Thrombopoietin Stimulates Endothelial Cell Motility and Neoangiogenesis by a Platelet-Activating Factor–Dependent Mechanism

Maria Felice Brizzi,* Edda Battaglia,* Giuseppe Montrucchio, Patrizia Dentelli, Lorenzo Del Sorbo, Giovanni Garbarino, Luigi Pegoraro, Giovanni Camussi

Abstract—In this study, we demonstrate that human umbilical cord vein–derived endothelial cells (HUVECs) expressed c-Mpl, the thrombopoietin (TPO) receptor, and that TPO activates HUVECs in vitro, as indicated by directional migration, synthesis of 1-alkyl-/1-acyl-platelet-activating factor (PAF) and interleukin-8 (IL-8), and phosphorylation of the signal transducers and activators of transcription (STAT) STAT1 and STAT5B. The observation that WEB 2170 and CV3988, 2 structurally unrelated PAF receptor antagonists, prevented the motility of HUVECs induced by TPO suggests a role of PAF as secondary mediator. Moreover, kinetic analysis of TPO-induced tyrosine phosphorylation of STAT demonstrated that STAT5B activation temporally correlated with the synthesis of PAF. PAF, in turn, induced a rapid tyrosine phosphorylation of STAT5B and PAF receptor blockade, by WEB 2170, preventing both TPO- and PAF-mediated STAT5B activation. The in vivo angiogenic effect of TPO, studied in a mouse model of Matrigel implantation, demonstrated that TPO induced a dose-dependent angiogenic response that required the presence of heparin. Moreover, the in vivo angiogenic effect of TPO was inhibited by the PAF receptor antagonist WEB 2170 but not by the anti–basic fibroblast growth factor neutralizing antibody. These results indicate that the effects of TPO are not restricted to cells of hematopoietic lineages, because TPO is able to activate endothelial cells and to induce an angiogenic response in which the recruitment of endothelial cells is mediated by the synthesis of PAF. Moreover, biochemical analysis supports the hypothesis that STAT5B may be involved in the signaling pathway leading to PAF-dependent angiogenesis. (Circ Res. 1999;84:785-796.)

Key Words: hemopoietic growth factor ■ STAT ■ platelet-activating factor ■ signal transduction ■ neovascularization

The c-mpl gene encodes a member of the hematopoietic receptor superfamily that shows, in the extracellular domain, high amino acid sequence homology with the erythropoietin receptor and with the common subunit (c) of the interleukin (IL)–3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors.1 The identification and the cloning of the c-Mpl ligand demonstrated the involvement of c-mpl in megakaryopoiesis.2,3 Thus it was termed thrombopoietin (TPO).2,3 More recently it has been shown that TPO can directly modulate the response of mature platelets to several stimuli and thereby their homeostatic potential.4 The in vitro and in vivo effects of TPO are mediated by the presence, on the target cells, of c-Mpl. TPO, as the other member of the hematopoietic receptor superfamily, is devoid of intrinsic tyrosine kinase activity. However, biochemical events triggered by ligand-receptor binding are characterized by tyrosine phosphorylation of multiple intracellular molecules,5 including Janus kinase 2 (JAK2) and members of the signal transducers and activators of transcription (STAT).6–8

The JAKs are nontransmembrane protein tyrosine kinases that are rapidly tyrosine phosphorylated on ligand binding and play a critical signaling function for cytokine receptors.9 One such function is the activation of STAT proteins, which are latent cytoplasmic proteins that undergo rapid tyrosine phosphorylation and dimerization following cytokine stimulation.9 As a consequence they acquire DNA-binding activity, translocate into the nucleus, bind to specific promoter elements, and control the expression of target genes.9 Several lines of evidence indicate that activation of the JAK/STAT signaling pathway elicits a number of responses, including changes in cell function, growth, differentiation,9 and epithelial tubule formation.10 Moreover, the observation that the JAK/STAT pathway regulates multiple developmental processes in Drosophila, including the formation of ectopic adult wing veins,11

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785
strongly suggests a role of this pathway in modulating new-vessel formation.

Although TPO has been initially defined as a lineage-specific late-acting growth factor, it has also been reported that this factor may exert effects on the erythroid and granulocytic population. Indeed, the hematopoietic progenitor cells express c-Mpl, and TPO has the ability to enhance proliferation of early erythroid progenitors and directly affects erythroid differentiation. Moreover, it has been recently demonstrated that TPO treatment also induces the functional activation of polymorphonuclear neutrophils. These observations suggest that TPO, beyond the maturation of the hematopoietic tissue homeostasis.

It has been suggested that interaction between endothelial cells and stem cells plays a critical role in hematopoiesis during embryogenesis and possibly in adults. Mammalian embryogenesis hematopoiesis is thought to begin in the yolk sac in the blood islands lined by endothelial cells. These endothelial cells are in close proximity to the developing stem cells and may provide the microenvironment required for stem cell proliferation and differentiation. Several lines of evidence suggest that the endothelium of yolk sac plays a major role in the induction of hematopoiesis in the early embryo. It has also been suggested that stromal cells capable of mediating hematopoiesis in fetal liver and subsequently in bone marrow have endothelial characteristics. Therefore, fetal hematopoiesis parallels neoangiogenesis, which has a critical role in the development of the embryo. These observations suggest a bidirectional interaction between endothelium and hematopoietic cells, with the generation of a microenvironment favorable to the development of hematopoietic processes and, conversely, with the production of angiogenic factors by cells of hematopoietic lineage. The neoangiogenesis involves a concerted sequence of events, including directional migration and proliferation of endothelial cells and canalization of solid endothelial cords penetrating in the tissue. A number of diffusible angiogenic factors have been recently characterized, including cytokines and mediators produced from the myeloid cell line. Some of these angiogenic factors, such as vascular endothelial growth factor (VEGF), IL-8, and platelet-activating factor (PAF), can also be produced by endothelial cells and possess an autocrine regulatory role on neoangiogenesis.

