Stat6 Activation Is Essential for Interleukin-4 Induction of P-Selectin Transcription in Human Umbilical Vein Endothelial Cells

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Abstract—Chronic upregulation of P-selectin expression on the surface of the endothelium has been observed in and likely contributes to a number of chronic inflammatory diseases, including atherosclerosis. Agonists of P-selectin expression fall into 2 categories: those that induce a very rapid, transient increase, lasting only hours, and those that induce prolonged upregulation lasting days. It is the latter group, which includes interleukin-4 (IL-4), that is likely to be a mediator of chronic P-selectin upregulation. The increase in P-selectin expression induced by IL-4 results from increased transcriptional activation of the P-selectin gene. The aim of this study was to deduce the postreceptor signaling pathway(s) giving rise to the prolonged increase in P-selectin expression induced by IL-4. We demonstrate the existence of 2 functional signal transducer and activator of transcription 6 (Stat6) binding sites on the P-selectin promoter and further demonstrate, by functional analysis of the P-selectin promoter, that binding of activated Stat6 to at least 1 site is essential for IL-4 induction of P-selectin transcription. Site 1 (nucleotide [nt] −142) bound Stat6 with a higher affinity than did site 2 (nt −229), and this difference was reflected functionally as constructs in which only site 1 was functional showed full IL-4 inducibility, whereas constructs in which only site 2 was functional showed only 40% of maximal IL-4 inducibility. IL-4 also induced prolonged activation of Stat6, which was contingent on the continuous presence of IL-4. The sustained activation of Stat6 induced by IL-4 is likely to be a key factor leading to the prolonged activation of the P-selectin promoter, thereby resulting in prolonged P-selectin upregulation. (Arterioscler Thromb Vasc Biol. 1999;19:1421-1429.)

Key Words: P-selectin ■ interleukin-4 ■ Stat6 ■ inflammation ■ chronic effects

The role of inflammation in the development of atherosclerosis is clearly established. The adhesion of circulating leukocytes, in particular, monocytes, to the endothelium and their subsequent migration through the endothelial barrier is a requisite step in the formation of fatty streaks, the precursors to atherosclerotic plaques. Members of the selectin family of adhesion receptors, when expressed on the surface of activated endothelial cells (ECs) during inflammation, mediate the capture of fast-rolling leukocytes, leading to leukocyte rolling on the vessel wall, a step necessary for the subsequent firm adhesion and extravasation of leukocytes (reviewed in References 1 and 2). P-selectin, a member of the selectin family of adhesion receptors, is a 140-kDa glycoprotein stored in the Weibel-Palade bodies of ECs3,4 and the α-granules of platelets and megakaryocytes.5 The importance of P-selectin in inflammation is aptly demonstrated by the decrease in numbers of rolling leukocytes in P-selectin-deficient mice, resulting in delayed and decreased leukocyte extravasation during acute inflammation.6 Increased expression of P-selectin in the blood vessels at sites of inflammation has been observed in a number of chronic inflammatory diseases such as rheumatoid arthritis,7 Graves disease,8 nasal polyps,9 and atherogenic plaques.10 In a recent study of P-selectin–null mice crossed with low density lipoprotein receptor–null mice (an established model of familial hypercholesterolemia11), the resulting double-knockout mice showed significantly smaller and fewer fatty streaks with delayed onset compared with their low density lipoprotein receptor–null parents. These results suggest that P-selectin plays an essential role in the initiation and perhaps also the progression of atherogenic plaques. Collectively, these observations suggest that a sustained increase in endothelial P-selectin expression may play a significant role in the maintenance of a chronic inflammatory state that could underpin the development of some chronic inflammatory diseases such as atherosclerosis. Little, however, is known about the mechanism(s) that leads to the prolonged upregulation of P-selectin expression at sites of chronic inflammation.

Two categories of agonist that bring about cell-surface elaboration of P-selectin have been described to date. Most known activators result in peak cell-surface expression in 10 minutes (eg, thrombin, histamine, and phorbol 12-myristate 13-acetate)12,13 or 2 to 4 hours (oxygen radicals14 and tumor...
necrosis factor-α [TNF-α] in the mouse). The half-life of the protein on the cell surface induced by agonists such as thrombin or phorbol 12-myristate 13-acetate is short, with a return to basal expression levels within several hours, even in the sustained presence of the agonist. This observation implies that there is a refractory period during which the cells are not responsive to further stimulation by the same agonist and also suggests that this class of agonist is more likely to be a mediator of acute inflammation.

