Peritoneal macrophages express both P-selectin and PSGL-1

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Macrophages, phagocytic cells involved in an early phase of host defense, are known to express the P-selectin ligand, PSGL-1. Here, we demonstrate that peritoneal macrophages isolated by peritoneal lavage of unchallenged mice express P-selectin on the extracellular surface of macrophages but is only partially colocalized with PSGL-1. P-Selectin is rapidly translocated from the macrophage plasma membrane to intracellular vesicles and to lysosomes. Peritoneal macrophages assemble into cell strings under flow conditions based upon macrophage–macrophage interactions mediated by P-selectin and PSGL-1. This is the first description of a leukocyte shown to express both P-selectin and PSGL-1.

Introduction

Macrophages, derived from monocytes, are an important component of the host defense system. In response to an inflammatory stimulus circulating monocytes migrate out of the bloodstream into sites of injury where they differentiate into inflammatory macrophages. Monocytes also continuously emigrate into peripheral tissues, even in the absence of any inflammatory stimulus (van Furth and Cohn, 1968). Recent experiments suggest that these monocytes can also become tissue macrophages (Randolph et al., 1998, 1999). Upon activation by immunologic stimuli, these resident macrophages exit the tissue and transmigrate to the lymphatic system for antigen presentation. Resident alveolar macrophages are able to translocate particulate antigens to the paracortical T cell area of draining lymph nodes (Thepen et al., 1993). During the antigen sensitization phase of contact hypersensitivity, resident dermal macrophages migrate to lymph nodes and contribute to the efficiency of sensitization (Sato et al., 1998).

Peritoneal macrophages are a population of resident mononuclear phagocytes of unknown function. In the first 2 h after induction of inflammation, the number of resident macrophages in the peritoneal cavity significantly decreases, suggesting their emigration out of the peritoneal cavity (Haskill and Becker, 1985; Barth et al., 1995). Activated resident peritoneal macrophages migrate into parathymic lymph nodes and to the gut-associated lymphoid tissues (Rosen and Gordon, 1990; Sminia et al., 1995; van Vugt et al., 1995; Bellingan et al., 1996). Adhesion molecules including L-selectin, VLA-4, Mac-1, LFA-1, and PSGL-1 that potentially mediate the interaction of monocytes under flow have been extensively studied (Luscinskas et al., 1994, 1996; Gerszten et al., 1998; Lim et al., 1998) in contrast to the interactions of macrophages with adhesive molecules under flow. Here, we demonstrate that mouse peritoneal macrophages adhere to VCAM-1 under conditions of laminar flow. We also demonstrate that homotypic interactions among macrophages occur in laminar flow and that these interactions are mediated by PSGL-1 and P-selectin on the surface of the peritoneal macrophages. P-Selectin expression has heretofore been thought to be restricted to platelets and endothelial cells. These studies are the first demonstration that a specific leukocyte subtype, resident peritoneal macrophages, can express functional P-selectin.

Results

Peritoneal macrophages bind to VCAM-1 during laminar flow

To determine if peritoneal macrophages bind to an adhesive surface under conditions of laminar flow, isolated mouse peritoneal leukocytes were perfused at 0.5 dynes/cm² over a
VCAM-1–coated surface in a parallel flow chamber. The binding was calcium dependent and was completely inhibited by the presence of 5 mM EDTA. Interaction between adherent and flowing cells results in linear arrays of peritoneal leukocytes parallel to the direction of flow (Fig. 1, A and B, WT). Leukocytes roll over an adherent cell and bind to it on the downstream side. The peritoneal leukocytes forming strings consist entirely of adherent macrophages. All the cells in the string-like clusters stain with F4/80 antibodies that recognize a macrophage-specific antigen (Fig. 1 A, inset, WT). Similar string-like clusters have been observed when monocytes are exposed to laminar flow over an adhesive surface (Luscinskas et al., 1994; Alon et al., 1996; Walcheck et al., 1996; Lim et al., 1998).

The secondary interactions that lead to string-like formations among monocytes rolling on and adhering to selectins, VCAM-1, or inflamed endothelium are blocked with anti–PSGL-1 or anti–L-selectin antibodies (Alon et al., 1996; Lim et al., 1998). To explore whether L-selectin and PSGL-1 mediate this phenomenon in macrophages, we used peritoneal leukocytes from PSGL-1 null or L-selectin null mice to evaluate the role of these adhesion molecules in secondary interactions among peritoneal macrophages adhering to a VCAM-1–coated surface. String formation was unaffected by the absence of L-selectin (Fig. 1, A and B, L"/"). In contrast string formation by peritoneal macrophages was essentially eliminated in the absence of PSGL-1 (Fig. 1, A and B, PSGL1"/"). Because P-selectin is the predominant PSGL-1 ligand in a variety of leukocyte cell adhesion processes in vivo (Yang et al., 1999; Hirata et al., 2000), we examined the properties of peritoneal leukocytes from P-selectin null mice during perfusion over a VCAM-1–coated surface. P-Selectin null leukocytes bind to the VCAM-1 surface but nucleation by adherent macrophages to form cell strings was decreased by 80–85% (Fig. 1, A and B, P"/"). These results indicate that string formation is independent of L-selectin at a shear stress of 0.5 dyn/cm², but requires P-selectin and/or PSGL-1.

