and antimouse IgG F(ab’)$_2$ fragment induced a moderate level of ROS production (Fig. 6). An increased level of ROS was produced in IL-8-treated neutrophils upon stimulation with the same combination of antibodies. The isotype-matched, normal IgG1, however, induced only basal levels of ROS production in

<table>
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<tr>
<th>IL-8 (50 ng/ml)</th>
<th>MβCD (10 mM)</th>
<th>Microaggregate formation$^*$ (%)</th>
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<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>20.6</td>
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<tr>
<td>+</td>
<td>–</td>
<td>28.7</td>
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<tr>
<td>–</td>
<td>+</td>
<td>21.4</td>
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<tr>
<td>+</td>
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<td>20.6</td>
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$^*$ Neutrophils associated with fluorescence-labeled and thrombin-activated platelets were measured by flow cytometry.
the presence or absence of the second antibody (data not shown). Immunofluorescence microscopy revealed that the treatment with PL1 and the F(ab')2 fragment of the second antibody elicited a more clustered distribution of PSGL-1 than treatment with PL1 alone (Fig. 7, A and B). The same combination of antibodies induced PSGL-1 to form a more compactly packed, cap-like structure on IL-8-treated neutrophils than did treatment with PL1 alone (Fig. 7, C and D). These results suggest that the redistribution of PSGL-1 induced by IL-8 potentiates cellular activation mediated by PSGL-1.

FIG. 7. The intracellular distribution of PSGL-1 on IL-8-treated or untreated neutrophils after the cross-linking with anti-PSGL-1 antibody. Neutrophils were treated with anti-PSGL-1 antibody (PL1, 10 μg/ml) for 10 min at 4°C. The cells were then treated with (B and D) or without (A and C) antiserum IgG F(ab')2 fragment (10 μg/ml) for 10 min at 4°C and subsequently with (C and D) or without (A and B) IL-8 (50 ng/ml) for 20 min at 37°C. After the cells were fixed with 1% formaldehyde, they were stained with Alexa Fluor 647-conjugated antiserum IgG. The nuclei were stained with PI (25 μg/ml). Confocal images of 15 serial sections with a thickness of 0.93 μm are superimposed. Original bar, 5 μm.

DISCUSSION

In the present study, we investigated the role of lipid microdomains in the redistribution of PSGL-1 on IL-8-treated neutrophils. PSGL-1 was shown to be translocated to form a cap-like structure when the neutrophils were stimulated with IL-8. This observation was consistent with the findings of our previous report [6] showing that sLeX carbohydrate epitopes were clustered after stimulation with cytokines. The redistribution of PSGL-1 was inhibited by treatment cholesterol-perturbing agents, MβCD or filipin (Fig. 1 and Table 1), and PSGL-1 was in fact recovered in the Brij 58-insoluble, low-density lipid microdomain (Fig. 2). Considering that MβCD treatment did not affect IL-8-induced activation of p38 MAPK [37] in neutrophils under our experimental conditions (Fig. 1E), it is strongly suggested that the translocation of PSGL-1 depends on the integrity of lipid microdomains. Although some of IL-8 signaling processes were reported to be inhibited by longer preincubation (37°C, 1 h) of neutrophils with MβCD [38], the conditions used in this study (37°C, 2 min) seem to preferentially affect organization of membrane microdomains. Handa and coworkers [26] reported that PSGL-1 and mucin-1 were located in lipid microdomains in T cells together with various signaling molecules, including CD45 and the Src family of tyrosine kinases. These microdomains were thought to constitute the functional machinery involved in adhesion and signal transduction in T cells. We reported previously that an intact actin cytoskeleton is required for the redistribution of β2-integrins [6]. Considering that the cytoplasmic tail of PSGL-1 was shown to interact with the ezrin-radixin-moesin family proteins, which serve as linking proteins between the plasma membrane and the actin cytoskeleton [39], it is reasonable to assume that the actin cytoskeleton regulates the motility of PSGL-1-containing lipid microdomains. Some lipid microdomain-associated proteins such as the high-affinity IgE receptor (FcεRI) [40], B cell antigen receptor [20], and LFA-1 [25] translocate into and out of lipid microdomains during cell activation. However, the localization of PSGL-1 in lipid microdomains was not altered by stimulation with IL-8; namely, PSGL-1 was translocated into the capping site without leaving the presence or absence of the second antibody (data not shown). Immunofluorescence microscopy revealed that the treatment with PL1 and the F(ab')2 fragment of the second antibody elicited a more clustered distribution of PSGL-1 than treatment with PL1 alone (Fig. 7, A and B). The same combination of antibodies induced PSGL-1 to form a more compactly packed, cap-like structure on IL-8-treated neutrophils than did treatment with PL1 alone (Fig. 7, C and D). These results suggest that the redistribution of PSGL-1 induced by IL-8 potentiates cellular activation mediated by PSGL-1.
the lipid microdomain. Not only the intracellular distribution observed by fluorescence microscopy (Fig. 4) but also the sensitivity to Triton X-100 and cholesterol-perturbing agents (Figs. 2 and 3) suggested that the type of microdomain containing PSGL-1 was distinct from the typical GEM. These results support the notion that lipid microdomains are heterogeneous in terms of their composition [41]. Schade and Levine [42] have recently proposed a scheme involving microdomains for T cell activation. According to their model, a microdomain composed of Lck tyrosine kinase is fused with another microdomain containing a substrate for the kinase upon T cell activation, resulting in the docking of the kinase with its substrate. In this case, the heterogeneity of the lipid microdomains dynamically regulates the T cell activation processes. LFA-1 is also thought to be distributed to lipid microdomains but not to those containing GM1 ganglioside and/or Thy-1 antigen in T cells [32]. In migrating T cells, PSGL-1 was shown to accumulate in the uropod, the rear end of moving cells, whereas other adhesion molecules, including integrins, were concentrated at the leading edge [43]. The redistribution of PSGL-1-containing microdomains is likely to play a role in the polarization of cells and PSGL-1-mediated signaling.

