Tumor Necrosis Factor Induces the Production of Urokinase-Type Plasminogen Activator by Human Endothelial Cells

By Victor W.M. van Hinsbergh, Eva A. van den Berg, Walter Fiers, and Gerard Dooijewaard

Endothelial cells play an important role in the regulation of fibrinolysis by the production of several key regulatory proteins; namely, the enzymes tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), and their inhibitor, plasminogen activator inhibitor-1 (PAI-1).1 In tissue sections of normal human blood vessels and in the conditioned medium of primary cultures of human umbilical vein endothelial cells, t-PA is found, but no u-PA is detected.2 Several types of cultured endothelial cells, such as bovine cells3 and serially propagated human endothelial cells,4 also produce u-PA. In bovine microvascular endothelial cells, the expression of u-PA activity is temporally and spatially correlated to the migration of these cells5 and depends on autocrine stimulation by basic fibroblast growth factor (b-FGF).6

The inflammatory mediators tumor necrosis factor (TNF), lymphotoxin, and interleukin-1 (IL-1) change the phenotype of endothelial cells in culture dramatically, as evidenced by changes in the immunologic properties and in the interaction with leukocytes,7 and in a shift of the hemostatic balance on the endothelial cell surface towards thrombogenicity.8 Parallel to these events, the production of PAI-1 is increased,9 which may impair the efficiency of the fibrinolytic process. The other hand, besides being secreted, active PAI-1 is deposited in the extracellular matrix of endothelial cells.10,11 Thus, the increase in PAI-1 production may also improve the protection of the cellular environment against uncontrolled plasminogen activator activity. Histologic studies by Kwaan and Astrap12 have demonstrated that during tissue repair and in experimental inflammation, plasminogen activator activity associated with the endothelium of the ingrowing capillaries is increased. The question of whether u-PA may play a role in these processes arises. Because the monokine TNF induce endothelial cell migration and neovascularisation in various experimental models,13 we investigated whether TNF and other cytokines could induce u-PA production by human endothelial cells. A concomitant induction of u-PA and PAI-1 might represent an additional aspect of the altered fibrinolytic properties of endothelial cells during inflammation.

MATERIALS AND METHODS

Materials. Human recombinant TNF27 and lymphotoxin (expressed in Chinese hamster ovary [CHO] cells) were gifts from Dr Jan Tavernier (Biogenet, Gent, Belgium). The TNF preparation contained 2.45 × 10^6 U/mg protein and less than 40 ng lipopolysaccharide (LPS) per mg protein; the specific activity of lymphotoxin was 1.6 × 10^8 U/mg protein. Human recombinant interleukin-6 (expressed in yeast cells) was prepared in the Laboratory of Molecular Biology, Gent; it was biologically active and had a specific activity of 1.4 × 10^6 U/mg protein as tested on 7TD1 cells. Human recombinant IL-1α (pl 5) and IL-1β (pl 7) were purchased from Genzyme (Haverhill, UK); polymyxin B, from Sigma (St Louis, MO). Rabbit and goat polyclonal anti-u-PA IgGs were raised in our laboratory; donkey anti-(goat IgG) IgGs conjugated with alkaline phosphatase were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). The pUK 0321 harboring a 1,023-bp fragment of the human u-PA cDNA was a gift of Wolf-Dieter Walter Fiers, and Gerard Dooijewaard. The 1-kb EcoRI to HindIII fragment of the human PAI-1 cDNA was a gift of Dr P. A. S. de Vries (Biocytex, Gent, Belgium). The pUK 0321 harboring a 1,023-bp fragment of the human u-PA cDNA was a gift of Wolf-Dieter Walter Fiers, and Gerard Dooijewaard. The 1-kb EcoRI to HindIII fragment of the human PAI-1 cDNA was a gift of Dr P. A. S. de Vries (Biocytex, Gent, Belgium). The pUK 0321 harboring a 1,023-bp fragment of the human u-PA cDNA was a gift of Wolf-Dieter Walter Fiers, and Gerard Dooijewaard. The 1-kb EcoRI to HindIII fragment of the human PAI-1 cDNA was a gift of Dr P. A. S. de Vries (Biocytex, Gent, Belgium).