To demonstrate that nucleation is mediated by interaction of P-selectin and PSGL-1, we perfused a mixture of PSGL-1 null and P-selectin null leukocytes over a VCAM-1–coated surface. Combining the PSGL-1 null and P-selectin null macrophages restored cell string formation (Fig. 1, A and B, P"/" + PSGL1"/""). Thus, PSGL-1 and P-selectin appear to be the major ligand–receptor pair mediating secondary interactions between resident peritoneal macrophages under these conditions.
Figure 2. **Mouse peritoneal macrophages express P-selectin.** Flow cytometry was performed on peritoneal cells freshly isolated from wild-type and P-selectin null mice. (A) Peritoneal leukocytes were labeled with a rat monoclonal anti–P-selectin antibody or with an isotype-matched rat IgG. Cells from wild-type mice labeled with anti–P-selectin antibody (shaded), cells from P-selectin null mice labeled with anti–P-selectin antibody (thin line); cells from wild-type mice labeled with isotype-matched rat IgG (thick line), cells from P-selectin null mice labeled with isotype-matched IgG (dashed line). (B) Peritoneal leukocytes were labeled simultaneously with FITC-conjugated rat monoclonal anti–P-selectin antibodies and with either PE-conjugated rat anti-F4/80 antibodies or PE-conjugated rat anti-CD2 antibodies. (C) Peritoneal cells labeled with FITC-conjugated anti–P-selectin antibodies and separated on a cell sorter according to P-selectin expression were stained with Wright-Giemsa stain and visualized by light microscopy. Left, unfractionated cells; center, P-selectin–positive cells; right, P-selectin–negative cells. (D) Proteins in the lysate from platelets (10⁸) and peritoneal leukocytes (4×10⁷) were separated by electrophoresis on 7% SDS-gels under reducing conditions and the proteins transferred to a PVDF membrane. P-Selectin was detected using rabbit antibodies to the cytoplasmic tail of P-selectin and HRP-conjugated goat anti–rabbit IgG. WT, wild-type mice; P⁻/⁻, P-selectin null mice; PSGL-1⁻/⁻, PSGL-1 null mice; hP, purified human P-selectin (0.1 µg); WEHI, lysate from 4×10⁷ WEHI cells; Pl, platelet lysate; Le, peritoneal leukocyte lysate. (E) Total mRNA was isolated from peritoneal leukocytes of wild-type (WT) or P-selectin null (P⁻/⁻) mice, the mRNA species separated by gel electrophoresis and transferred to a PVDF membrane. The P-selectin band was detected using radiolabeled probe complementary to exon 3 of mouse P-selectin. (F) Peritoneal leukocytes were labeled simultaneously with FITC-conjugated rat monoclonal anti–P-selectin antibodies and with PE-conjugated rat anti–L-selectin antibodies, PE-conjugated anti–PSGL-1 antibodies, PE-conjugated anti–Mac1 antibodies or PE-conjugated rat anti–VLA-4 antibodies.
Resident macrophages isolated from the mouse peritoneum have surface P-selectin

To date P-selectin has been observed only in platelets and endothelial cells (Hsu-Lin et al., 1984; Stenberg et al., 1985; Berman et al., 1986; Bonfanti et al., 1989; McEver et al., 1989). We used flow cytometry to confirm the presence of P-selectin on peritoneal leukocytes. Monoclonal rat anti–P-selectin antibodies bound peritoneal leukocytes (Fig. 2 A) indicating that the mouse peritoneum contains a population of P-selectin–positive leukocytes in the absence of an inflammatory stimulus. In control experiments with peritoneal macrophages from P-selectin–deficient mice, no cell labeling was observed.

To confirm that the peritoneal cells bearing P-selectin are macrophages, we studied the interaction of these cells with antibodies specific for several leukocyte populations. P-selectin was expressed only by F4/80-positive cells, a population that corresponds to a macrophage subset of leukocytes (Fig. 2 A) indicating that the mouse peritoneum contains a population of P-selectin–positive leukocytes in the absence of an inflammatory stimulus. In control experiments with peritoneal macrophages from P-selectin–deficient mice, no cell labeling was observed.

