

# Monocyte Tethering by P-Selectin Regulates Monocyte Chemotactic Protein-1 and Tumor Necrosis Factor- $\alpha$ Secretion

## Signal Integration and NF- $\kappa$ B 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- $\alpha$  (TNF- $\alpha$ ) 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- $\kappa$ B (NF- $\kappa$ B), a transcription factor required for expression of MCP-1, TNF- $\alpha$ , 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)<sup>1</sup> 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-1 $\beta$  (IL-1 $\beta$ ) and TNF- $\alpha$ , 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  $\beta$ 1 integrins on the monocyte surface (4) and involves rapid phosphorylation of proteins on tyrosine residues (3, 5). Specific transcription factors, including NF- $\kappa$ B, 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-1 $\beta$  and TNF- $\alpha$ , 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<sup>2</sup> 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).

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Received for publication 29 November 1994 and in revised form 9 January 1995.

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.

*J. Clin. Invest.*

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0021-9738/95/05/2297/07 \$2.00

Volume 95, May 1995, 2297–2303

2. Elstad, M. R., F. S. Cowley, T. R. LaPine, R. P. McEver, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman, manuscript submitted for publication.

Immunopurified membranous P-selectin and mAb against P-selectin [ $F(ab')_2$  of mAb G1 and  $F(ab')_2$  of mAb S12] were prepared and characterized as described (14–16). The monoclonal antibody against intercellular adhesion molecule-3 (ICAM-3) (26E3D-1) (reference 17) was a gift from Dr. Michael Gallatin (ICOS Corp., Seattle, WA). Recombinant human TNF- $\alpha$  and anti-human TNF- $\alpha$  were from R&D systems (Minneapolis, MN). Recombinant human monocyte chemoattractant protein-1 (MCP-1) and anti-human MCP-1 were from Genzyme Corp. (Cambridge, MA). A double-stranded  $\kappa$ B consensus oligonucleotide was from Promega Corp. (Madison, WI).

**Cells.** Chinese hamster ovary (CHO) cells stably transfected with cDNA for P-selectin were prepared and maintained as previously described (18). Peripheral blood monocytes were isolated by counter-current 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  $\mu$ l of HBSS containing 10  $\mu$ g/ml of human serum albumin in HBSS (HSA), anti-ICAM-3, or 2.5  $\mu$ g/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 ( $1 \times 10^6$ /ml) resuspended in serum-free M199 medium were added to the wells in the presence of buffer (HSA), PAF (1 nM to 1  $\mu$ M), or LPS (1 ng/ml to 1  $\mu$ g/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 ( $1 \times 10^6$ /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- $\alpha$  concentrations were measured as described above. Cell-free supernatants from CHO cells did not release detectable MCP-1 or TNF- $\alpha$  in the absence of monocytes.

In selected experiments, 10  $\mu$ g/ml of  $F(ab')_2$  of mAb G1 or  $F(ab')_2$  of mAb S12 was added to the wells after the final washing 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- $\kappa$ B translocation to the nucleus.** Translocation of the nuclear transcription factor, NF- $\kappa$ B, 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 indicated otherwise. Nuclear extracts were isolated according to the method of Kitchens et al. (21). Nuclear protein (1  $\mu$ g, 1–4  $\mu$ l) was mixed with a reaction mixture that contained, in 10  $\mu$ l of final volume, 1  $\mu$ l BSA, 2  $\mu$ l buffer (20% glycerol, 5 mM MgCl<sub>2</sub>, 2.5 mM EDTA, 2.5 mM DTT, 50 mM Tris-HCl, 0.25 mg/ml of poly[dI-dC]), and balance ( $\mu$ l) of dH<sub>2</sub>O. After incubating the reaction mixture at 25°C for 10 min, we added 1  $\mu$ l of a <sup>32</sup>P-labeled  $\kappa$ B 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  $\mu$ l of gel loading 10 $\times$  buffer (250 mM Tris-HCl, 0.2% bromophenol 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 $\times$  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  $\kappa$ B

oligonucleotide, added to the reaction mixture before the addition of <sup>32</sup>P-labeled  $\kappa$ B consensus oligonucleotide, competitively inhibited its association with nuclear NF- $\kappa$ B (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- $\kappa$ B p65 used at a dilution of 1:100 (1  $\mu$ g/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 by Normarski interference contrast optics using a Zeiss Axioplan light microscope (Thornwood, NY).

