

Interleukin 4 or Oncostatin M Induces a Prolonged Increase in P-Selectin mRNA and Protein in Human Endothelial Cells

By Longbiao Yao, Junliang Pan, Hendra Setiadi, Kamala D. Patel, and Rodger P. McEver

From the W.K. Warren Medical Research Institute, Departments of Medicine and Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, and Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Summary

During acute inflammation, P-selectin is transiently mobilized from Weibel-Palade bodies to the surface of histamine-activated endothelial cells, where it mediates rolling adhesion of neutrophils under hydrodynamic flow. During chronic or allergic inflammation, sustained expression of P-selectin on the endothelial cell surface has been observed. We found that the cytokines interleukin 4 (IL-4) or oncostatin M (OSM) induced a five- to ninefold increase in P-selectin messenger RNA (mRNA) in human umbilical vein endothelial cells (HUVEC) that persisted as long as 72 h. IL-4 elevated P-selectin mRNA by increasing its transcription rate rather than by prolonging its already long half-life. Stimulation of P-selectin transcription by IL-4 or OSM required new protein synthesis and tyrosine phosphorylation of cellular proteins. Tumor necrosis factor α , IL-1 β , lipopolysaccharide, or IL-3 did not increase P-selectin mRNA in HUVEC, and did not augment the IL-4-induced increase in P-selectin transcripts. IL-4 or OSM increased P-selectin protein on the cell surface as well as in Weibel-Palade bodies. Under flow conditions, neutrophils rolled on P-selectin expressed by IL-4-treated HUVEC, and even more neutrophils rolled on P-selectin after IL-4-treated HUVEC were stimulated with histamine. These data demonstrate that IL-4 or OSM stimulates endothelial cells to synthesize more P-selectin over prolonged periods. The increased expression of P-selectin may facilitate the emigration of leukocytes into sites of chronic or allergic inflammation.

Leukocyte emigration into lymphoid tissues and sites of inflammation is mediated by the sequential engagement of adhesion molecules. Molecules that transmit signals to leukocytes, endothelial cells, or platelets regulate the expression and/or function of the adhesion molecules. Specific combinations of signaling and adhesion molecules may control the onset and duration of leukocyte recruitment, as well as the subclasses of leukocytes that are mobilized, during acute, chronic, or allergic inflammation (1).

The binding of selectins to cell-surface carbohydrate ligands mediates the initial tethering and rolling of leukocytes on the vessel wall under conditions of hydrodynamic flow (2). Subsequent leukocyte activation promotes stable adhesion through engagement of β 2 integrins with Ig-like counterreceptors such as intercellular adhesion molecule (ICAM)-1¹ and ICAM-2 (1). Depending on their activa-

tion state, the α 4 integrins on mononuclear cells, eosinophils, and basophils mediate both tethering and stable adhesion through interactions with the Ig counterreceptors, vascular cell adhesion molecule (VCAM)-1, and mucosal addressin cell adhesion molecule-1 (3, 4).

TNF- α , IL-1 β , and LPS participate in acute inflammation, where neutrophils preferentially accumulate, but they may also be present at sites of chronic inflammation. These mediators stimulate endothelial cells to transcribe mRNAs encoding E-selectin, ICAM-1, and VCAM-1 (5). In vivo, the selective expression of VCAM-1 is associated with the accumulation of mononuclear leukocytes or eosinophils during chronic or allergic inflammation (6–13). IL-4, a cytokine expressed by CD4⁺ T cells, basophils, and mast cells (14–17), is frequently detected at the same sites (18, 19). In vitro, IL-4 induces human umbilical vein endothelial cells (HUVEC) to synthesize VCAM-1, but not E-selectin or ICAM-1. VCAM-1 mediates adhesion of mononuclear cells, eosinophils, and basophils, but not neutrophils, to the endothelial cell surface (20–22). Under shear stress, monocytes attach and roll on IL-4-activated HUVEC through

¹Abbreviations used in this paper: HUVEC, human umbilical vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; OSM, oncostatin M; STAT, signal transducer and activator of transcription; VCAM-1, vascular cell adhesion molecule-1.

interactions with VCAM-1 and with a ligand for L-selectin (23). These data suggest that IL-4 promotes emigration of mononuclear cells or eosinophils during chronic and allergic inflammation, in part, by inducing the selective expression of VCAM-1 on the endothelium. Another potential mediator of chronic inflammation is oncostatin M (OSM), which is secreted by monocytes and activated T cells (24). OSM stimulates release of plasminogen activator activity from bovine endothelial cells (25) and expression of IL-6 in HUVEC (26). It is not known whether OSM alters the expression of adhesion molecules on endothelial cells.

P-selectin is stored in the membranes of the α granules of platelets (27, 28) and the Weibel-Palade bodies of endothelial cells (29, 30). Upon stimulation of these cells by agonists such as thrombin, histamine, and complement components, P-selectin is rapidly mobilized to the plasma membrane, where it mediates the adhesion of neutrophils, monocytes, eosinophils, basophils, and subsets of lymphocytes (31–36). The protein is then rapidly internalized (37, 38) and targeted to lysosomes for degradation (39) or, in some cases, resorted into secretory granules (40). This rapid but self-limited surface expression enables P-selectin to mediate leukocyte tethering to the vessel wall during the earliest stages of acute inflammation (41, 42).

Persistent expression of P-selectin on the endothelial cell surface has also been observed in tissues with chronic or allergic inflammation such as rheumatoid synovium (43), atherosclerotic plaque (44), and nasal polyp (45). In vitro, TNF- α or LPS induces a two- to fourfold increase in the steady-state levels of P-selectin transcripts in murine and bovine endothelial cells (46–48). This increased synthesis of P-selectin may saturate the sorting pathway into secretory granules, leading to direct delivery of the protein to the cell surface (47). In vivo, TNF- α or LPS also increases the levels of P-selectin mRNA and protein in endothelial cells of mice and rats (46, 48–50). Both in vitro and in vivo, however, the increased mRNA levels peak within 2–4 h and decline to basal levels within 12–24 h after addition of TNF- α or LPS. Thus, it is not clear whether the effects of these mediators are sufficient to account for the persistent and selective expression of P-selectin in certain tissues with chronic and allergic inflammation. Furthermore, unlike its effects in cultured rodent and bovine endothelial cells, TNF- α does not increase P-selectin mRNA in HUVEC (51). These data suggest that mediators other than TNF- α and LPS might increase the transcription of P-selectin mRNA during some inflammatory responses.

In this study, we demonstrate that either IL-4 or OSM causes a sustained accumulation of P-selectin mRNA and protein in cultured HUVEC. This mechanism to increase expression of P-selectin may facilitate the emigration of leukocytes into sites of chronic or allergic inflammation.

