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# Signaling Function of PSGL-1 in Neutrophil: Tyrosine-Phosphorylation-Dependent and c-Abl-Involved Alteration in the F-Actin-Based Cytoskeleton

**Xueqing Ba, Cuixia Chen, Yanguang Gao, and Xianlu Zeng***

Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, Peoples Republic of China

**Abstract** P-selectin glycoprotein ligand-1 (PSGL-1) is the best-characterized selectin ligand that has been demonstrated to mediate leukocytes rolling on endothelium and leukocytes recruitment into inflamed tissue in vivo. In addition to its direct role in leukocyte capturing, PSGL-1 also functions as a signal-transducing receptor. The present work showed that after cross-linking of PSGL-1 with KPL1, an anti-PSGL-1 monoclonal antibody, PSGL-1 linked to the cytoskeleton and became a detergent-insoluble component in activated neutrophils. The antibody cross-linking led to the polymerization and redistribution of F-actin-based cytoskeleton, and this alteration of cytoskeleton was spatiotemporally related to the polarization of PSGL-1. PSGL-1's polarization was cytoskeleton-dependent because it was eliminated by cytochalasin B. Furthermore, the polymerization and redistribution of F-actin filaments were tyrosine-phosphorylation-dependent since the alteration of F-actin-based cytoskeleton was severely blocked by genistein, a universal tyrosine kinase inhibitor. STI571, a small molecule inhibitor for cytoplasmic tyrosine kinase c-Abl, also inhibited the alteration of F-actin-based cytoskeleton, and c-Abl was redistributed to where F-actin concentrated in the activated neutrophils. The results suggested that cross-linking of PSGL-1 induces the phosphorylation-dependent and c-Abl-involved alteration of F-actin-based cytoskeleton in neutrophils. J. Cell. Biochem. 94: 365–373, 2005. 

**Key words:** PSGL-1; signaling function; cytoskeleton; c-Abl

The migration of leukocytes from the blood vessel into inflammatory tissue is a multiple-step process, which is initiated by the capture of leukocytes from the bloodstream and followed by their rolling along the surface of endothelial cells [Kansas, 1996; Zimmerman et al., 1996]. The rolling process is mainly mediated by selectins, a special family of cell adhesion molecules including L-, P-, and E-selectin, and their ligands. L-selectin is constitutively expressed on most types of leukocytes; E-selectin is expressed on the activated endothelium, and P-selectin is expressed on the activated platelets and endothelium. PSGL-1, the best-characterized selectin ligand, is constitutively expressed on the microvilli of almost all types of leukocytes [Moore et al., 1992]. A series of in vivo studies have proved that PSGL-1 is a physiologically relevant ligand for P-, E-, and L-selectin [Norman et al., 2000; Hirata et al., 2002; Xia et al., 2002; Hicks et al., 2003; Sperandio et al., 2003].

In recent years, accumulating data have demonstrated that in addition to its direct role in leukocyte capturing, PSGL-1 also functions as a signal-transducing receptor. The engagement of PSGL-1 with bivalent monoclonal antibody (mAb) against PSGL-1 or immobilized P-selectin induced rapid tyrosine phosphorylation of several proteins in human neutrophils, including Ras and ERK1/2 family of mitogen-activated protein (MAP) kinases, and the engagement of PSGL-1 with mAb was sufficient to activate human neutrophils to secrete IL-8.

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*Correspondence to: Xianlu Zeng, Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, Peoples Republic of China.
E-mail: zengx779@nenu.edu.cn

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This secretion could be blocked by tyrosine kinase inhibitor genistein [Hidari et al., 1997]. Soluble P-selectin-IgG triggered genistein-sensitive and $\beta_2$-integrin-dependent homomorphous aggregation, suggesting that PSGL-1 initiate tyrosine-dependent signals in polymorphonuclear neutrophil (PMN) and up-regulate the function of Mac-1 [Evangelista et al., 1999]. The rolling of neutrophils on E-selectin induced signal transduction and resulted in MAP-kinase-pathway-dependent activation of $\beta_2$-integrin [Simon et al., 2000]. The adhesion of T cells to P-selectin induced tyrosine phosphorylation of the $pp^{125}$ focal adhesion kinase (FAK) [Haller et al., 1997]. Recently, some reports have elicited that PSGL-1's redistribution after neutrophils activation was related to the interaction of its cytoplasmic tail with ERM (ezrin/radixin/moesin) complex [Alonso-Lebrero et al., 2000; Serrador et al., 2002; Snapp et al., 2002; Urzainqui et al., 2002]. Additionally, PSGL-1 engagement could induce tyrosine phosphorylation of non-receptor tyrosine kinase Syk and SRE-dependent transcriptional activity [Urzainqui et al., 2002]. The combination of all these reports clearly demonstrated that PSGL-1 functions as a signaling receptor on leukocytes. However, there are no direct data showing the alteration of cytoskeleton in the activated neutrophils caused by antibody cross-linking and the mechanism by which the alteration of cytoskeleton is affected.

In this study, we investigated the association of PSGL-1 with detergent-insoluble cytoskeleton as well as the polymerization and redistribution of F-actin in activated leukocytes caused by cross-linking of PSGL-1. Results showed that the polymerization and redistribution of F-actin is spatiotemporally related with the polarization of PSGL-1, and the F-actin's dynamics is tyrosine-phosphorylation-dependent and c-Abl-involved.

