Monocyte Tethering by P-Selectin Regulates Monocyte Chemotactic Protein-1 and Tumor Necrosis Factor-α Secretion

Signal Integration and NF-κB Translocation

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Abstract

Adhesion molecules that tether circulating leukocytes to endothelial cells may also transduce or modulate outside-in signals for cellular activation, providing an initial regulatory point in the inflammatory response. Adhesion of human monocytes to P-selectin, the most rapidly expressed endothelial tethering factor, increased the secretion of monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) by the leukocytes when they were stimulated with platelet-activating factor. Increased cytokine secretion was specifically inhibited by G1, an anti-P-selectin mAb that prevents P-selectin from binding to its ligand (P-selectin glycoprotein ligand-1) on myeloid cells. Moreover, tethering by P-selectin specifically enhanced nuclear translocation of nuclear factor-κB (NF-κB), a transcription factor required for expression of MCP-1, TNF-α, and other immediate-early genes. These results demonstrate that P-selectin, through its ligands on monocytes, may locally regulate cytokine secretion in inflamed tissues. (J. Clin. Invest. 1995. 95:2297–2303.) Key words: selectin • cytokines • immediate-early (IE) genes • platelet-activating factor • signaling

Introduction

The adhesion of leukocytes is critical in physiologic and pathologic inflammation (1) and initiates responses that parallel contact-dependent events in embryonic development, tissue repair, and neoplasia (2). In human monocytes, a subset of blood leukocytes that are secretory and phagocytic in tissues, signals are delivered that induce expression of immediate-early (IE) response genes when the cells adhere to plastic or to extracellular matrix proteins (3). Some of the IE genes code for inflammatory cytokines. Adhesion of monocytes to plastic or to purified immobilized extracellular proteins differentially induces patterns of gene expression depending on the specific surface (3). While mRNA accumulates in these adherent monocytes, the cytokines themselves, which include interleukin-1β (IL-1β) and TNF-α, are not secreted unless there is a "second stimulus" such as bacterial lipopolysaccharide (LPS) (3). Thus, surface adhesion may cause futile activation of IE genes in monocytes unless there is coordinate action of a signaling molecule that stimulates completion of the synthetic process. Adhesion-dependent induction of mRNA for IE gene products is mediated by β integrins on the monocyte surface (4) and involves rapid phosphorylation of proteins on tyrosine residues (3, 5). Specific transcription factors, including NF-κB, are also required (3).

In contrast to interactions of monocytes with extracellular matrix proteins, little is known about cell-cell interactions that influence signaling of cytokine genes. Ligation of LFA-3 on monocytes by purified, immobilized CD2 caused secretion of IL-1β and TNF-α, suggesting that adhesion of T cells to monocytes via this ligand pair transduces signals and serves as a "physiologic trigger" for cytokine generation (6). Contact between monocytes and stimulated endothelial cells is a critical control point in inflammation and occurs before the adhesion of monocytes to matrix proteins or to T cells at extravascular sites. P-selectin, the adhesion factor most rapidly expressed by endothelial cells (1, 7), tethers human monocytes* and is present on endothelial surfaces after oxidant attack (8–11) and in atherosclerotic and rheumatoid lesions (12, 13). In this study we explored the generation of cytokines by monocytes adherent to P-selectin.

Methods

Antibodies and reagents. Hanks’ balanced salt solution (HBSS) and M199 were from Whittaker M. A. Bioproducts (Walkersville, MD), and human serum albumin (25%) was from Miles Laboratories, Inc. (Elkhart, IN). Fatty acid-free bovine serum albumin (BSA), LPS, and polymyxin B sulfate were from Sigma Chemical Co. (St. Louis, MO). Platelet-activating factor (PAF) was from Avanti Polar Lipids (Birmingham, AL).

1. Abbreviations used in this paper: CHO, Chinese hamster ovary; EMSA, electrophoretic mobility shift assay; ICAM-3, intercellular adhesion molecule-3; IE, immediate-early; MCP-1, monocyte chemotactic protein-1; PAF, platelet-activating factor; VCAM-1; vascular cell adhesion molecule-1.


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Recombinant human TNF-α and anti-human TNF-α were from R&D Systems. Recombinant human mAb was from Promega Corp. (Madison, WI). Peripheral blood monocytes were isolated by elutriation previously (18). Peripheral blood monocytes were isolated by elutriation previously (18). Peripheral blood monocytes were isolated by elutriation previously (18).

**Cells.** Chinese hamster ovary (CHO) cells stably transfected with cDNA for P-selectin were used for P-selectin and anti-P-selectin as described (18). Peripheral blood monocytes were isolated by countercurrent elutriation as described previously (19).