The aim of the present study was to evaluate whether TPO is able to induce angiogenesis. We found that human umbilical cord vein–derived endothelial cells (HUVECs) express c-Mpl and that TPO activates HUVECs in vitro, as indicated by directional migration, tubulogenesis, synthesis of PAF and IL-8, and phosphorylation of STAT1 and STAT5B. In vivo, TPO induced an angiogenic effect in a mouse model in which Matrigel was used for the delivery of mediators. These in vitro and in vivo effects of TPO were inhibited by the PAF-receptor antagonist, WEB 2170, suggesting a role of PAF as secondary mediator in TPO-induced angiogenesis.

Materials and Methods

Reagents
Matrigel was obtained from Becton Dickinson Labware. Synthetic 1-alkyl-C16 PAF (1-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) (1-alkyl-PAF) was obtained from Bachem Feinchemikalien. 1-Acyl-C16 PAF (1-palmitoyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) (1-acyl-PAF) was obtained by acetylation of 1-palmitoyl-sn-glycerol-3-phosphorylcholine with acetic anhydride and dimethylformamide as previously described. CV 3988, a structural analog of PAF that acts as specific PAF antagonist was from Takeda Chemical Industries. WEB 2170, a triazolodiazepine (hetrazepinoic) with potent and specific PAF–receptor antagonist activity, was obtained from Boehringer Ingehelm KG. Diazepam, a benzodiazepine without a significant PAF-inhibitory activity, was obtained from Roche S.p.A. Anti-mouse T-cell serum, anti-L3T4 and anti-LY2 monoclonal antibodies (mAbs), anti-MAC-1 FITC-conjugated mAb, and the correspondent irrelevant isotypic IgG controls were purchased from Cedarlane. FITC-conjugated IgG and anti-mouse IgG were from Cappel Laboratories. Rabbit anti-human von Willebrand factor (vWF), rabbit IgG, and anti-rabbit IgG mAb (RG-96) were obtained from Merck. μ Parasil HPLC columns were provided from the Millipore chromatographic division (Waters). RPMI 1640 (GIBCO) and bovine calf serum (BCS) were from HyClone Laboratories. Human TPO, a gift from Genzyme, was used throughout all experiments. Murine versus human TPO cDNA sequences revealed 71% sequence identity; however, human TPO was active in an assay using murine megakaryocytic progenitors. The presence of endotoxin contamination of TPO preparation was tested by the Limulus amebocyte assay, and the concentration was <0.1 ng/mL. Sepharose-protein A and phospholipase C from Bacillus cereus were purchased from Sigma Chemical Co. Nitrocellulose filters, horseradish peroxidase–conjugated protein A, molecular-weight markers, [α-32P]dCTP, and the chemiluminescence reagent were from Amersham. Poly(dI·dC):poly(dI·dC) was obtained from Pharmacia.

Antisera
Polyclonal anti–c-Mpl antisera raised in rabbit immunized with a glutathione S-transferase (GST)– fusion protein containing the amino acid sequence corresponding to residues 518 to 541 (GenBank accession No. M90103) was obtained as previously described. Anti-c-Mpl antisera was affinity purified on a column of Sepharose covalently cross-linked with the GST-fusion protein. Specificity of anti-c-Mpl antisera was assessed by competition experiments. One hundred micrograms of affinity-purified Igs were sequentially preadsorbed (twice) with 1.5 mg of GST-fusion protein covalently cross-linked with the Sepharose-protein A for 1 hour at 4°C. The recovered serum samples were used individually to immunoprecipitate platelet lysates. Polyclonal antibodies to p91 and polyclonal rabbit anti-c-mpl were purchased from Upstate Biotechnology and from Affinity Research Products Ltd., respectively. Anti-STAT5A (L-20), anti-STAT5B (G-2), and anti-STAT5B (C-17) antisera were purchased from Santa Cruz Biotechnology, Inc.

Western Blot Analysis and Immunoprecipitation Studies
HUVEC monolayers that had been tightly confluent for a minimum of 24 hours were washed twice with PBS; serum starved for 4 hours at 37°C in endotoxin-free medium M199 containing 1% BSA, PBS (30% vol/vol), sodium orthovanadate 0.2 mmol/L, and EDTA 1 mmol/L; and incubated without or with TPO (20 ng/mL) or PAF (10 nmol/L) at 37°C for the time indicated. Protein concentration of HUVEC lysates obtained as previously described was determined.
by the Bradford technique, and the protein content of the samples was normalized to 250 mg/sample by appropriate dilution with lysis buffer. The samples were then adsorbed by antisera coupled to Sepharose-protein A, and bound proteins were eluted and processed as previously described. 24

Preparation of Nuclear Extract and Gel Retardation Assay
Nuclear extracts from untreated and from TPO- and PAF-treated HUVECs were prepared as described by Sadowski and Gilman. 23 The oligonucleotides used that corresponded to the prolactin-inducible element of the β-casein promoter (PIE) were G GGG GGA CTT CTT GGA ATT AAG GGA and G GGG TCC CTT AAT TCC AAG AAG TTC. Those that used that corresponded to the serum-inducible element (SIE) of c-fos were G GGG GAT TTC CGG TAA ATC and G GGG GAT TTA CGG GAA ATG. 24 The annealed oligonucleotides were labeled by filling in the overhanging ends with Klenow fragment in the presence of [α-32P]dCTP. Gel retardation reactions were performed as previously described. 24

In Vitro PAF Synthesis by Endothelial Cells
HUVECs were prepared, grown, and characterized as described previously. 27 In standard PAF synthesis assays, HUVECs at the third or fourth passage, maintained for 24 hours in DMEM without FCS, were stimulated in 1 mL of Iscove's medium containing 0.25% BSA for various periods of time with TPO.