## MATERIALS AND METHODS

### Reagents and Antibodies

Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden). KPL1 (the anti-PSGL-1 mAb, mouse IgG1, sc-13535), E-10 (the anti-VCAM-1 mAb, mouse IgG1, sc-13160), H-300 (the anti-c-Abl polyclonal antibody, rabbit IgG, sc-13076), non-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (sc-3696), FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (sc-3699), and TRITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (sc-3796) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FITC-conjugated goat anti-rabbit IgG (H + L) and TRITC-conjugated goat anti-rabbit IgG (H + L) were from Jackson Laboratory (Bar Harbor, ME). Cytochalasin B, FITC-conjugated Phalloidin, lysophosphatidylcholine, and genistein (a universal tyrosine kinases inhibitor) were purchased from Sigma. STI571 (a inhibitor to non-receptor tyrosine kinase c-Abl), was a gift of Novartis Pharma Schweiz AG (Basel, Switzerland).

### Neutrophil Isolation

Neutrophils were isolated by standard procedures after sedimentation of erythrocytes by dextran T-500 and centrifugation of leukocytes over Ficoll-hypaque gradients [Berton et al., 1992]. After lysis of contaminating erythrocytes with hypotonic saline (0.17 M Tris, 0.16 M $NH_4Cl$, pH 7.2), neutrophils were washed and resuspended in cold PBS containing 0.1% bovine serum albumin (BSA). The isolated neutrophils were stored at 4°C until use. More than 95% of the isolated cells were polymorphonuclear leukocytes, and viability was determinate to be >98% by trypan blue exclusion.

### Antibody Cross-Linking

The isolated neutrophils were resuspended in PBS with 0.5% BSA and 20 mM glucose, ligated with KPL1 at a final concentration of 10 μg/ml at 4°C for 20 min, and interacted with $F(ab')_2$ fragment of goat anti-mouse IgG at a final concentration of 20 μg/ml at 4°C for another 20 min, and then incubated at 37°C for 10 min to make PSGL-1 clustered. As the control, neutrophils were ligated with E-10, an anti-VCAM-1 mAb instead of KPL1. Vascular cell adhesion molecule-1 (VCAM-1) is a molecule expressed on the endothelium but not on the surface of neutrophils. In some other experiments, to detect the distribution of PSGL-1 on the surface of neutrophils before or after antibody cross-linking, non-fluorescent secondary antibody was replaced by FITC- or TRITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG. To test the inhibition of cytochalasin B to the polarization of PSGL-1, cells were pre-incubated with cytochalasin B at a final concentration of 100 μM, a potent concentration previously used to inhibit the redistribution of carbohydrate ligands of leukocytes [Nagata

et al., 2000] at 4°C for 30 min before PSGL-1 was cross-linked with primary antibody and secondary antibody.

### Detergent Extraction of Soluble Membrane Proteins

To extract soluble membrane proteins, cells were gently resuspended in a modified cytoskeletal stabilizing buffer (CSB) (0.2% Trion X-100, 50 mM NaCl, 2 mM $MgCl_2$, 0.22 mM EGTA, 13 mM Tris (pH 8.0), 1 mM PMSF, 10 mM iodoacetamide, and 2% fetal bovine serum (FBS)), incubated at room temperature for 15 min, and washed gently with the above buffer without Triton X-100. Pellets (containing the insoluble F-actin-based cytoskeleton and the proteins linked) were gently resuspended in PBS containing 1% formaldehyde and analyzed by flow cytometry.

### Flow Cytometry

To detect the level of PSGL-1 on the surface of differently treated neutrophils, the isolated neutrophils were incubated with KPL1 (or E-10 in control experiments) at a final concentration of 10 μg/ml for 30 min at 4°C, and subsequently incubated with FITC-conjugated $F(ab')_2$ fragment goat anti-mouse IgG for another 30 min at 4°C. In the absence of detergent treatment, the level of PSGL-1 on the surface of these resting cells was considered as a basal control. Triton X-100 detergent extraction was performed after or before antibody cross-linking to PSGL-1. The basal level of PSGL-1 and the level of remained cytoskeleton-associated PSGL-1 were valued by the fluorescence of the samples.

The quantification of polymerized F-actin within neutrophils was performed as previously described [Redmond et al., 1994; Simon et al., 1999]. Briefly, the resting or antibody-cross-linked neutrophils were fixed and permeabilized with lysophosphatidylcholine (0.1 mg/ml final concentration) in buffered formalin. After a 5-min incubation at 37°C, cells were stained with $3.3 \times 10^{-7}$ M FITC-conjugated phalloidin for 20 min at room temperature and washed to eliminate the excessive FITC-conjugated phalloidin. Cells were examined on a FACScan, and values were expressed as relative fluorescence index (RFI) by dividing the fluorescence of the experimental group by the value for the unstimulated control cells. For those inhibitor-blocking experiments, the inhibitors (genistein or STI571) were added in to interact with cells for 30 min at 4°C before interaction of PSGL-1 with primary antibody and secondary antibody. The data shown were expressed as RFI by dividing the values of inhibitor-treated groups by that of the cells only treated with equivalent volume of DMSO.