**Measurements of cytokine secretion.** 16-mm plastic wells (Costar Corp., Cambridge, MA) were left uncoated or were incubated overnight at 4°C with 300 μl of HBSS containing 10 μg/ml of human serum albumin in HBSS (HSA), anti-ICAM-3, or 2.5 μg/ml of P-selectin as described previously (7). After the plates were blocked with 5 mg/ml of HSA (4 h at 25°C), monocytes (1 x 10^6/ml) resuspended in serum-free M199 medium were added to the wells in the presence of buffer (HSA), PAF (1 nM to 1μM), or LPS (1 ng/ml to 1 μg/ml). The cells were incubated on each surface for 8 h unless otherwise indicated. After this incubation period, the supernatants were removed and centrifuged at 10,000 rpm in a microcentrifuge for 10 min. The cell-free supernatants were collected and stored at -70°C for subsequent analysis.

Transfected or wild-type CHO cells were grown to confluence, and monocytes (1 x 10^6/ml) in M199 medium were added to each monolayer. After the addition of vehicle (HSA), PAF, or LPS, the supernatants were collected at the end of 8 h and MCP-1 and TNF-α concentrations were measured as described above. Cell-free supernatants from CHO cells did not release detectable MCP-1 or TNF-α in the absence of monocytes.

In selected experiments, 10 μg/ml of F(ab')2, of mAb G1 or F(ab')2, of mAb S12 was added to the wells after the final wash step and before the addition of cells as described previously (16).

**Adherence studies.** Monocyte adherence to uncoated plastic or surfaces coated with P-selectin, albumin, or anti-ICAM-3 was determined by a modification of our method for neutrophil adherence (20), as described.

**Assays for NF-κB translocation to the nucleus.** Translocation of the nuclear transcription factor, NF-κB, from cytoplasm to the nucleus was examined using electrophoretic mobility shift assays (EMSA) and immunocytochemistry. EMSA. Monocytes adherent to immobilized P-selectin or control surfaces were stimulated with vehicle (HSA), PAF, or LPS for 2 h unless otherwise indicated. Nuclear extracts were isolated according to the method of Kitchens et al. (21). Nuclear protein (1 μg, 1–4 μl) was mixed with a reaction mixture that contained, in 10 μl of final volume, 1 μl BSA, 2 μl buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 50 mM Tris-HCl, 0.25 mg/ml of poly(dI-dC)), and balance (μl) of dH2O. After incubating the reaction mixture at 25°C for 10 min, we added 1 μl of a 32P-labeled κB consensus oligonucleotide with the following sequence:

5'-AGT TGA GGG GAC TTT CCC AGG C-3'

3'-TCA ACT CCC CTG AAA GGG TCC G-5'

The mixture was incubated at room temperature for 20 min, the reaction was stopped by adding 1 μl of gel loading 10× buffer (250 mM Tris-HCl, 0.2% bromphenol blue, 0.2% xylene cyanol, and 40% glycerol), and the reaction mixture was loaded onto a nondenaturing, 4% acrylamide (80:1 acrylamide to bisacrylamide) gel that had been “prerun” for 30 min. Electrophoresis was performed for 3 h at 100 V in 0.5× TBE running buffer. The gel was dried onto paper under vacuum at 80°C for 1 h and visualized by exposure to high performance autoradiography film (Amersham Corp., Arlington Heights, IL). Unlabeled κB oligonucleotide, added to the reaction mixture before the addition of 32P-labeled κB consensus oligonucleotide, competitively inhibited its association with nuclear NF-κB (not shown).

**Immunocytochemical analysis.** Monocytes adherent to P-selectin or to control surfaces were examined at 2 h unless indicated otherwise. The primary antibody was a rabbit polyclonal IgG against NF-κB p65 used at a dilution of 1:100 (1 μg/ml). A Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) was used for detection as described previously (9, 10, 22). Control slides included omission of the primary antibody or omission of the secondary antibody. Slides were viewed and photographed with Normarski interference contrast optics using a Zeiss Axioplan light microscope (Thornwood, NY).