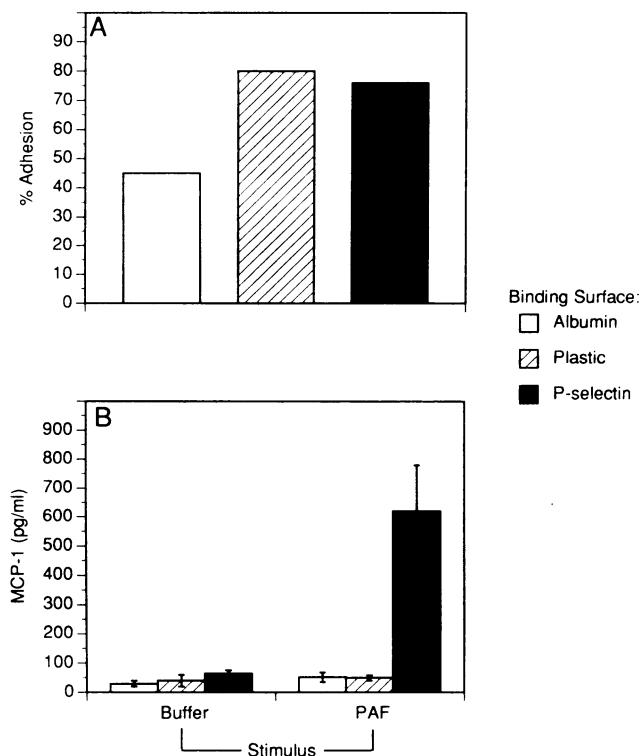
**Enzyme-linked immunoassay (ELISA).** MCP-1 and TNF- $\alpha$  concentrations were measured using minor modifications of ELISAs previously described by Evanoff et al. (23). Both ELISAs were specific and did not crossreact with other cytokines tested (i.e., IL-8, GM-CSF, RANTES, or macrophage inflammatory protein-1 $\alpha$ ).

## 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,<sup>3</sup> 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,<sup>2</sup> 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,<sup>2</sup> 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 $\pm$ 6 pg/ml). In contrast, PAF (1 nM to 1  $\mu$ M) 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,<sup>3</sup> 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) polymixin B (10  $\mu$ g/ml), an LPS inactivating agent, was present in all experimental conditions; (b) our purified P-selectin contained < 1 ng/ml of LPS, a

3. Weyrich, A. S., and G. A. Zimmerman, unpublished experiments.

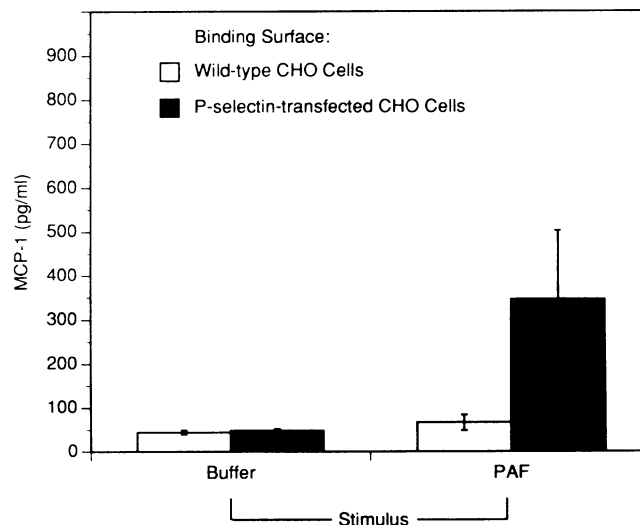


**Figure 1.** Adhesion of monocytes to purified, immobilized P-selectin enhances MCP-1 secretion in response to PAF. (A) Radiolabeled monocytes were incubated on immobilized P-selectin or control surfaces and adhesion was measured after 8 h. The figure represents the mean of two experiments. (B) Monocytes were allowed to adhere to P-selectin or to control surfaces and then were treated with control buffer or with PAF (100 nM). Secretion of MCP-1 was measured after an 8-h incubation. The figure represents the mean  $\pm$  SE of four experiments.

concentration that did not increase MCP-1 secretion ( $< 60$  pg/ml) over basal levels in the presence of polymixin B; and (c) costimulation with PAF (1  $\mu$ M) and LPS (100 ng/ml) did not increase MCP-1 secretion ( $57 \pm 5$  pg/ml) by monocytes adherent to control surfaces (albumin or plastic) in the presence of polymixin B. Thus, the regulatory effects of P-selectin on MCP-1 (Fig. 1 B) secretion were not due to cryptic activation of the monocytes by LPS.