In Vitro Endothelial Cell Proliferation and Migration Assay
Proliferative activity of HUVECs was assayed by direct cell count as previously described. 28 Migration of HUVECs or CDC-HMEC-1 cells, kindly provided by Francisco J. Candal, was performed in Boyden's chambers as previously described. 29 Endothelial cells that passed across the polycarbonate filter after addition in the lower compartment of the chamber of vehicle alone (saline containing 0.25% BSA), PAF (10 nmol/L), or TPO (1 to 10 ng/mL) were counted at 200 magnification. The involvement of PAF-specific receptors in endothelial cell migration was evaluated by endothelial-cell treatment with 3 μmol/L WEB 2170 22 or 5 μmol/L CV3988, 21 which are 2 chemically unrelated specific PAF-receptor antagonists.

In vitro angiogenesis was studied on Matrigel-coated surface as described by Montesano and Orci. 30 TPO was either incorporated in Matrigel or added to the medium. To evaluate cell viability, trypan blue exclusion staining was performed at the end of the experiments.

Murine Angiogenesis Assay
Female C57 mice were used at 6 to 8 weeks of age. Matrigel (8.13 mg/mL), in liquid form at 4°C, was mixed with the experimental substances and injected (0.5 mL) into the abdominal subcutaneous tissue of mice along the peritoneal midline. 31, 32 At various times after injection and daily for 6 days, mice were killed and gels were recovered and processed for histology and immunohistochemistry as previously described. 23 Vessel area and total Matrigel area were planimetrically assessed from stained sections, as described by Kibbey et al. 33 We considered vessels only those structures possessing a patent lumen and containing red blood cells. Results were expressed as percentage ± SEM of the vessel area relative to the total Matrigel area. Animal procedures conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985).

In Vivo Experimental Protocol
The angiogenic effect of various doses of TPO (1, 5, 10, 15, and 40 ng) in 0.5 mL of Matrigel was studied at different times and in the absence or presence of 10 U/mL heparin. In selected experiments, the effect of WEB 2170, a tetrahydroisopioic benzodiazepine with specific PAF-receptor antagonist activity (IC50 = 0.3 μmol/L on in vitro platelet aggregation), 22 on TPO-induced angiogenesis was evaluated. WEB 2170 was included in the Matrigel plug (final concentration, 250 ng/mL) and injected intraperitoneally (10 mg/kg) 30 minutes before the subcutaneous injection and daily for 6 days. Diazepam, a nonhetrazepinoic benzodiazepine with absent or minimal activity as a PAF antagonist when tested on in vitro platelet aggregation (IC50 = 260 μmol/L), 23 was used instead of WEB 2170 as control. To evaluate whether the angiogenic effect of TPO may depend on local production of bFGF, 10 mg/mL of anti-bFGF neutralizing antibody were added into Matrigel before injection. In preliminary experiments, this dose of antibody was found to inhibit the angiogenesis induced by 50 ng bFGF.

The direct angiogenic effect of synthetic PAF and of PAF extracted and purified from Matrigel plugs of mice challenged with TPO was also studied.

Assay and Quantification of PAF
PAF extracted and purified by the Matrigel plugs obtained from mice or from cultured HUVECs was measured by bioassay after extraction and purification by TLC and HPLC. 34 Characterization of bioactive PAF was performed by comparison with synthetic PAF according to previously described criteria. 34 To study the incorporation of radioactive precursors, 5 × 103 HUVECs were incubated in 1 mL RPMI 1640 for 30 minutes with 30 mCi [14C]acetate (from Amity) before stimulation. 24 Cell extracts were fractionated by TLC on aluminum-sheet silica-gel plates (silica gel 60, F254, 0.2-mm thickness; Merck) using a chloroform/methanol/acetic acid/water solvent (50:25:8:4) by volume. The plates were cut into 1-cm sections, and the radioactivity of each was measured. Radiolabeled PAF was used as a standard. To discriminate between the presence of an ester or ether group at the sn-1 position, the 1-radyl-2-[3H]acetyleglycerols, obtained by phospholipase C treatment, were acetylated at position 3 by incubation for 16 hours at 37°C with 0.5 mL of acetic anhydride and 0.1 mL of pyridine. 35

Immunofluorescence Study
For the study of TPO-induced STAT5B nuclear translocation, coverslip-attached HUVECs were fixed for 5 minutes in 3% paraformaldehyde in PBS (pH 7.4) containing 2% sucrose and permeabilized with HEPES–Triton X-100 buffer (20 mmol/L HEPES, pH 7.4, 300 mmol/L sucrose, 50 mmol/L NaCl, 1 mmol/L MgCl2, and 0.5% Triton X-100). STAT5B was detected by indirect immunofluorescence with a mouse monoclonal anti-STAT5B IgG1 (G-2; Santa Cruz) and a rabbit FITC-conjugated anti-mouse IgG as secondary antibody (Cedarlane). As control, the primary antibody was substituted by an irrelevant isotypic control mouse IgG (Cedarlane).

Results
Expression of TPO Receptor on HUVECs
To evaluate c-Mpl expression by HUVECs, cell lysates from HUVECs or platelets were immunoprecipitated with an anti-c-Mpl antiserum, raised against the intracellular domain of the receptor, or with a preimmune serum. The eluted proteins were subjected to 8% SDS-PAGE, and the filter was immunoblotted with the same antiserum. As shown in Figure 1A, the anti–c-Mpl antiserum was able to recognize TPO receptor both in platelets and in HUVECs immunoprecipitated with the anti–c-Mpl antiserum but not with the preimmune serum. The specificity of the anti–c-Mpl antiserum was assessed by immunoblotting experiments. As shown in Figure 1B, after 2 steps of absorption the immunoprecipitated band corresponding to the c-Mpl protein was barely detectable.