Recently, another group of agonists were discovered that induced cell-surface expression with kinetics very different from that induced by the agonists described earlier. These are the cytokines interleukin-3 (IL-3), IL-4, and oncostatin-M (OsM), products of activated T cells and monocytes usually present at chronic inflammatory sites. We showed that IL-3 can upregulate cell-surface P-selectin expression on cultured human umbilical vein ECs (HUVECs) for a prolonged period, beginning ~16 hours after stimulation and lasting at least 4 days. The increase in cell-surface P-selectin expression was accompanied by an equivalent persistent increase in its mRNA level. IL-4 and OsM also caused prolonged expression of both P-selectin mRNA and cell-surface P-selectin on ECs with similar kinetics to IL-3 (Reference 17 and this study). The unusual kinetics of induction of P-selectin expression by IL-3, OsM, and IL-4 suggests that they are likely to be mediators of increased P-selectin expression seen in chronic inflammatory diseases and raise intriguing questions regarding the nature of the intracellular signaling pathway(s) utilized. Binding of IL-4 to its receptor results in activation of several intracellular signaling pathways, including the phosphatidylinositol-3-kinase and the Janus kinase (Jak)/signal transducers and activators of transcription (STAT) pathways. Signaling through the Jak/STAT pathway occurs by a tyrosine phosphorylation cascade and whereby Jak tyrosine kinases activated by tyrosine phosphorylation in turn phosphorylate the STAT family of transcription factors, leading to STAT dimerization and ultimately, translocation into the nucleus.

IL-4 stimulation results in activation of Stat6 in most cell types, including HUVECs. IL-4 suppression of TNF-α-induced E-selectin expression on HUVECs also occurs through Stat6 activation. We therefore hypothesized that P-selectin gene transcription in response to IL-4 stimulation would probably occur through activation of Stat6. The proximal P-selectin promoter contains 2 putative Stat6-binding sites, and in this study, we demonstrate that they are both functional and that the binding of Stat6 to at least 1 Stat6 site is essential for IL-4 induction of P-selectin gene transcription. We also found that IL-4 not only induced the prolonged expression of P-selectin but, surprisingly, also induced a similarly prolonged activation of the transcription factor Stat6.

Methods

Cell Culture, Reagents, and Antibodies (Abs)

HUVECs were extracted by collagenase treatment according to a modified version of the procedure of Wall et al as previously described. Cells were either used 2 to 5 days after establishment of culture by harvesting with trypsin (0.05%)-EDTA (0.02%) (Gibco-BRL, Life Technologies) for replotting or passaged further. After replating, EC growth supplement (Collaborative Research) at 25 mg · mL⁻¹ and heparin were included in the culture medium. All reagents used in the growth, passaging, and stimulation of HUVECs were made up under endotoxin-free conditions and contained between 10 and 100 pg · mL⁻¹ endotoxin as ascertained by the Limulus amoebocyte assay. Typically cells between passages 1 and 4 were used.

Stat6 antisera was a gift from Dr James Ihle, St Jude Children’s Hospital, Memphis Tenn; recombinant human (rh) IL-4 was a gift of Immunex Corp, Seattle Wash; rhIL-3 was a gift from Dr Christopher Bagley; rhOsM was a gift from Drs Richard Simpson and Robert Moritz of the Ludwig Institute, Melbourne, Australia; and affinity-purified polyclonal P-selectin antibody (Ab) was a gift from Dr Michael Berndt, Baker Medical Research Institute, Prahran, Australia. The anti-activin mitogen-activated protein kinase (MAPK) polyclonal Ab was obtained from Promega, and the MAPK polyclonal Ab (anti-ERK1 and 2) was from Zymed.

Synthetic Oligonucleotides and Plasmid Constructs

The following oligonucleotides were used as probes or competitors in electrophoretic mobility shift assays (EMSA): (1) site 1 probe, 5'-agentTAATCTTCTAGGAAGGGGg-3'; (2) site 2 probe, 5'-agentCATCTTTAGGAAGGGGg-3'; (3) Ie-5'-agentGCAGTTCCCAA- GACATGg-3'; (4) mut, 5'-agentGCAGTTCCGTTGCTACTG-3'; and (5) β-cas, 5'-agentAGTATTGGAAGGATCAATTCCg-3'. (The lower-case letters represent the sequences added to generate an Hind III restriction site at the 5' end and an Sal I site at the 3' end.) Site 1 and site 2 sequences were from the P-selectin promoter, and the Ie core Stat6 sequence (italicized) was from the immunoglobulin η (Ie) promoter that was previously shown to bind Stat6. The mut sequence contained mutations in the site 1 core sequence that rendered it unable to bind Stat6 (data not shown). The β-cas sequence is the interferon-γ activation site–like sequence (GAS) element from the β-casein promoter, which binds Stat5 and acts as a general STAT binding site (Y.K.-G. et al, unpublished observations, 1998, and Reference 27).