### Immuno-Fluorescence Microscopy

To detect the influence of antibody cross-linking on the cytoskeleton, after ligation of PSGL-1 with KPL1 and clustering with non-conjugated secondary antibody (or TRITC-conjugated secondary antibody), neutrophils were fixed with 1% paraformaldehyde for 15 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS (containing 5 mM EDTA and 2% FBS) for 5 min. After washing with PBS, cells were stained with $3.3 \times 10^{-7}$ M FITC-conjugated phalloidin for 20 min at room temperature and washed to eliminate the excessive FITC-conjugated phalloidin. To investigate the localization of non-receptor tyrosine kinase c-Abl in resting and activated neutrophils, after ligation with KPL1 and TRITC-conjugated secondary antibody, neutrophils were fixed, permeabilized, and then incubated with H-300, an anti-c-Abl polyclonal antibody, at a final concentration of 10 μg/ml for 30 min at room temperature, washed with PBS, and incubated with FITC-conjugated goat anti-rabbit IgG (H + L) for another 30 min, then washed with PBS. To dually label F-actin and c-Abl in activated neutrophils, cells were fixed and permeabilized, and followed by staining c-Abl with H-300 and TRITC-conjugated secondary antibody and staining F-actin with FITC-conjugated phalloidin. All these stained cells were fixed in PBS containing 1% paraformaldehyde and investigated under the fluorescence microscope.

## RESULTS

### After Cross-Linking, PSGL-1 Associates With Cytoskeleton and Becomes a Detergent-Insoluble Component

In order to determine whether PSGL-1 interacts with cytoskeleton in the neutrophils activated by cross-linking with antibody, detergent extraction analyses were performed. Isolated neutrophils were stained with KPL1 and FITC-conjugated secondary antibody at 4°C, the fluorescence level of these resting cells was

considered as a basal control. Other aliquots of the isolated neutrophils were extracted with Triton X-100 either before or after antibody cross-linking. The detergent-insoluble fractions were fixed and analyzed by flow cytometry. As shown in Figure 1, the positive percentage of the cells without detergent treatment was 97.4% (Fig. 1B); When the cells were treated with detergent after antibody cross-linking, the positive percentage was 79.9% (Fig. 1D), indicating that most of PSGL-1 still remained on the surface of neutrophils, but when the cells were treated with detergent before antibody cross-linking, the positive percentage decreased to 23.8% (Fig. 1F), indicating that most of PSGL-1 were lost after detergent extraction. Above

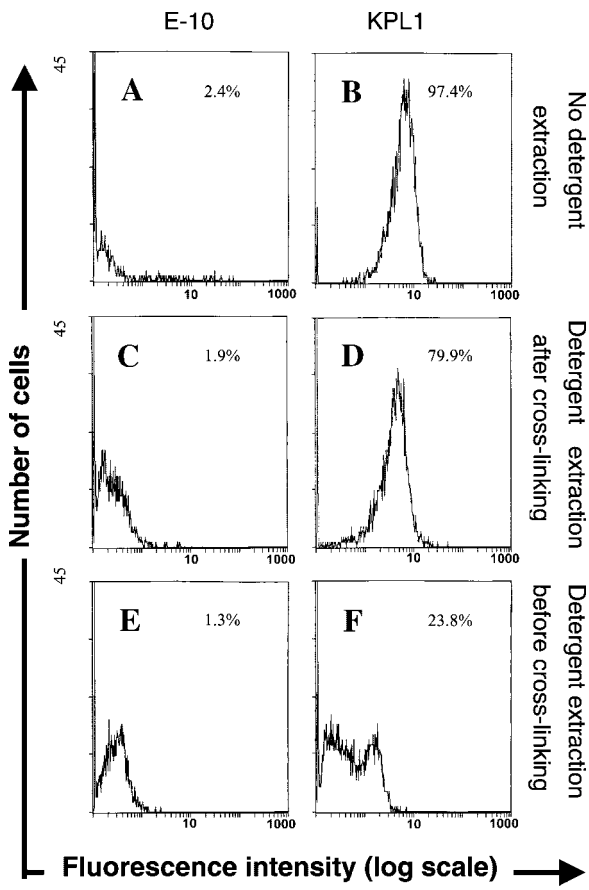


**Fig. 1.** Antibody cross-linking induces PSGL-1 association with the detergent-insoluble cytoskeleton. Antibody cross-linking and detergent extraction were performed as described in Materials and Methods. Neutrophils ligated with E-10, an anti-VCAM-1 primary antibody, and fluorescent secondary antibody were detected as negative controls for setting of data collection gates under different conditions (left panels, **A**, **C**, **E**), and the data of right panels (**B**, **D**, **F**) represented the percentages of positive cells on which PSGL-1 was remained under different conditions. Each experiment was repeated three times with similar results.

data suggested that PSGL-1 is not a constitutive component associating with the cytoskeleton on the resting neutrophils. Whereas, on the neutrophils activated by antibody cross-linking, PSGL-1 becomes one of the fractions binding to the detergent-insoluble cytoskeleton.