To evaluate the specificity of TPO receptor engagement by the ligand, we studied the ability of TPO to affect the signaling pathway of other receptors similar to c-Mpl, such as the IL-3 receptor. 1 Unstimulated and TPO- or IL-3–stimulated HUVECs were immunoprecipitated with an antiserum to the β signal–transducing subunit of the IL-3 receptor that
is also shared by the GM-CSF and the IL-5 receptors. As indicated by the antiphosphotyrosine immunoblot shown in Figure 2, TPO, unlike IL-3, was unable to induce phosphor-

**Figure 1.** c-Mpl is specifically expressed on HUVECs. A, Expression of TPO receptor on HUVECs and platelets. Platelets (lanes 1 and 2) and HUVECs (lanes 3 and 4) were lysed and immunoprecipitated (IP) with an anti–c-Mpl antiserum (lanes 2 and 4) or a preimmune serum (lanes 1 and 3). B, Specificity of the antiserum to c-Mpl. Lysates from platelets were immunoprecipitated with the untreated anti–c-Mpl antiserum (lane 1) or with the antiserum to c-Mpl sequentially preadsorbed 1 time (lane 2) or 2 times (lane 3) with the GST-fusion protein and covalently cross-linked with Sepharose-protein A, as described in Materials and Methods. The eluted proteins were subjected to 8% SDS-PAGE and electrophoretically transferred to nitrocellulose filters, and the filters were immunoblotted (IB) with the anti–c-Mpl antiserum. The position of c-Mpl is indicated. No detection of c-Mpl was observed when a nitrocellulose filter was immunoblotted, in parallel, using Ig from preimmune serum as a negative control (data not shown).

**Figure 2.** Effect of TPO and IL-3 on the activation of the GM-CSF/IL-3/IL-5 receptor common β subunit. HUVECs were incubated in the absence (lane 1) or in the presence of IL-3 (10 ng/mL) (lane 2) or TPO (10 ng/mL) (lane 3) for 7 minutes, lysed, and immunoprecipitated (IP) with an antiserum to the GM-CSF/IL-3/IL-5 receptor common β subunit. The eluted proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were probed (IB) with the antiphosphotyrosine antibody (anti–P-tyr, top) and reprobed with the anti-β antiserum (bottom). Four individual experiments were performed with similar results. The positions of the tyrosine-phosphorylated common β subunit are indicated.

**TABLE 1.** Effects of TPO and bFGF on HUVEC Proliferation

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>Number of Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>TPO</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>5.6</td>
</tr>
<tr>
<td>6</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Cell proliferation was assessed at the indicated times by direct cell counts of triplicate wells. The results represent the mean of 3 individual experiments.

In Vitro Growth and Migration of HUVECs

To evaluate cell proliferation, HUVECs were stimulated every 2 days with various concentrations of TPO or with bFGF as positive control. As shown in Table 1, TPO did not induce any significant increase in cell number, whereas bFGF stimulated cell growth. In contrast, TPO, added to the lower well of the Boyden’s chamber, induced a dose-dependent migration of HUVECs across the 5-mm (pore size) gelatin-coated polycarbonate filters (Figure 3A). The extent of migratory response observed at 10 ng/mL concentration of TPO was similar to that induced by 10 ng/mL bFGF or 10 nmol/L PAF. When TPO was also added to the upper well of the Boyden’s chamber, it failed to induce a significant transmembrane migration (number of cell migrated: with 10 ng/mL TPO, 21.55±1.24; 10 nmol/L PAF, 18±3.2; and 10 ng/mL bFGF, 50.2±7.95; in unstimulated control, 17.22±6.64). Similar results were obtained using CDC-HMEC-1 microvascular human endothelial cells (data not shown). As shown in Figure 3B, heparin and polymyxin B did not affect HUVEC migration. These results indicate that the motogenic effect of TPO is independent of the synthesis of a heparin-binding growth factor and that it is not due to endotoxin contamination. Pretreatment with WEB 2170 and CV 3988, 2 structurally unrelated specific PAF-receptor antagonists, inhibited TPO-induced migration (Figure 3B). Heat-inactivated TPO did not stimulate HUVEC migration. Moreover, when HUVECs were plated on a Matrigel-coated surface, TPO induced a rapid formation of cordlike structures of endothelial cells (Figure 4). Cells move from their initial uniform pattern of dispersal to form a series of cell clusters joined by long, multicellular processes, previously defined as a “tube formation.” Cordlike structures of endothelial cells appeared within 1 hour in TPO-stimulated HUVECs (Figure 4B). A similar extent of cordlike structures formation was observed both in experiments in which TPO was incorporated into the Matrigel and in those in which TPO was added to the incubation medium. In untreated controls spontaneous cordlike structures of endothelial cells were absent at 1 hour (Figure 4A) and started to be detectable after 3 hours (data not shown). Cell viability of endothelial cells was >95% as evaluated by trypan blue exclusion assay. No
changes in cell viability were observed when cells were stained before and at the end of experiments. The formation of cordlike structures of endothelial cells was an energy-requiring mechanism, as it was abrogated by 10–2 mol/L NaN₃, an inhibitor of the oxidative phosphorylation and glycolysis (data not shown). The ability of the microvascular human endothelial cells, CDC-HMEC-1, to form cordlike structures was also evaluated. As shown in Figure 4, cordlike structure formation in Matrigel was present after 1 hour of TPO stimulation (Figure 4D), whereas it was absent in unstimulated CDC-HMEC-1 cells (Figure 4C).