Materials and Methods

Reagents and Antibodies. The anti-human P-selectin mAbs S12 and G1 were characterized as described (32, 52). Human rIL-4, murine IL-4, human OSM, and human IL-3 were purchased

from R & D Systems, Inc. (Minneapolis, MN). Recombinant human TNF- α , human IL-1 β , and Nutridoma SP serum-free medium were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). LPS from *Salmonella typhosa*, cycloheximide, LPS-free gelatin, polymyxin B, and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO). Herbimycin A and genistein were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). All other reagents were obtained from Fisher Scientific (Pittsburgh, PA) unless noted otherwise.

Cell Culture. HUVEC were isolated and cultured in medium 199 containing 20% human serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml of streptomycin as described (53). Cells were grown to confluence on LPS-free, gelatin-coated tissue culture plastic. Passage 1 cells were used in all experiments illustrated. However, similar responses to cytokines were observed with cells studied after two or three passages. Cytokines or pharmacologic agents, dissolved in fresh medium, were added to the confluent cells for the time intervals and at the final concentrations indicated in the text. As a control, fresh medium lacking the cytokine or pharmacologic agent was added to other cells. In other experiments, Nutridoma SP serum-free medium, in the presence or absence of a cytokine, was added to the cells. As determined by Northern blot analysis, HUVEC responded similarly to cytokines whether they were dissolved in fresh medium 199 containing human serum or dissolved in Nutridoma SP serum-free medium.

Human lung microvascular endothelial cells were obtained from Clonetics (San Diego, CA) and cultured in medium supplied by the manufacturer (modified MCDB medium supplemented with 5% FBS, 12 ng/ml bovine brain extract, 10 ng/ml human epidermal growth factor, 1 ng/ml hydrocortisone, 50 μ g/ml gentamicin, and 50 ng/ml amphotericin). Passage 4 or 5 cells were used in all experiments illustrated. Murine bEnd.3 endothelioma cells (47) were grown in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human microvascular endothelial cells and bEnd.3 cells were treated with cytokines as described for HUVEC, except that murine IL-4 was used instead of human IL-4 to treat bEnd.3 cells. Cells used in all experiments were determined to be free of mycoplasma, as measured by a PCR-based detection system (Stratagene, San Diego, CA).

Northern Blot Analysis. Northern blot analysis of total RNA isolated from confluent endothelial cells was performed as described (54). The 32 P-labeled probes were a 1.4-kb SmaI fragment of human P-selectin cDNA (54), a BstXI-linearized plasmid containing a human VCAM-1-Ig cDNA chimera (55), a 2.9-kb XbaI fragment of human E-selectin cDNA (56), a BamHI-linearized plasmid containing the entire CHO-B cDNA (57), and a HindIII-linearized CDM8 plasmid containing murine P-selectin cDNA (46). Quantification of RNA was performed by densitometry using a Model GS-670 imaging densitometer (Bio Rad Laboratories, Hercules, CA). The scans were normalized according to the levels of CHO-B transcripts, which did not change when endothelial cells were treated with cytokines.

Nuclear Run-on Analysis. HUVEC were incubated in fresh medium in the presence or absence of 20 ng/ml of IL-4 for 10 h at 37°C. Cell nuclei were isolated and nuclear run-on assays were performed as described (58). Equal amounts of 32 P-labeled, newly transcribed RNA from unstimulated or IL-4-stimulated HUVEC were hybridized with target DNAs that were immobilized on Hybond-N membranes using a Bio Rad slot-blot apparatus. The target DNAs were the SmaI fragment of human P-selectin cDNA, the linearized plasmid containing the CHO-B cDNA, or,

as a negative control, the pIBI20 plasmid (IBI Laboratories, New Haven, CT) lacking any cDNA insert.

Binding of ^{125}I -S12 to HUVEC. Binding of ^{125}I -labeled mAb S12 to fixed or nonfixed HUVEC monolayers was performed as described (37, 59).

Quantification of P-Selectin by ELISA. HUVEC in six-well plates were lysed with 500 μl of 0.1 M sodium phosphate, pH 8.0, 1% Triton X-100, 1.5 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF, 5 $\mu\text{g}/\text{ml}$ pepstatin A, 21 μM leupeptin, and 27 mM iodoacetamide. The lysates were centrifuged at 10,000 g for 10 min. P-selectin antigen in the supernatants was measured by ELISA, using recombinant soluble P-selectin as standard (60).

Adhesion of Neutrophils to HUVEC under Static or Flow Conditions. Adhesion of human neutrophils to HUVEC under static conditions was performed as described (32, 53). Neutrophil attachment to HUVEC under hydrodynamic flow conditions was measured in a parallel-plate flow chamber as described (61, 62).

Results

Stimulation of HUVEC with IL-4 Increases mRNA for P-Selectin and VCAM-1, but Not for E-Selectin. To determine whether IL-4 affected the amount of P-selectin mRNA in HUVEC, we treated confluent cell monolayers with 20 ng/ml of human rIL-4 for various intervals. Steady-state levels of P-selectin mRNA were measured by Northern blot analysis of equivalent amounts of RNA isolated from each group of cells. As demonstrated previously (54), unstimulated HUVEC expressed mRNA for P-selectin (Fig. 1 A). IL-4 markedly increased the levels of P-selectin mRNA. Transcripts did not accumulate until 10 h after the addition of IL-4; mRNA levels reached a maximum after 24 h and remained at this level for at least 32 h (Fig. 1 A). In other experiments, mRNA remained at maximal levels for up to 72 h after addition of IL-4 (data not shown). The membrane was rehybridized with a probe encoding CHO-B, a ubiquitously expressed transcript that does not change its levels when cells are stimulated (57). Quantification of the P-selectin transcripts, normalized for the amount of CHO-B mRNA, indicated that IL-4 increased P-selectin mRNA by seven- to ninefold after 24–32 h (Fig. 1 B). IL-4 increased P-selectin mRNA in a concentration-dependent manner, with near-maximal levels achieved at a concentration of 10 ng/ml (data not shown). The effect of IL-4 was not caused by contamination with LPS, since boiled IL-4 did not increase P-selectin transcripts. Indeed, exogenous LPS did not alter P-selectin mRNA (Fig. 1 A).

Rehybridization of the membrane with probes for E-selectin and VCAM-1 indicated that unstimulated HUVEC did not express transcripts for either molecule. IL-4 induced the accumulation of mRNA for VCAM-1, but not for E-selectin, confirming previous observations (63). Transcripts for VCAM-1 increased more rapidly than those for P-selectin (Fig. 1 A). LPS markedly increased mRNA for both E-selectin and VCAM-1.