**Transfected cells expressing P-selectin potentiate MCP-1 secretion by monocytes.** To determine if P-selectin presented by cellular surfaces also regulates MCP-1 secretion by adherent monocytes, we incubated the leukocytes with transfected CHO cells that express P-selectin (18, 24). Monocyte adherence to these cells is increased dramatically over adhesion to wild-type cells and is blocked by the anti-P-selectin mAb, G1.<sup>2</sup> Monocytes adherent to CHO cells expressing P-selectin did not secrete greater amounts of MCP-1 than did those incubated with control cells in the absence of additional stimulation (Fig. 2). However, stimulation with PAF induced MCP-1 secretion by monocytes tethered to cellular P-selectin (Fig. 2). In contrast, there was no enhanced secretion by monocytes on wild-type CHO cells.

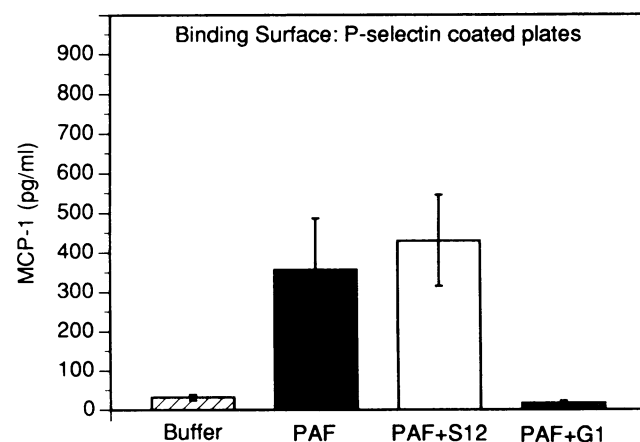
**Enhanced secretion of MCP-1 by monocytes is inhibited by a blocking antibody to P-selectin.** We examined the specificity of enhanced MCP-1 secretion by monocytes tethered to P-selectin using the F(ab')<sub>2</sub> of mAb G1, which specifically blocks



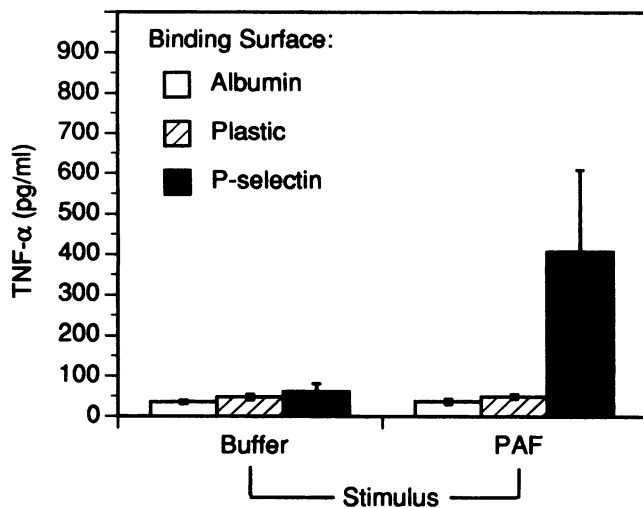
**Figure 2.** Adhesion of monocytes to transfected CHO cells that express P-selectin enhances MCP-1 secretion in response to PAF. Monocytes were added to wild-type CHO cells or transfected CHO cells that express P-selectin. PAF (1  $\mu$ M) or control buffer was added, and secretion of MCP-1 was measured after an 8-h incubation. The figure represents the mean  $\pm$  SE of four experiments.

monocyte adhesion to purified P-selectin<sup>2</sup> 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')<sub>2</sub> of a nonblocking anti-P-selectin mAb, S12, did not (Fig. 3).