To confirm that the peritoneal cells bearing P-selectin are macrophages, we observed the interaction of these cells with antibodies specific for several leukocyte populations. P-selectin was expressed only by F4/80-positive cells, a population that corresponds to a macrophage subset of leukocytes (Fig. 2 B). In contrast, peritoneal leukocytes that were positive for P-selectin were negative for CD2 that is expressed on peripheral B cells, T cells, and natural killer cells (Fig. 2 B).

To confirm that the P-selectin–positive cells were macrophages, we observed their morphology by light microscopy. Peritoneal cells were isolated and the P-selectin–positive and –negative fractions separated on a FACsorter™ with the aid of P-selectin antibodies. The total cell population (Fig. 2 C, left) and the fractionated cells were stained with Wright-Giemsa stain and analyzed by light microscopy. P-Selectin–positive cells had the morphological features of macrophages (Fig. 2 C, center), whereas P-selectin–negative cells appeared to be lymphocytic or plasmacytic in origin (Fig. 2 C, right). Thus, the P-selectin–positive cells isolated from the peritoneal cavity of mice appear to be macrophages.

We examined lysates of peritoneal leukocytes by Western blot analysis to confirm the presence of P-selectin and to determine its molecular weight. The P-selectin in peritoneal leukocytes was compared with P-selectin in lysates from mouse platelets and P-selectin purified from human platelets. The results of this analysis confirmed the presence of P-selectin on mouse peritoneal macrophages (Fig. 2 D). Anti–P-selectin antibodies stained bands of ~140,000 molecular weight in platelet lysates and in peritoneal leukocyte lysates from wild-type and PSGL-1–deficient mice. P-Selectin m-RNA was also identified in peritoneal leukocytes from wild-type mice, but not P-selectin null mice, by Northern blot analysis (Fig. 2 E).

L-selectin is expressed on neutrophils, monocytes, and most lymphocytes, whereas PSGL-1 is expressed on essentially all blood leukocytes. We explored whether L-selectin and PSGL-1 were coexpressed with P-selectin on the surface of peritoneal macrophages. A subset of P-selectin bearing peritoneal macrophages also express L-selectin, whereas essentially all of the P-selectin bearing macrophages also express PSGL-1 (Fig. 2 F). P-Selectin bearing peritoneal macrophages express the integrins MAC-1 and VLA-1, important for monocyte trafficking (Fig. 2 F).
P-Selectin is synthesized in the peritoneal macrophage

P-Selectin is present on the surface of activated platelets and activated endothelial cells (Hsu-Lin et al., 1984; Stenberg et al., 1985; Sims et al., 1988; Bonfanti et al., 1989; McEver et al., 1989). We explored whether peritoneal macrophage P-selectin might arise from contamination with activated platelets or platelet-derived or endothelial cell–derived microparticles bound to the PSGL-1 bearing leukocytes. Neither a platelet-specific marker, GPIIb, nor an endothelial marker, VE-cadherin, is observed on the surface of peritoneal leukocytes by flow cytometry (Fig. 3 A). Similarly, mRNA for GPIIb or VE-cadherin was not detectable in peritoneal leukocytes by RT-PCR analysis of peritoneal cells (Fig. 3 B). However, using RT-PCR we were able to amplify a portion of P-selectin cDNA including the coding sequence from the lectin domain to the transmembrane domain, indicating that macrophage-associated P-selectin is the membrane bound and not the soluble form of the protein (Fig. 3 C; Johnston et al., 1990). We did not detect any mRNA for von Willebrand factor, which resides within the same intracellular compartments as P-selectin in endothelial cells and platelets (Fig. 3 C; Bonfanti et al., 1989).

To directly demonstrate P-selectin synthesis by peritoneal macrophages, plastic-adherent macrophages from wild-type and P-selectin null mice, as well as CHO cells expressing P-selectin and untransfected CHO cells, were grown in media containing [35S]methionine and [35S]cysteine. A band of radiolabeled protein of the proper molecular weight was iso-
labeled from wild-type peritoneal macrophages and CHO cells expressing P-selectin using rabbit antibodies against the cytoplasmic tail of P-selectin (Fig. 3 D). Using this antibody no radiolabeled protein was isolated from peritoneal macrophages of P-selectin null mice or from untransfected CHO cells. Together, these results support the conclusion that peritoneal P-selectin is derived from peritoneal leukocytes and is not of platelet or endothelial cell origin.

**P-Selectin undergoes internalization in macrophages**

To address more precisely where P-selectin and PSGL-1 localize within peritoneal macrophages, we stained unfixed cells with anti–P-selectin antibodies labeled with Texas red and anti–PSGL-1 antibodies labeled with Alexa 488. Confocal microscopic analyses of stained macrophages show only small areas of colocalization of P-selectin and PSGL-1 (Fig. 4 A). Staining for PSGL-1 appears primarily on the periphery of the cell. P-Selectin is localized primarily in granule-like structures that appear to be beneath the cell surface although some P-selectin was observed on the cell surface. The granular appearance of the staining for P-selectin suggested that much of the P-selectin might be internalized.

