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Human IL-3 Stimulates Endothelial Cell Motility and Promotes In Vivo New Vessel Formation

Patrizia Dentelli,* Lorenzo Del Sorbo,† Arturo Rosso,* Annarita Molinar,* Giovanni Garbarino,* Giovanni Camussi,* Luigi Pegoraro,2* and Maria Felice Brizzi*

Angiogenesis is a critical process for growth of new capillary blood vessels from preexisting capillaries and postcapillary venules, both in physiological and pathological conditions. Endothelial cell proliferation is a major component of angiogenesis and it is regulated by several growth factors. It has been previously shown that the human hematopoietic growth factor IL-3 (hIL-3), predominantly produced by activated T lymphocytes, stimulates both endothelial cell proliferation and functional activation. In the present study, we report that hIL-3 is able to induce directional migration and tube formation of HUVEC. The in vivo neoangiogenic effect of hIL-3 was also demonstrated in a murine model in which Matrigel was used for the delivery of the cytokine, suggesting a role of hIL-3 in sustaining neoangiogenesis. Challenge of HUVEC with hIL-3 lead to the synthesis of platelet-activating factor (PAF), which was found to act as secondary mediator for hIL-3-mediated endothelial cell motility but not for endothelial cell proliferation. Consistent with the role of STAT5 proteins in regulating IL-3-mediated mitogenic signals, we herein report that, in hIL-3-stimulated HUVEC, the recruitment of STAT5A and STAT5B, by the β common (βc) subunit of the IL-3R, was not affected by PAF receptor blockade. The Journal of Immunology, 1999, 163: 2151–2159.

The new capillary blood vessel formation, in adult life, takes place generally from preexisting vessels, in direct response to tissue demands, by true sprouting or by splitting angiogenesis (1, 2). In healthy adults, angiogenesis does not normally occur, except during the female ovarian cycle. However, neoangiogenesis may occur in several pathophysiological conditions, including wound healing, chronic inflammatory diseases, and solid tumors (1, 2). In response to angiogenic stimuli, endothelial cells degrade the extracellular matrix, migrate into the perivascular space, proliferate, and align themselves into patent blood vessels. The regulation of angiogenesis is determined by the combined effects of positive and negative signals, simultaneously transduced by numerous receptors expressed on endothelial cells (1, 2). Several lines of evidence indicate that the vascular endothelial cell growth factor and the other members of its family are crucial in promoting embryonic and adult angiogenesis (1–4). Besides these factors, a number of diffusible angiogenic factors have been recently characterized, including a vast array of cytokines and mediators produced by neoplastic cells (5). Some of these factors, such as platelet-activating factor (PAF)3 (6) and IL-8 (7), can also be produced by endothelial cells and possess an autocrine regulatory role on neoangiogenesis. We have previously demonstrated that the human hematopoietic growth factor IL-3 (hIL-3) was able to induce endothelial cell proliferation (8), indicating a potential physiopathological role of this cytokine in sustaining the neoangiogenic process. At variance with the canonical endothelial cell growth factors that act by activating tyrosine kinase receptors, hIL-3 mediates its biological effects throughout a heterodimeric receptor (IL-3R) comprised of a ligand-binding-specific α subunit and of a common transducing β (βc) subunit, which does not possess an intrinsic tyrosine kinase domain (9, 10). However, activation of IL-3R is associated with the tyrosine phosphorylation of several molecules, including the member of the Janus family, JAK2, and the transcriptional factors, termed STATs (11–14). STAT proteins are latent cytoplasmic transcriptional factors that, upon activation, regulate expression of target genes (14–17). Among members of the STAT family, STAT5 consists of two highly related proteins, STAT5A and STAT5B (18). Although STAT5 was originally identified as a transcriptional factor activated by prolactin in the lactating mammary gland (19), several lines of evidence indicate that STAT5 can also be activated by other cytokines (14–17), including IL-3 (18). Moreover, besides the prolactin-inducible element (PIE) of the β casein gene, other genes have been identified as target for STAT5 in IL-3-stimulated cells (18).