**Adhesion of monocytes to P-selectin facilitates stimulated secretion of TNF- $\alpha$ .** To determine if adhesion of monocytes to P-selectin alters expression of other cytokines, we measured TNF- $\alpha$  secretion. TNF- $\alpha$  release by monocytes adherent to im-



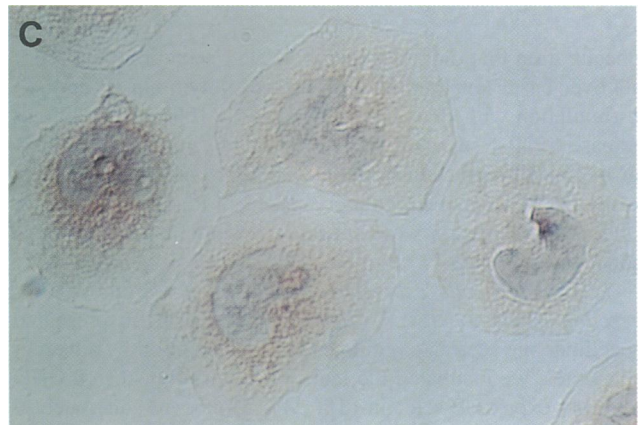
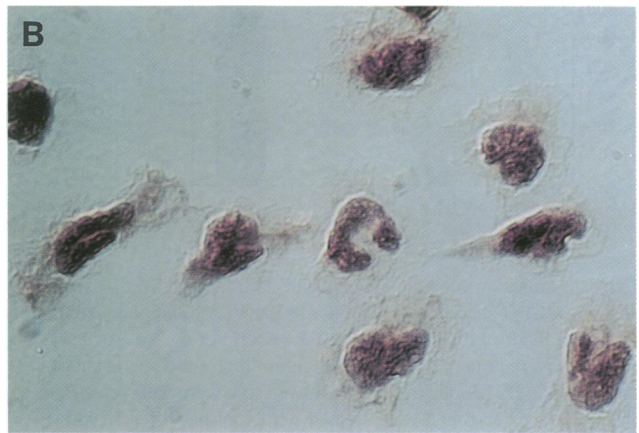
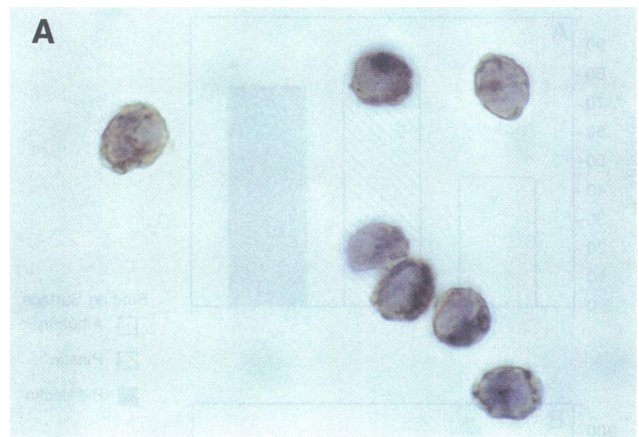
**Figure 3.** An anti-P-selectin antibody inhibits stimulated MCP-1 secretion by adherent monocytes. P-selectin was immobilized on plastic wells as described in Methods and Fig. 1 followed by addition of the F(ab')<sub>2</sub> of mAb G1 (10  $\mu$ g/ml) or the F(ab')<sub>2</sub> of mAb S12 (10  $\mu$ g/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  $\pm$  SE of three experiments.



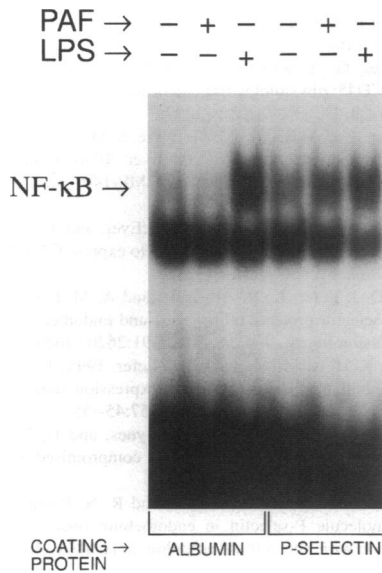
**Figure 4.** Adhesion of monocytes to P-selectin enhances stimulated secretion of TNF- $\alpha$ . Monocytes were allowed to adhere to immobilized P-selectin albumin or to uncoated plastic followed by treatment with control buffer or PAF (100 nM) as described in Methods and Fig. 1. TNF- $\alpha$  secretion was measured after 8 h. The figure represents the mean  $\pm$  SE of five 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.<sup>3</sup> 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- $\alpha$  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- $\alpha$  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- $\alpha$  secretion in response to PAF (fourfold greater than nonstimulated controls). PAF did not induce TNF- $\alpha$  secretion by monocytes adherent to wild-type CHO cells.