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characterized as previously described. Confluent cultures from umbilical artery and vein were used after three passages (split ratio, 3:1), unless otherwise mentioned; confluent foreskin microvascular endothelial cells were used after 12 to 14 passages. Endothelial cell conditioned media were obtained by incubating confluent cells at 37°C for the indicated period of time in M199 medium supplemented with 10% human serum, 20 mmol/L HEPES, and penicillin/streptomycin. In serum-free incubations, the human serum was replaced by 0.03% pyrogen-free human serum albumin. Conditioned media were immediately centrifuged and stored at -20°C until use. After incubation, the cells were washed three times with phosphate-buffered saline and scraped in a small volume (0.5 mL/35 mm dish) of 0.5% Triton X-100 in distilled water and stored at -20°C until use. Trasylol (20 KIU/mL) was added immediately after incubation to the samples to be used for biological immunoassay of pro-u-PA. To determine the polarity of u-PA production, endothelial cells from umbilical vein (primary) or aorta (after five passages) were released with trypsin/EDTA and seeded in confluent density on fibronectin-coated polycarbonate filters (6 cm²; 0.4 μm pore size) in the Transwell system (Costar, Cambridge, MA). In this system, the endothelial cell monolayer separates two compartments: an upper compartment of 0.65 mL on its apical site and a lower compartment of 2.35 mL on its basolateral site. The cells were maintained under standard culture conditions and were used after 5 days. The characteristics of these monolayers have been described previously. To determine the polarity of the secretion of various proteins, the cells were incubated for 24 hours at 37°C under 5% CO₂/95% air atmosphere in M199 medium (without phenol red) supplemented with 10% human serum, 20 mmol/L HEPES, and penicillin/streptomycin. The indicated amount of TNF was added to both compartments. As a control of the quality of the monolayer, horseradish peroxidase (5 μg/mL) was added to the upper compartment, and its passage into the lower compartment was measured. Assays. Levels of t-PA antigen were measured by an enzyme immunoassay (Imulysate) purchased from Biopool (Umeå, Sweden), according to the manufacturer’s description. In this assay, t-PA and t-PA:PAI-1 complexes are detected with similar efficiency. The u-PA antigen was measured with a sandwich enzyme-linked immunosorbent assay (ELISA) essentially as described by Binnema et al., using donkey anti-(goat-IgG) IgGs immobilized with alkaline phosphatase instead of conjugated rabbit anti-goat IgG. The assay measures the u-PA antigen irrespective of its molecular form, ie, the inactive proenzyme single-chain u-PA (sc-u-PA), the active two-chain u-PA (tc-u-PA), and the u-PA in complex with PAI-1. The plasmin-activatable u-PA activity and active tc-u-PA activity were measured by a biological immunos assay (BIA). In the first step of this assay, u-PA, irrespective of its molecular form, is singled out by the same rabbit polyclonal anti-u-PA IgGs immobilized on microtiter plates as used in the u-PA antigen ELISA. In the next step, the plasminogen activator activity of the immuno-immobilized material is measured before (active tc-u-PA) and, in separate wells, after a 1-hour activation step of the immuno-immobilized material with 1 μM/mL human plasmin (active sc-u-PA plus plasmin-activatable sc-u-PA). The plasminogen activator activity was assayed in a two-step cascade with plasminogen and S-2251 essentially as described for t-PA and was read as the difference of PA activity with and without inhibitor rabbit anti-u-PA IgGs present. The plasmin-activatable u-PA in conditioned media and cell extracts of endothelial cells represents sc-u-PA, because (1) no u-PA activity was found in samples without plasmin treatment, and (2) no u-PA activity was liberated in this assay from the u-PA:PAI-1 complex upon plasmin treatment. Responses in the BIA and ELISA were correlated via calibration with sc-u-PA purified from human fibroblasts, and calibration of u-PA antigen occurred as described.
Considerable variation in u-PA production was observed between various cultures. The amount of u-PA in the 24-hour conditioned medium of cells treated with 500 U/mL TNF (55 ± 39 ng u-PA/mL) was 15 ± 11-fold higher (range, 5- to 36-fold) than that in their nontreated counterparts (4 ± 2 ng u-PA/mL) (mean ± SD of 14 different cultures of umbilical artery and vein endothelial cells, after two or three passages). During the same time period, the overall protein synthesis estimated by incorporation of 35S-methionine did not change significantly; 24 hours after addition of 500 U/mL TNF, the overall protein synthesis was 97% ± 17% of the value obtained with nonstimulated cells (mean ± SD, nine cultures). The stimulation of u-PA production by TNF did not depend on the presence of serum proteins. In the 24-hour, 10% serum-containing conditioned media of three independent cultures, the basal and 500 U/mL TNF-stimulated u-PA levels were 4 ± 2, and 49 ± 36 ng u-PA/mL, respectively; while in their serum-free counterparts, they amounted to 5 ± 2 and 60 ± 45 ng u-PA/mL (mean ± SD). The basal and increased u-PA production could be inhibited by addition of cycloheximide or actinomycin D (data not shown).