### Cross-Linking of PSGL-1 Induces the Polymerization and Redistribution of F-Actin

In order to determine whether cross-linking of PSGL-1 can lead to the alteration of cytoskeleton in neutrophils, isolated neutrophils were cross-linked with primary and secondary antibodies, fixed, permeabilized, and stained with FITC-conjugated Phalloidin. In control experiment, neutrophils were ligated with E-10, an anti-VCAM-1 mAb, instead of KPL1 as the primary antibody. We tested the fluorescence of the control and experimental groups by flow cytometry and expressed the value by RFI. The results showed antibody cross-linking induced an approximately 1.5-fold increase in the amount of F-actin compared with the control (Fig. 2-I). The fluorescence for F-actin in majority of control cells was faint, obscure and homogeneous (Fig. 2-II, A), whereas, in most KPL1-treated neutrophils, the fluorescence for F-actin was more intensive than that in the resting cells and redistributed to the periphery or the leading edge of the cell (Fig. 2-II, B). The results indicated that cross-linking of PSGL-1 can induce the polymerization and the redistribution of F-actin in neutrophils.

### Alteration of F-Actin-Based Cytoskeleton in the Activated Neutrophils Caused by Cross-Linking of PSGL-1 Is Phosphorylation-Dependent and c-Abl-Involved

Because protein tyrosine kinases play important roles in the transduction of extracellular signals, here in the present study we raised a query if protein tyrosine kinases affect the alteration of cytoskeleton in the activated neutrophils. To test this, neutrophils were treated with genistein before they were incubated with antibodies. According to the concentrations of this compound used to inhibit different functions of leukocytes in previous studies [Hidari et al., 1997; Kelly et al., 1998], we set a range of concentration of genistein from 1 to 200 μM (1, 10, 20, 100, and 200 μM) and tested the inhibition of this compound to the assembly of F-actin by flow cytometry. The results showed that genistein exhibited no

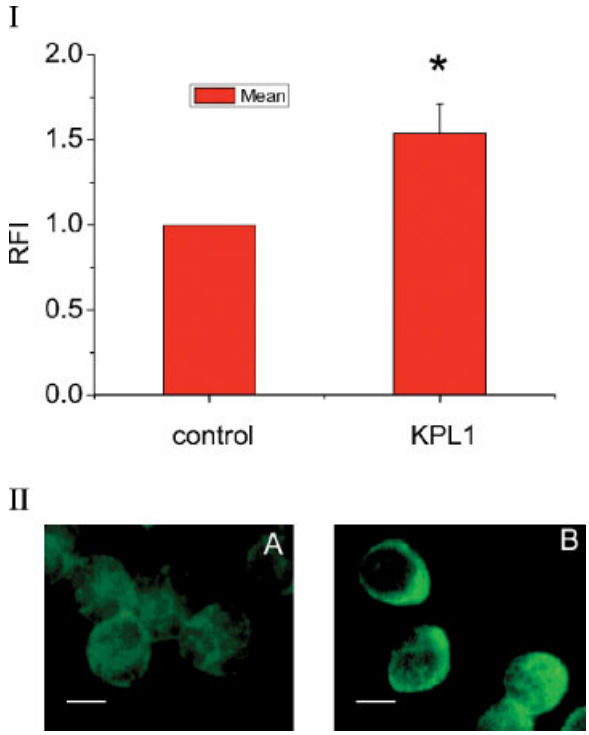


**Fig. 2.** Cross-linking of PSGL-1 induces the polymerization and the redistribution of F-actin. **I**: Neutrophils were incubated with KPL1 and cross-linked with non-fluorescent secondary antibody. F-actin was stained by FITC-conjugated phalloidin. The amount of F-actin was quantified by flow cytometry. The values were expressed as relative fluorescence index (RFI) by dividing the fluorescence of the experimental group by the value for the control cells. The significant difference of values was determined by ANOVA (*, $P < 0.01$), and the data represents the mean ± SD of three experiments. **II**: Neutrophils were cross-linked with KPL1 and non-fluorescent secondary antibody. F-actin was visualized by staining with FITC-conjugated phalloidin. **A**: The fluorescence for F-actin in the neutrophils absent of ligation with KPL1 but ligated with E-10. **B**: The fluorescence for F-actin in the neutrophils cross-linked with KPL1 and non-fluorescent secondary antibody. Each image is representative of three independent experiments with cells from different donors. Bar, 8 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

