Interleukin-4 Stimulates Expression of Urokinase-Type-Plasminogen Activator in Cultured Human Foreskin Microvascular Endothelial Cells

By Johann Wojta, Marisa Gallicchio, Hans Zoellner, Enrico L. Filoni, John A. Hamilton, and Katherine McGrath

The effect of interleukin-4 (IL-4) on the fibrinolytic system of human microvascular and macrovascular endothelial cells in culture was studied. Only foreskin microvascular endothelial cells (EC) responded to IL-4 treatment with a dose- and time-dependent increase in urokinase-type plasminogen activator (u-PA) expression. u-PA was increased significantly in HCMEC in the presence of IL-4, whereas tissue-type plasminogen activator (t-PA) levels remained unaffected when HCMEC were treated with IL-4. These findings were also reflected in the specific mRNA levels as determined by Northern blotting. u-PA-specific mRNA increased significantly in HCMEC in the presence of IL-4, whereas t-PA mRNA and PAI-1-specific mRNA in HFMEC and u-PA-specific mRNA in human saphenous vein EC (HSVEC) remained unaffected by IL-4 treatment. Our findings suggest a role for IL-4 in the process of angiogenesis, in addition to its known proliferative effect on human microvascular EC, by increasing the fibrinolytic potential of such EC, thereby facilitating extracellular proteolysis and cell migration.

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ENDOTHELIAL CELLS (EC) play a key role in regulating fibrinolysis. Human EC express both types of plasminogen activators (PAs), the urokinase-type PA (u-PA), and the tissue-type PA (t-PA), as well as their inhibitor, plasminogen activator inhibitor type 1 (PAI-1).

The balance between PAs and PAI-1 is regulated by a wide variety of stimuli (for a review see the paper by Schleef and Loskutov and Van Hinsbergh). Whereas t-PA seems to be mainly responsible for intravascular clot lysis, u-PA seems to be involved in processes requiring extracellular proteolysis such as angiogenesis, cell migration, invasion, and metastasis. In bovine microvascular EC, u-PA expression is upregulated by the angiogenic agent basic fibroblast growth factor (b-FGF), and correlates with cell migration. Recently, tumor necrosis factor-α (TNF-α), which induces EC migration and neovascularization, has been shown to stimulate the production of u-PA.

The cytokine interleukin-4 (IL-4) has been identified as a potent mitogen for human microvascular EC. Furthermore, IL-4, which is produced by activated T cells, mast cells, and bone marrow (BM) stromal fibroblasts, counteracts pyrogen-induced downregulation of thrombomodulin in human EC, thereby protecting the EC surface against procoagulant changes. IL-4 has also been shown to modulate the fibrinolytic potential of monocytes by stimulating t-PA production. The aim of this study was to investigate whether IL-4 could also influence the fibrinolytic potential of human EC.

MATERIALS AND METHODS

Cell culture. Human foreskin microvascular EC (HFMEC) were isolated according to the method of Jackson et al. Briefly, specimens of human neonatal foreskin were cut into 5-mm cubes and incubated for 40 minutes at 37°C in 0.3% trypsin, 1% EDTA in Hanks' Balanced Salt Solution (HBSS). Thereafter, the tissue was washed three times with HBSS and microvascular segments were squeezed from the cut edges by downward pressure with the flat side of a scalpel blade. The suspension was pushed through a 100-μm nylon mesh and centrifuged for 5 minutes at 200g. The cells were resuspended in 80 mL of HBSS containing 5% supplemented calf serum (SCS) (Hyclone, Logan, UT) and incubated with 20 μL of Dynabeads (10^8 beads/mL) (Dynal, Oslo, Norway) coated with Ulex europaeus agglutinin (UEA) (Sigma, St Louis, MO) for 10 minutes at 4°C with end-over-end rotation. The beads were collected with a magnetic particle concentrator (MPC) (Dynal) and the supernatant was discarded. Thereafter, EC bound to the beads were washed five times with resuspending with 2 mL of HBSS containing 5% SCS, mixing by end-over-end rotation for 1 minute followed by separation using the MPC for 1 minute. Thereafter, the EC were resuspended in M199 containing 20% SCS, 50 μg/mL EC growth supplement (EGCS) (prepared as described previously), and 5 U/mL heparin (Fisons, Castle Hill, New South Wales, Australia) and plated in Petri dishes (100 mm) (Costar, Cambridge, MA) coated with gelatin (Biomed, Richmond, CA). After 5 to 8 days HFMEC reached confluence and were subcultured using a split ratio of 1:3. Human umbilical vein EC (HUVEC), human umbilical artery EC (HUAEC), and human saphenous vein EC (HSVEC) were isolated by mild collagenase treatment following the method of Glimbrone et al. HUVEC, HUAEC, and HSVEC were grown in gelatin-coated Petri dishes, using M199 containing 20% SCS, 50 μg/mL.