**Results**

**Adhesion of monocytes to immobilized P-selectin potentiates stimulated secretion of MCP-1.** We first measured secretion of cytokines as the endpoint most relevant to the inflammatory state in vivo. There was little or no secretion of MCP-1 or of several other cytokines coded by IE genes when monocytes were incubated in suspension without stimulation, consistent with the observation that there is little or no mRNA for cytokines in resting monocytes (3). Incubation of monocytes on immobilized P-selectin did not induce secretion of MCP-1 above the levels found when the cells were incubated on plastic or albumin, even though there was robust adherence (Fig. 1 A), and the levels of MCP-1 released were not greater than those of monocytes incubated in suspension without stimulation (not shown). The conditions we used to coat the surfaces with P-selectin result in maximal adhesion of monocytes, and increasing the concentration of P-selectin by 10-fold in the buffer used to coat the wells did not further increase the secretion of MCP-1. These findings indicated that tethering of the cells to P-selectin does not alone induce MCP-1 secretion. We then asked if adhesion of monocytes to P-selectin alters MCP-1 secretion in response to a known agonist. We chose PAF, a biologically active phospholipid, because it is coexpressed with P-selectin on endothelial surfaces and mediates juxtacrine activation of tethered leukocytes (20, 24). Also, synthesis of PAF by monocytes is enhanced by tethering to P-selectin under some conditions, and PAF and MCP-1 may have synergistic autocrine effects (25). PAF was a weak or ineffective agonist for monocyte IE gene expression when the cells were stimulated in suspension (MCP-1 = 50±6 pg/ml). In contrast, PAF (1 nM to 1 μM) induced a 10-fold increase in MCP-1 secretion by monocytes adherent to P-selectin (Fig. 1 B). There was no enhancement of PAF-induced MCP-1 secretion in monocytes adherent to the control surfaces.

Although monocytes were potently stimulated by LPS, contamination of our P-selectin preparation by LPS was excluded as the cause for enhancement of MCP-1 secretion (Fig. 1 B) by the following: (a) polymyxin B (10 μg/ml), an LPS inactivating agent, was present in all experimental conditions; (b) our purified P-selectin contained < 1 ng/ml of LPS, a

monocyte adhesion to purified P-selectin and also blocks binding of P-selectin to high-affinity ligands purified from myeloid cells (14, 26). G1 inhibited MCP-1 secretion from PAF-stimulated monocytes adherent to immobilized P-selectin, whereas the F(ab')2 of a nonblocking anti1-P-selectin mAb, S12, did not (Fig. 3).

Adhesion of monocytes to P-selectin facilitates stimulated secretion of TNF-α. To determine if adhesion of monocytes to P-selectin alters expression of other cytokines, we measured TNF-α secretion. TNF-α release by monocytes adherent to immobilized P-selectin was greater than that of monocytes adherent to control surfaces (Fig. 3). The figure represents the mean±SE of three experiments.

Figure 3. An anti-P-selectin antibody inhibits stimulated MCP-1 secretion by adherent monocytes. P-selectin was immobilized on plastic wells as described in Methods and Fig. 1 followed by addition of the F(ab')2 of mAb G1 (10 μg/ml) or the F(ab')2 of mAb S12 (10 μg/ml). Monocytes were then added, PAF (100 nM) or control buffer was added, and secretion of MCP-1 was measured after 8 h. The figure represents mean±SE of three experiments.
mobilized P-selectin in the absence of additional stimulation was very low and was not greater than that by monocytes incubated on albumin or plastic (Fig. 4) or adherent to fibronectin or laminin. This again suggests that adhesion to P-selectin is insufficient to directly signal cytokine secretion. When stimulated with PAF, monocytes adherent to P-selectin secreted levels of TNF-α that were eightfold greater than those of monocytes adherent to albumin or plastic (Fig. 4). Exposure of the adherent cells to PAF for periods as short as 30 min followed by removal of the incubation medium was sufficient to elicit TNF-α secretion, indicating that brief contact with the signaling molecule is sufficient for cytokine production (not shown). Adherence of monocytes to CHO cells that express P-selectin also elicited an increase in TNF-α secretion in response to PAF (fourfold greater than nonstimulated controls). PAF did not induce TNF-α secretion by monocytes adherent to wild-type CHO cells.

**P-selectin facilitates NF-κB translocation in adherent monocytes.** Translocation of a transcription factor, NF-κB, to the nucleus is a key mechanism in adhesion-dependent induction of a variety of IE genes (3) and is required for MCP-1 (27) and TNF-α (28) expression. We examined the distribution of NF-κB in adherent monocytes using microscopic immunolocalization of an antibody against the p65 subunit (29) and by EMSA. Monocytes tethered by P-selectin remained round, indicating that a signal for cellular spreading had not been delivered, and NF-κB was concentrated in the cell cytoplasm with minimal nuclear staining (Fig. 5 A). EMSA demonstrated that there was a small, but discernible, increase in nuclear NF-κB (p50-p65) in monocytes adherent to purified P-selectin compared with those on immobilized albumin (Fig. 6). Consistent with this small increase, MCP-1 release was minimal (Fig. 1 B). When monocytes tethered to P-selectin were stimulated with PAF, NF-κB was robustly translocated to the nucleus as shown by EMSA (Fig. 6) and immunocytochemistry (Fig. 5 B). This was accompanied by dramatic cell spreading and polarization.