In the present study, we evaluated the ability of hIL-3 to promote new vessel formation. We demonstrate that, in vitro, hIL-3 stimulated directional migration and tubulogenesis of both HUVEC and of murine endothelial cells, and in vivo, hIL-3 was able to induce neoangiogenesis in a murine model of Matrigel implantation. Moreover, consistent with the role of STAT5 proteins in regulating IL-3-mediated cell proliferation, we demonstrate that STAT5A and STAT5B were recruited by the IL-3R in HUVEC stimulated with hIL-3.

Materials and Methods

Reagents

M199 medium (endotoxin-tested), BSA, protein A-Sepharose, FITC-conjugated anti-rabbit IgG and anti-mouse IgG were all from Sigma (St. Louis, MO). Bovine calf serum (endotoxin-tested) was obtained from HyClone...
Annealed oligonucleotide was labeled by filling in the overhanging ends of 3'-GGG TCC CTT AAT TCC AAG AAG TCC-5' from Affinity Research Products (Nottingham, U.K.). Anti-STAT5A (L-20) and Poly(dI-dC):poly(dI-dC) were obtained from Sigma. PY20 anti-phosphotyrosine Ab was obtained from Becton Dickinson Labware (Bedford, MA). Anti-hIL-3R antisera coupled to protein A-Sepharose, and bound proteins were eluted with the correspondent irrelevant isotypic IgG controls were purchased from Cedarlane (Ontario, Canada). Anti-mouse T cell serum, anti-STAT5A and anti-STAT5B antisera (described in Trier et al. (28), TGF-β and hIL-3 STIMULATES NEOANGIOGENESIS, TGF-β in the Matrigel is in the latent form, which is biologically inactive. At various times, mice were subsequently killed and gels were recovered and processed for histology. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support. Part of tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin were studied by light microscopy. Other sections, obtained from frozen tissue cut with a cryostat, were stained for nonspecific esterase activity (33) or processed for immunofluorescence microscopy, performed as previously described (6). Vessel area and the total Matrigel area were planimetrically assessed from stained sections, as described by Kibble et al. (34). Considered vessels were only those structures possessing a patent lumen and containing RBC. Results were expressed as percentage ± SEM of the vessel area to the total Matrigel area. Animal procedures conformed to the Guide for Care and Use of Laboratory Resources (National Institutes of Health publication no. 93-23, revised 1985).

Cells

Endothelial cells were isolated from HUVEC within 4 h of delivery by Trypsin treatment (0.1%, dilution cultured in M199 with 10% bovine calf serum and 10 ng/ml of basic fibroblast growth factor (bFGF). HUVEC were characterized by morphologic criteria and positive immuno- fluorescence for factor VIII Ag. Contamination with blood leukocytes was assessed by immunofluorescence analysis using an anti-CD45 Ab. They were used at early passage (II-III). M-07e cells were grown as previously described (22). Polymyoma T-transformed endothelial cell line (HEnd), which has previously been shown to behave in vitro as normal endothelial cell (23, 24), was used as murine endothelium. 32Dcl3 murine hemopoietic cell line was kindly provided by Dr. G. Rovera (Wistar Institute, Philadelphia, PA).

Western blot analysis and immunoprecipitation studies

HUVEC monolayer, which had been tightly confluent for a minimum of 24 h, was washed twice with PBS and serum-starved, in endothelin-free medium, M199 supplemented with 1% BSA, PBS (30% v/v), 0.2 mM sodium orthovanadate, and 1 mM EDTA, for 4 h at 37°C, and incubated with or without hIL-3 (20 ng/ml), PAF (10 nM/L), or WEB 2170 (3 μM/L) for the indicated time. Protein concentration of HUVEC lysates, obtained as previously described (21), 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 protein A-Sepharose, and bound proteins were eluted and processed as previously described (21).

Preparation of nuclear extract and gel retardation assay

Nuclear extracts from untreated and hIL-3-treated M-07e cells and untreated and hIL-3-treated HUVEC were prepared by Nonident P-40 lysis, as described by Sadowsky and Gilman (25). The oligonucleotides used were: PIE sense 5'-GGGG GGA CTT GGA ATT AAG GGA-3' and PIE antisense 3'-GGGG TCC CTT GGA ATT AAG GGA-5'. The annealed oligonucleotide was 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 (21).