The cross-linking of cell surface PSGL-1 on neutrophils by antibodies induces the tyrosine phosphorylation of membrane proteins, the activation of the MAPK cascade, and cytokine production, thus suggesting that PSGL-1 transduces signals leading to cellular activation [9–11, 34, 35]. However, PSGL-1 itself is not phosphorylated and possesses neither known enzymatic activity nor target sequences that bind to signaling molecules in its cytoplasmic tail. It is therefore likely that signaling molecules associated with PSGL-1-containing microdomains are involved in signal transduction. In the present study, the production of ROS in neutrophils induced by the cross-linking of PSGL-1 was potentiated by pretreatment of the cells with IL-8 (Fig. 6). The potentiation of ROS production accompanied the redistribution of PSGL-1 to form a more compactly accumulated cluster (Fig. 7). The clustering of PSGL-1-containing lipid microdomains may facilitate the close association of signaling molecules and efficient signal transduction, thereby leading to ROS production. It remains to be clarified in future studies which signaling molecules are associated with PSGL-1-containing lipid microdomains and which mediate signaling processes for ROS production in neutrophils.

The adhesion of neutrophils to thrombin-activated platelets was enhanced by the treatment with IL-8 (Fig. 5). The redistribution of PSGL-1 on neutrophil membranes may induce a high-density interaction with P-selectin on activated platelets and thus stabilize the formation of microaggregates composed of these cells. Platelet-neutrophil microaggregates have been detected frequently in the circulation under pathophysiologic conditions such as inflammatory and atherosclerotic diseases [44–48]. Neutrophils with clustered PSGL-1 on their surface may be stimulated efficiently by activated platelets via P-selectin-mediated adhesion. Conversely, it was reported that the polarized distribution of PSGL-1 on neutrophils to some extent weakened the adhesion of neutrophils to a cell monolayer expressing P-selectin [13]. The adhesion mediated by P-selectin has been shown to be influenced greatly by various conditions including shear stress, the density of receptors and ligands, and the flexibility of the cells [49, 50]. Thus, the adhesion of neutrophils to activated platelets and the endothelium might be regulated dynamically by the redistribution of PSGL-1 on cell membranes. In conclusion, the present findings strongly suggest that the redistribution of PSGL-1 requires the integrity of the lipid microdomain and modulates P-selectin-mediated neutrophil adhesion and signaling processes for cellular activation.

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Itoh et al. Redistriution of PSGL-1 and lipid microdomain 1421