Stimulation of HUVEC with OSM Increases mRNA for P-Selectin, but Not for E-Selectin or VCAM-1. Although OSM is known to stimulate HUVEC through interactions with a high affinity receptor (26), its ability to affect expres-

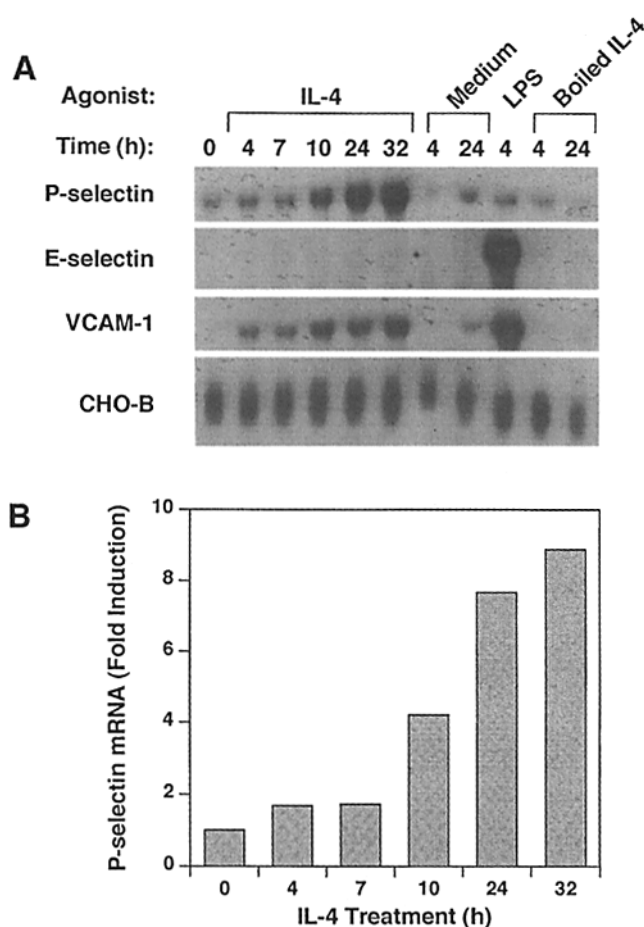


Figure 1. Stimulation of HUVEC with IL-4 increases mRNA for P-selectin and VCAM-1 but not for E-selectin. (A) Confluent monolayers of HUVEC were treated with fresh medium containing 20 ng/ml IL-4 or with fresh medium alone. To ensure that the observed effects of IL-4 were not caused by contaminating LPS, some cells were treated with IL-4 that was boiled to inactivate the cytokine, or with 10 ng/ml of exogenously added LPS. After the indicated time, total RNA was isolated, and 20 μg of RNA from each group of cells was electrophoresed and then transferred to a Hybond-N membrane for Northern blot analysis. The same membrane was sequentially hybridized with the indicated cDNA probes. The mobilities of the hybridized transcripts corresponded to published values. (B) The IL-4-induced increase in P-selectin mRNA was quantified by densitometric scanning. The level of P-selectin mRNA in each lane was normalized according to the level of CHO-B mRNA, which was not affected by IL-4. The data are representative of three independent experiments.

sion of adhesion molecules on these cells has not been studied. We treated HUVEC with 10 ng/ml of OSM for various intervals and then measured the levels of mRNA for P-selectin, E-selectin, and VCAM-1 by Northern blot analysis (Fig. 2). OSM markedly increased transcripts for P-selectin in a manner much like that observed for IL-4. Transcripts did not accumulate until 10 h after addition of OSM, reached a maximum after 24–32 h, and remained at this level for up to 72 h. Densitometric analysis indicated that OSM increased mRNA levels for P-selectin by five- to sixfold. OSM increased P-selectin transcripts in a concentration-depen-

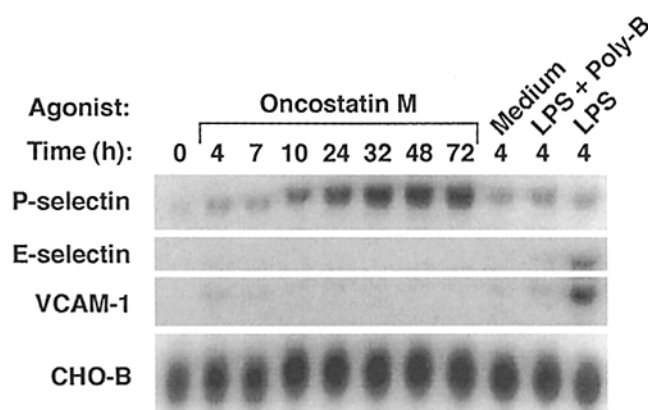


Figure 2. Stimulation of HUVEC with OSM increases mRNA for P-selectin and VCAM-1, but not for E-selectin. HUVEC were treated with fresh medium in the presence or absence of 10 ng/ml of OSM, 10 ng/ml of LPS, or 10 ng/ml of LPS inactivated with 10 μ g/ml of polymyxin B. After the indicated time, total RNA was isolated and analyzed by Northern blotting, as described in Fig. 1. The data are representative of three independent experiments.

dent manner, with near-maximal effects at a concentration of 10 ng/ml (data not shown). OSM did not induce accumulation of mRNA for E-selectin. Unlike IL-4, OSM also did not increase the levels of VCAM-1 mRNA (Fig. 2).

Costimulation of HUVEC with IL-4 Plus TNF- α , IL-1 β , LPS, or IL-3 Does Not Further Increase mRNA for P-Selectin. Costimulation of HUVEC with IL-4 plus TNF- α increases VCAM-1 RNA to a much higher level than that induced by either individual cytokine (63). We stimulated HUVEC with TNF- α , IL-1 β , LPS, or IL-3 for various intervals. Other cells were treated with IL-4, alone or in combination with one of the other agonists. We then measured the levels of mRNA for P-selectin and VCAM-1 (Fig. 3). As reported previously (6, 63, 64), TNF- α increased VCAM-1 mRNA, and TNF- α combined with IL-4 increased VCAM-1 mRNA significantly more than the level produced by either cytokine alone (Fig. 3 A). The combination of IL-4 with IL-1 β or LPS produced a similar synergistic increase in VCAM-1 mRNA (Fig. 3, B and C). In contrast, TNF- α , IL-1 β , or LPS did not increase P-selectin mRNA and had little or no effect on the IL-4-induced increase in P-selectin transcripts (Fig. 3, A-C). In fact, these agonists sometimes decreased P-selectin mRNA levels after 10–24 h (Fig. 3, A and C). IL-3 had little or no effect on VCAM-1 or P-selectin mRNA and did not augment the increases in these transcripts induced by IL-4 (Fig. 3 D).