The P-selectin promoter fragment from nucleotides (nt's) −270 to −13 was generated by polymerase chain reaction (PCR) amplification of genomic DNA and specific 5' and 3' primers based on a previously published sequence and subcloned into the chloramphenicol acetyltransferase (CAT) reporter vector, pBLCAT3, for use in transient transfection assays. Mutations introduced into the Stat6 sites were also generated by PCR with oligonucleotides containing the mutated sequence as primers. All constructs were sequenced to ensure that no unintended mutations had been introduced.

Nuclear Extract Preparation and EMSA

Nuclear extracts were prepared from 2 × 10⁶ cells at 4°C according to the protocol of Mui et al. EMSAs were performed in a total volume of 15 μL containing 10 mmol/L Tris-HCl (pH 8.0), 100 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L DTT, 0.5 mg/mL BSA, 10% glycerol, 1 mmol/L PMSF, 2 μg poly(dI-dC), and 2 × 10⁶ counts per minute (~0.8 ng) of ³²P-labeled probe. The reactions were initiated by the addition of ~9 μg of nuclear extract (in a volume of 2 to 5 μL) and allowed to incubate at room temperature for 20 minutes before electrophoretic separation on a 5% polyacrylamide gel in 0.25 × Tris-borate-EDTA buffer. In competition assays, unlabeled oligonucleotides were preincubated with nuclear extract in the reaction mix 10 minutes before the addition of labeled probe. Similarly, in Ab supershift assays, anti-Stat6 or preimmune serum was preincubated with nuclear extract in the reaction mix for 1 hour at 4°C before the addition of labeled probe.
Transient Transfections and CAT Assays
HUVECs were transiently transfected using lipofectin (BRL-Gibco, Life Technologies), according to the manufacturer’s instructions, with 6 μl lipofectin reagent and 1 μg plasmid in 1 mL Opti-MEM medium for 10h per well in 6-well tissue-culture dishes. Cells were harvested 48 hours after transfection, and the CAT assays were then performed. CAT assays were carried out as previously described.30 The various acetylated forms of [14C]chloramphenicol were detected by using a PhosphorImager (Molecular Dynamics) and quantitated by using ImageQuant software (Molecular Dynamics).

Flow Cytometry to Detect Cell-Surface P-Selectin Expression
To determine cell-surface expression of P-selectin, IL-4 stimulation was carried out in a defined medium (Opti-MEM) containing 2% FCS instead of HUVEC medium containing 20% FCS, which has been used in all other experiments described. Stimulating HUVECs in the presence of a high FCS concentration resulted in a 3- to 4-fold higher basal P-selectin expression and hence, apparently less IL-4 stimulation.

Flow cytometry was used to detect cell-surface P-selectin expression as previously described16 using a rabbit polyclonal Ab against human platelet P-selectin at 10 ng/mL.1 Before cell fixation, all solutions used with HUVECs contained 0.2% (wt/vol) NaN3 to prevent reinternalization of P-selectin from the cell surface. The polyclonal P-selectin Ab had previously been shown to bind specifically to P-selectin and did not cross-react with E-selectin expressed on the surface of Chinese hamster ovary cells transfected with the E-selectin CDNA (data not shown). Cells were washed twice at 4°C before the addition of FITC-conjugated sheep anti-rabbit IgG Fab; secondary Ab (Silenus, Victoria, Australia) at a 1:80 dilution. The nuclei were incubated on ice for 30 minutes; washed once; and then fixed in 1% formaldehyde, 2% (wt/vol) glucose, and 0.02% (wt/vol) NaN3 in PBS. Flow cytometry was carried out using an EPICS Profile II (Coulter); 10 000 cells per group were analyzed. The background mean fluorescence intensity was determined by using rabbit nonimmune serum.