*P-selectin facilitates NF- $\kappa$ B translocation in adherent monocytes.* Translocation of a transcription factor, NF- $\kappa$ B, 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- $\alpha$  (28) expression. We examined the distribution of NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B (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- $\kappa$ B 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 5.** Tethering of monocytes by P-selectin enhances nuclear translocation of NF- $\kappa$ B. (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.



**Figure 6.** Adhesion of monocytes to purified, immobilized P-selectin enhances nuclear translocation of NF- $\kappa$ B measured by EMSA. Monocytes were allowed to adhere to immobilized albumin (lanes 1–3) or to immobilized P-selectin (lanes 4–6) in the presence of buffer, PAF (100 nM), or LPS (1  $\mu$ g/ml) for 2 h. Nuclear extracts were then prepared, and binding of NF- $\kappa$ B (p50-p65) in these samples to a consensus  $\kappa$ B oligonucleotide was assayed by EMSA as described in Methods. The figure is representative of three experiments.

(Fig. 5 B) as well as release of MCP-1 (Fig. 1 B) 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 or no nuclear translocation assessed by microscopic analysis (not shown), consistent with results using EMSA (Fig. 6). We also 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<sup>3</sup> or when stimulated by PAF (Fig. 5 C), although activation signals had been delivered as indicated by cellular spreading (Fig. 5 C). Similarly, secretion of MCP-1 by monocytes bound to anti-ICAM-3 and stimulated with PAF was at background levels (48 $\pm$ 9 pg/ml;  $n = 4$ ) (compare with Fig. 1 B).

## 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 stimulated endothelial cells or by platelets, which also express it,<sup>4</sup> can potentially influence cytokine generation early in in-

flammatory 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. 1 B 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, I $\kappa$ B, and rapidly translocates to the nucleus. This event is followed by binding of NF- $\kappa$ B to  $\kappa$ B 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. 5). 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

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- $\kappa$ B may be one mechanism by which adhesion to P-selectin amplifies MCP-1 and TNF- $\alpha$  secretion by stimulated monocytes. Enhanced NF- $\kappa$ B 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  $\alpha_4/\beta_1$ -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-1 $\alpha$  after 24 h (36). Presumably, this occurred by binding of  $\beta_2$  (CD11/CD18) integrins on the monocyte plasma membranes to ICAM-1, although outside-in signaling through  $\beta_2$  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-1 $\beta$  or TNF- $\alpha$  secretion (6). Taken together, however, these observations suggest that binding of P-selectin to ligands on monocytes, together with ligation of  $\beta_1$  or  $\beta_2$  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.

## Acknowledgments

We thank Michael Gallatin (ICOS Corp.) for providing mAb 26E3D-1; Kurt Albertine (University of Utah Health Sciences Center Research Microscopy Facility) for helpful discussions regarding immunocytochemistry techniques; Donelle Benson, Ruth Ann Green, Angela Le, and Margaret Vogel for help with leukocyte isolation and cell culture; and Michelle Bills for preparation of the manuscript.

This work was supported by the Nora Eccles Treadwell Foundation and grants from the National Institutes of Health (HL-44525, HL-07636, and HL-45510).

## References

- Zimmerman, G. A., S. M. Prescott, and T. M. McIntyre. 1992. Endothelial cell interactions with granulocytes: tethering and signaling molecules. *Immunol. Today*. 13:93–100.
- Hynes, R. O., and A. D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell*. 68:303–322.
- Juliano, R. L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577–585.
- Yurochko, A. D., D. Y. Liu, D. Eierman, and S. Haskill. 1992. Integrins

as a primary signal transduction molecule regulating monocyte immediate-early gene induction. *Proc. Natl. Acad. Sci. USA*. 89:9034–9038.