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ECSG, and 5 U/mL heparin as described above and subcultured using a split ratio of 1:3.

The cells were confirmed as endothelial by their "cobblestone" morphology, positive staining with anti-von Willebrand Factor (vWF) VIII antibodies, and uptake of immunofluorescent-labeled acetylated low-density lipoprotein (LDL). All EC used in this study were between passage 2 and 4.

Cytokines. Recombinant human IL-4 (rh IL-4) expressed in Escherichia coli was obtained from DNAX (Palo Alto, CA) and had a specific activity of 10^7 U/mg. Recombinant human TNF-α (rh TNF-α) expressed in E. coli was obtained from Boehringer-Ingelheim (Sydney, Australia) and had a specific activity of 6 × 10^6 U/mg. A rat anti-IL-4 monoclonal antibody (MoAb) (MP4-25D2-11) blocking the activity of the cytokine (5 ng block 5 U of IL-4) was obtained from Schering Plough (Bloomfield, NJ).

IL-4 treatment. EC, grown to confluence in 6-or 24-well dishes (Costar) were rinsed twice with HBSS containing 0.1% Triton X-100. Lysates were processed as described above.

Assay for u-PA activity and antigen. u-PA activity and antigen in conditioned media and cell lysates was determined by an enzyme-linked immunosorbent assay (ELISA) as described recently. To characterize the molecular form of u-PA present in the conditioned media and cell lysates of HFMEC, a method similar to the one described by Van Hinsbergh et al. was used.

Briefly, a polyclonal anti-u-PA antibody immobilized to an ELISA plate was used to bind u-PA present in the samples. After washing, u-PA bound to the antibody was incubated for 1 hour at 37°C with 100 µL/well of phosphate-buffered saline (PBS) containing 1 mU/mL plasmin to activate single-chain u-PA (scu-PA) into two-chain u-PA (tcu-PA). Duplicate wells were incubated for 1 hour at 37°C with 100 µL/well of PBS to account for the amount of tcu-PA present without plasmin activation. Thereafter, activity and antigen were measured as described.

Assay for t-PA antigen. t-PA antigen in conditioned media and cell lysates was measured by an ELISA using MoAbs as described previously.

Assay for PAI-1 antigen. PAI-1 antigen in the samples was quantified by an ELISA. Briefly, a monoclonal anti-PAI-1 antibody (5PAI12) that recognizes active PAI-1, latent PAI-1, and PAI-1 in complex with t-PA was immobilized to an ELISA plate and used to bind the PAI-1 contained in the sample. A second peroxidase-labeled monoclonal anti-PAI-1 antibody (3PAI5) that also recognizes active and latent PAI-1 as well as PAI-1 in complex with t-PA was used to quantify the amount of bound PAI-1. Purified melanoma PAI-1^a was used as a calibration standard.