Western Blotting to Detect MAPK Activation
Untreated or cytokine-treated HUVECs (2×105 cells) were washed in PBS containing 1 mMol/L sodium vanadate and then lysed in lysis buffer (20 mMol/L Tris-Cl, pH 8.0; 160 mMol/L NaCl; 1 mMol/L CaCl2; 1 mMol/L Na3-EDTA; 1% Triton X-100; 1 mMol/L leupeptin; 200 mMol/L sodium vanadate; 1 mMol/L NaF; 2 mMol/L PMSF; 25 mMol/L benzamide; and 5 mMol/L β-glycerophosphate). The cell lysate was collected and centrifuged. The supernatant was added to a 1/3 volume of 20% SDS, 20% sucrose, 6% croton acid, and 50 mMol/L β-mercaptoethanol. The proteins were separated by electrophoresis on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Active MAPK was detected with a 1/20 000 dilution of anti-ERK Ab to detect total MAPK. Active MAPK was detected with a 1/20 000 dilution of anti-ERK Ab to detect total MAPK.

Results
P-Selectin Promoter Binds Stat6 Induced by IL-4
Two putative Stat6-binding sites (Figure 1), fitting the consensus sequence TTCNNNNGAA,27 were identified in the proximal promoter of the P-selectin gene28 at nt -142 (denoted as site 1) and -229 (site 2). Scanning 5 kb of the upstream flanking sequence failed to reveal additional Stat6 sites. The binding characteristics of these putative Stat6 sites were investigated by EMSA using either site 1 or site 2 oligonucleotide probes with either unstimulated or IL-4-treated HUVEC nuclear extracts (Figure 2). Both putative Stat6 sites bound a factor that was induced by treatment with IL-4 for 15 minutes. Two lines of evidence suggest that this factor is Stat6. First, the binding was completely abolished by the addition of a cold competitor oligonucleotide (denoted Ie) containing the Stat6-binding site of the Ie promoter, which had previously been demonstrated to efficiently bind Stat6.25 In contrast, an oligonucleotide probe with a mutation in the Stat6 consensus sequence (denoted mut) was unable to compete for binding to either site 1 or site 2 probes even when added at 100-fold molar excess. This finding suggests that the IL-4-inducible factor was specifically binding to both of the canonical Stat6 sequences of the P-selectin promoter. Second, incubation in the presence of an anti-Stat6-specific Ab resulted in a supershift that was not observed when nonimmune serum was used (Figure 2). This result was again observed with both site 1 and site 2 probes, confirming that the factor binding to both sites was indeed Stat6.

During EMSA with the site 1 and 2 probes, it was noted that the site 1 probe gave a more intense band than did the site
At Least 1 Stat6 Site on the P-Selectin Promoter Is Required for IL-4 Induction

The induction of cell-surface P-selectin by IL-4 has previously been shown to be accompanied by an increase in transcriptional activity of the P-selectin gene. \(^\text{17}\) Having established that the P-selectin promoter has 2 Stat6-binding sites, their role in mediating IL-4–induced transcription of the P-selectin gene was investigated. A set of promoter-CAT reporter constructs (Figure 4a) was made and transiently transfected into HUVECs, and the CAT activity in transfec-
tants that were either untreated or treated with IL-4 was determined. Transfectants carrying the parent construct p–270-CAT, which contains the proximal P-selectin promoter from –270 to –13 and includes both Stat6-binding sites, showed a 2.5±0.4-fold (mean±SEM) increase in CAT activity when treated with IL-4 compared with untreated transfectants (Figure 4b). The increase in CAT activity observed in transfec-
tants stimulated with IL-4 suggests that the P-selectin promoter from –270 to –13 contains the elements that confer IL-4 responsiveness. In contrast, trans-
fectants with reporter-CAT constructs driven by an unrelated promoter, such as the thymidine kinase promoter, did not exhibit any increase in CAT activity when stimulated with IL-4 (Figure 4b).

To establish that the Stat6-binding sites are critical for mediating IL-4 induction of P-selectin promoter activity, mutations that rendered them unable to bind activated Stat6 were introduced into either 1 or both Stat6 sites. The mutations introduced were at the 3’ end of the consensus sequence, altering it from TTTCCATGGAA to TTTNNNCTT. Owing to 2 different problems that arose, 2 sets of constructs containing wild- type Stat6 sites and 2 sets containing site 1 mutations were made. First, the cloning strategy used to obtain site 1 and the double mutants resulted in a 2-bp (CG) insertion at nt –120 of the P-selectin promoter. A control construct (p–270CG-CAT) was there-

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**Figure 3.** Site 1 is a higher-affinity Stat6-binding site than is site 2. a, EMSA using the site 1 probe to detect activated Stat6 in HUVEC extract. HUVECs were either untreated or treated with IL-4 at 20 ng/mL for the indicated time before being harvested, and nuclear extracts were made. Assays were performed either in the absence of any cold competitor or in the presence of various molar excesses of either site 1 or site 2 cold competitors as indicated. b, EMSA as described in panel a, except that the site 2 probe was used to detect activated Stat6. Data shown are representative of 2 experiments.