Highly confluent primary cultures of umbilical artery and vein release hardly any u-PA antigen in the supernatant medium. As is shown in Table 2, these cells contain a small amount of u-PA associated with the cells, and they respond to TNF by a fivefold or more increase in the synthesis of u-PA antigen. An increase in u-PA antigen production was also observed when endothelial cells were incubated with lymphotoxin (Table 2), a lymphokine that binds to the same receptor as TNF. TNF and lymphotoxin at 500 U/mL concentrations were nearly equally effective in enhancing u-PA production by primary endothelial cell cultures.

Subcultured endothelial cells from adult human vena cava, aorta, and carotid artery vary considerably in their basal rate of u-PA production, depending upon their vascular origin and their in vitro age. Despite the large variation in basal u-PA production, the relative increase in u-PA production induced by TNF and lymphotoxin was similar to that observed with umbilical artery and vein endothelial cells (data not shown).

**Induction of u-PA mRNA by TNF.** Total RNA isolated from untreated and TNF-stimulated endothelial cells was analyzed by Northern blotting to establish whether the increase in u-PA antigen was paralleled by an increase in the level of u-PA mRNA. Figure 3A shows the autoradiogram of a representative experiment with umbilical vein endothelial cells. The u-PA mRNA level was already elevated 2 hours after addition of 500 U/mL TNF was markedly increased after 6 and 8 hours incubation with TNF. The u-PA mRNA level declined again 24 hours after addition of TNF. A 5- to 20-fold increase in u-PA mRNA was estimated by densitometric scanning in three independent cultures of umbilical vein and artery endothelial cells that had been incubated for 6 hours with 500 U/mL TNF. The blots were rehybridized with the PAI-1 mRNA probe and, as a control, with the GAPDH mRNA probe (Fig 3A). The PAI-1 mRNA level increased continuously during incubation with TNF and became highly elevated after 24-hour incubation period. In various experiments, the GAPDH mRNA level was not influenced by TNF. In Fig 3B it is shown that TNF also did not change the t-PA mRNA content of the cells.

**Polarity of u-PA secretion.** To investigate whether the basal or stimulated u-PA secretion proceeds in a polarized way, we cultured human umbilical vein and aorta endothelial cells on fibronectin-coated polycarbonate filters. The cells were seeded in a very high density, and in 4 days, the cells formed a tight monolayer, the characteristics of which have been described previously in detail. The integrity of every
monolayer was verified by measuring the passage of peroxidase from the apical site towards the basolateral site of the cell monolayer. In early passage umbilical vein endothelial cells, the basal production of u-PA showed no or only a slight polarity towards the basolateral side (Fig 4). Similarly, the production of t-PA antigen, PAI activity, and PAI-1 antigen showed no polarity. However, after addition of 500 U/mL TNF, the increased production of u-PA was totally found at the basolateral side of the cells, whereas the increased production of PAI activity and PAI-1 antigen was equally divided over the two compartments (Fig 4).