Northern blot analysis. Northern blot analysis was performed using HFMEC from two donors and HSVEC and HUAEC from a single donor, respectively, cultured in 6-well plates with each well having a surface area of 9.6 cm^2. Cells in triplicate wells were treated with rh IL-4 or rh TNF-α. Total cellular RNA from triplicates was extracted and pooled by the guanidine isothiocyanate/cesium chloride method. Agarose gel electrophoresis was used to size fractionate RNA, using a formaldehyde containing 1.4% (wt/vol) agarose gel. RNA was transferred from the gel to a Bio Trace HP membrane (Gelman Sciences, Ann Arbor, MI) using a transfer buffer of 1.5 mol/L NaCl and 0.5 mmol/L NaOH. Hybridizations were performed overnight, using cDNA fragment probes encoding u-PA, t-PA, and PAI-1 (pTPE14, kindly provided by Dr W.D. Schleunning, Berlin, Germany), PAI-1 (DNA fragment corresponding to bases 1 to 1249 of human PAI-1 cDNA, kindly provided by Dr D.J. Loskutoff, La Jolla, CA), and the "housekeeping" gene GAPDH (pRGAPDH-1, kindly provided by Dr O. Bernard, Melbourne, Australia). The respective plasmids were digested with their respective enzymes (Sma-I BamHI for the u-PA, EcoRI-BamHI for the t-PA, EcoRI for the PAI-1, and Pst I for the GAPDH-probes, respectively) at 37°C for 2 hours, run on a 1% agarose gel, and isolated onto NA-45 diethylaminoethyl (DEAE) membrane (Schleicher and Schuell, Dassel, Germany) and then eluted using high salt at 70°C for 1 hour. Probes were labeled by random priming with [32P]adenosine triphosphate (ATP) (Amersham, North Ryde, New South Wales, Australia) and added to a 50% (vol/vol) formamide hybridization buffer to achieve a minimum specific activity of 2 × 10^8 cpm/mL. Following hybridization, membranes were washed in reducing concentrations of salt sodium citrate (SSC) to a final concentration of 0.2× SSC/0.1% sodium dodecyl sulfate (SDS) (wt/vol) at 65°C. Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at ~70°C. Films were scanned using an LKB 2202 Ultra Scan Laser Densitometer (LKB, Uppsala, Sweden) to quantify differences in mRNA expression.

Statistical analysis. Data were compared statistically by using a Student's t-test for paired observations. The P values calculated were based on differences between values of IL-4-treated cells and corresponding control values at the respective time points.

RESULTS

As can be seen from Fig 1, u-PA antigen increased significantly in the conditioned media in a dose-dependent manner when HFMEC were incubated for 24 hours with increasing amounts of rh IL-4. Maximum effects were achieved at a concentration of rh IL-4 of 200 U/mL (control; 3.0 ± 0.8 ng/10^5 cells/24 h [n = 6]; 200 U/mL IL-4: 6.7 ± 0.8 ng/10^5 cells/24 h [n = 6] [P < .001]).
When HFMEC were stimulated for different time periods in the presence or absence of 200 U/mL rh IL-4, a time-dependent increase in u-PA antigen and u-PA activity in the conditioned media was observed. When aliquots of the same samples were preincubated with plasmin as described in the Materials and Methods section, u-PA activity increased up to sixfold. U-PA antigen and activity (only determined after plasmin treatment) also increased in the cell lysates in the presence of IL-4 (Fig 2).

When HFMEC were incubated for 8 hours in the presence or absence of IL-4 together with either cycloheximide (10 μg/mL) or actinomycin D (2 μg/mL), u-PA antigen accumulation was completely blocked in the conditioned media of IL-4-stimulated cells as well as in the conditioned media of unstimulated cells (data not shown). Cell viability was not affected by such treatment as judged by trypan blue exclusion.

As can be seen from Table 1, the stimulating effect of rh IL-4 on u-PA production by HFMEC could be abolished by boiling, but was not affected when rh IL-4 was added to the cells together with polymyxin B. These results indicate that the stimulating effect on u-PA production was not caused by lipopolysaccharide (LPS) contamination of the IL-4 preparation used. The stimulating effect of rh IL-4 on u-PA production by HFMEC was abolished when IL-4 was preincubated for 30 minutes at room temperature with a rat monoclonal antihuman IL-4 antibody at a concentration of 1.0 μg/mL. Nonimmune rat IgG at the same concentration did not influence the increase in u-PA production.

When HFMEC obtained from different donors were exposed to 200 U/mL of rh IL-4, a similar stimulating effect on u-PA production by these different batches of HFMEC was seen. However, u-PA production in EC from large vessels, eg, HUVEC and HSVEC, was not affected by rh IL-4. In contrast, TNF-α used as a positive control, because of its known stimulatory effect on u-PA expression in EC, increased u-PA production significantly.