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**Figure 4.** IL-4–induced increase in P-selectin promoter activity is dependent on at least 1 Stat6 site. a, Schematic of P-selectin promoter–CAT constructs used in transient transfection assays in HUVECs. p–270-CAT is the parent construct containing the authentic P-selectin promoter sequence from –270 to –13. Locations of the 2 Stat6-binding sites are shown boxed; nt within the boxes indicates the authentic (wild-type) Stat6 sequence for the respective site, and mut indicates that the respective Stat6 site has been mutated to render the site unable to bind Stat6. Authentic site 1 and site 2 sequences are TTCATGGAA and TTTCCATGGAA, respectively. Mutant site 1 sequences were either TTCATGGAA and TTCATGGAA (5’

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mutation), and the mutant site 2 sequence is TTCCATGCTT (italicized letters are altered nt’s). In the construct in which both sites were mutated [pS6(1/2)-CAT], the site 1 sequence has the 3’ mutation. The thick bar indicates the position of major transcription start sites (italicized letters are altered nt’s). In the construct in which both sites were mutated [pS6(1/2)-CAT], the site 1 sequence has the 3’ mutation. The thick bar indicates the position of major transcription start sites (italicized letters are altered nt’s).
fore generated that contained the 2-bp insertion and wild-type Stat6 sites to ensure that the 2-bp insertion itself did not impair IL-4 responsiveness. Table 1 shows that the 2-bp insertion had no significant effect on IL-4 induction of P-selectin promoter activity. Because introduction of the site 1 mutation [pS6(1)3'-CAT] also resulted in mutation of an overlapping putative ets transcription factor–binding site (see Figure 1), a second site 1 mutant construct [pS6(1)5'-CAT] with the mutations introduced at the 5' end of the Stat6-binding site (AAAGNNNGAA) was made. A comparison of the IL-4 inducibility showed no significant difference between the 2 site 1 mutants (Table 2).

In the overall analysis (Figure 4), transfection data obtained from the 2 constructs with wild-type Stat6 sites and the 2 site 1 mutants were pooled and collectively referred to as p−270-CAT and pS6(1)-CAT, respectively. Simultaneously mutating both Stat6 sites [pS6(1/2)-CAT] resulted in complete loss of IL-4 induction of P-selectin promoter activity. Mutation of site 1 alone [pS6(1)] led to a significant but partial loss of IL-4 stimulation (1.6±0.1-fold, *P<0.05). This value represents a 60% decrease in IL-4 inducibility compared with p−270-CAT, which contained both authentic Stat6 sites. However, mutation to site 2 alone had no significant effect on IL-4 stimulation of P-selectin promoter activity (2.7±0.4-fold, *P=0.7). These data suggest that IL-4 stimulation of P-selectin promoter activity was dependent on having at least 1 active Stat6 binding site on the P-selectin promoter. They also suggest that site 1 alone is sufficient for full stimulation by IL-4, whereas site 2 alone results in only partial stimulation.

IL-4 Induces Both Prolonged Cell-Surface P-Selectin Expression and Stat6 Activation

The time course of IL-4 induction of cell-surface P-selectin expression was examined by flow cytometry using a polyclonal Ab raised against P-selectin. IL-4 induced a 3-fold increase in cell-surface P-selectin expression that peaked ∼24 hours after IL-4 stimulation and was sustained for at least 72 hours after stimulation (see Figure 6b). Therefore, the kinetics of IL-4–induced Stat6 activation was examined by EMSA using the site 1 oligonucleotide probe from the P-selectin promoter. Figure 5 shows that activation of Stat6 by IL-4 was very rapid, occurring within 15 minutes of IL-4 addition. Surprisingly, activation of Stat6, like that of cell-surface P-selectin expression, was also prolonged, lasting at least 48 hours (Figure 5).

**TABLE 1. Effect of CG Insertion on IL-4 Inducibility**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fold Induction by IL-4 (Mean±SEM)</th>
<th>No. of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>p−270-CAT</td>
<td>2.68±0.44</td>
<td>3</td>
</tr>
<tr>
<td>p−270CG-CAT</td>
<td>2.67±0.22</td>
<td>3</td>
</tr>
</tbody>
</table>

*P=0.99 (Student’s t test).