![Figure 4](image1)  
**Figure 4.** Adhesion of monocytes to P-selectin enhances stimulated secretion of TNF-α. Monocytes were allowed to adhere to immobilized P-selectin albumin or to uncoated plastic followed by treatment with control buffer or PAF (100 nM) as described in Methods and Fig. 1. TNF-α secretion was measured after 8 h. The figure represents the mean±SE of five experiments.

![Figure 5](image2)  
**Figure 5.** Tethering of monocytes by P-selectin enhances nuclear translocation of NF-κB. (A) Monocytes were allowed to adhere to immobilized P-selectin for 2 h in the absence of an additional stimulus, followed by incubation with an antibody to p65 and staining as described in Methods. Adjustment of the focal plane allowed visualization of blue nuclei with little nuclear staining by the anti-p65 antibody. (B) Monocytes adherent to P-selectin were stimulated with PAF (100 nM), and staining with anti-p65 was done after a 2-h incubation as described above. (C) Plastic wells were coated with anti-ICAM-3 as described in Methods. Monocytes were added, and staining using the anti-p65 antibody was done after a 2-h incubation in the presence of PAF (100 nM). A–C are representative results of four experiments.
flammary processes. Cell-cell interactions at vascular interfaces may establish levels or patterns of IE gene expression before the time monocytes encounter matrix proteins or other signals in the extravascular milieu.

Activation of leukocytes after adhesion to P-selectin is controversial (31). Earlier, it was reported that binding of purified P-selectin to polymorphonuclear leukocytes (PMNs) inhibits stimulated activation responses (32, 33), suggesting that it delivers negative signals. In contrast, we found that tethering of PMNs to purified P-selectin, or to transfected cells that express P-selectin, allows rapid activation events (inside-out signaling of $\beta_2$ integrins, polarization, priming for enhanced granular secretion) to proceed and is important for efficient activation of PMNs by juxtacrine signals at the surfaces of stimulated endothelial cells (20, 24). We then explored activation of monocytes and found that their adhesion to P-selectin, via constitutive surface ligands, enhances secretion of MCP-1 and TNF-$\alpha$ when the cells are stimulated with PAF (Fig. 1B and Fig. 4). Specific inhibition by mAb G1 (Fig. 3) suggests that the critical ligand on the monocyte surface may be P-selectin glycoprotein ligand-1 (34), since mAb G1 inhibits binding of P-selectin to purified P-selectin glycoprotein ligand-1 (26). Adhesion of monocytes to P-selectin caused them to be responsive to PAF, whereas adhesion to other surfaces did not (Figs. 1 and 4), and PAF was not effective as an agonist when monocytes were in suspension. This suggests that a major consequence of adhesion of monocytes to P-selectin is integration, or amplification, of signals delivered through the serpentine receptor for PAF and, potentially, through other such receptors. While mean levels of MCP-1 and TNF-$\alpha$ were slightly higher when monocytes were tethered to P-selectin in the absence of PAF, the values were not consistently greater than the background levels found when monocytes were incubated on control surfaces. This suggests that binding of P-selectin to ligands on the monocyte is by itself insufficient to induce the coordinate events that lead to MCP-1 and TNF-$\alpha$ secretion without the action of a signaling molecule such as PAF. In contrast, it was reported recently that P-selectin directly signals expression of tissue factor (mRNA and surface activity), albeit at much lower levels than when the monocytes were stimulated with LPS (35). Activation of specific genes and processing of their products in monocytes is differentially regulated by adhesion (3); therefore, it is possible that expression of proteins other than MCP-1 and TNF-$\alpha$ is directly induced by tethering to P-selectin.