5. Lin, T. H., A. Yurochko, L. Kornberg, J. Morris, J. J. Walker, S. Haskill, and R. L. Juliano. 1994. The role of protein phosphorylation in integrin-mediated gene induction in monocytes. *J. Cell Biol.* 126:1585–1593.

6. Webb, D. S. A., Y. Shimizu, G. A. Van Seventer, S. Shaw, and T. L. Gerrard. 1990. LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. *Science* (Wash. DC). 249:1295–1297.

7. Geng, J.-G., M. P. Bevilacqua, K. L. Moore, T. M. McIntyre, S. M. Prescott, J. M. Kim, G. A. Bliss, G. A. Zimmerman, and R. P. McEver. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* (Lond.). 343:757–760.

8. Patel, K. D., G. A. Zimmerman, S. M. Prescott, R. P. McEver, and T. M. McIntyre. 1991. Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. *J. Cell Biol.* 112:749–759.

9. Weyrich, A. S., X.-L. Ma, D. J. Lefer, K. H. Albertine, and A. M. Lefer. 1993. In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J. Clin. Invest.* 91:2620–2629.

10. Weyrich, A. S., M. Buerke, K. H. Albertine, and A. M. Lefer. 1995. Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of ischemic feline myocardium. *J. Leukocyte Biol.* 57:45–55.

11. Mayadas, T. N., R. C. Johnson, H. Rayburn, R. O. Hynes, and D. D. Wagner. 1993. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell*. 74:541–554.

12. Johnson-Tidey, R. R., J. L. McGregor, P. R. Taylor, and R. N. Poston. 1994. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1. *Am. J. Pathol.* 144:952–961.

13. Grober, J. S., B. L. Bown, H. Ebling, B. Athey, C. B. Thompson, D. A. Fox, and L. M. Stoolman. 1993. Monocyte-endothelial adhesion in chronic rheumatoid arthritis. In situ detection of selectin and integrin-dependent interactions. *J. Clin. Invest.* 91:2609–2619.

14. Moore, K. L., N. L. Stults, S. Diaz, D. F. Smith, R. D. Cummings, A. Varki, and R. P. McEver. 1992. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J. Cell Biol.* 118:445–456.

15. Norgard, K. E., K. L. Moore, S. Diaz, N. L. Stults, S. Ushiyama, R. P. McEver, R. D. Cummings, and A. Varki. 1993. Characterization of a specific ligand for P-selectin on myeloid cells. *J. Biol. Chem.* 268:12764–12774.

16. Ushiyama, S., T. M. Laue, K. L. Moore, H. P. Erickson, and R. P. McEver. 1993. Structural and functional characterization of monomeric soluble P-selectin and comparison with membrane P-selectin. *J. Biol. Chem.* 268:15229–15237.

17. El-Gabalawy, H., M. Gallatin, R. Vazeux, G. Peterman, and J. Wilkens. 1994. Expression of ICAM-R (ICAM-3), a novel counter-receptor for LFA-1, in rheumatoid and nonrheumatoid synovium. *Arthritis Rheum.* 37:846–854.

18. Disdier, M., J. H. Morrissey, R. D. Fugate, D. F. Bainton, and R. P. McEver. 1992. Cytoplasmic domain of P-selectin (CD62) contains the signal for sorting into the regulated secretory pathway. *Mol. Biol. Cell.* 3:309–321.

19. Elstad, M. R., S. M. Prescott, T. M. McIntyre, and G. A. Zimmerman. 1988. Synthesis and release of platelet-activating factor by stimulated human mononuclear phagocytes. *J. Immunol.* 140:1618–1624.

20. Lorant, D. E., K. D. Patel, T. M. McIntyre, R. P. McEver, S. M. Prescott, and G. A. Zimmerman. 1991. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J. Cell Biol.* 115:223–234.

21. Kitchens, R. L., R. J. Ulevitch, and R. S. Munford. 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J. Exp. Med.* 176:485–494.

22. Feuillard, J., M. Korner, C. Fourcade, A. Costa, J.-L. Binet, P. Debre, and M. Raphael. 1994. Visualization of the endogenous NF- $\kappa$ B p50 subunit in the nucleus of follicular dendritic cells in germinal centers. *J. Immunol.* 152:12–21.