Synthesis of PAF and IL-8 by TPO-Stimulated HUVECs
Since several cytokines were shown to induce PAF synthesis by HUVECs, the ability of TPO to stimulate the synthesis of PAF was studied. As shown in Table 2, HUVECs synthesized PAF after stimulation with TPO in an amount comparable to that induced by tumor necrosis factor-α (TNF-α) and thrombin, but with a distinct time course. PAF synthesized after TPO stimulation remained cell associated, being undetectable in the cell-free supernatant. Using radioactive acetate as a substrate for PAF synthesis, we found that PAF detected after stimulation with TPO was newly synthesized. The TLC analysis of lipid fractions extracted 30 minutes after addition of TPO to HUVECs preincubated with [³H]acetate demonstrated the presence of a main peak of radioactivity that comigrated with synthetic [³H]C₁₆-PAF. This peak was absent in the lipid fractions extracted from unstimulated HUVECs. The heterogeneity of acetylated glycerophospholipids produced by HUVECs has been extensively documented.³⁶ Therefore, to evaluate the molecular species of PAF produced by TPO-stimulated HUVECs, the ³H-labeled lipids extracted and purified by TLC were separated by TLC. The radioactive products appeared in 2 distinct regions of chromatogram, corresponding to the retention front of radiolabeled 1-palmitoyl-2,3-diacylglycerol (peak I; 24 000 ± 650 cpm) and 1-O-hexadecyl-2,3-diacylglycerol (peak II; 15 325 ± 720 cpm).

Moreover, we evaluate the release of IL-8 by HUVECs to investigate whether TPO was also able to stimulate the production of a polypeptide mediator. TPO induced IL-8 production by HUVECs with an early release detectable after 2 hours followed by a production that was sustained up to 24 hours (Figure 5). Heat-inactivated TPO failed to induce PAF synthesis and IL-8 production.

TPO Induces STAT1 and STAT5B but Not STAT5A Tyrosine Phosphorylation in HUVECs
The involvement of the JAK/STAT pathway in TPO-induced HUVEC activation was evaluated by the analysis of STAT1, STAT5A, and STAT5B tyrosine phosphorylation. Unstimulated and TPO-stimulated HUVECs were immunoprecipitated with an anti-STAT1 antiserum and immunoblotted the...
an antiphosphotyrosine antibody. Kinetic analysis of STAT1 tyrosine phosphorylation, reported in Figure 6A, demonstrated that both tyrosine phosphorylation and dephosphorylation of STAT1 occurred very rapidly in TPO-stimulated HUVECs (STAT1 tyrosine phosphorylation peaked 5 minutes after TPO stimulation), suggesting a direct effect of TPO in mediating the activation of this STAT protein. When the same time-course experiments were performed using a specific antiserum to STAT5A (L-20, Santa Cruz) or STAT5B (G-2, Santa Cruz), we found that STAT5B, but not STAT5A, became tyrosine phosphorylated on TPO stimulation (Figure 6B). By contrast, in experiments not reported, when TPO was added to the growth factor–dependent M-07e, cells either STAT5A or STAT5B became tyrosine phosphorylated. Moreover, kinetic analysis of STAT5B activation demonstrated a delayed and persistent STAT5B tyrosine phosphorylation detectable from 30 to 90 minutes (Figure 6B and 6C).

### TPO-Mediated STAT5B but Not STAT1 Tyrosine Phosphorylation Is Abrogated by the PAF-Receptor Antagonist WEB 2170

The delayed tyrosine phosphorylation of STAT5B observed after TPO stimulation suggested that this effect of TPO might be indirect. The finding that HUVEC activation is associated with the neosynthesis of several mediators, including PAF and IL-8, led us to evaluate the ability of the specific PAF-receptor antagonist, WEB 2170, or the IL-8 blocking antibody, to prevent TPO-mediated STAT5B tyrosine phosphorylation. Unstimulated HUVECs or HUVECs stimulated for 30 minutes with TPO, pretreated or not pretreated with WEB 2170, were immunoprecipitated with a specific antiserum to STAT5B (G-2, Santa Cruz) and immunoblotted with an anti-phosphotyrosine antibody. The results shown in Figure 7A demonstrate that tyrosine phosphorylation of STAT5B is completely abrogated by pretreatment of HUVECs with the PAF-receptor antagonist WEB 2170. The effect of WEB 2170 was specific for STAT5B, as it was unable to prevent STAT1 tyrosine phosphorylation occurring 5 minutes after TPO addition (Figure 7B). In contrast, the neutralizing anti–IL-8 antibody did not affect tyrosine phosphorylation of STAT5B induced by TPO (data not shown).

### PAF Induces STAT5B but Not STAT5A Tyrosine Phosphorylation

The above results, demonstrating the effect of the PAF-receptor antagonist, WEB 2170, in preventing TPO-mediated STAT5B tyrosine phosphorylation, prompted us to investigate the effect of PAF on STAT5A and STAT5B activation. Unstimulated and 1-alkyl-PAF–stimulated HUVECs were immunoprecipitated with an antiserum to STAT5A (L-20, Santa Cruz) or STAT5B (G-2, Santa Cruz) and immunoblotted with an anti-phosphotyrosine antibody. The results reported in Figure 8 demonstrated that PAF induced a rapid (peaking after 5 minutes) STAT5B, but not STAT5A, tyrosine phosphorylation. We also compared the effect on STAT5B activation of 1-alkyl-PAF with that of 1-acyl-PAF. As shown in Figure 9, when 1-acyl-PAF was used, at the same concentration of 1-alkyl-PAF, no tyrosine phosphorylation of STAT5B was detected.