In vitro endothelial cell growth assay

Proliferative activity of HUVEC was assayed by direct cell count, as previously described (8). Moreover, cell proliferation was also assessed using the colorimetric assay based on the MTT dye reaction (Boehringer Mannheim, Milan, Italy).

In vitro endothelial cell migration assay

Migration of HUVEC was performed in Boyden’s chambers. Endothelial cells that passed across the filter (8-μm pore size) after addition in the lower compartment of the chamber of the vehicle alone (saline containing 0.25% BSA), bFGF (10 ng/ml), hIL-3 (20 ng/ml), or hIL-3 + anti-hIL-3 Ab (20 ng/ml of cytokine with 20 μg/ml of Ab, preincubated for 30 min at room temperature) were counted. A possible involvement of PAF-specific receptors in endothelial cell migration was evaluated by HUVEC pretreatment with 3 μM/L of WEB 2170, a chemically unrelated specific PAF receptor antagonist (20). The specificity of the anti-hIL-3 Ab was assessed by adding in the lower compartment of the chamber bFGF + anti-hIL-3 Ab or PAF + anti-hIL-3 Ab. Chemotaxis of HUVEC in Boyden’s chambers was performed as previously described (27).

Murine angiogenesis assay

Female C57 mice were used at 6–8 wk of age. Angiogenesis was assayed as growth of blood vessels from s.c. tissue into a solid gel of basement membrane containing the test sample (28, 29). 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 s.c. tissue of mice, along the peritoneal midline. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. The Matrigel used was extracted according to the procedure described by Taub et al. (30), that has been previously shown to efficiently deplete Matrigel of bFGF, epidermal growth factor, insulin-like growth factor, and platelet-derived growth factor (31, 32). The content of bFGF and IL-8 measured by ELISA (Quantikine, R&D System, Minneapolis MN) was <0.1 pg/ml and <3.0 pg/ml, respectively. The content of TGF-β was 1 ng/ml as detected by ELISA (Quantikine, R&D System). The Matrigel was prepared as previously described (33). Considered vessels were only those structures possessing a patent lumen and containing RBC. Results were expressed as percentage ± SEM of the vessel area to the total Matrigel area. Animal procedures conformed to the Guide for Care and Use of Laboratory Resources (National Institutes of Health publication no. 93-23, revised 1985).

In vivo experiment protocol

The angiogenic effect of hIL-3 (20 ng/ml) in 0.5 ml of Matrigel was studied in the presence or in the absence of 64 U/ml heparin. In selected experiments, the effect on hIL-3-induced angiogenesis of WEB 2170, a triazolodiazepine (hetrazepinoic) with potent and specific PAF-receptor antagonist activity (IC50 = 0.3 mM on in vitro platelet aggregation) (35) and of an anti-hIL-3 Ab was evaluated. WEB 2170 was included in the Matrigel plug (final concentration 250 ng/ml) and injected i.p. (10 mg/kg) 30 min before the s.c. injection and daily for 6 days. The anti-hIL-3 Ab, used at 20 μg/ml, was included in the Matrigel plug.

Assay and quantification of PAF

PAF bioactivity, tested after extraction (36) and purification by TLC and HPLC (37), was characterized by comparison with synthetic PAF according to the following criteria: 1) induction of platelet aggregation by a pathway-independent of both ADP- and arachidonic acid/thromboxane A2-mediated pathway; 2) specificity of platelet aggregation as inferred from the inhibitory effect of PAF receptor antagonist WEB 2170 (3 μM/L); 3) TLC and HPLC chromatographic behavior and physicochemical characteristics, such as inactivation by strong bases and 5 min heating in boiling water. The methods used were previously described in detail (36).

Immunofluorescence study

To study the nuclear translocation of the activated STAT5A and STAT5B, coverslip-attached HUVEC were fixed for 30 min in 3% paraformaldehyde in PBS (pH 7.4), containing 2% sucrose and permeabilized with HEPES-NaCl-MgCl2 solution (0.5% Triton X-100 buffer (20 mM/L HEPES (pH 7.4), 300 mM/L sucrose, 50 mM/L NaCl, 3 mM/L MgCl2, and 0.5% Triton X-100). STAT5A and anti-STAT5B antisera (described in Antisera section) and a FITC-conjugated goat anti-rabbit IgG or anti-mouse IgG, respectively, as secondary Abs. As control, the primary antisera were substituted by preimmune rabbit serum or an irrelevant isotypic control mouse IgG.