To begin to explore mechanisms by which adhesion to P-selectin influences cytokine gene expression in monocytes, we focused on its effect on the transcription factor, NF-$\kappa$B. In resting cells, NF-$\kappa$B is located in the cytoplasm (29). With an appropriate activating stimulus, it is released from an inhibitory factor, IxB, and rapidly translocates to the nucleus. This event is followed by binding of NF-$\kappa$B to kB sequences in promoter and enhancer regions of a variety of genes, where it regulates their transcription together with other factors (29). NF-$\kappa$B is a critical regulator of IE genes in monocytes (3) and is required for MCP-1 (27) and TNF-$\alpha$ expression (28). We found that adhesion of monocytes to P-selectin alone caused a small but consistent translocation of NF-$\kappa$B (p50-p65); however, nuclear translocation was enhanced dramatically when the cells were stimulated with PAF (Fig. 4). We have not yet examined mRNA levels for MCP-1 and TNF-$\alpha$, but the degree of NF-$\kappa$B translocation in control and PAF-stimulated cells correlated

(Fig. 5B) as well as release of MCP-1 (Fig. 1B) and TNF-$\alpha$ (Fig. 4). PAF-induced nuclear translocation of NF-$\kappa$B was evident when monocytes adhered to P-selectin for periods as short as 30 min, indicating that brief contact between P-selectin and its ligands on monocytes is sufficient to facilitate NF-$\kappa$B translocation and, potentially, to initiate cytokine generation (not shown). Incubation of monocytes on albumin-coated surfaces caused little (not shown). Incubation of monocytes on albumin-coated surfaces caused little. We then examined the possibility that interaction with surfaces nonspecifically facilitates NF-$\kappa$B translocation in monocytes using a different approach. An immobilized mAb against ICAM-3, a surface molecule present on resting monocytes (30), gave cell binding that was equal to that on immobilized P-selectin (82% of monocytes adhered to anti-ICAM-3 compared with 78% to immobilized P-selectin; n = 2). However, there was no translocation of NF-$\kappa$B in monocytes adherent to anti-ICAM-3 in the basal state or when stimulated by PAF (Fig. 5C), although activation signals had been delivered as indicated by cellular spreading (Fig. 5C). Similarly, secretion of MCP-1 by monocytes bound to anti-ICAM-3 and stimulated with PAF was at background levels (48±9 pg/ml; n = 4) (compare with Fig. 1B).

**Discussion**

Our studies demonstrate that adhesion of monocytes to P-selectin alters events that are critical for the expression of IE genes and for secretion of products of these genes, inflammatory cytokines. Thus, adhesion of monocytes to P-selectin presented by immobilized endothelial cells or by platelets, which also express it, can potentially influence cytokine generation early in in-

4. We have found that stimulated platelets enhance NF-$\kappa$B translocation in monocytes, and cytokine secretion, by a P-selectin-dependent mechanism. Weyrich, A. S., M. R. Elstad, R. P. McEver, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman, manuscript in preparation.
with the magnitude of cytokine generation (Figs. 1–4). This suggests that facilitated transfer of NF-κB may be one mechanism by which adhesion to P-selectin amplifies MCP-1 and TNF-α secretion by stimulated monocytes. Enhanced NF-κB translocation was evident in stimulated monocytes 30 min after adhesion to P-selectin suggesting that brief contact with P-selectin, which occurs when the leukocytes interact with endothelial cells during transmigration in vivo, may be sufficient to induce this component of the response.

When monocytes adhere to inflamed endothelium, it is possible that P-selectin acts in a costimulatory fashion with other adhesion molecules such as ICAM-1 or vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 is proposed to regulate IE gene expression by binding to α4β1-integrin on monocytes (4). Although surface expression of P-selectin is often more transient than that of VCAM-1 and occurs in response to a different spectrum of agonists (1), it is present for prolonged periods after oxidant attack (8–11) and in atherosclerotic and rheumatoid lesions (12, 13), as is VCAM-1. Interaction of monocytes with ICAM-1, which is constitutively present and is upregulated on stimulated endothelial cells, caused generation of macrophage inflammatory protein-1α after 24 h (36). Presumably, this occurred by binding of β2 (CD11/CD18) integrins on the monocyte plasma membranes to ICAM-1, although outside-in signaling through β2 integrins is not thought to be sufficient to induce IE genes (4) and adhesion of monocytes to purified ICAM-1 did not alone signal IL-1β or TNF-α secretion (6). Taken together, however, these observations suggest that binding of P-selectin to ligands on monocytes, together with ligation of β1 or β2 integrins on their surfaces, may influence IE genes in concert, setting specific patterns of expression. In T lymphocytes, a soluble P-selectin chimera differentially altered GM-CSF and IL-8 secretion when a ligand for P-selectin was first induced by culture and the cells were then costimulated for 48 h with an anti-T cell receptor antibody (37). For monocytes, coordinate stimulation may occur when they bind to P-selectin and to ligands for integrins, or by adhesion to P-selectin and action of a signaling molecule such as PAF.

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