### TPO- and PAF-Induced Nuclear Translocation of STAT Proteins

The nuclear translocation of STAT1 and STAT5B was studied by gel retardation assay, evaluating the formation of SIE or PIE complexes in nuclear extract from untreated and TPO- or PAF-treated HUVECs. The results reported in Figure 10A demonstrated the presence of a band of SIE-binding activity that was specifically blocked by competition with unlabeled SIE, but not with the unrelated oligonucleotides corresponding to the PIE sequence. That STAT1 was present in the TPO-induced DNA-protein complex was evident from a supershift analysis in which an antibody to

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**TABLE 2. Time Course of PAF Synthesis and Release by HUVECs Stimulated with TPO**

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>TNF (10 ng/mL)</th>
<th>TPO (10 ng/mL)</th>
<th>Thrombin (2 U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell Associated</td>
<td>Released</td>
<td>Cell Associated</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6.5±1.94</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.75±2.15</td>
</tr>
<tr>
<td>30</td>
<td>0.25±0.03</td>
<td>0.2±0.01</td>
<td>4.55±18.7</td>
</tr>
<tr>
<td>1 hour</td>
<td>0.5±0.012</td>
<td>0.15±0.09</td>
<td>0</td>
</tr>
<tr>
<td>4 hours</td>
<td>5.8±0.24</td>
<td>3.2±1.12</td>
<td>0</td>
</tr>
<tr>
<td>12 hours</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

PAF was extracted and purified from the cell pellet (cell-associated PAF) or the supernatants (released PAF) of 15×10⁶ HUVECs stimulated with 10 ng/mL TPO or 10 ng/mL TNF. Unstimulated controls were always <0.1 ng/mL as cell-associated PAF and negative as released PAF. Results are expressed as mean±SEM ng/mL PAF of 3 experiments.
STAT1, but not STAT3, specifically caused a mobility shift of the SIE-containing complex (Figure 10B). These data were consistent with the inability of TPO to induce STAT3 activation (data not shown). The results reported in Figure 11A demonstrated the presence of a band of PIE-binding activity in the nuclear extracts from both TPO- and PAF-treated HUVECs that were completely blocked by adding an excess of unlabeled PIE. Supershifted experiments demonstrated that the anti-STAT5B (C-17, Santa Cruz) but not the anti-STAT5A antiserum altered the mobility shift of the PIE complex, indicating the presence of STAT5B in this complex (Figure 11B). As control, nuclear extracts from TPO-stimulated M-07e cells were preincubated with an anti-STAT5A or an anti-STAT5B antiserum, and the results (not reported) demonstrated the presence of both proteins in the TPO-induced PIE complex.

Nuclear translocation of STAT5B was also evaluated by immunofluorescence. As shown in Figure 12A, unstimulated HUVECs showed a peripheral cytoplasmic staining for STAT5B. By contrast, a nuclear staining of STAT5B was observed after incubation both with TPO (Figure 12B and 12C) and PAF (Figure 12D), with a time course that paralleled the corresponding tyrosine-phosphorylation analysis. Controls with

Figure 6. Kinetic analysis of STAT1 and STAT5B tyrosine phosphorylation in TPO-treated HUVECs. HUVECs were incubated in the absence or in the presence of TPO (10 ng/mL) for the indicated times, lysed, and immunoprecipitated (IP) with the anti-STAT1 antiserum (A) or with the anti-STAT5A or anti-STAT5B antiserum (B). The eluted proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were probed (IB) with the anti-phosphotyrosine antibody (top) and reprobed with the indicated antiserum (bottom). Three individual experiments were performed with similar results. The positions of STAT1 and STAT5B are indicated. C, Tyrosine phosphorylation of STAT5B was estimated by densitometric scanning of the film with an LKB2202 Ultrascan laser densitometer.

Figure 7. WEB 2170 inhibits TPO-induced STAT5B but not STAT1 tyrosine phosphorylation. A, Cell lysates from unstimulated (−) and TPO-stimulated (10 ng/mL, for 30 minutes) or WEB 2170-stimulated (3 μmol/L) (alone or in combination) HUVECs were subjected to immunoprecipitation (IP) with an anti-STAT5B antiserum. B, Cell lysates from unstimulated (−) and TPO-stimulated (10 ng/mL, for 5 minutes) or WEB 2170-stimulated (3 μmol/L) (alone or in combination) HUVECs were subjected to immunoprecipitation (IP) with an antiserum to STAT1. The eluted proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were probed (IB) with the anti-phosphotyrosine antibody (anti-P-tyr, top) and reprobed with the anti-STAT5B (A) or anti-STAT1 antiserum (B) (bottom). Three individual experiments were performed, and similar results were obtained. The positions of STAT5B and STAT1 are indicated.

Figure 8. Kinetic analysis of STAT5B tyrosine phosphorylation in PAF-treated HUVECs. HUVECs were incubated in the absence or in the presence of 1-alkyl-PAF (10 nmol/L) for the indicated times, lysed, and immunoprecipitated (IP) with the anti-STAT5B or anti-STAT5A antiserum as indicated. The eluted proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were probed (IB) with the anti-phosphotyrosine antibody (anti–P-tyr, top) and reprobed with the anti-STAT5B or anti-STAT5A antiserum (bottom). The position of STAT5B is indicated (left).
irrelevant isotypic IgG1, used instead of the anti-STAT5B monoclonal IgG1, were negative (data not shown).