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that hIL-3 acts as proliferative and trophic factor for central cho-

dritic neurons in vitro and in vivo. Therefore, we evaluated

whether hIL-3 was also able to induce directional migration of

cells from the murine endothelial cell line H.end. The results, re-

ported in Table I, demonstrated that hIL-3 elicited chemotaxis of

H.end and that this effect was prevented by a blocking anti-hIL-3

Ab. We have also comparatively evaluated binding of hIL-3 on

H·end and on HUCVE. As shown in Fig. 2, both H·end (Fig.

2A) and HUCVE (Fig. 2B) exhibited a significant hIL-3-binding

(40% H·end and 50% HUCVE) when cells were incubated with hIL-3

(thick line). No staining was observed by incubating cells with

IL-1β (continuous line) or with preimmune rabbit IgG (dotted

line). The ability of hIL-3 to sustain the growth of the IL-3-de-

pendent murine hemopoietic cell line 32Dcl3 was also evaluated.

Consistent with previous reports (39), we found that hIL-3 was

ineffective on the proliferative activity of this cell line (data not

shown). Accordingly, the result reported in Fig. 2C demonstrates

that 32Dcl3 cells are unable to bind hIL-3.

**In vivo angiogenic effect of hIL-3**

Figs. 3 and 4 show the results of experiments performed to eval-

uate the in vivo angiogenic effect of hIL-3. Matrigel containing 64

U/ml of heparin and 20 ng/ml hIL-3, 20 ng/ml hIL-3 plus anti-
hIL-3 Ab, 20 ng/ml hIL-3 plus WEB 2170, or sterile saline, used

as vehicle for hIL-3, was injected s.c. into mice. After 6 days, mice

were killed, and the Matrigel plugs were excised and processed for

histologic and morphometric analyses (Fig. 4). As shown in Fig. 4,

hIL-3, in the presence of heparin, was able to induce neoangiogen-

esis, while this angiogenic response was absent in Matrigel

containing hIL-3 without heparin (data not shown). Several cana-

lized vessels containing erythrocytes and leukocytes were ob-

served in the Matrigel plugs (Fig. 4, B–D). Some of the vessels

formed microaneurysmatic structures (Fig. 4, B and C); others

were surrounded by inflammatory cells (Fig. 4D). This effect was

absent in control mice treated with heparin plus saline (Fig. 4A).

Moreover hIL-3-induced neoangiogenesis was significantly

reduced in mice injected with Matrigel containing hIL-3 plus anti-
hIL-3 Ab (Fig. 4E) or hIL-3 plus WEB 2170 (Fig. 4F). Similar

results were obtained when mice IL-3 (mIL-3) or mIL-3 plus anti-
mIL-3 Ab was injected s.c. into mice (data not shown). These in

vivo results are consistent with the observation that hIL-3 binds to

murine endothelial cells and stimulates their in vitro migration.

**Synthesis of PAF by hIL-3-stimulated HUCVE**

The ability of hIL-3 to stimulate the synthesis of PAF was evalu-

ated. As shown in Fig. 5, HUCVE synthesized PAF after stimu-

lation with hIL-3. PAF synthesized after hIL-3 stimulation re-

mained all cell-associated, being undetectable in the cell-free

supernatant. Using radioactive acetate as substrate for PAF syn-

thesis, we found that PAF, detected after stimulation with hIL-3,

was newly synthesized. The TLC analysis of lipid fractions ex-

tracted 4 h after addition of hIL-3 to HUCVE (Fig. 5) preincubated

with WEB 2170, significantly reduced both PAF- and hIL-3-induced

directional migration of HUVEC (Fig. 1), suggesting a role of PAF as

secondary mediator. The specificity of the anti-hIL-3 Ab was dem-

onstrated by its inability to block bFGF- or PAF-induced cell mi-

gration (data not shown).