Macrophages were stained with P-selectin either at 0°C or at 37°C. Labeling of peritoneal macrophages with rat anti–P-selectin antibody was strongly dependent on temperature (Fig. 4 B). Cells incubated at 37°C are more strongly labeled with anti–P-selectin than cells labeled at 0°C, suggesting accumulation of P-selectin–specific antibody within the cell. There is no temperature-dependent difference in staining of peritoneal macrophages with the isotype matched IgG. To distinguish surface-bound anti–P-selectin antibodies from those that have been internalized, cells labeled with anti–P-selectin antibodies were exposed to buffer at pH 2.3 or to buffer at pH 7.0. When cells stained with anti–P-selectin antibodies on ice were exposed to buffer at pH 2.3, staining due to anti–P-selectin was reduced almost to the background level observed with isotype matched IgG (Fig. 4 C).

In contrast, peritoneal macrophages labeled with anti–P-selectin antibodies at 37°C retained considerable anti–P-selectin antibody when exposed to buffer at pH 2.3 (Fig. 4 C). These results suggest that P-selectin is actively internalized at 37°C and that bound anti–P-selectin antibody is internalized with its target antigen.

Incubation of peritoneal leukocytes with FITC-conjugated dextran results in colocalization of P-selectin with FITC-dextran particles, a fluid phase marker. Colocalization is likely within endocytic vesicles and lysosomes (Fig. 4 D).

In electron micrographs of cell sections from macrophages incubated at 0°C before fixation, P-selectin detected with nanogold-conjugated antibodies is localized to the external face of the plasma membrane and to the internal face of the membrane of intracellular vesicles (Fig. 5 A). Gold particles were not observed on the peritoneal macrophages of P-selectin–deficient mice. In cell sections from macrophages incubated at 37°C, P-selectin is also detected in intracellular compartments (Fig. 5, B and C). To evaluate the nature of these compartments, cells stained with anti–P-selectin and anti–LAMP-1 antibodies were examined by confocal microscopy. Confocal images show that P-selectin is partially colocalized with the lysosomal marker, LAMP-1, in macrophages (Fig. 5 D).

**Expression of P-selectin by stimulated macrophages**

Surface expression of P-selectin in endothelial cells is regulated by two different mechanisms (Weller et al., 1992). Activation of endothelial cells with a secretagogue leads to translocation of P-selectin from the Weibel-Palade body membrane to the cell surface. Activation of endothelial cells with a cytokine leads to de novo P-selectin synthesis followed by P-selectin surface expression. Therefore, we investigated whether activation of resident peritoneal macrophages by cytokines alters P-selectin surface expression. Macrophages are activated through a two-stage mechanism, a priming stage and a triggering stage (Adams and
Figure 6. Expression of P-selectin by activated macrophages. Resident peritoneal macrophages were stimulated with 20 ng/ml of IFNγ and/or 100 ng of LPS for 24 h. The level of P-selectin expression on the surface of resting or stimulated macrophages was determined by FACS®. Mean fluorescence of P-selectin signal was determined as the difference between signal obtained after staining of macrophages with FITC-labeled anti-P-selectin antibodies and FITC-labeled control IgG (black bars). The concentration of TNFα in the culture supernatants was measured by ELISA (white bars). Values represent mean ± SD.

Hamilton, 1987). We determined the level of P-selectin surface expression on peritoneal macrophages after 24 h of stimulation with IFNγ, LPS or IFNγ, and LPS. The extent of macrophage stimulation was determined by measuring secretion of TNFα into the cell supernatant. Activation of resident peritoneal macrophages with either agent or the combination of agents did not alter the level of P-selectin on the macrophage plasma membrane (Fig. 6). In contrast, the secretion of TNFα was significantly stimulated after activation of macrophages with LPS with or without IFNγ.

Aggregation of peritoneal macrophages

Expression of both an adhesion receptor and its physiological ligand by the same cell can potentially promote formation of cell aggregates. Therefore, we analyzed the number of cell aggregates in peritoneal lavage harvested from P-selectin null, PSGL-1 null, and wild-type mice. When determined from cells maintained at 37°C, the percentage of cells in aggregates in isolated peritoneal lavage from PSGL-1 (8%) and P-selectin (7%) null mice were equivalent to the percentage observed in wild-type mice (11%). Because we demonstrated temperature-dependent internalization of macrophage surface-bound P-selectin, we compared the number of cell aggregates in peritoneal lavage after incubation of cells at 0°C. Incubation of wild-type peritoneal cells at 0°C significantly increased calcium-dependent cell aggregation (Fig. 7 A). This cell aggregation is P-selectin mediated and was not affected by the blocking of the function of another PSGL-1 ligand, L-selectin (Fig. 7 A).