To investigate whether active protein synthesis is required for induction of u-PA mRNA accumulation by IL-4, HFMEC were incubated for 4, 8, and 12 hours in serum-free media without any addition, with 200 U/mL rh IL-4, with 10 μg/mL cycloheximide, and a combination of IL-4 and cycloheximide at concentrations indicated above. Furthermore, HFMEC were incubated for 4 hours in serum-free media containing 2 μg/mL actinomycin D or actinomycin D at the same concentration in combination with 200 U/mL of rh IL-4, to investigate whether the IL-4-induced increase in u-PA mRNA could occur in the absence of transcription. Total cellular RNA from such treated cells was prepared and used for Northern blotting experiments for u-PA-specific mRNA (Fig 4A). u-PA mRNA was quantified by densitometry (Fig 4B) as described in the Materials and Methods section. Values are given in ng/10^5 cells (A and B) and in IU/10^5 cells (C and D) and represent means ± SD of six independent wells. 0.1 IU corresponds to 1.0 ng in the combined activity-antigen–ELISA used. Where absent, SD was smaller than symbol size. (*) P < .001; (**) P < .005.
and Methods section. As can be seen, when used separately, both IL-4 and cycloheximide caused a transient increase in u-PA mRNA, resulting in a 2-fold and in a 10-fold stimulation of u-PA mRNA, respectively. However, when cycloheximide was used in combination with IL-4, a further increase of u-PA mRNA (up to 20-fold) was observed. It is noteworthy that this increase when compared with cycloheximide-treated cells is similar to the twofold increase in u-PA mRNA caused by IL-4 alone. No increase in u-PA mRNA was observed when HFMEC were treated with IL-4 in combination with actinomycin D. Similar results were seen in an experiment performed as above with HFMEC isolated from a different donor.

**DISCUSSION**

It has been shown recently that cytokines such as TNF-α, lymphotixin, and IL-1 can modulate the fibrinolytic capacity of EC by stimulating the expression of PAI-1 and u-PA. In this report we present evidence that yet another cytokine, IL-4, increases u-PA expression in human microvascular EC. The stimulating effect of IL-4 on u-PA antigen production by HFMEC was dose- and time-dependent with a maximum effect achieved with a dose of 200 U/mL of IL-4. u-PA activity in the conditioned media of such cells also increased in the presence of IL-4. However, the respective activity values increased sixfold when aliquots of the samples were pretreated with plasmin. This indicates the u-PA is released by HFMEC both in the absence and presence of IL-4 predominantly in its inactive single-chain form scu-PA. It is also noteworthy that between 80% and 100% of the total u-PA antigen present in the conditioned media of HFMEC under control conditions and in HFMEC treated with IL-4, respectively, was active after plasmin treatment. However, in a recent study only 16% of the u-PA could be activated by plasmin when u-PA expression was stimulated by TNF-α in human EC. The u-PA activity increased 2.5-fold to 3-fold in cell lysates and conditioned media. This increase is similar to that caused by angiogenic stimuli like b-FGF and 12-0-tetradecanoyl-phorbol-13-acetate (TPA). The increase in u-PA antigen caused by IL-4 requires both protein and RNA synthesis, as indicated by the fact that it is completely blocked by cycloheximide and actinomycin D.

The stimulating effect of IL-4 on u-PA production by HFMEC is unlikely to be caused by LPS contamination of the IL-4 preparation used, because it was not affected when