IL-4 Does Not Detectably Affect the Half-life of P-Selectin mRNA, but Does Increase the Transcription of P-Selectin mRNA. To determine whether IL-4 increased the steady-state levels of P-selectin mRNA by prolonging its half-life,

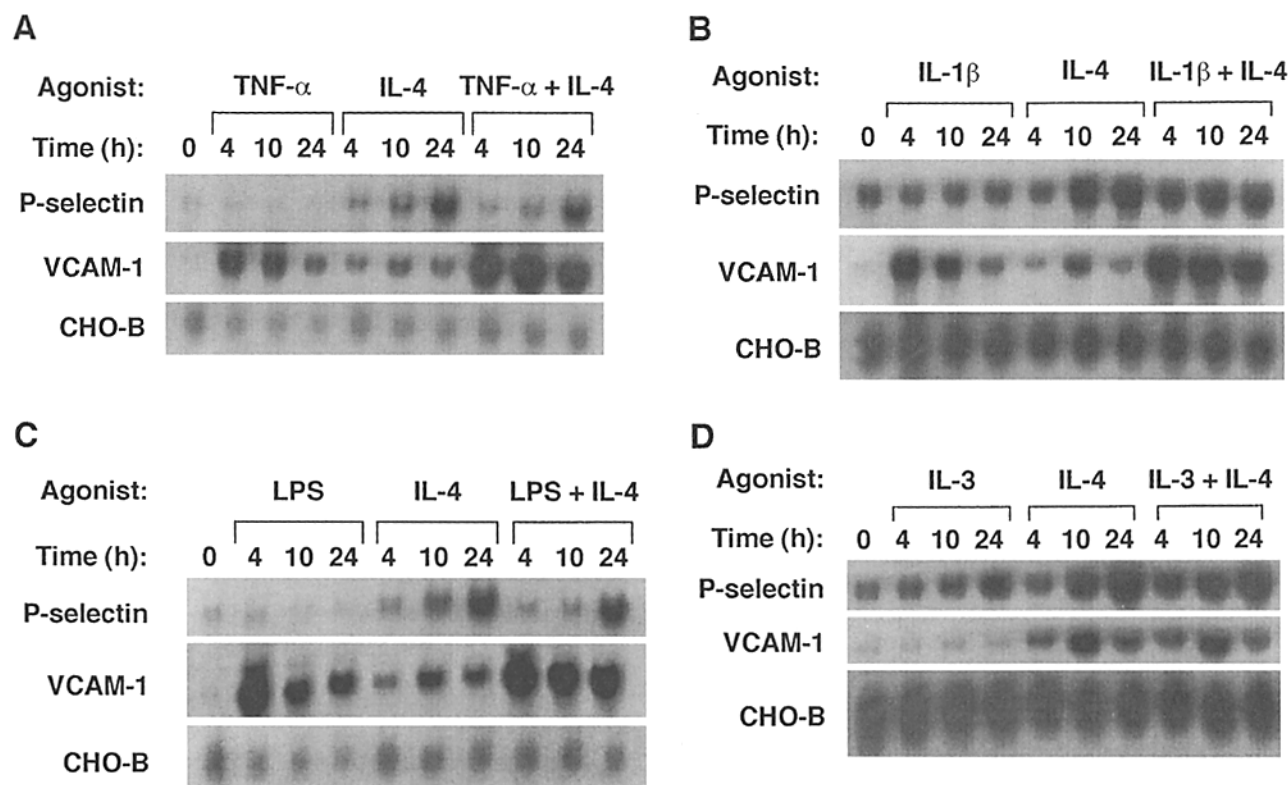


Figure 3. Costimulation of HUVEC with IL-4 plus TNF- α , IL-1 β , LPS, or IL-3 does not further increase mRNA for P-selectin. HUVEC were treated with (A) TNF- α (100 U/ml), (B) IL-1 β (100 U/ml), (C) LPS (10 ng/ml), or (D) IL-3 (10 ng/ml). Other HUVEC were treated with 20 ng/ml of IL-4, alone, or with the indicated mediator. After the indicated time, total RNA was isolated and analyzed by Northern blotting, as described in Fig. 1. The data are representative of two (A–C) or three (D) experiments.

we pretreated HUVEC with medium in the presence or absence of IL-4 for 24 h, and then blocked further transcription by adding actinomycin D. At intervals up to 12 h, the remaining P-selectin mRNA was detected by Northern blot analysis and quantified by densitometry of the blots (Fig. 4). The half-life of P-selectin transcripts in both control (medium only) and IL-4-treated cells exceeded 12 h. These results indicate that P-selectin mRNA has a long half-life in both control and IL-4-treated HUVEC.

When IL-4 and actinomycin D were added simultaneously to HUVEC, there was no increase in P-selectin mRNA (data not shown). To confirm that IL-4 increased the transcription of P-selectin mRNA, we used a nuclear run-on assay. Treatment of HUVEC with IL-4 for 10 h significantly increased the transcription of mRNA for P-selectin (Fig. 5). Densitometric analysis indicated that IL-4 increased the transcription of P-selectin mRNA by four- to fivefold (data not shown).

Inhibitors of Tyrosine Kinases Block the IL-4- and OSM-induced Increases in P-Selectin mRNA. IL-4 and OSM bind to a class of cytokine receptors that transduce signals, in part, through tyrosine phosphorylation of a group of tyrosine kinases and transcription molecules that associate with the ligand-occupied receptors (65, 66). We pretreated HUVEC with two tyrosine kinase inhibitors, genistein and herbimycin A, which suppress IL-4- and OSM-induced tyrosine phosphorylation in HUVEC (67, 68). Both inhibitors blocked the increase in P-selectin mRNA induced by IL-4 or OSM, and also slightly reduced the level of constitutively expressed P-selectin mRNA (Fig. 6). This result

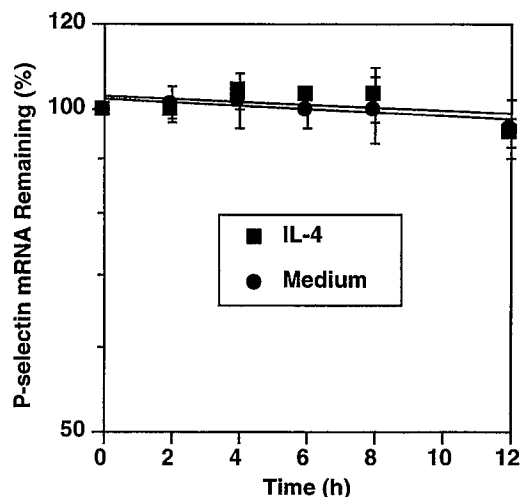


Figure 4. P-selectin mRNA has a long half-life in both control and IL-4-stimulated HUVEC. HUVEC were pretreated with fresh medium or with fresh medium plus 10 ng/ml of IL-4 for 24 h. Actinomycin D (5 μ g/ml) was then added to block further transcription. After the indicated time, total RNA was isolated, and P-selectin mRNA was quantified by Northern blotting and densitometry. The mRNA level before addition of actinomycin D (time 0) was assigned a value of 100%, and subsequent values were normalized relative to this value. The data represent the mean \pm SD of three independent experiments for both control and IL-4-treated cells.