**TABLE 2. Comparison of Mutations in the 5’ and 3’ Ends of the Proximal Stat6 Site (Site 1) on IL-4 Responsiveness**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fold Induction by IL-4 (Mean±SEM)</th>
<th>No. of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>p−S6(1)3' CAT</td>
<td>1.52±0.05</td>
<td>3</td>
</tr>
<tr>
<td>p−S6(1)5' CAT</td>
<td>1.74±0.10</td>
<td>3</td>
</tr>
</tbody>
</table>

*P=0.34 (Student’s t test).

Figure 5. IL-4 induces prolonged activation of Stat6.

**a** EMSA using the site 1 oligonucleotide probe was used to detect activated Stat6. HUVECs were treated with IL-4 at 20 ng/mL for the indicated times before being harvested, and nuclear extracts were made. Arrowhead indicates the position of the Stat6 band.

**b** Pooled data from 3 separate HUVEC lines. The gels were scanned using a PhosphorImager and the Stat6 bands quantitated.

**Prolonged Activation of Stat6 and Prolonged P-Selectin Expression Both Require the Continuous Presence of IL-4**

The prolonged activation of Stat6 after IL-4 stimulation suggested incomplete receptor downregulation after ligand binding or a long-lived signal generated after IL-4 binding to its receptor. To differentiate between these 2 possibilities, we treated HUVECs with IL-4 for 23 hours, after which IL-4 was thoroughly removed by 3 changes of medium. The cells were then further incubated in the absence of IL-4 for 1 or 3 hours before nuclear extracts were made. The presence of activated Stat6 was detected with an oligonucleotide probe to site 1. After 23 hours of IL-4 stimulation, there was a significant level of activated Stat6 present (Figure 6a). One hour after removal of IL-4, there was little change in the level of activated Stat6 remaining, but 3 hours after removal of IL-4, no detectable levels of activated Stat6 were observed. In the controls to which IL-4 was added again for a further 3 hours after the wash, there was no detectable decrease in activated Stat6 (Figure 6a). These results suggest that the continuous presence of IL-4 is necessary for prolonged activation of Stat6.

A similar approach was followed to assess whether removal of IL-4 also results in a shortened duration of P-selectin expression on the cell surface. Removal of IL-4 after 24 hours of stimulation resulted in a 75% loss of cell-surface P-selectin and a complete return to basal expression 48 hours afterward (Figure 6b). Thus, like Stat6 activation, continuous stimulation by IL-4 for 72 hours or replacement of IL-4 into the medium for a further 48 hours after washing out the initial stimulant is necessary for maintenance of prolonged cell-surface expression. However, unlike downregulation of activated Stat6, a complete return to basal levels was not observed until at least 24 hours after removal of IL-4. This delay in the kinetics of P-selectin downregulation...
compared with the downregulation of activated Stat6 is most likely due to the long half-life of P-selectin mRNA.17

OsM and IL-3 Do Not Activate Stat6

Because OsM and IL-3 also induce expression of P-selectin on HUVECs,16,17 we investigated whether these cytokines utilize the same signaling pathway as IL-4. HUVECs were treated for 30 minutes with OsM or IL-3, and the activation of Stat transcription factors was analyzed by EMSA with the GAS element of the $\beta$-casein promoter. The $\beta$-casein GAS element contains the consensus sequence TTCNNNGAA, which is a general Stat- binding sequence.27 OsM was found to induce 1 prominent complex (Figure 7a) that was identified to be Stat1 by a Stat1 Ab supershift (Figure 7b) and by its relative mobility on gels (data not shown). Occasionally, a weaker Stat3 band was observed after OsM stimulation (Figure 7b), but Stat1-Stat3 heterodimers have not been observed. Stat1 and Stat5 both bind to a GAS element with the canonical sequence TTCNNNGAA, of which the Stat6-binding site is a variant. Because there were no sequence motifs in the P-selectin promoter fitting the canonical sequence of the Stat1-Stat5 GAS element, we investigated whether the 2 Stat6 sites present could bind Stat1 or Stat5. Figure 7a shows that although OsM induced a large activation of Stat1, none of it was able to bind to either of the Stat6 sites.

IL-3 was found to activate Stat5, which also did not to bind to either Stat6 site (data not shown). Thus, OsM and IL-3 induce P-selectin expression by a pathway different from that used by IL-4 and that does not involve Stat6 activation.

In addition to the Jak/STAT pathway, both OsM and IL-3 also activate the ras-MAPK pathway in several different cell types.31,32 To investigate whether OsM or IL-3 also activated MAPK in HUVECs, an Ab specific for active MAPK (phosphorylated p44 Erk and p42 Erk) was used in Western blot analysis. Figure 7c shows that both OsM and IL-3 induced MAPK activation 15 minutes after their addition, with residual active MAPK still detectable 24 hours afterward, although the levels were markedly reduced. In contrast, this was not observed when cells were stimulated with IL-4.