**hIL-3R analysis**

The presence of the hIL-3R on murine H.end and HUCVE was evaluated by cytofluorometric analysis by assessing hIL-3 binding to the putative murine or human receptors using a technique previously described (38). hIL-3 binding to the putative receptor on the murine cell line or HUCVE was evaluated following blockade of nonspecific sites by incubation with heat-inactivated human serum for 15 min. A total of 2×10⁶ cells, in 100 μl of staining buffer (PBS containing 2% heat-inactivated human serum and 0.1% sodium azide), was sequentially incubated for 1 h with hIL-3 (20 ng/ml), IL-1β (10 U/ml), or a preimmune rabbit IgG for 30 min with the rabbit polyclonal anti-hIL-3 Ab, and for 30 min with FITC-conjugated goat anti-rabbit IgG. All incubations were performed at 4°C in staining buffer, and cells were washed twice between incubations. The stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

**Results**

hIL-3 stimulates in vitro directional migration of HUCVE

We investigated the effect of hIL-3 on directional migration of HUCVE. As shown in Fig. 1, when hIL-3 was added at a concentration of 20 ng/ml, in the lower compartment of the Boyden’s chamber, migration of HUCVE, across the 8-μm pore-size gelatin-coated polycarbonate filters, was observed. The hIL-3-induced HUCVE migration was similar to that induced by 10 ng/ml of bFGF. The motogenic activity of hIL-3 on HUCVE was also assayed in the presence of an anti-hIL-3 Ab. The results reported in Fig. 1 demonstrated that preincubation of hIL-3 with the anti-hIL-3 Ab significantly decreased the hIL-3-mediated HUCVE migration, whereas no effect was observed with the Ab alone. Moreover, pre-
treatment of HUCVE with the PAF receptor antagonist, WEB 2170, significantly reduced both PAF- and hIL-3-induced directional

migration of HUCVE (Fig. 1), suggesting a role of PAF as secondary mediator. The specificity of the anti-hIL-3 Ab was dem-

onstrated by its inability to block bFGF- or PAF-induced cell mi-

gration (data not shown).

hIL-3 binds to murine endothelial cells and stimulates their in vitro migration

Despite the low sequence homology between hIL-3 and murine IL-3 (29% identity) (39), Kamegai et al. (40) have demonstrated that hIL-3 acts as proliferative and trophic factor for central cho-
with $[^3]$H]-acetate demonstrated the presence of one main peak of radioactivity that comigrated with synthetic $[^3]$H]-C16-PAF (data not shown). This peak was absent in the lipid fractions extracted from unstimulated HUVEC.

**Effects of hIL-3 on HUVEC proliferation**

To investigate the role of PAF in mediating hIL-3-induced endothelial cell proliferation, the effects of hIL-3 in the presence or in the absence of WEB 2170 was evaluated. As shown in Table II, in the absence of growth factor, no significant increase in endothelial cell number was detected. By contrast, as previously reported (8), hIL-3 was able to support a 3-fold increase of the seeded cell number after 7 days of culture. Moreover, the addition of WEB 2170 did not prevent hIL-3-induced endothelial cell proliferation. A 5-fold increase of endothelial cells was observed when bFGF, the reference growth factor, was added to the cultures. Similar

**FIGURE 2.** Detection of hIL-3 receptor by flow cytometry on murine H·end, HUVEC, and 32Dcl3 hemopoietic cell line. The binding of hIL-3 was assayed at 4°C by incubating murine H·end (A) or HUVEC (B) with IL-1β (continuous line), with preimmune rabbit IgG (dotted line) or with hIL-3 (thick line) and then with the anti-hIL-3 Ab, as described in Materials and Methods. Three experiments were performed with similar results. C, 32Dcl3 cells were incubated with preimmune rabbit IgG (dotted line) or with hIL-3 (continuous line) and then with the anti-hIL-3 Ab. Similar results were obtained in three individual experiments.