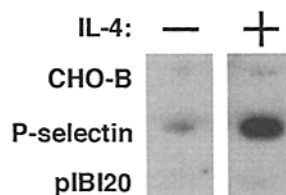


Figure 5. IL-4 increases the transcription of P-selectin mRNA. Cell nuclei were isolated from HUVEC that were pretreated with fresh medium in the presence or absence of 20 ng/ml of IL-4 for 10 h. Equal amounts of 32 P-labeled nuclear RNA from unstimulated and IL-4-stimulated cells were hybridized with unlabeled P-selectin cDNA, CHO-B cDNA, and a control pIBI20 plasmid that were immobilized on a Hybond-N membrane with a slot blot apparatus. The data are representative of three independent experiments.

supports a role for tyrosine phosphorylation in the cytokine-induced expression and perhaps the constitutive expression of P-selectin mRNA.

The IL-4- and OSM-induced Accumulation of P-Selectin mRNA in HUVEC Requires Protein Synthesis. The lag period required for HUVEC to accumulate P-selectin mRNA in response to IL-4 differs from the early accumulation of transcripts from immediate early genes, which cytokines activate without a requirement for new protein synthesis (5, 65, 66). To determine whether the IL-4-induced increase in P-selectin mRNA required protein synthesis, we stimulated HUVEC with IL-4 in the presence or absence of cycloheximide. The cycloheximide-treated cells did not increase P-selectin mRNA in response to IL-4 (Fig. 7). In contrast, these cells expressed higher

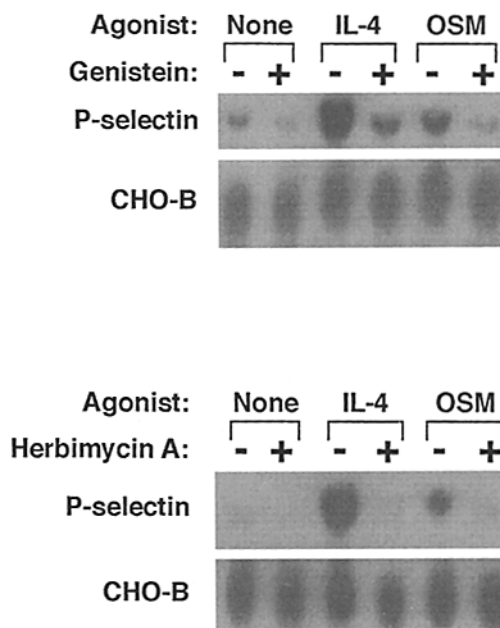


Figure 6. Inhibitors of tyrosine kinases block the IL-4- and OSM-induced increases in P-selectin mRNA. HUVEC were preincubated at 37°C with genistein (100 μ M) for 1 h or with herbimycin A (1 μ M) for 24 h. Control cells were not treated with either agent. The cells were then incubated for 12 h in the presence or absence of 20 ng/ml IL-4 or 10 ng/ml OSM. Genistein or herbimycin A were maintained at the same concentrations during this 24-h incubation. Total RNA was then isolated and analyzed by Northern blotting as in Fig. 1. The data are representative of three independent experiments.

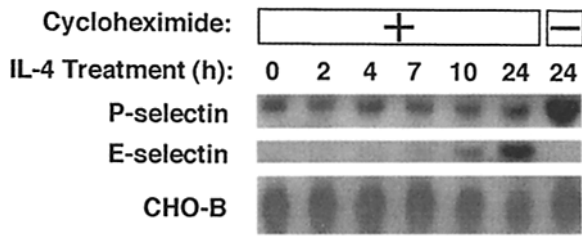


Figure 7. The IL-4-induced accumulation of P-selectin mRNA in HUVEC requires protein synthesis. HUVEC were treated with fresh medium containing 20 ng/ml IL-4 in the presence or absence of 10 μ g/ml of cycloheximide. After the indicated time, total RNA was isolated and analyzed by Northern blotting, as described in Fig. 1. The data are representative of three independent experiments.

E-selectin mRNA levels, presumably because they could not synthesize new $\text{I}\kappa\text{B}-\alpha$ to retain heterodimeric NF- κB complexes in the cytoplasm (69, 70). These results demonstrate that, to fully activate the P-selectin gene, IL-4 must first induce the synthesis of one or more proteins in HUVEC. OSM also required protein synthesis to increase P-selectin mRNA in HUVEC (data not shown).

Stimulation of HUVEC with IL-4 or OSM Increases P-Selectin Protein. To determine whether the IL-4-induced increase in P-selectin mRNA led to an increase in P-selectin protein, we treated HUVEC with or without IL-4 for 24 h and then measured the amount of P-selectin protein with three different assays (Fig. 8). Using an ELISA, we found that IL-4 increased P-selectin in cell lysates by nearly three-fold (Fig. 8 A). We next compared the levels of surface and total P-selectin by measuring binding of ^{125}I -labeled S12, an mAb to P-selectin, to fixed, nonpermeabilized or permeabilized cells. IL-4 increased both the surface and total levels of P-selectin, although only a small fraction of the total P-selectin was on the surface in both control and treated cells (Fig. 8 B). We then added ^{125}I -S12 to live cells at 37°C, which allowed cumulative binding of the antibody to P-selectin molecules cycling between endosomes and the plasma membrane (37, 38). IL-4 treatment increased the amount of P-selectin in these two compartments. In both control and IL-4-treated cells, stimulation with histamine further elevated P-selectin, consistent with fusion of Weibel-Palade membranes with the plasma membrane (Fig. 8 C). These data demonstrate that IL-4 increases the level of P-selectin constitutively present on the cell surface, as well as the quantity of P-selectin that can be mobilized to the plasma membrane from Weibel-Palade bodies. As measured with the same assays, OSM also increased P-selectin protein in HUVEC (data not shown).

More Neutrophils Adhere to P-Selectin on IL-4-stimulated HUVEC under Both Static and Flow Conditions. We next asked whether the IL-4-induced increase in P-selectin protein enhanced the adhesion of neutrophils to HUVEC. We used neutrophils because they do not express functional $\alpha 4$ integrins, except in response to certain stimuli (71). Therefore, neutrophils do not interact with VCAM-1 (6, 64, 72), which is expressed on IL-4-activated HUVEC (20-23). In