To demonstrate that PSGL-1 is the primary P-selectin ligand in macrophage aggregation, we examined the temperature-dependent aggregation of a mixture of P-selectin null and PSGL-1 null cells. Significant temperature and calcium ion–dependent macrophage aggregation was observed in the mixture of P-selectin null and PSGL-1 null cells (Fig. 7 B) indicating the role of PSGL-1 as the P-selectin ligand in this process.

Discussion

P-Selectin is localized in the membranes of the α granules of platelets and the Weibel-Palade bodies of endothelial cells. Upon cell activation P-selectin is translocated to the plasma membrane of these cells (Stenberg et al., 1985; Berman et al., 1986; Bonfanti et al., 1989; McEver et al., 1989). P-Selectin on the surface of activated platelets and endothelial cells is available to interact with its ligand on myeloid cells (Larsen et al., 1989; Hamburger and McEver, 1990). The P-selectin ligand on the surface of myeloid cells has been identified as PSGL-1 (Moore et al., 1992; Sako et al., 1993). PSGL-1 has also been demonstrated on subsets of T lymphocytes (Moore and Thompson, 1992). These adhesive molecules mediate tethering of leukocytes to P-selectin expressed by stimulated endothelial cells and platelets.

Here, we demonstrate that peritoneal macrophages isolated from normal mice synthesize and express a functional form of P-selectin. This is the first example of a leukocyte that produces P-selectin. In contrast to platelets and endothelial cells, peritoneal macrophage P-selectin is constitutively expressed on the plasma membrane. We have demonstrated the presence of P-selectin on the cell surface by flow cytometry, by fluorescence and electron microscopy. However, distribution of P-selectin and PSGL-1 on the cell surface, as analyzed by fluorescence microscopy, reveals that, although there is some colocalization of the two proteins, PSGL-1 appears on the cell surface membrane, whereas most of the P-selectin is intracellular.

In cells with regulated secretory granules sequences within the cytoplasmic tail of P-selectin target this protein to the storage granules (Disdier et al., 1992; Koedam et al., 1992).
Sequences within the C1 and C2 domains of the P-selectin cytoplasmic tail have been implicated in delivery of P-selectin to secretory granules during expression in heterologous cells (Modderman et al., 1998; Blagoveshchenskaya et al., 1999). After the stimulation of endothelial cells, P-selectin is redistributed to the plasma membrane from storage granules and then rapidly internalized (Subramanian et al., 1993; Steiadi et al., 1995; Hattori et al., 1989). When P-selectin is expressed in heterologous cells lacking regulated secretory granules, it is transported to the cell surface and then rapidly endocytosed for degradation (Green et al., 1994; Blagoveshchenskaya et al., 1998b). The cytoplasmic tail of P-selectin contains a lysosomal targeting signal in the C1 domain and lysosomal avoidance signals in the C2 domain (Blagoveshchenskaya et al., 1998a,b). Together these signals regulate P-selectin transport among early and late endosomes and lysosomes (Blagoveshchenskaya et al., 1998a). These same sequences within the P-selectin cytoplasmic tail likely also regulate distribution of the protein in peritoneal macrophages. In the absence of secretory granules, macrophages direct P-selectin to the plasma membrane. However, P-selectin is found primarily in vesicular compartments within peritoneal macrophages. Colocalization with dextran particles places P-selectin in endosomes, whereas colocalization with LAMP-1 places P-selectin in lysosomes. These results reflect the pathways of P-selectin expression and transport established in model systems of cells lacking regulated secretory organelles. Although de novo P-selectin synthesis is observed in endothelial cells after cytokine stimulation, macrophage activation does not appear to alter P-selectin surface expression.