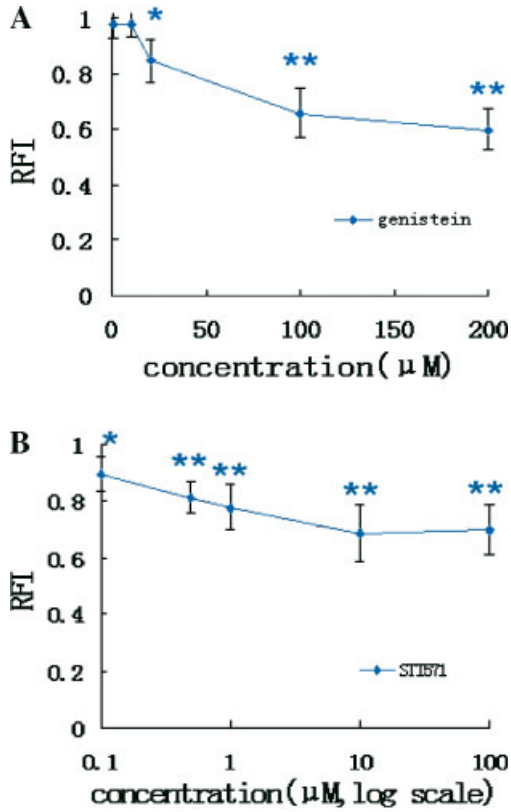


**Fig. 3.** The effect of inhibitors at different concentration on the assembly of F-actin. **A**: Neutrophils were preincubated with genistein at different concentrations (1, 10, 20, 100, and 200 μM) for 30 min at 4°C and then cross-linked with antibodies. The group treated with equivalent volume of DMSO, without genistein, was considered as a positive control, and the values of inhibitor-treated groups were expressed as RFI by dividing their fluorescence by the value for the control cells. Each data point represents the mean ± SD of three independent experiments, and the significant difference from the control was determined by ANOVA (*, $P < 0.05$, **, $P < 0.01$). **B**: Neutrophils were preincubated with STI571 at different concentrations (0.1, 0.5, 1, 10, 100 μM) for 30 min at 4°C and then cross-linked with antibodies. The group treated with equivalent volume of DMSO, without STI571, was considered as a positive control, and the values of inhibitor-treated groups were expressed as RFI by dividing their fluorescence by the value for the control cells. Each data point represents the mean ± SD of three independent experiments and the significant difference from the control was determined by ANOVA (*, $P < 0.05$, **, $P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibitory activity to assembly of F-actin at concentrations lower than 10 μM, and when cells were treated with genistein at higher concentrations, the assembly of F-actin was inhibited in a dose-dependent manner (Fig. 3A). At the concentration of 200 μM, the amount of F-actin nearly decreased by 40% and was similar to that in the resting cells directly fixed after isolation (not shown).

Among the numerous non-receptor tyrosine kinases, c-Abl family is concerned with dynamics of cytoskeleton because of its F-actin-binding domain in the structure of the kinase. To address the role of c-Abl in the assembly of F-actin, neutrophils were treated with STI571 before they were activated by antibody cross-linking. STI571 is a potent and specific blocker of c-Abl with an $IC_{50}$ of ~650 nM for inhibition of kinase activity [Tanis et al., 2003]. So we tested the effect of STI571 on the assembly of F-actin in neutrophils induced by cross-linking of PSGL-1 in a range of concentrations from 0.1 to 100 μM (0.1, 0.5, 1, 10, and 100 μM). The results showed

that the inhibitory activity of STI571 to the assembly of F-actin kept at a maximal level of 30% decrease and exhibited no more increase when the concentrations were higher than 10 μM, and in a range of concentrations lower than 10 μM, the compound inhibited the assembly of F-actin in a dose-dependent manner (Fig. 3B). Incubation of neutrophils with genistein at concentrations up to 200 μM or with STI571 at concentrations up to 100 μM did not result in any increase in cell lysis or death in our experiments (not shown). Taken together, the results indicated that the alteration of F-actin-based cytoskeleton in neutrophils caused by antibody cross-linking is phosphorylation-dependent and c-Abl-involved.

### Polarization of PSGL-1 Is Spatiotemporally Related With the Alteration of F-Actin in the Neutrophils Activated by Antibody Cross-Linking

We first investigated the redistribution of PSGL-1 on the neutrophils activated by cross-linking with antibody. The isolated neutrophils were incubated with KPL1 at 4°C for 30 min and FITC-conjugated secondary antibody at 4°C for another 30 min. A part of cells were directly fixed to detect the distribution of PSGL-1 on the resting neutrophils, another part of cells were incubated at 37°C for 10 min to lead PSGL-1 clustered, and then the redistribution of PSGL-1 on the activated neutrophils were detected. Under a fluorescence microscope, we observed that PSGL-1 was evenly distributed on the surface of resting neutrophils (Fig. 4I,A), whereas, on most activated neutrophils the distribution of PSGL-1 were polarized (Fig. 4I,B). The results indicated that antibody cross-linking can induce the redistribution of PSGL-1. In order to determine whether the cytoskeleton is necessary for the polarization of PSGL-1, neutrophils were exposed to cytochalasin B at 100 μM of final concentration before antibody cross-linking. As a result, the polarization of PSGL-1 was severely inhibited (Fig. 4I,C), suggesting that the redistribution is cytoskeleton-dependent.