In Vivo Angiogenic Effect of TPO

Figures 13 and 14 show the results of experiments performed to evaluate the angiogenic effect of TPO in Matrigel containing 64 U/mL heparin and various concentrations of TPO, TPO plus WEB 2170, or sterile saline used as vehicle for TPO, which were injected subcutaneously into mice. The histological and morphometric analyses of Matrigel plugs were performed upon euthanizing the mice 6 days later. TPO induced a dose-dependent angiogenic response that was absent in controls containing heparin plus saline (Figure 13A). TPO-induced angiogenesis was significantly reduced in mice treated with Matrigel containing TPO and WEB 2170 (250 ng/mL) followed by daily intraperitoneal injection of WEB 2170 (10 mg/kg per day) (Figure 13B). When diazepam was used instead of WEB 2170, no significant inhibition of TPO-induced angiogenesis was observed (Figure 13B). In contrast, neutralizing antibody against the bFGF did not significantly reduce TPO-induced angiogenesis. In the absence of heparin, TPO induced cell migration within Matrigel but not formation of canalized vessels (Figures 13B and 14C). The optimal angiogenic effect of TPO required a concentration of 64 U/mL heparin. The same concentration was required for the optimal angiogenic effect of bFGF or VEGF. The requirement of heparin for the angiogenic effect of TPO was also indicated by the effect of protamine, which completely abrogated the angiogenesis induced by 10 ng/mL TPO (Figure 13B). These results suggest that the angiogenic effect of TPO depends on the production of heparin-binding growth factors. Indeed, it has been recently reported that TPO stimulates the production of VEGF. The penetration into the Matrigel of cords of vWF-positive endothelial cells was observed after 6 and 24 hours of implantation of TPO-containing Matrigel (data not shown). Canalization of vessels progressively increased in the following days and reached its
maximum at day 6, with formation of microaneurismatic (Figure 14A) or linear (Figure 14B) structures containing red blood cells and leukocytes, which were indicative of functional vessels. Sections of the gel were stained with anti-vWF antibody to confirm the presence of endothelial cells in association with the vessels (Figure 14B, inset). Infiltration into the Matrigel of T lymphocytes (reactive with a pan-T mAb), CD4-positive T lymphocytes (reactive with anti-L3/T4 mAb), CD8-positive T lymphocytes (reactive with anti-Ly2 mAb), and polymorphonuclear neutrophils was never observed. The staining of Matrigel sections for aspecific esterase or for Mac-1 antigen showed that monocytes were confined within the lumen of neoformed vessels but were absent within the Matrigel. As shown in Figures 13B and 14D, WEB 2170 markedly reduced the neovascularization of Matrigel induced by TPO. In Matrigel containing as control either 64 U/mL heparin alone or heparin plus heat-inactivated TPO, angiogenesis was never observed (data not shown).

In some experiments Matrigel was submitted to PAF extraction at 6 hours, 24 hours, and 6 days after the beginning of the experiment. PAF was detected at 6 and 24 hours in mice injected with Matrigel containing TPO but not in controls (Figure 15). When the animals were injected with Matrigel containing an amount of synthetic 1-alkyl-PAF (5 ng/mL), an angiogenic response was observed (28±9.1 mean area percent; 5 experiments). In contrast, 1-acyl-PAF (5 ng/mL) induced a slight migration of endothelial cell into the Matrigel but not the formation of canalized vessels (5.2±1.8 mean area percent; 5 experiments).

**Discussion**

The aim of the present study was to evaluate whether endothelium can represent a target for TPO. The results obtained demonstrate that HUVECs express functional TPO receptors and that TPO is able to activate HUVECs in vitro and to stimulate angiogenesis in vivo via the synthesis of PAF.

The formation of new vessels involves a sequence of events including directional migration and proliferation of endothelial cells, as well as canalization of solid endothelial cords penetrating into the tissue. A number of soluble factors that may act with autocrine or paracrine mechanisms have been found to modulate these events. In the present study we demonstrate that TPO may engage in some of the physiological events involved in the angiogenesis. The in vitro study demonstrated that HUVECs express c-Mpl and that receptor activation by TPO promotes directional migration of HUVECs. In contrast, no proliferation was observed. Other angiogenic factors, such as TNF, were shown to promote endothelial cell motility without a direct stimulation of cell division. This suggests the involvement of secondary mediators in the full development of the angiogenic process. Indeed, it has been shown that the angiogenic effect of TNF is mediated by the production of PAF and nitric oxide. In the present study we observed that TPO stimulated the synthesis of PAF and of IL-8. Despite the fact that both of these mediators possess angiogenic properties, the experi-

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**Figure 12.** Nuclear translocation of STAT5B in HUVECs stimulated by TPO. Immunofluorescence micrograph showing the pattern of distribution of STAT5B in HUVECs unstimulated (A) or stimulated for 20 (B) or 40 (C) minutes with 10 ng/mL TPO or for 20 minutes with 10 nmol/L 1-alkyl-PAF (D). Unstimulated HUVECs showed a peripheral cytoplasmic staining, whereas nuclear staining of STAT5B started to be detectable after 20 minutes and was maximal after 40 minutes of incubation with TPO. In contrast, nuclear staining of STAT5B was already detectable after 20 minutes of incubation with 1-alkyl-PAF.

**Figure 13.** Angiogenic effect of TPO in vivo. Matrigel containing 10 U/mL heparin was mixed with various doses of TPO (1 to 40 ng/mL) or with vehicle alone (control) and was injected subcutaneously into mice (A). After 6 days mice were killed, and the Matrigel plugs were excised and processed for light and immunofluorescence microscopy. Quantitation of neovascularization was performed on hematoxylin-eosin–stained histological sections as described in Materials and Methods, and results were expressed as percentage±SEM of the vessel area to the total Matrigel area. Each individual experimental group included 10 mice. ANOVA with Newman-Keuls test was performed. *P<0.05, control vs 1, 5, 15, or 40 ng/mL TPO. B, Effect of TPO alone (15 ng/mL) or in the presence of heparin (64 U/mL), heparin plus protamine (+ protamine, 50 mg/mL), WEB 2170, diazepam; or anti-FGF antibody (10 mg/mL), WEB 2170 (final concentration, 250 ng/mL) or diazepam (final concentration, 15 µg/mL) was included in the Matrigel plug and injected intraperitoneally (WEB 2170: 10 mg/kg; diazepam: 0.5 mg/kg) 30 minutes before the subcutaneous injection and daily for 6 days. Anti-bFGF neutralizing antibody was included in Matrigel before injection.