Secondary leukocyte–leukocyte interactions under flow conditions have been demonstrated previously for monocytes flowing over surfaces coated with P-selectin, E-selectin, or TNF-α–treated endothelial cells (Lim et al., 1998) and for neutrophils flowing over surfaces coated with E-selectin, P-selectin, L-selectin, PNA, VCAM-1, cytokine-stimulated endothelial cells, and PSGL-1 or neutrophil monolayers (Alon et al., 1996; Walcheck et al., 1996). L-selectin has been demonstrated to mediate the secondary interactions resulting in string-like cell formations for both monocytes and neutrophils under hydrodynamic shear stress of 1.5–3.0 dynes/cm² (Alon et al., 1996; Walcheck et al., 1996; Lim et al., 1998). PSGL-1 has been demonstrated to be the counter ligand for this interaction for both neutrophils and monocytes (Walcheck et al., 1996; Lim et al., 1998). However, macrophages are not found in blood but in theafferent lymph. The shear stress in the lymphatic system is much less than that in blood. Shear stress above a threshold level is critical for the optimal interaction of L-selectin with its ligands (Finger et al., 1996). At 0.7 dynes/cm² wall shear stress, neutrophils flowing over a P-selectin–coated surface do not form string-like formations; secondary accumulation of neutrophils does not occur at this wall shear stress as this is an L-selectin–mediated interaction (Alon et al., 1996). Failure to observe a contribution of L-selectin to peritoneal macrophage string formation in our system may be a result of insufficient wall shear stress to induce L-selectin–PSGL-1 binding. In contrast, P-selectin does not require shear above a critical threshold to promote and maintain interactions with its counterreceptor (Finger et al., 1996).

The functional significance of our observation that peritoneal macrophages express both the receptor and the counterreceptor, P-selectin and PSGL-1, remains unknown. We have demonstrated that at low temperature, when P-selectin internalization is slowed, homotypic peritoneal macrophage aggregates form (Fig. 7). It is possible that under certain physiologic conditions, P-selectin expression on the macrophage surface is stabilized. The presence of P-selectin and PSGL-1 on the same cell would potentially yield macrophage aggregates within the peritoneum. These peritoneal macrophages would not only adhere to each other but also to neutrophils, monocytes, and subsets of T lymphocytes. These cell aggregates may be part of a host defense system that clears the peritoneum of bacteria or other microbial particles. These aggregates may nucleate the formation of granulomas or possibly participate in the formation of giant cell granulomas.

Alternatively, P-selectin on the macrophage surface may be important in the immune response. Specifically, P-selectin and PSGL-1 may represent a ligand pair that contributes to the binding energy between macrophages and T lymphocytes. The interaction of antigen presenting cells with the lymphocyte T cell receptor complex is of low affinity, and several other ligand pairs are known to support both cell–cell interaction as well as cell signaling in the immunologic synapse (Lee et al., 1998; Grakoui et al., 1999). It remains plausible that in the case of peritoneal macrophage–T lymphocyte interaction, P-selectin on the macrophage participates in recognition and binding of T cell PSGL-1. LPS-induced up-regulation of P-selectin mRNA has been observed in both human dendritic cells and in Kupffer cells (Essani et al., 1995; Baltathakis et al., 2001) suggesting the potential for similar mechanisms in these cells.

In summary, these results suggest a functional role for the dual appearance of P-selectin and PSGL-1 on the peritoneal macrophage. These adhesion molecules may be important for microbial defense or for antigen presentation and immune response. In vivo experiments using genetically modified mice will offer an approach to testing these hypotheses.

Materials and methods

Mice

L-selectin–deficient mice were a gift from T. Tedder (Duke University, Durham, NC). P-selectin–deficient mice and C57BL/6J mice were obtained from Jackson Laboratory. PSGL-1–deficient mice were prepared as described previously (Yang et al., 1999) and backcrossed five generations with C57BL/6J. All mice were 8–12 wk old when used for the described experiments. All experimental procedures on animals were approved by the Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Reagents

Rat antibodies specific to murine PSGL-1 (2PH1), P-selectin (RB40.34), L-selectin (MEL-14), CD41 (MWReg30), CD11b (M1/70), CD49D (MFR4.B), CD2 (RM2–5), and polyclonal rabbit anti–P-selectin antibodies were obtained from BD Biosciences. F4/80 antibodies were obtained from Serotec Ltd. Goat anti–VE-cadherin antibodies (C19) were obtained from Santa Cruz Biotechnology, Inc. Colloidal gold-conjugated goat anti–rat antibodies (10 or 18 nm particles) were obtained from Jackson Immunoresearch Laboratories. Rabbit anti–P-selectin cytoplasmic tail antibodies were prepared as described previously (Chong et al., 1994). The soluble form of mouse VCAM-1 was from R&D Systems. The soluble form of recombinant human PSGL-1, a gift from Genetics Institute (Andover, MA), was characterized in our laboratory (Croce et al., 1998). P-Selectin was purified from human platelets (Larsen et al., 1992). Texas red was obtained from Molecular
ular Probes. Wright-Giemsa solution and low endotoxin BSA were obtained from Sigma-Aldrich.

**Isolation of resident leukocytes from mouse peritoneum**

Mice were killed and their peritoneal contents collected by flushing with 10 ml of ice-cold PBS containing 2 mM EDTA and 10 U/ml of heparin. Total leukocyte counts were determined using a counter (model T980; Beckman Coulter).