**FIGURE 3.** Angiogenic effect of hIL-3 in vivo. Matrigel containing 64 U/ml heparin was mixed at 4°C with one of the following reagents: vehicle alone (control), 20 ng/ml hIL-3, hIL-3 plus anti-hIL-3 Ab, anti-hIL-3 Ab, hIL-3 plus WEB 2170, bFGF, or bFGF plus WEB 2170, WEB 2170, PAF, or PAF plus WEB 2170. Matrigel was then injected s.c. into mice. After 6 days, mice were killed and the Matrigel plugs were excised and processed for light microscopy. Quantitation of neovascularization was performed on hematoxylin-eosin-stained histologic sections, as described in Materials and Methods, and results were expressed as percentage ± SD of the vessel area to the total Matrigel area. Each individual experimental group included five mice. ANOVA with Newman Keul’s multicomparison test was performed: Control vs the experimental groups (*, p < 0.05); hIL-3 vs hIL-3 + anti-hIL-3 Ab or vs hIL-3 + WEB 2170, and PAF vs PAF + WEB 2170: $, p = 0.05$; bFGF vs bFGF + WEB 2170, NS.

**FIGURE 4.** Histological analysis of Matrigel plugs. Hematoxylin-eosin-stained sections of Matrigel containing 64 U/ml heparin plus vehicle alone (A) or 20 ng/ml hIL-3 (B–D) or hIL-3 plus anti-hIL-3 Ab (20 μg/ml) (E) or hIL-3 plus WEB 2170 (3 μM/L) (F). Typical microaneurysmatic structures (D and C) and a canalized linear vessel (D) containing RBC and leukocytes are seen. D, The neoformed vessel is surrounded by inflammatory cells. E and F, The inhibitory effect on neovascularization of anti-hIL-3 Ab and WEB 2170, respectively. (A, C–F: ×250; B: ×150).
results were obtained when cell proliferation was assessed by MTT colorimetric assay (Table II).

**STAT5A and STAT5B are activated in response to hIL-3 and form a stable complex with the β-c subunit of the hIL-3R**

Receptor activation leads to the recruitment of several transducing molecules, including members of the STAT family (14–17). It has been shown that STAT5 proteins are implicated in IL-3-induced cell proliferation (18). We thus investigated the ability of hIL-3 to induce STAT5 protein activation in endothelial cells. To this end, kinetic analysis of STAT5A and STAT5B tyrosine phosphorylation in HUVEC stimulated with hIL-3 was performed. As shown in Fig. 6, A and B, both STAT5A and STAT5B became rapidly tyrosine phosphorylated upon hIL-3 treatment; however, STAT5A tyrosine phosphorylation was no more detectable after 15 min, while STAT5B tyrosine phosphorylation was still detectable after 30 min of hIL-3 stimulation.

Upon ligand stimulation, the activated STAT proteins tightly interact, through their SH2 domains, with specific receptor’s phosphotyrosine residues (14–17). Therefore, we addressed the ability of the hIL-3-activated STAT5A and STAT5B to be recruited by the activated IL-3R β- subunit. The results of coimmunoprecipitation experiments shown in Fig. 7 demonstrate that both STAT5A (A) and STAT5B (B) are physically associated with the βc subunit of the IL-3R and that this interaction depends on ligand stimulation.

**PIE complex formation in hIL-3-treated HUVEC**

Early studies on mammary gland tissue demonstrated that STAT5 was able to recognize a specific DNA sequence located upstream of the β casein promoter, PIE (19). Therefore, to extend the analysis of STAT5 protein activation in HUVEC, we evaluated, by gel retardation assay, the formation of a DNA-protein complex with the PIE sequence in nuclear extracts from untreated and hIL-3-treated cells. The results shown in Fig. 8 indicate that hIL-3 stimulation led to the formation of a PIE complex in HUVEC. That the hIL-3-induced DNA-binding complex contained both STAT5 proteins was evident from supershifted experiments in which Abs to STAT5A and STAT5B were able to induce a mobility shift of the PIE complex (Fig. 8B). As control for STAT5 protein activation, hIL-3-stimulated M-07e cells were used (Fig. 8, A and B).