To investigate the relationship between the polarization of PSGL-1 and the redistribution of F-actin, the resting and activated neutrophils that had been stained with KPL1 and TRITC-conjugated secondary antibody were sequentially labeled with FITC-conjugated phalloidin. Results showed that the fluorescence for F-actin was homogeneous in the resting neurophils

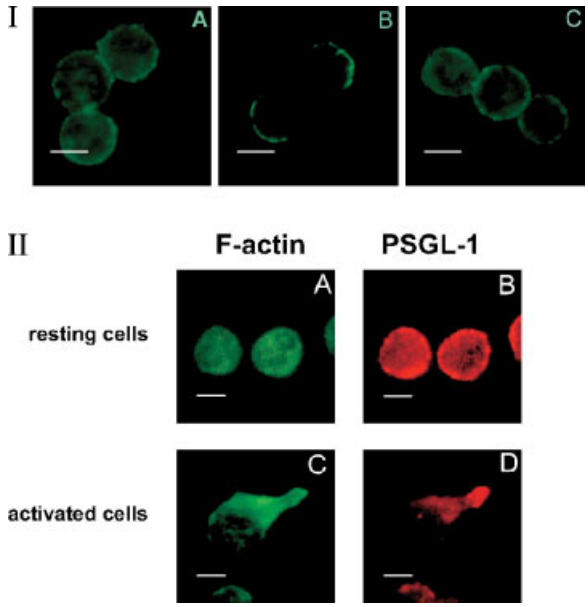


**Fig. 4.** The polarization of PSGL-1 is spatiotemporally related with the alteration of F-actin. **I**: The isolated neutrophils were incubated with KPL1 at 4°C for 30 min and FITC-conjugated secondary antibody at 4°C for another 30 min. A part of cells were directly fixed, and another part of cells were incubated at 37°C for 10 min to lead PSGL-1, clustered, then fixed. **A**: PSGL-1 on the resting neutrophils. **B**: PSGL-1 on the activated neutrophils. **C**: PSGL-1 on the cells treated with cytochalasin B before they were incubated with antibodies. **II**: Neutrophils were cross-linked with KPL1 and TRITC-conjugated secondary antibody followed by staining for F-actin with FITC-conjugated phalloidin. **A**: F-actin in the resting neutrophils. **B**: PSGL-1 on the surface of the same resting neutrophils. **C**: F-actin in the antibody-cross-linked cells. **D**: PSGL-1 on the surface of the same antibody-cross-linked cells. Each image is representative of three independent experiments with cells from different donors. Bar, 8 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

while PSGL-1 was evenly distributed on the surface of cells (Fig. 4II,A,B). When the neutrophils were activated by antibody cross-linking, F-actin was redistributed and PSGL-1 was polarized, and the redistribution of F-actin was spatiotemporally related with the polarization of PSGL-1 (Fig. 4II,C,D) in the activated neutrophils. Considering this relationship as well as the achieved results from above experiments (the polarization of PSGL-1 is cytoskeleton-dependent and PSGL-1 binds to cytoskeleton after activation), we deduced that the polarization of PSGL-1 is under the direction of F-actin-based cytoskeleton.

### Cross-Linking of PSGL-1 Induces the Re-Localization of c-Abl in Neutrophils

We further focused on the behavior of c-Abl in neutrophils before and after activation caused

by antibody cross-linking. To investigate the relationship between the localization of c-Abl and the distribution of PSGL-1, after ligation of PSGL-1 with KPL1 and TRITC-conjugated secondary antibody at 4°C, some cells were incubated at 37°C for 10 min to make PSGL-1 clustered, and then fixed, permeabilized, and stained with anti-c-Abl primary antibody and FITC-conjugated secondary antibody, other resting cells were directly fixed, permeabilized, and stained. The results showed that c-Abl was approximatively uniform within the resting cells while PSGL-1 was evenly distributed (Fig. 5I,A,B), whereas after activation caused by antibody cross-linking, more intensive fluorescence representing c-Abl emitted at the periphery or inclined to an end of the cell where PSGL-1 polarized (Fig. 5I,C,D). The behavior of c-Abl in the activated neutrophils was similar to that of F-actin. To determine the relationship between the redistribution of F-actin and c-Abl, the activated neutrophils were dually labeled. The results showed that c-Abl was redistributed and re-localized to where F-actin concentrated (Fig. 5II,A,B). Taken together, the results suggested the re-localization of c-Abl is closely related with the alteration of F-actin-based cytoskeleton.