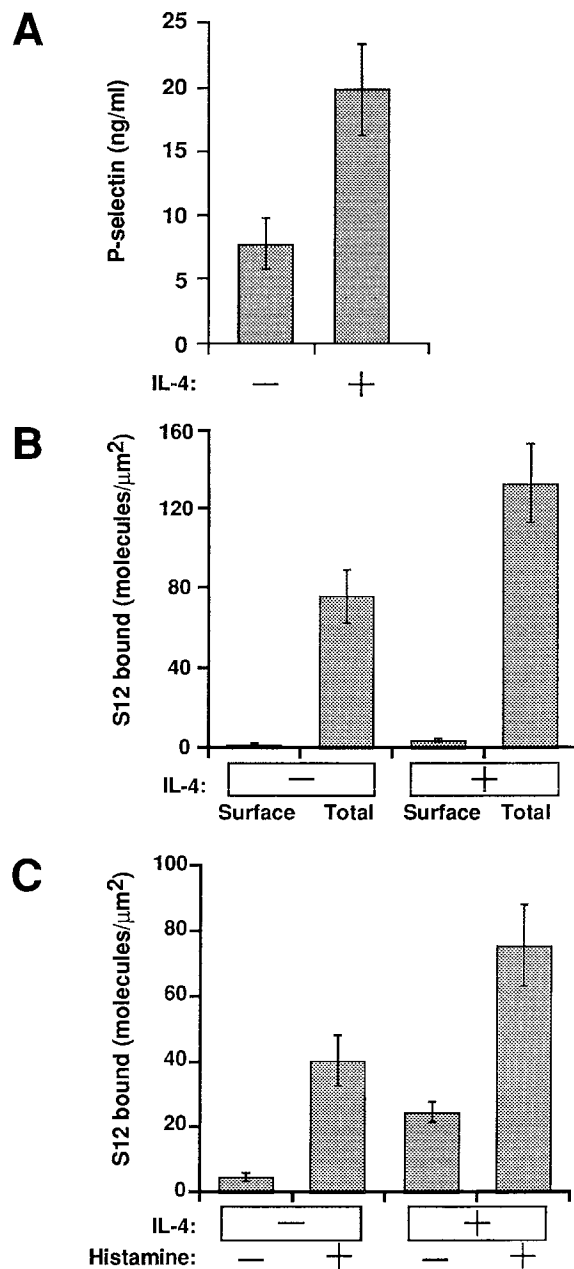


Figure 8. Stimulation of HUVEC with IL-4 increases P-selectin protein. HUVEC were preincubated with fresh medium in the presence or absence of 20 ng/ml IL-4 for 24 h. (A) Cells were lysed in buffer containing Triton X-100, and the amount of P-selectin in the lysate was measured by ELISA. (B) Cells were fixed with 1% paraformaldehyde. A saturating concentration (1 μ g/ml) of ^{125}I -S12, an anti-P-selectin mAb, was added in the absence or presence of 0.2% saponin to measure, respectively, surface or total P-selectin. (C) Live cells treated with or without 10^{-5} M histamine were incubated with 1 μ g/ml ^{125}I -S12 for 30 min at 37°C to measure P-selectin cycling between endosomes and the plasma membrane. For each assay, the data represent the mean \pm SEM of determinations from duplicate wells performed in each of five independent experiments.

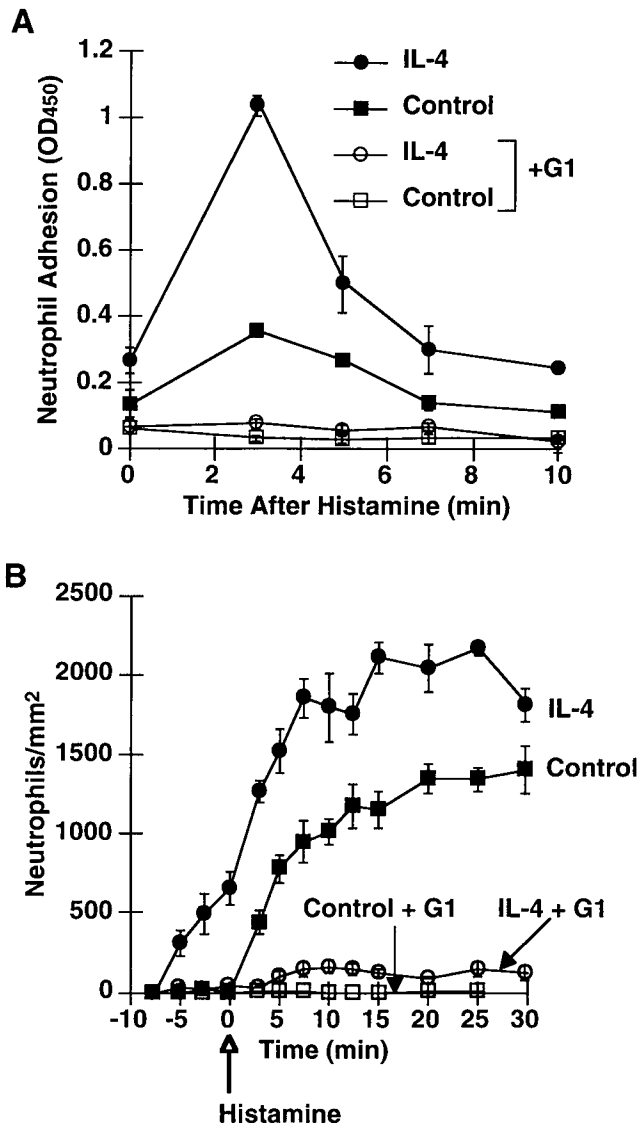


Figure 9. More neutrophils adhere to P-selectin on IL-4-stimulated HUVEC under both static and flow conditions. Confluent HUVEC were pretreated with fresh medium in the presence or absence of 20 ng/ml of IL-4 for 24 h. (A) HUVEC in 24-well dishes were washed twice with HBSS and then incubated at 37°C with 2×10^{-5} M histamine in HBSS/HSA in the presence or absence of the blocking anti-P-selectin mAb G1. After the indicated time, the buffer was replaced with 5×10^5 neutrophils in HBSS/HSA in the presence or absence of mAb G1. After 5 min at 37°C, the nonadherent cells were removed by washing twice with HBSS. The number of adherent neutrophils was quantified with a myeloperoxidase assay. The data represent the mean \pm range of duplicate determinations, and are representative of three independent experiments. (B) HUVEC in 35-mm dishes were washed twice with HBSS and then inserted into a parallel-plate flow chamber. Neutrophils (10^6 /ml in HBSS/HSA) were perfused through the chamber at a wall shear stress of 1.5 dyn/cm² in the presence or absence of mAb G1. After 10 min, 2×10^{-5} M histamine was added to the perfusate. At each time point, the number of attached neutrophils was measured by viewing six random 20 \times fields. The data represent the mean \pm SD of the attached cells in each field, and are representative of three independent experiments.

static adhesion assays, few neutrophils adhered to control HUVEC (Fig. 9 A). As observed previously (32, 73), neutrophils adhered transiently to HUVEC stimulated with histamine; adhesion peaked 3 min after addition of histamine and then rapidly declined to basal levels. More neutrophils adhered to IL-4-treated HUVEC, even in the absence of histamine, and significantly more neutrophils transiently adhered after stimulation with histamine. Neutrophils did not attach to unstimulated HUVEC under conditions of hydrodynamic flow (Fig. 9 B). However, neutrophils rolled on HUVEC immediately after the monolayer was stimulated with histamine, confirming previous results (74). Neutrophils rolled on IL-4-treated HUVEC, even in the absence of histamine, and significantly more neutrophils rolled on HUVEC after stimulation with histamine (Fig. 9 B). Neutrophil adhesion under both static and flow conditions was blocked by G1, an mAb to P-selectin, indicating that the observed adhesion was to P-selectin. These results demonstrate that neutrophils attach and roll on P-selectin that is constitutively expressed on the surface of IL-4-treated HUVEC. Treatment with histamine mobilizes additional P-selectin from Weibel-Palade bodies to the cell surface, promoting attachment of even more neutrophils.