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**Figure 12.** Nuclear translocation of STAT5B in HUVECs stimulated by TPO. Immunofluorescence micrograph showing the pattern of distribution of STAT5B in HUVECs unstimulated (A) or stimulated for 20 (B) or 40 (C) minutes with 10 ng/mL TPO or for 20 minutes with 10 nmol/L 1-alkyl-PAF (D). Unstimulated HUVECs showed a peripheral cytoplasmic staining, whereas nuclear staining of STAT5B started to be detectable after 20 minutes and was maximal after 40 minutes of incubation with TPO. In contrast, nuclear staining of STAT5B was already detectable after 20 minutes of incubation with 1-alkyl-PAF. Magnification, ×600.
ments with the PAF-receptor antagonists and with anti–IL-8 blocking antibody indicate that the directional migration elicited in vitro by TPO was mainly PAF dependent. Several studies demonstrated that HUVECs can synthesize various molecular species of PAF with different biological properties.\(^3\) PAF synthesized after TPO stimulation contained \(76\%\) and \(37\%\) of 1-acyl-PAF and 1-alkyl-PAF, respectively. The study of the angiogenic potential of these PAF analogues suggests that the 1-alkyl derivative of PAF mainly accounts for the angiogenic activity observed in vivo.

The activation of HUVECs by TPO was also supported by biochemical studies indicating the involvement of STAT proteins. The STAT are cytosolic transcriptional factors that are tyrosine phosphorylated following ligand stimulation.\(^9\) They form homo- or heterodimers and translocate into the nucleus, where they bind and activate specific DNA sequences.\(^9\) Several lines of evidence indicate that TPO treatment both in proliferating cells and in terminally differentiated elements, such as platelets and neutrophils, leads to the activation of the STAT signaling pathway.\(^6\)–\(^8\) Among the STAT proteins STAT1, STAT3, and STAT5 seem to be strictly related to TPO-mediated receptor activation.\(^6\)–\(^8\) Our results demonstrate a rapid and transient tyrosine phosphorylation of STAT1 with a consequent formation of an SIE complex on TPO treatment. Unlike other STAT proteins, STAT5 consists of 2 proteins, which are referred to as STAT5A and STAT5B.\(^9\) These 2 proteins are encoded from 2 distinct yet highly homologous genes and can be differently activated by a wide range of cytokines.\(^9\) We found that TPO-mediated HUVEC activation is associated with STAT5B but not with STAT5A tyrosine phosphorylation. These data are consistent with the observed nuclear translocation of STAT5B and with the formation of a PIE complex containing STAT5B only. Similar patterns of STAT activation are triggered by a number of cytokine receptors sharing structural features with c-Mpl, including erythropoietin, GM-CSF, IL-3, and IL-5 receptors.\(^1\) Moreover, it has been recently reported that activation of c-Mpl in an erythroleukemia cell line may affect the signaling pathway of the GM-CSF/IL-3/IL-5 receptors via the phosphorylation of their common \(\beta\) subunit.\(^40\) However, our finding that TPO was unable to activate the signaling through the common \(\beta\)
bryogenesis, and cell differentiation. It has recently been shown that PAF directly stimulates in vitro migration of endothelial cells and promotes in vivo angiogenesis by a heparin-dependent mechanism. Several lines of evidence indicate that PAF acts as secondary mediator of angiogenesis induced by TNF and hepatocyte growth factor, as PAF is actively synthesized in vivo during the angiogenic processes and a PAF-receptor antagonist significantly reduced the angiogenic response to these cytokines. The present observation that PAF is synthesized by HUVECs stimulated in vitro with TPO and in vivo within Matrigel during TPO-induced neoangiogenesis, as well as the observation that a specific PAF-receptor antagonist, WEB 2170, inhibits in vitro HUVEC migration and, in vivo, the angiogenic process, suggests that this phospholipid acts as a secondary mediator also for TPO. PAF may act as a mediator of cell-to-cell communication involved in the amplification of the signal triggered by TPO by determining the production of other endothelial-derived heparin-dependent angiogenic factors that may account for the in vivo endothelial cell proliferation. Indeed, PAF has been shown to induce the expression within Matrigel of several angiogenic factors, including VEGF and its specific receptor flk-1. On the other hand, it has been recently shown that TPO triggers the production of VEGF in c-Mpl-expressing cells. Therefore, one can speculate that PAF may stimulate migration of endothelial cells while VEGF may provide the signal for cell proliferation. Further studies are needed to investigate the pathophysiological role of TPO in angiogenesis. The observation that fetal hematopoiesis parallels neoangiogenesis suggests a bidirectional interaction between endothelial and hematopoietic cells. In this context, TPO may contribute to generate a microenvironment favorable to a coordinated development of hematopoiesis and angiogenesis.

In conclusion, the results of the present study indicate that TPO is able to activate endothelial cells and to induce an angiogenic response that is mediated by the synthesis of PAF. Moreover, biochemical analysis supports the hypothesis that STAT5B may be involved in the signaling pathway leading to PAF-dependent angiogenesis. The failure to demonstrate a defect in vasculogenesis/angiogenesis by targeted inactivation of either c-mpl or STAT5B genes may be explained by the redundancy of angiogenic mechanisms acting in vivo. To further investigate the role of STAT5B-dependent signaling pathway in vasculogenesis and neoangiogenesis, generation of transgenic mice with a specific endothelial promoter containing a dominant negative STAT5B construct, is under development in our laboratory.

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References


Thrombopoietin Stimulates Endothelial Cell Motility and Neoangiogenesis by a Platelet-Activating Factor–Dependent Mechanism

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