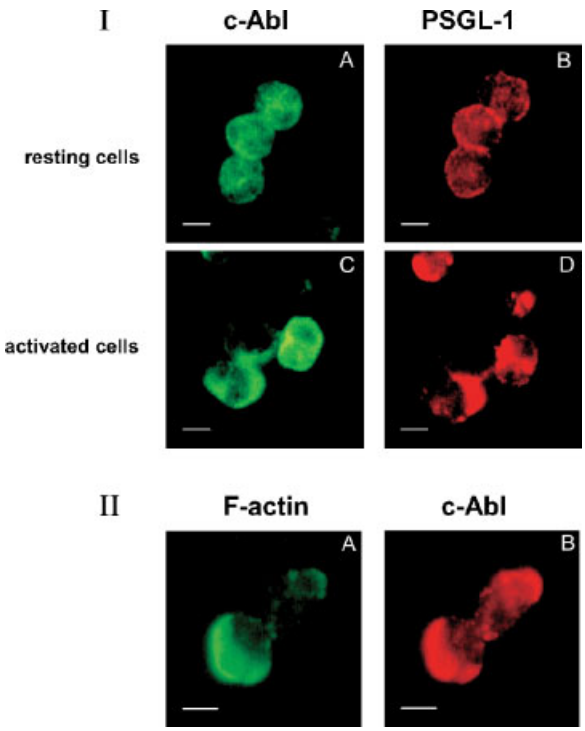


**Fig. 5.** After antibody cross-linking, the non-receptor tyrosine kinase c-Abl was redistributed and re-localized to the region where F-actin concentrated. **I**: Neutrophils were cross-linked with KPL1 and TRITC-conjugated secondary antibody, and followed by staining for c-Abl with H-300, an anti-c-Abl primary antibody, and FITC-conjugated secondary antibody. **A**: c-Abl in the resting cells. **B**: PSGL-1 on the surface of the same resting neutrophils. **C**: The distribution of c-Abl in the antibody-cross-linked cells. **D**: PSGL-1 on the surface of the same antibody-cross-linked cells. **II**: Neutrophils were cross-linked with KPL1 and non-fluorescent secondary antibody. After cross-linking, cells were dually stained (successively FITC-conjugated phalloidin representing F-actin and TRITC-conjugated secondary antibody representing c-Abl). **A**: The distribution of F-actin in the activated cells. **B**: The distribution of c-Abl in the same activated cells. Each image is representative of three independent experiments with cells from different donors. Bar, 8 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

## DISCUSSION

During the rolling adhesion of leukocytes, selectins and their receptors expressed on the surface of leukocytes act not only as anchor molecules to arrest fleetly pass-by leukocytes to the activated endothelial cells, but also as signaling molecules to initiate intracellular signals, activate leukocytes, and facilitate their further firm adhesion [Vestweber and Blanks, 1999]. Among numerous signaling events, the alteration of F-actin-based cytoskeleton is an important one because the cytoskeleton is an essential element to form cell membrane ruffling, and uropoda as well, which are the structures to facilitate leukocytes firmly adhere to vascular wall. It has been shown that triggering of L-selectin led to a 10-fold increase of actin filament polymerization via activation of the small G protein Rac-2 [Brenner et al., 1997] and cross-linking of L-selectin induced actin assembly and co-localization with $CD_{18}$ [Simon et al., 1999]. L-selectin and PSGL-1 are both localized at the microvilli of leukocytes and both contribute to the activation of leukocytes during rolling adhesion. The alteration of cytoskeleton caused by cross-linking of PSGL-1 has not yet been reported, despite some recent important studies in which it has been suggested that PSGL-1 linked to cytoskeleton through its cytoplasm tail after leukocytes activation [Alonso-Lebrero et al., 2000; Serrador et al., 2002; Snapp et al., 2002; Urzainqui et al., 2002]. In the present report, we showed that PSGL-1 bound to cytoskeleton after antibody cross-linking and the cross-linking of PSGL-1 led to the polymerization and redistribution of F-actin. Further, the alteration of F-actin-based cytoskeleton was spatiotemporally

related to the polarization of PSGL-1. Cytochalasin B eliminated the polarization of PSGL-1, suggesting the polarization is cytoskeleton-dependent. Our results suggested that antibody cross-linking induces the alteration of F-actin, and the polarization of PSGL-1 is under the direction of F-actin-based cytoskeleton.

Cytoplasmic protein tyrosine kinases play important roles in the transduction of extracellular signals. In the present work, the treatment of cells with genistein severely blocked the alteration of cytoskeleton in the activated neutrophils, indicating that the alteration of F-actin-based cytoskeleton caused by cross-linking of PSGL-1 is tyrosine-phosphorylation-dependent. Among the tyrosine kinases, c-Abl is distinct in binding directly to F-actin. The non-receptor tyrosine kinase c-Abl is both localized in the nucleus and the cytoplasm and can shuttle between the nucleus and the cytoplasm due to its three nuclear location signals and one nuclear export signal [Woodring et al., 2003]. Nuclear c-Abl was reported to be related to transcription regulation [Shaul, 2000; Vigneri and Wang, 2001], and the cytoplasmic c-Abl is located to the dynamic region of cytoskeleton and appears to regulate the assembly of F-actin polymers into different structures depending on the extracellular signals. Here in our study, we observed that c-Abl was redistributed and re-localized to the periphery or an end of the neutrophil where F-actin concentrated after activation. Additionally, STI571 obviously blocked the alteration of F-actin-based cytoskeleton, suggesting the implication of c-Abl in the dynamics of cytoskeleton in the activated neutrophils. Taken together, the results proposed that cross-linking of PSGL-1, a signaling molecule contributing to the activation of neutrophils during rolling adhesion, can induce a phosphorylation-dependent and c-Abl-involved alteration of F-actin-based cytoskeleton in neutrophils.

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## REFERENCES

Alonso-Lebrero JL, Serrador JM, Dominguez-Jimenez C, Barreiro O, Luque A, del Pozo MA, Snapp K, Kansas G, Schwartz-Albiez R, Furthmayr H, Lozano F, Sanchez-Madrid F. 2000. Polarization and interaction of adhesion molecules P-selectin glycoprotein ligand 1 and intercellular adhesion molecule 3 with moesin and ezrin in myeloid cells. Blood 95:2413–2419.

Berton G, Laudanna C, Sorio C, Rossi F. 1992. Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils. J Cell Biol 116:1007–1017.