**Macrophage binding studies**

25 µl mouse recombinant VCAM-1 in 50 mM sodium bicarbonate containing 100 mM NaCl, pH 8.5, at a final concentration of 5 g/ml was coated on a polystyrene dish and incubated overnight at 4°C. The dish was washed three times with PBS and blocked with BSA (20 mg/ml in PBS) for 2 h at 37°C. A VCAM-1–coated plate was assembled in a parallel plate laminar flow chamber (Glycotech) and mounted on the stage of an inverted phase-contrast microscope (model Labovert FS; Leitz). To generate substrate. Peritoneal cells (10⁶ cells/ml) were perfused at 0.5 dyn/cm² shear stress over a surface coated with VCAM-1. After 5 min of perfusion, free-bound ligands were visualized with a 10× objective and videotaped using a digital color video camera (model SSC-S320; Sony) and a video recorder (model HS-U79; Mitsubishi).

String formation by peritoneal macrophages mediated by secondary macrophage–macrophage interactions was studied on VCAM-1–coated substrate. Peritoneal cells (10⁶ cells/ml) were diluted in a binding buffer (HBSS containing BSA and where indicated, EDTA) to the desired concentration and perfused through the flow chamber at the indicated shear stress. Cell interactions with the surface-bound ligands were visualized with a 10× objective and videotaped using a digital color video camera (model SSC-S320; Sony) and a video recorder (model HS-U79; Mitsubishi).

**Flow cytometric analysis and FACSorting™**

Peritoneal cells were isolated, washed, and resuspended in 200 µl of FACSM® solution. After incubation for 5 min with 5 µl of anti-CD16/CD32 antibodies to minimize nonspecific binding, cells were stained for 30 min at 4°C with fluorescently labeled antibodies to the indicated cell surface antigens and analyzed by flow cytometry.

**Fluorescent microscopy**

For flow studies, peritoneal cells were labeled with Texas red–conjugated F4/80 antibodies or with calcine. After washing, labeled cells were mixed at a 1:1 ratio to a final concentration of 10⁶ cells/ml. Cells were perfused for 5 min over the VCAM-1–coated surface. Adherent cells were washed with PBS, fixed for 15 min with 4% PFA solution, and examined by fluorescent microscopy. Excitation was at either 488 or 621 nm on a fluorescence microscope (model AX70; Olympus) equipped with a 40× water immersion objective.

For confocal microscopy, live or fixed Leucoperm permeabilized cells were stained with 20 µg/ml of antigen-specific mAbs directly conjugated to Texas red, Alexa 488, or FITC. After staining for 40 min at the indicated temperature, cells were washed twice with PBS at 4°C and examined microscopically using an 100× oil immersion objective.

**Northern blot and RT-PCR analysis of gene expression**

Peritoneal cells (8 × 10⁵) in RPMI medium were plated on a 60 × 15-mm tissue culture dish. After a 2-h incubation, nonadherent cells were removed by washing with 2 ml EDTA in PBS. Total RNA from adherent cells (>98% macrophages) was isolated using TRIzol reagent (Life Technologies). RNA from Northern blots and RT-PCR were performed by the manufacturer’s protocol (Ambion). The P-selectin probe corresponding to exon 3 of P-selectin was radiolabeled by PCR using [32P]dCTP incorporation.

For RT-PCR analysis, RNA from total mouse peritoneal leukocytes, lung, and bone marrow was isolated and the mRNA reverse transcribed using oligo (dT)12–15 primers and SuperScript II reverse transcriptase (Life Technologies). The resulting cDNAs were used for amplification of mouse proteins. The gene-specific primers were: W/F, forward (2647–2673); 5′-CTTGGAGCAGTTATCGGGAAGAAATG-3′ and reverse (4163–4137); 5′-AGGCCGATCTCATCACCTGGAAGGTTCTC-3′; P-selectin, forward (311–333); 5′-GGACCTGGTGGGAACAAAAATG-3′ and reverse (2170–2145); 5′-TTGTAAGAAGGCTGACACCCCAAAG-3′; GPIIIb forward (720–749), 5′-ATTGGACACATCATCCTACGACTGACCCG-3′ and reverse (998–982), 5′-CGAGGCCGACATATAGGAACATCTG-3′; VCAM-1, forward (224–249), 5′-CGCTGGCCCACACTGTCGAAGATACG-3′ and reverse (452–427); 5′-TGACAGTAGACCTGGAAGGTTGTCTC-3′.