23. Evanoff, H. L., M. D. Burdick, S. A. Moore, S. L. Kunkel, and R. M. Strieter. 1992. A sensitive ELISA for the detection of human monocyte chemoattractant protein-1 (MCP-1). *Immunol. Invest.* 21:39–45.

24. Lorant, D. E., M. K. Topham, R. E. Whately, R. P. McEver, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 1993. Inflammatory roles of P-selectin. *J. Clin. Invest.* 92:559–570.

25. Locati, M., D. Zhou, W. Luini, V. Evangelista, A. Mantovani, and S. Sozzani. 1994. Rapid induction of arachidonic acid release by monocyte chemoattractant protein-1 and related chemokines. Role of Ca<sup>2+</sup> influx, synergism with platelet-activating factor and significance for chemotaxis. *J. Biol. Chem.* 269:4746–4753.

26. Moore, K. L., S. F. Eaton, D. E. Lyons, H. S. Lichenstein, R. R. Cummings, and R. P. McEver. 1994. The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetylglucosamine. *J. Biol. Chem.* 269:23318–23327.

27. Ueda, A., K. Okuda, S. Ohno, A. Shirai, T. Igarashi, K. Matsunaga, J. Fukushima, S. Kawamoto, Y. Ishigatsubo, and T. Okubo. 1994. NF- $\kappa$ B and Sp1

regulate transcription of the human monocyte chemoattractant protein-1 gene. *J. Immunol.* 153:2052–2063.

28. Ziegler-Heitbrock, H. W. L., T. Sternsdorf, J. Liese, B. Belohradsky, C. Weber, A. Wedel, R. Schreck, P. Bauerle, and M. Strobel. 1993. Pyrrolidine dithiocarbamate inhibits NF- $\kappa$ B mobilization and TNF production in human monocytes. *J. Immunol.* 151:6986–6993.

29. Liou, H.-C., and D. Baltimore. 1993. Regulation of the NF- $\kappa$ B/rel transcription factor and I $\kappa$ B inhibitor system. *Curr. Opin. Cell Biol.* 5:477–487.

30. Vazeux, R., P. A. Hoffman, J. K. Tomita, E. S. Dickenson, R. L. Jasman, T. St. John, and W. M. Gallatin. 1992. Cloning and characterization of a new intercellular adhesion molecule ICAM-R. *Nature (Lond.)* 360:485–488.

31. McEver, R. P. 1994. Selectins. *Curr. Opin. Immunol.* 6:75–84.

32. Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas. 1990. Prevention of activated neutrophil adhesion to endothelium by soluble GMP-140. *Science (Wash. DC)* 249:414–417.

33. Wong, C. S., J. R. Gamble, M. P. Skinner, C. M. Lucas, M. C. Berndt,

and M. A. Vadas. 1991. Adhesion protein GMP140 inhibits superoxide anion release by human neutrophils. *Proc. Natl. Acad. Sci. USA.* 88:2397–2401.

34. Sako, D., X.-J. Chang, K. M. Barone, G. Vachino, H. M. White, G. Shaw, G. M. Veldman, K. M. Bean, T. J. Ahern, B. Furie, D. A. Cumming, and G. R. Larsen. 1993. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell.* 75:1179–1186.

35. Celi, A., G. Pellegrini, R. Lorenzet, A. De Blasi, N. Ready, B. C. Furie, and B. Furie. 1994. P-selectin induces the expression of tissue factor on monocytes. *Proc. Natl. Acad. Sci. USA.* 91:8767–8771.

36. Lukacs, N. W., R. M. Strieter, V. M. Elnor, H. L. Evanoff, M. Burdick, and S. L. Kunkel. 1994. Intercellular adhesion molecule-1 mediates the expression of monocyte-derived MIP-1 $\alpha$  during monocyte-endothelial interactions. *Blood.* 83:1174–1178.

37. Damle, N. K., K. Klussman, M. T. Dietsch, N. Mohagheghpour, and A. Aruffo. 1992. GMP-140 (P-selectin/CD62) binds to chronically stimulated but not resting CD4<sup>+</sup> T lymphocytes and regulates their production of proinflammatory cytokines. *Eur. J. Immunol.* 22:1789–1793.