Nuclear translocation of STAT5 proteins was also evaluated by immunofluorescence. As shown in Fig. 9, unstimulated HUVEC showed a peripheral cytoplasmic staining for STAT5A (A) and STAT5B (B), by contrast a nuclear staining in cells stimulated with hIL-3 indicates the ability of STAT5A (B) and STAT5B (D) to migrate into the nucleus. Controls, with preimmune rabbit serum or with an irrelevant isotypic mouse IgG, used instead of the specific anti-STAT5 antisera, were negative (data not shown).

### Table II. Effects of bFGF, hIL-3, and hIL-3 + WEB 2170 on HUVEC proliferation

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>Number of Cells</th>
<th>MTT</th>
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<tbody>
<tr>
<td></td>
<td>Controls bFGF hIL-3 hIL-3 + WEB</td>
<td>Controls bFGF hIL-3 hIL-3 + WEB</td>
</tr>
<tr>
<td>3</td>
<td>7 16 14 14</td>
<td>0.123 0.232 0.175 0.172</td>
</tr>
<tr>
<td>5</td>
<td>11 26 18 18</td>
<td>0.095 0.605 0.305 0.290</td>
</tr>
<tr>
<td>7</td>
<td>7 34 22 20</td>
<td>ND ND ND ND</td>
</tr>
</tbody>
</table>

[a] Cells were resuspended at $5 \times 10^6$ ml in M199 with the addition of 20% BCS, seeded in 24-well plates, and allowed to adhere. After 24 h, the medium was replaced with M199 additioned with 15% BCS, and cells were incubated for different times with or without bFGF (10 ng/ml) or hIL-3 (20 ng/ml) or hIL-3 + WEB 2170 (3 μM/L). Cell proliferation was assessed at the indicated times by direct counts of triplicate wells or by MTT colorimetric assay. The results represent the mean of three individual experiments.

[b] Number of cells $\times 10^4$.

[c] Optical density at 580 nm.
WEB 2170, a PAF receptor antagonist, does not abrogate activation of STAT5A and STAT5B in HUVEC stimulated with hIL-3

Our finding that hIL-3-induced angiogenesis is mediated by the synthesis of PAF led us to investigate the role of this soluble mediator on STAT5 protein activation in response to hIL-3. Unstimulated and hIL-3-stimulated HUVEC, pretreated or not with WEB 2170, were immunoprecipitated with specific antisera to STAT5A and STAT5B. That WEB 2170 was unable to prevent IL-3-mediated STAT5A and STAT5B activation was demonstrated by the anti-phosphotyrosine immunoblot shown in Fig. 10, A and B, respectively. In conclusion, these data indicate that the effect of hIL-3 on STAT5 protein activation does not depend on the synthesis of PAF.