IL-4 Increases P-Selectin mRNA in Murine Endothelial Cells and in Human Lung Microvascular Endothelial Cells. To determine whether IL-4 affected P-selectin mRNA levels in endothelial cells other than HUVEC, we added IL-4 to murine bEnd.3 endothelioma cells and to human lung microvascular endothelial cells. The amounts of P-selectin or VCAM-1 mRNA were then measured by Northern blot analysis. We used murine IL-4 to stimulate bEnd.3 cells because of the restricted species specificity of IL-4 for its receptor (75). We also used a murine P-selectin cDNA probe (46) to measure P-selectin mRNA in bEnd.3 cells. Constitutively expressed P-selectin mRNA was detected in murine bEnd.3 cells, but not in human lung microvascular endothelial cells (Fig. 10). IL-4 increased P-selectin mRNA levels in bEnd.3 cells. The kinetics of accumulation after IL-4 treatment were different than those observed in HUVEC; transcripts were detected after 4 h, peaked at 10 h, and persisted at this level for at least 24 h. IL-4 increased P-selectin mRNA in human lung microvascular endothelial cells; the kinetics of accumulation were similar to those in HUVEC, although much less P-selectin mRNA accumulated relative to total RNA. IL-4 also modestly increased VCAM-1 mRNA in the human lung microvascular endothelial cells. TNF- α increased P-selectin mRNA in bEnd.3 cells, consistent with previous results with other murine endothelioma cells (46). However, TNF- α did not increase P-selectin mRNA in human lung microvascular endothelial cells, although it markedly increased VCAM-1 transcripts. These data demonstrate that IL-4 increases P-selectin mRNA in murine endothelial cells and human lung microvascular endothelial cells as well as in HUVEC. In contrast, TNF- α increases P-selectin mRNA in murine endothelial cells, but not in the human endothelial cells examined.

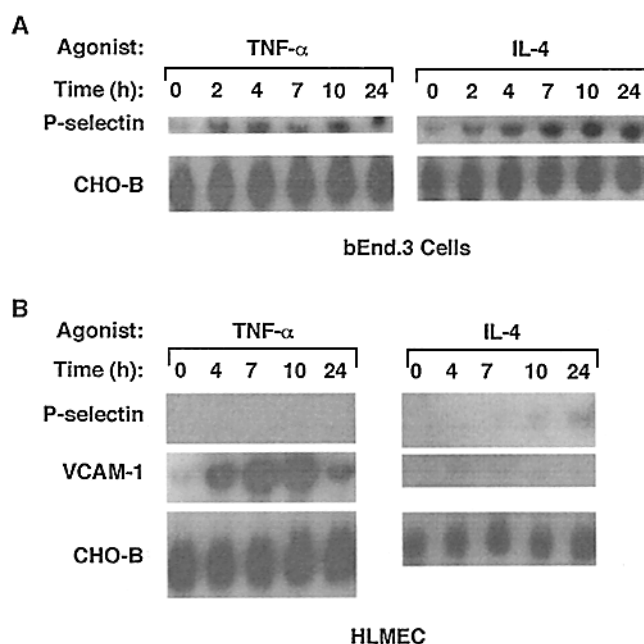


Figure 10. IL-4 increases P-selectin mRNA in murine endothelial cells and in human lung microvascular endothelial cells. Murine bEnd.3 endothelioma cells and human lung microvascular endothelial cells (HLMEC) were treated with 100 U/ml TNF- α or 20 ng/ml of IL-4. After the indicated time, total RNA was isolated and analyzed by Northern blotting, as described in Fig. 1. The data are representative of four independent experiments for bEnd.3 cells and two independent experiments for the HLMEC.

Discussion

Megakaryocytes and venular endothelial cells constitutively synthesize P-selectin and then store it in the α granules of platelets and the Weibel-Palade bodies of endothelium (27-30). Histamine and other secretagogues rapidly mobilize P-selectin to the cell surface, where it mediates the initial tethering and rolling of neutrophils on the vessel wall during acute inflammation (32, 41, 76). Endothelial cells stimulated with secretagogues normally limit the time that P-selectin can function by clearing it from the surface by endocytosis (37, 38). However, P-selectin has also been observed on the luminal surface of endothelial cells in tissues with chronic or allergic inflammation (43-45). In these conditions, there must be mechanisms that prolong the surface expression of P-selectin. We found that IL-4 and OSM, two cytokines that are elaborated during chronic or allergic inflammation, increased P-selectin mRNA in cultured endothelial cells for as long as 72 h. Some of the newly translated P-selectin protein reached the cell surface, where it mediated tethering of neutrophils under static or flow conditions. These data suggest that IL-4 and OSM provide a mechanism to signal sustained expression of P-selectin on the endothelial cell surface during inflammation.

In HUVEC, IL-4 increased the steady-state level of P-selectin mRNA by accelerating its transcription. Because P-selectin transcripts have a long half-life, even a brief increase in the transcription rate could ensure a prolonged ac-

cumulation of P-selectin mRNA. HUVEC pretreated with inhibitors of tyrosine kinases did not increase P-selectin transcripts in response to IL-4 or OSM. This is consistent with known signaling mechanisms for these cytokines, which include rapid tyrosine phosphorylation of Janus kinases and signal transducer and activator of transcription (STAT) proteins that associate with the cytoplasmic domains of ligand-occupied receptors (65, 66). IL-4 and OSM bind to distinct receptors, and, in cells studied to date, IL-4 causes phosphorylation of Stat6, whereas OSM causes phosphorylation of Stat3 (65, 66). However, Stat3 and Stat6 can bind to the same regulatory element in some genes, providing a potential mechanism for the induction of P-selectin expression by either IL-4 or OSM (66, 77). Furthermore, both cytokines might phosphorylate common STATs not yet described, or signal through shared mechanisms that do not involve Stats (78). Both IL-4 and OSM required new protein synthesis to increase P-selectin mRNA, suggesting that the cytokines functioned indirectly by activating the gene(s) for one or more other proteins that then activated the P-selectin gene. IL-4 also increased P-selectin mRNA in murine endothelioma cells. In these cells, transcripts accumulated and then declined somewhat earlier than in HUVEC. We have not yet determined whether IL-4 requires new protein synthesis to increase P-selectin mRNA in murine endothelial cells.