### Table 1. Characterization of the Effect of IL-4 on u-PA Production by HFMEC

<table>
<thead>
<tr>
<th>u-PA Antigen</th>
<th>Control</th>
<th>IL-4 (200 U/mL)</th>
<th>TNF-α (10^-8 mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFMEC 1*</td>
<td>2.1 ± 0.21</td>
<td>5.3 ± 0.71</td>
<td>21.0 ± 2.93t</td>
</tr>
<tr>
<td>HFMEC 2*</td>
<td>3.0 ± 0.83</td>
<td>6.7 ± 0.82</td>
<td>NT</td>
</tr>
<tr>
<td>HFMEC 3</td>
<td>2.2 ± 0.04</td>
<td>5.3 ± 0.37</td>
<td>NT</td>
</tr>
<tr>
<td>HFMEC 4</td>
<td>1.1 ± 0.37</td>
<td>2.8 ± 0.12</td>
<td>10.4 ± 1.57t</td>
</tr>
<tr>
<td>HFMEC 5</td>
<td>1.4 ± 0.26</td>
<td>4.1 ± 0.39</td>
<td>6.8 ± 0.52t</td>
</tr>
<tr>
<td>HFMEC 6</td>
<td>2.3 ± 0.44</td>
<td>4.0 ± 0.58</td>
<td>7.6 ± 0.58t</td>
</tr>
<tr>
<td>HSVEC</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.01</td>
<td>20.0 ± 1.13t</td>
</tr>
<tr>
<td>HSVEC</td>
<td>0.3 ± 0.05</td>
<td>0.3 ± 0.04</td>
<td>5.3 ± 0.87t</td>
</tr>
</tbody>
</table>

### Table 2. Effect of IL-4 on u-PA Production by HFMEC, HUVEC, and HSVEC

<table>
<thead>
<tr>
<th>u-PA Antigen</th>
<th>Control</th>
<th>IL-4 (200 U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFMEC 1</td>
<td>2.3 ± 0.2</td>
<td>218.3 ± 38.1</td>
</tr>
<tr>
<td>HUVEC</td>
<td>1.6 ± 0.2^*</td>
<td>208.3 ± 36.5</td>
</tr>
<tr>
<td>HSVEC</td>
<td>2.0 ± 0.4</td>
<td>169.8 ± 21.1</td>
</tr>
<tr>
<td>HFMEC 2</td>
<td>1.3 ± 0.2^*</td>
<td>174.3 ± 20.7</td>
</tr>
<tr>
<td>HUVEC</td>
<td>7.8 ± 0.8</td>
<td>364.1 ± 46.6</td>
</tr>
<tr>
<td>HSVEC</td>
<td>5.8 ± 0.4^*</td>
<td>415.9 ± 42.4</td>
</tr>
<tr>
<td>HFMEC 3</td>
<td>3.6 ± 0.5</td>
<td>184.7 ± 28.2</td>
</tr>
<tr>
<td>HUVEC</td>
<td>3.1 ± 0.3</td>
<td>222.8 ± 32.5</td>
</tr>
</tbody>
</table>

### Table 3. Effect of IL-4 on t-PA and PAI-1 Antigen Production by HFMEC, HUVEC, and HSVEC

<table>
<thead>
<tr>
<th>t-PA Antigen</th>
<th>Control</th>
<th>IL-4 (200 U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFMEC 1</td>
<td>2.3 ± 0.2</td>
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<td>HSVEC</td>
<td>5.8 ± 0.4^*</td>
<td>415.9 ± 42.4</td>
</tr>
</tbody>
</table>

Confluent monolayers of HFMEC were incubated for 24 hours in the absence or presence of 200 U/mL untreated rh IL-4, 200 U/mL rh IL-4 boiled for 45 minutes, and 200 U/mL rh IL-4 in the presence of polymyxin B (1 μg/mL). Confluent monolayers of HFMEC 5 were incubated for 24 hours in the absence or presence of 200 U/mL untreated rh IL-4, 200 U/mL rh IL-4 preincubated for 30 minutes at room temperature with a rat monoclonal antihuman IL-4 antibody (MP4-25D2-11) at a concentration of 1.0 μg/mL, or 200 U/mL rh IL-4 preincubated as described above with nonimmune rat IgG at a concentration of 1.0 μg/mL. u-PA antigen in the conditioned media was determined as described in the Materials and Methods section. Values are given in ng/10^6 cells/24 h and represent means ± SD of six independent wells for HFMEC 1 and 2 and means ± SD of three independent wells for HUVEC and HSVEC.

* P < .05.
IL-4 was added to the cells together with polymyxin B, but it was abolished completely when IL-4 was boiled before addition to the cells. Furthermore, the increase in u-PA production by HFMEC could be blocked when IL-4 was preincubated with a specific antihuman IL-4 antibody.