Discussion

Angiogenesis depends on the tightly regulated growth of blood vessels that can “turn on” and “turn off” within a brief period. When blood vessels grow unabated, angiogenesis becomes pathologic and sustains the progression of many neoplastic and nonneoplastic diseases (1–3). Indeed, angiogenesis is implicated in the
the results presented here demonstrate that hIL-3 can bind to human hematopoietic cells. On the other hand, it has been also demonstrated that hIL-3 has no detectable activity on the human ligand (39). These observations are consistent with the low homology between the murine and human receptor (9). In accordance with the low homology between the murine and human receptor (9), in contrast, the ligand-binding (α) subunit (30% identity) of the human receptor is a transducing subunit, shared with the GM-CSF and the IL-5 receptor (46), showing extensive sequence homology to the murine receptor (47) revealed a low homology with the human α subunit (30% identity) (9), in accordance with the low homology between the murine and the human ligand (39). These observations are consistent with the inability of hIL-3 to stimulate proliferation and colony formation of murine hemopoietic cells (Ref. 39, and our unpublished observations) and with the failure of hIL-3 to bind to (Fig. 2C) murine hemopoietic cells. On the other hand, it has been also demonstrated that hIL-3, as the murine IL-3, is a trophic factor for mouse central cholinergic neurons (40), indicating that, in tissues different from bone marrow, hIL-3 can bind to the mouse receptor. Indeed, the results presented here demonstrate that hIL-3 can bind to murine endothelial cells and is able to promote a motogenic response. Therefore, we evaluated whether hIL-3 was also able to elicit an angiogenic response in an experimental murine model. These in vivo studies demonstrated that hIL-3-containing Matrigel injected s.c. in mice led to neoangiogenesis and that, for a full expression of the in vivo angiogenic properties, the presence of heparin was required (data not shown). The hIL-3 dependency of the in vivo angiogenic process was confirmed by the inhibitory effect of anti-hIL-3 blocking Ab. Some of the neo-formed vessels contained or were surrounded by inflammatory cells consistently with the observation that hIL-3 triggers the expression of adhesion molecules by endothelial cells (8, 43). Moreover, we found that a specific PAF receptor antagonist, WEB 2170, was able to inhibit HUVEC migration in vitro and the angiogenic process in vivo, suggesting that PAF may act as a mediator of cell-to-cell communication of signaling induced, on endothelial cells, by hIL-3. These results are reminiscent of those observed with other polypeptide mediators that require the synthesis of PAF for the full expression of their angiogenic properties (48, 49). Indeed, we demonstrated that stimulation of HUVEC with hIL-3 leads to the synthesis of PAF that peaks after 4 h of treatment, as previously reported for IL-1 and TNF-α (50). The role of PAF in angiogenesis is mainly related to its motogenic activity on endothelial cells, while the proliferative response required for new vessel growth is sustained by the production of other endothelial-derived heparin-dependent angiogenic factors (48). In agreement with these observations, we found that only the motogenic activity but not the proliferative effect of hIL-3 was prevented by a specific PAF receptor antagonist. Therefore, in this context, hIL-3 may directly sustain the proliferative activity of endothelial cells, whereas IL-3-mediated PAF release may account for their migration. The observation that patients treated with hIL-3 showed an increase of endothelial cell number in bone marrow microenvironment (51) supports the possibility that the proliferative effect of hIL-3 on endothelial cells can also be operative in vivo. hIL-3 binding to its receptor triggers a cascade of intracellular protein tyrosine phosphorylations, including members of the STAT family (14–17). Although the functional role in specific cellular program of some STAT proteins has been demonstrated by targeting disruption of their genes, for the other members, considerable discussion centers on whether individual STAT proteins play a role in differentiation, proliferation, or both (15–18). We found that in hIL-3-stimulated HUVEC both STAT5A and STAT5B are rapidly tyrosine phosphorylated and recruited by the β3-transducing subunit of the hIL-3R before migrating into the nucleus to bind the target gene. Activation of STAT5 has been initially related to its ability to bind the β3 casein promoter; however, more recent observations demonstrate that STAT5 can also interact with other DNA elements (18), suggesting that the role of STAT5 is not exclusively related to the lactating mammary genes. Indeed, STAT5 can interfere with the expression of the IL-3-mediated activation of early response genes (18). Similarly, a dominant negative STAT5 inhibits IL-3-stimulated cell proliferation (52). These observations, together with the finding that a constitutive activation of STAT5 correlated with the transition from IL-3-dependent to IL-3-independent growth of Ba/F3 cell line (53), suggest a role of STAT5 in regulating IL-3-induced mitogenic signals. It is then conceivable to assume that, also in endothelial cells, the activation of STAT5 proteins in response to hIL-3 may be associated with proliferative signals. Inhibition of the transcriptional factor NF-κB has been shown to block tubular morphogenesis of human microvascular endothelial cells induced by oxidative stress, suggesting a role of this transcriptional factor in regulating some events of neoangiogenesis
(54). Since PAF can activate NF-κB (55, 56), it is possible that NF-κB may contribute to the proangiogenic effect of PAF. IL-3 does not directly activate NF-κB (57); however, it may activate this transcriptional factor indirectly via the synthesis of PAF.

In conclusion, these results demonstrate that hIL-3 possesses angiogenic properties both in vitro and in vivo in a murine model of Matrigel implantation, suggesting a potential role of hIL-3 in sustaining new vessel formation in pathophysiological processes where T lymphocytes are involved. Moreover, the analysis of the mechanisms involved in hIL-3-induced angiogenic response indicates that, while endothelial cell migration depends on a PAF-mediated pathway, proliferation of endothelial cells is directly elicited by hIL-3.

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References