Unlike IL-4 or OSM, the mediators TNF- α , IL-1 β , LPS, or IL-3 had little or no effect on P-selectin mRNA in HUVEC. Furthermore, they did not augment the IL-4-induced accumulation of P-selectin RNA. In fact, TNF- α or LPS sometimes decreased the level of P-selectin mRNA after 10-24 h, as observed previously (51). Whether this decline in transcripts reflects reduced synthesis or increased turnover is unknown. The inability of TNF- α to elevate P-selectin mRNA is in contrast to a modest increase in P-selectin antigen reported on the surface of HUVEC treated with TNF- α for 6 h, although P-selectin mRNA levels were not measured in that study (79). It was recently reported that IL-3 induces a 1.9-fold increase in P-selectin mRNA in HUVEC (80). This small increase in P-selectin mRNA by IL-3 was occasionally observed in our experiments, but it was much less than that induced by IL-4 or OSM.

In contrast to their lack of effect on P-selectin mRNA in HUVEC, TNF- α or LPS clearly increase P-selectin mRNA in cultured murine and bovine endothelial cells (46). In vivo, these mediators also increase P-selectin mRNA and protein levels in rodent endothelial cells (46, 48-50). P-selectin transcripts reach a maximum 2-4 h after stimulation and then decline to basal levels within 12-24 h (46, 48, 49). It is possible that TNF- α , IL-1 β , and LPS increase human P-selectin mRNA levels in vivo, but not in HUVEC or the human microvascular endothelial cells that we examined in vitro. However, P-selectin mRNA levels decline in the atria of patients after cardiopulmonary bypass, whereas mRNA levels for E-selectin and ICAM-1 increase (51). Furthermore, LPS injected intradermally in rhesus monkeys results in loss of P-selectin antigen in venular endothelial cells, whereas the antigen for E-selectin appears (81).

Thus, TNF- α , IL-1 β , or LPS may not affect the P-selectin gene in primates in the same manner as the P-selectin gene in species such as mice and rats. These mediators mobilize heterodimeric NF- κ B proteins from the cytoplasm to the nucleus, where they bind to κ B elements of responsive genes encoding proteins such as E-selectin, ICAM-1, and VCAM-1 (5). The promoter of the human gene for P-selectin has a κ B element, but it binds only homodimers of p50 or p52 (82). This suggests that TNF- α , IL-1 β , and LPS either do not activate the human P-selectin gene, or regulate it by a mechanism different than that used for genes with more conventional κ B elements. It will be informative to compare the putative regulatory elements in the promoters of the murine and human P-selectin genes. If there are differences in how this gene is regulated across species, functions ascribed to P-selectin in some animal models of inflammation may not apply to humans.

HUVEC do not constitutively synthesize VCAM-1, but they do express VCAM-1 in response to IL-4 (20–22, 63). TNF- α , IL-1 β , or LPS also increase VCAM-1 mRNA, but with different kinetics than does IL-4 (63; this study). Any of these mediators combined with IL-4 enhances and prolongs the accumulation of VCAM-1 transcripts (63; this study). This synergy results from transcriptional activation of the VCAM-1 gene by TNF- α (and probably IL-1 β and LPS) plus prolongation of the otherwise short half-life of VCAM-1 mRNA by IL-4 (63). In vivo, IL-4 may be most effective in inducing VCAM-1 expression when low levels of another mediator such as TNF- α are also present (63). In contrast, IL-4 may increase expression of P-selectin, even in the absence of such additional mediators. Like IL-4, OSM increases P-selectin mRNA, but unlike IL-4, it does not induce VCAM-1 mRNA. OSM might selectively increase the expression of P-selectin in the presence of low levels of TNF- α that are insufficient to induce transcription of VCAM-1. IL-4 suppresses the induced expression of E-selectin and ICAM-1 by TNF- α (83). Thus, the relative levels of IL-4, OSM, and other mediators such as TNF- α may differentially regulate the expression of P-selectin, VCAM-1, and other adhesion molecules during chronic or allergic inflammation.

IL-4 or OSM also elevated P-selectin protein in HUVEC, although not to the same degree as P-selectin mRNA. The relatively limited increase in protein probably reflects its complex subcellular trafficking. After synthesis, P-selectin is sorted in the *trans*-Golgi network for delivery to secretory granules, where it has a relatively long half-life (39). The protein that is not sorted is delivered to the plasma membrane, where it is rapidly internalized in clathrin-coated pits (37, 38). The half-life of missorted P-selectin is

short because it is efficiently delivered from endosomes to lysosomes for degradation (39). Although some P-selectin molecules may recycle from endosomes to the *trans*-Golgi network and be resorted (40), in vivo immunohistochemical studies suggest that this is a relatively minor pathway (81). An increase in the level of newly synthesized P-selectin may saturate the sorting pathway, increasing the fraction of molecules that travel directly to the cell surface and are then degraded. Thus, a large increase in P-selectin mRNA might produce a relatively limited increase in protein.

IL-4-treated HUVEC expressed more P-selectin on the plasma membrane as well as in Weibel-Palade bodies, where it could be mobilized to the cell surface by histamine. Under static conditions, IL-4 effected only a small increase in neutrophil adhesion to P-selectin in the absence of histamine. Previous studies did not note this increase, perhaps because it was modest compared to the increased adhesion of eosinophils, monocytes, and T cells to VCAM-1 on IL-4-treated HUVEC (21, 22). Under flow conditions, the increase in P-selectin was sufficient to mediate rolling of neutrophils in the absence of histamine, and even more neutrophils attached after the addition of histamine. It is not yet known whether these densities of P-selectin mediate attachment of flowing eosinophils, monocytes, or T cells. These leukocytes express α 4 integrins that interact with VCAM-1 (64, 72, 84), which could stabilize attachment mediated by even low densities of P-selectin. Other inducible molecules such as a ligand for L-selectin may enhance tethering under flow (23). IL-4 and OSM might also have important “priming” functions. The cytokines could chronically increase the steady-state levels of P-selectin on the plasma membrane. Subsequent challenges that generate histamine, thrombin, or complement could mobilize an additional larger pool of P-selectin to the cell surface. Pathologic mediators such as oxygen-derived radicals might further increase surface expression of P-selectin by interfering with endocytosis (85).

In the multistep model of leukocyte recruitment, the sequential interactions of different adhesion molecules are regulated by specific signaling molecules. Different combinations of adhesion and signaling molecules may control the classes of leukocytes that emigrate into sites of inflammation, and the kinetics of their accumulation (1). The inflammatory cytokines IL-4 and OSM have many signaling functions (14–17, 24–26). We found that they also induce a sustained increase in the expression of P-selectin in endothelial cells. This may provide a mechanism for P-selectin to mediate leukocyte adhesion, not only during acute inflammation, but also during chronic and allergic inflammation.

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Address correspondence to Rodger P. McEver, M.D., W.K. Warren Medical Research Institute, University of Oklahoma Health Sciences Center, 825 N.E. 13th Street, Oklahoma City, OK 73104.

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