**Western blot analysis**

500 µl of mouse blood was diluted twice with HBSS and 2 mM EDTA. Platelet-rich plasma was isolated by centrifugation at 400 g for 7 min. The platelets from platelet-rich plasma were washed twice with PBS and treated with lysis buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 10 mM sodium phosphate, and 10 mM sodium pyrophosphate) containing protease inhibitors (benzamidine, aprotinin, leupeptin, pepstatin A, and PMSF). Mouse peritoneal cells were treated with lysis buffer. The proteins from the cell lysates were separated under the reducing conditions on 7% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked for 2 h with 3% BSA in PBS and rabbit antibodies specific for P-selectin cytoplasmic tail were added to a final concentration of 10 µg/ml. After 2 h of incubation, the membrane was washed and bound antibodies were detected using goat anti–rabbit IgG conjugated to peroxidase.

**Internalization assay**

Freshly isolated peritoneal cells were first incubated for 40 min at 0°C in FACSM® solution to inhibit endocytosis. After incubation, cells were stained with FITC-labeled monoclonal anti–P-selectin antibodies or isotype-matched control IgG for 30 min at the indicated temperature. After washing with PBS at 4°C, half of the sample was incubated for 2 min in PBS, pH 7.0, at 4°C. The other half of the sample was treated with low pH buffer (500 mM NaCl, 0.2 N acetic acid, pH 2.3). The optimal pH for stripping antibody from the cell surface was determined using 10 µm polyethylene beads (Polysciences Inc.) with covalently bound mouse P-selectin. After incubation at low and neutral pH, cells were washed twice with PBS at 4°C and the mean fluorescence was determined by flow cytometry on a FACSCaliber™ after gating on the macrophage population.

**EM**

Peritoneal leukocytes were washed twice with FACSM® solution and incubated for 30 min at 0°C with rat anti–P-selectin antibodies. The cells were incubated for 30 min at either 0°C or 37°C with goat anti-IgG on 10- or 18-nm gold particles. Cells were washed twice with PBS and fixed for 15 min in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Fixed cells were pelleted and resuspended in 5% low gelling temperature sea plaque agarose. Agarose blocks were fixed after in 1% osmium tetroxide in cacodylate buffer for 1 h and stained enbloc with 2% uranyl acetate in water overnight. Blocks were washed, dehydrated, infiltrated in Epon 812 polyethylene oxide, and polymerized for 48 h at 60°C. 80-nm sections were placed on formvar-coated nickel grids, counterstained with uranyl acetate and lead citrate, and viewed on an electron microscope (model JEM 100CX; JEOL).

**Cell aggregation studies**

Leukocytes (2.5 × 10⁵) isolated from the peritoneum of wild-type mice, P-selectin null mice, or PSGL-1 null mice were resuspended in 50 µl of HBSS containing 2 mM CaCl₂. Cells were incubated at the indicated temperature in the presence or absence of 10 µg/ml blocking antibodies or 5 mM EDTA for 1 h. Aggregates were observed and quantified by phase-contrast microscopy using a 10× objective. Aggregates were defined as containing two or more cells.

**Measurement of P-selectin expression and TNFα production in stimulated macrophages**

Resident peritoneal cells were incubated on dishes for 4 h. Nonadherent adherent macrophages were cultured with 20 ng/ml IFNγ and/or 100 ng/ml LPS for 24 h. Adherent cells were harvested by scraping dishes and stained with FITC-labeled rat anti–P-selectin antibodies or FITC-labeled isotype matched control IgG. Stained cells were...
analyzed on a FACSCalibur™ flow cytometer. Concentrations of TNFα in the culture supernatants were determined by ELISA (R&D Systems).

Metabolic radiolabeling
CHO cells stably transfected with human P-selectin (2×10⁵) or 5×10⁵ resident peritoneal macrophages from wild-type or P-selectin-deficient mice were grown in 150 μl/Cd [35S]methionine/cysteine containing DME supplemented with 10% FCS for 14 h. Cells were washed three times with cold PBS, and adherent macrophages and CHO cells harvested by scraping. Cells were sedimented by centrifugation and lysed in buffer containing 1% Triton X-100, 0.1% SDS, 0.5% DOC, 50 mM Tris, pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.02% NaN₃, and protease inhibitors. After a 1-h incubation at 4°C, cell lysates were clarified by centrifugation at 10,000 g for 15 min. Supernatants were preclared with protein G-Sepharose after a 4-h incubation with 10 μg/ml rabbit nonimmune IgG. Cell lysates were incubated overnight at 4°C with rabbit antibodies directed against the cytoplasmic tail of P-selectin (5 μg/ml). Immunoprecipitates were collected by incubation of cell lysates with protein G-Sepharose (Amersham Biosciences) for 4 h at 4°C. Supernatant was removed and the Sepharose beads washed three times with lysis buffer containing 500 mM NaCl. Bound protein was eluted from Sepharose beads by incubation with SDS sample buffer containing mercaptoethanol. Eluted proteins were separated by SDS-PAGE. Autoradiographs were analyzed using an imager (model Typhoon 9400; Amersham Biosciences).

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