Discussion

P-selectin on ECs has been shown to be a major determinant of inflammatory responses. Two mechanisms of control of cell-surface P-selectin expression have been delineated that appear to function at 2 extremes of the time course: 1 that results in rapid upregulation within minutes of stimulation, utilizing only preformed protein stored in Weibel-Palade bodies and lasting only several hours, and the other, taking several hours to increase P-selectin expression, through a
sustained increase in gene transcription lasting several days. Recently, we and others have identified a number of cytokines (References 16, 17, and 33 and this study) as possible mediators of the latter mechanism of chronic P-selectin expression on endothelium by virtue of the following: (1) they are products of activated T cells and monocytes, cells characteristic of chronic inflammatory sites and (2) they induce prolonged expression of P-selectin on ECs in vitro by stimulating P-selectin gene transcription. One such cytokine is IL-4, a product of activated T cells, mast cells, and eosinophils that is present at sites of chronic and allergic inflammation.

The signal transduction pathway(s) leading to prolonged P-selectin expression is largely unknown. One pathway common to all 3 cytokines that induce prolonged P-selectin expression is the Jak/STAT pathway. The identification of 2 canonical Stat6-binding sequences on the proximal P-selectin promoter at positions −229 and −142 led us to postulate that IL-4 stimulation of P-selectin transcription might occur through activation of Stat6. We established, by EMSA and with the use of unlabeled oligonucleotide competitors and an anti-Stat6–specific Ab, that both of these putative Stat6 sites could indeed specifically bind Stat6 activated by IL-4. Our data also demonstrated that Stat6 binding to at least 1 Stat6-binding site was obligatory for inducing transcription of the P-selectin gene after IL-4 stimulation, since simultaneous inactivation of both Stat6 sites led to complete loss of IL-4 inducibility.

Northern blot analysis (data not shown) showed a 2- to 3-fold increase in P-selectin mRNA as early as 3 hours after IL-4 addition. This is consistent with the increase in P-selectin promoter activity (Figure 4b) induced by IL-4 and also with the kinetics of Stat6 activation. The steady-state level of P-selectin mRNA continued to increase linearly over time up to between 16 and 24 hours, when a plateau was reached. The amount of mRNA at 24 hours varied between 8- and 10-fold above basal levels. Yao et al17 had previously investigated the kinetics of induction of P-selectin mRNA after IL-4 stimulation and found a similar response, except that no increase was observed between the 3- to 7-hour time point. This result, together with the requirement for protein synthesis to upregulate P-selectin mRNA, could be indicative of an indirect effect of IL-4. However, the long, slow, linear increase in steady-state mRNA to the plateau level, as we have observed, is consistent with a small increase in transcriptional rate induced by IL-4 coupled with a slow turnover rate of mRNA. This, together with the data showing that Stat6 binding to the P-selectin promoter is essential for IL-4 induction of P-selectin transcription, suggests that IL-4–activated Stat6 is acting directly on the P-selectin promoter to increase its transcription rate.

Activation of Stat6 occurs through tyrosyl phosphorylation and is thus independent of de novo protein synthesis. Indeed, the presence of cycloheximide for 24 hours did not significantly alter the amount of Stat6 binding to the site 1 probe (data not shown). Yao et al17 had previously reported and our data (not shown) confirm that the increase in mRNA induced by IL-4 was cycloheximide-sensitive, suggesting that de novo protein synthesis was also essential for induction of mRNA synthesis. We therefore postulate that in addition to Stat6 activation, de novo synthesis of a second transcription factor is also required for IL-4 induction of P-selectin transcription.

The 2 Stat6-binding sites on the P-selectin promoter have different properties. They differ by 3 nt’s in the variable region of the canonical sequence, which results in site 1’s having a much greater affinity for Stat6 than does site 2. This suggests that Stat6 would preferentially occupy site 1 in this promoter. The difference in Stat6 binding affinity is also reflected in the function of the 2 sites: mutations that left only site 1 functional showed full IL-4 inducibility compared with the native promoter, whereas when site 2 only was functional, IL-4 inducibility was reduced to <50%. It is unclear what the role of site 2 is. One possibility is that Stat6 bound to site 2 could interact with other transcription factors to affect their activity. Alternatively, we hypothesize that it could be a binding site for other Stat proteins and thereby participate in transducing signals from other cytokine receptors to the P-selectin promoter.