Brenner B, Gulbins E, Busch GL, Koppenhoefer U, Lang F, Linderkamp O. 1997. L-selectin regulates actin polymerisation via activation of the small G-protein Rac2. Biochem Biophys Res Commun 231:802–807.

Evangelista V, Manarini S, Sideri R, Rotondo S, Martelli N, Piccoli A, Totani L, Piccardoni P, Vestweber D, de Gaetano G, Cerletti C. 1999. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: Role of PSGL-1 as a signaling molecule. Blood 93:876–885.

Haller H, Kunzendorf U, Sacherer K, Lindschau C, Walz G, Distler A, Luft FC. 1997. T cell adhesion to P-selectin induces tyrosine phosphorylation of pp125 focal adhesion kinase and other substrates. J Immunol 158:1061–1067.

Hicks AE, Nolan SL, Ridger VC, Hellewell PG, Norman KE. 2003. Recombinant P-selectin glycoprotein ligand-1 directly inhibits leukocyte rolling by all 3 selectins in vivo: Complete inhibition of rolling is not required for anti-inflammatory effect. Blood 101:3249–3256.

Hidari KI, Weyrich AS, Zimmerman GA, McEver RP. 1997. Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils. J Biol Chem 272:28750–28756.

Hirata T, Furie BC, Furie B. 2002. P-, E-, and L-selectin mediate migration of activated CD8+ T lymphocytes into inflamed skin. J Immunol 169:4307–4313.

Kansas GS. 1996. Selectins and their ligands: Current concepts and controversies. Blood 88:3259–3287.

Kelly SA, Goldschmidt-Clermont PJ, Milliken EE, Arai T, Smith EH, Bulkley GB. 1998. Protein tyrosine phosphorylation mediates TNF-induced endothelial-neutrophil adhesion in vitro. Am J Physiol 274:H513–H519.

Moore KL, Stults NL, Diaz S, Smith DL, Cummings RD, Varki A, McEver RP. 1992. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J Cell Biol 118:445–456.

Nagata K, Tsuji T, Matsushima K, Hanai N, Irimura T. 2000. Redistribution of selectin counter-ligands induced by cytokines. Int Immunol 12:487–492.

Norman KE, Katopodis AG, Thoma G, Kolbinger F, Hicks AE, Cotter MJ, Pockley AG, Hellewell PG. 2000. P-selectin glycoprotein ligand 1 supports rolling on E- and P-selectin in vivo. Blood 96:3585–3591.

Redmond T, Tardif M, Zigmond SH. 1994. Induction of actin polymerization in permeabilized neutrophils. J Biol Chem 269:21657–21663.

Serrador JM, Urzainqui A, Alonso-Lebrero JL, Cabrero JR, Montoya MC, Vicente-Manzanares M, Yanez-Mo M, Sanchez-Madrid F. 2002. A juxta-membrane amino acid sequence of P-selectin glycoprotein ligand-1 is involved in moesin binding and ezrin/radixin/moesin-directed targeting at the trailing edge of migrating lymphocytes. Eur J Immunol 32:1560–1566.

Shaul Y. 2000. c-Abl: Activation and nuclear targets. Cell Death Differ 7:10–16.

Simon SI, Cherapanov V, Nadra I, Waddell TK, Seo SM, Wang Q, Doerschuk CM, Downey GP. 1999. Signaling functions of L-selectin in neutrophils: Alterations in the cytoskeleton and colocalization with CD18. J Immunol 163:2891–2901.

Simon SI, Hu Y, Vestweber D, Smith CW. 2000. Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. J Immunol 164:4348–4358.

Snapp KR, Heitzig CE, Kansas GS. 2002. Attachment of the PSGL-1 cytoplasmic domain to the actin cytoskeleton is essential for leukocyte rolling on P-selectin. Blood 99: 4494–4502.

Sperandio M, Smith ML, Forlow SB, Olson TS, Xia L, McEver RP, Ley K. 2003. P-selectin glycoprotein ligand-1 mediates L-selectin-dependent leukocyte rolling in venules. J Exp Med 197:1355–1363.

Tanis KQ, Veach D, Duewel HS, Bornmann WG, Koleske AJ. 2003. Two distinct phosphorylation pathways have additive effects on Abl family kinase activation. Mol Cell Biol 23:3884–3896.

Urzainqui A, Serrador JM, Viedma F, Yanez-Mo M, Rodriguez A, Corbi AL, Alonso-Lebrero JL, Luque A, Deckert M, Vazquez J, Sanchez-Madrid F. 2002. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. Immunity 17:401–412.

Vestweber D, Blanks JE. 1999. Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev 79:181–213.

Vigneri P, Wang JY. 2001. Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of BCR-ABL tyrosine kinase. Nat Med 7:228–234.

Woodring PJ, Hunter T, Wang JY. 2003. Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. J Cell Sci 116(Pt 13):2613–2626.

Xia L, Sperandio M, Yago T, McDaniel JM, Cummings RD, Pearson-White S, Ley K, McEver RP. 2002. P-selectin glycoprotein ligand-1-deficient mice have impaired leukocyte tethering to E-selectin under flow. J Clin Invest 109:939–950.

Zimmerman GA, McIntyre TM, Prescott SM. 1996. Adhesion and signaling in vascular cell–cell interactions. J Clin Invest 98:1699–1702.