In contrast to TNF-α and IL-1, which increase u-PA in EC of both microvascular and macrovascular origin, IL-4 appears to stimulate u-PA production only in foreskin microvascular but not in macrovascular EC. Six different preparations of HFMEC responded to IL-4 treatment with an increase in u-PA antigen, whereas u-PA antigen production by HUVEC and HSVEC remained unaffected. These findings were also reflected in the levels of u-PA-specific mRNA. As shown by Northern blotting, u-PA mRNA increased significantly in HFMEC treated with IL-4 whereas no changes were observed in u-PA mRNA prepared from IL-4-treated HSVEC, HUVEC, or HUAEC. It is noteworthy that microvascular EC and at least one type of macrovascular EC (HUVEC) express receptors for IL-4, and that both cell types have been shown to respond to IL-4 with increased proliferation. However, IL-4 is a potent mitogen for microvascular EC, whereas its mitogenic effect on HUVEC is weak.

When HFMEC were treated with cycloheximide, u-PA mRNA increased transiently. A similar effect of cycloheximide on u-PA mRNA has been shown in HT 1080 fibrosarcoma cells. These findings suggest that unstable proteins may exist that cause either the suppression of u-PA transcription or the degradation of u-PA mRNA. The existence of short-lived transcriptional repressors acting on the u-PA gene has been suggested. Furthermore, it has been shown recently that protein synthesis inhibition stabilizes u-PA mRNA in calcitonin stimulated porcine renal epithelial LLC-PK cells. The twofold increase of u-PA mRNA caused by IL-4 in the presence of cycloheximide suggest that de novo protein synthesis is not required. This is similar to the induction of u-PA mRNA in mouse keratinocytes caused by epidermal growth factor (EGF). Inhibition of the stimulating effect of IL-4 on u-PA mRNA by actinomycin D suggests that it is dependent on de novo gene transcription.

Furthermore, IL-4 exclusively stimulated u-PA expression, whereas PAI-1 remained unaffected and t-PA antigen was slightly decreased. This is in contrast to the simultaneous stimulation of u-PA and PAI-1 in EC by IL-1 and TNF-α, which could mean that an increase in the fibrinolytic potential of the EC brought about by an increased expression of u-PA could at least be partially counterbalanced by the simultaneous increase in PAI-1. In contrast, the IL-4-induced increase in u-PA, because not accompanied by an increase in PAI-1, would be reflected in a proportional increase in the fibrinolytic potential of the cell.

The upregulation of u-PA expression in HFMEC by IL-4 is in contrast to its stimulating effect on t-PA production in monocytes. These findings indicate that IL-4 can modulate the fibrinolytic system differently depending on the target cells.

It has been shown recently that IL-4 is a potent mitogen for human microvascular EC, whereas its mitogenic effect on HUVEC was only moderate. u-PA synthesis has been shown to vary during cell cycle in mouse keratinocytes and fibroblasts stimulated with serum or EGF. Therefore, it is possible that the increase in u-PA expression in HFMEC is a consequence of changes in the cell cycle induced by the mitogenic properties of IL-4. The induction of u-PA by IL-4 is similar to the stimulation of u-PA by TNF, β-FGF, and phorbol myristate acetate (PMA) that also induce angiogenesis. u-PA is thought to be involved mainly in extracellular, spatially controlled proteolysis, via binding to its receptor, which is present on the surface of many cell types including EC. This is supported by the fact that u-PA expression correlates with migration in EC.
In conclusion, our data give evidence that IL-4, besides its known effects on EC proliferation and on their coagulant and adhesive properties, also modulates the fibrinolytic system of HFMEC. Our findings suggest a possible role for IL-4 in the process of angiogenesis by inducing not only proliferation but also by facilitating extracellular proteolysis by increasing the fibrinolytic potential of the EC via increased expression of u-PA. Finally, further studies will be required to clarify if the effect of IL-4 on the fibrinolytic system is specific for dermal microvascular EC or if it is also seen in microvascular endothelial cells from other vascular beds.

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Interleukin-4 stimulates expression of urokinase-type-plasminogen activator in cultured human foreskin microvascular endothelial cells

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