IL-316 and OsM17 are 2 other cytokines that induce P-selectin expression and also activate the Jak/STAT pathway. IL-3 activates Stat5 in HUVECs (E.I. Korpelainen, et al, unpublished observations, 1995), whereas OsM predominantly activated Stat1, with lower levels of Stat3 observed occasionally. Stats 1, 3, and 5 bind a TTCNNNGAA consensus sequence that is not present in the P-selectin promoter. We therefore investigated whether they could bind to site 1 and 2 sequences and found no complexes binding to either Stat6 site when HUVECs were stimulated with IL-3 or OsM. The lack of binding sites for Stats1 and 5 on the P-selectin promoter and the lack of activation of Stat3 by OsM suggests that upregulation of P-selectin expression by IL-3 and OsM is unlikely to occur through Stat activation. In addition to activation of the Jak/STAT pathway, both IL-3 and OsM also activated MAPK in HUVECs. One transcription factor that is a potential downstream target after gp130–ras-MAPK activation is nuclear factor (NF)–IL-6.34 The canonical NF—IL-6 binding sequence is TT/GNNNAAT/G,35 and a sequence fitting this consensus is present at −118 on the P-selectin promoter. It remains to be seen whether this is the mechanism by which IL-3 and OsM upregulate P-selectin expression.

Three different observations are particularly relevant in the sustained increase in P-selectin expression induced by IL-4. First, we show here that IL-4 induced prolonged activation of Stat6 itself, with elevated levels of Stat6 detectable by EMSA as long as 48 hours after IL-4 stimulation. This was somewhat surprising because the activation of STATs has often been observed to be rapid and transient. The consequence of sustained activation of Stat6 after IL-4 stimulation would be a prolonged duration of increased synthesis of P-selectin mRNA. Second, P-selectin mRNA itself has an intrinsically long half-life.17 Third, our studies also showed that both prolonged Stat6 activation and prolonged P-selectin expression are contingent on the continuous presence of IL-4. Removal of IL-4 after stimulation led to a rapid loss of activated Stat6 followed by a slower loss of cell-surface P-selectin expression. The requirement for a continuous presence of IL-4 to sustain Stat6 activation suggests that continuous receptor-mediated events were necessary. It also suggests that removal of IL-4 or its source may be an effective way of downmodulating P-selectin expression at sites of chronic inflammation.
Finally, IL-4 appears to have different immunomodulatory effects on the endothelium that are mediated through different signaling pathways and mechanisms. IL-4 upregulates the expression of P-selectin and augments TNF-α–induced vascular cell adhesion molecule-1 (VCAM-1) expression but inhibits TNF-α induction of E-selectin expression. IL-4 upregulates P-selectin expression by increasing transcription through the Jak/Stat6 pathway (this study), whereas the VCAM-1 promoter does not contain any Stat6-binding sites. VCAM-1 upregulation occurs by stabilization of its mRNA through an as-yet-undefined signaling pathway. On the other hand, IL-4 also inhibits TNF-α induction of E-selectin expression through a Jak/Stat6 pathway, but this is effected through competition with NF-κB binding. The Stat6-binding site on the E-selectin promoter overlaps the NF-κB site, which mediates TNF-α induction, such that Stat6 binding results in competition with NF-κB binding. In the E-selectin promoter, Stat6 binding after IL-4 stimulation of HUVECs does not result in transactivation of promoter activity. The reason for this is unknown, but 1 suggestion is that the necessary component for Stat6 to interact with the basal transcriptional machinery is not present. It is presently unclear whether transactivation is a prerequisite for IL-4 induction of P-selectin gene transcription or whether the increase in transcription occurs because Stat6 binding is competing away a repressor.

The reasons for the different immunomodulatory effects of IL-4 are not entirely understood, but they may serve to determine the leukocyte subtypes that extravasate at different stages of the inflammatory response. In the acute stages of inflammation, when TNF-α is present, upregulation of E-selectin expression and IL-8 production may facilitate neutrophil extravasation. As inflammation progresses to the transition stages when T-cell infiltration occurs, IL-4 is produced, switches the selectin elaboration on the endothelium from E- to P-selectin, and also enhances and prolongs VCAM-1 expression. The combination of P-selectin and VCAM-1 would facilitate monocytic infiltration over a more prolonged period. At later stages, if the source of IL-4 persists, then there exists the possibility for chronic inflammation to develop. However, sites of inflammation are often polyclonal Ab to P-selectin, and Jenny Drew for preparation of HUVECs.

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