In human aorta endothelial cells (after six passages), the apical and basolateral secretion of u-PA antigen were 10 ± 1 and 12 ± 6 ng u-PA/mg cell protein, respectively, under control conditions. Although there was a preferential stimulation of the basolateral secretion as compared with the apical secretion after addition of TNF to the cells, the polarity of the stimulated u-PA secretion was much less than in the early passage umbilical vein endothelial cells. During 24-hour stimulation with 500 U/mL TNF, the basolateral secretion was enhanced sevenfold to 85 ± 13 ng u-PA/mg cell protein, whereas the increase in apical secretion was twofold less (41 ± 5 ng u-PA/mg cell protein, mean ± SD of three filters). Since umbilical cord endothelial cells lost their ability to form tight monolayers after two passages, we have not been able to establish whether human endothelial cells lose this polarity during subculturing.

Plasmin activatable u-PA. By immunoprecipitation of metabolically-labeled u-PA antigen from endothelial cell-conditioned medium, we have previously demonstrated the presence of two molecular forms of u-PA: one at 55 Kd, which probably represents the sc-u-PA (pro-urokinase), and the other at 95 Kd. The latter represents the u-PA:PAI-1 complex because it could also be precipitated by anti-PAI-1 IgG. Because the fluororadiograms were too faint for reliable quantification of these molecular species, we quantified sc-u-PA by BIA. To that end, u-PA was singled out from the conditioned media and cell extracts by anti-u-PA IgG fixed to microtiter wells, washed, activated with plasmin, and washed again. Finally, u-PA activity was assayed spectrophotometrically with a synthetic substrate. Without plasmin treatment, no free u-PA activity could be demonstrated in the samples, probably because the large amounts of PAI-1 in the conditioned medium and extracellular matrix of the endothelial cells irreversibly eliminated tc-u-PA. Table 3 shows that the amount of plasmin-activatable u-PA increased from 0.17 IU u-PA per well in the conditioned medium of nonstimulated cells to 1.11 IU u-PA per well in the supernatant of TNF-stimulated cells. The increase in

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Table 2. Effect of TNF and Lymphotoxin on Cellular and Secreted u-PA Antigen Produced by Primary Cultures of Human Endothelial Cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>u-PA Antigen (ng/10^6 cells)</th>
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<tbody>
<tr>
<td></td>
<td>Conditioned Medium</td>
</tr>
<tr>
<td>None</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>TNF (500 U/mL)</td>
<td>0.51 ± 0.36</td>
</tr>
<tr>
<td>Lymphotoxin (500 U/mL)</td>
<td>0.39 ± 0.14</td>
</tr>
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</table>

Highly confluent primary cultures of umbilical artery and vein endothelial cells were incubated for 24 hours in M199 medium-10% human serum with and without 500 U/mL TNF or 500 U/mL lymphotoxin. u-PA antigen was assayed by ELISA in the conditioned medium and in 0.5% Triton X-100 cell extracts prepared after incubation. In parallel wells, cell density and protein content of the cultures was determined. The data represent the mean ± SD of two different cultures. Cell density of these cultures was 0.4 ± 0.2 × 10^5 cells/cm^2; protein content was 24 ± 2 μg cell protein/cm^2.
Fig 3. Induction of u-PA mRNA by TNF in human umbilical vein endothelial cells. (A, upper panel) Autoradiograph of $^{32}$P-u-PA cDNA hybridized to a Northern blot of RNA fractionated by formaldehyde-agarose gel electrophoresis. Total RNA preparations were made as described in Materials and Methods from endothelial cells that had been incubated for various incubation periods without (lanes 1 through 5) or with 500 U/mL TNF (lanes 6 through 10) in M199 medium supplemented with 10% human serum. The blots were cleaned and rehybridized with $^{32}$P-PAI-1 cDNA (middle panel showing two PAI-1 mRNA species of 3.0 and 2.3 kb), and subsequently cleaned and rehybridized with $^{32}$P GAPDH cDNA (A, lower panel). (B) Autoradiograph of $^{32}$P-u-PA cDNA (left) and $^{32}$P-t-PA cDNA (right) hybridized to a Northern blot of total RNA of another culture of umbilical vein endothelial cells that had been incubated for 6 hours in M199 medium-10% human serum without (−) or with (+) 500 U/mL TNF.

total u-PA antigen and, hence, in nonactivatable u-PA was much higher. In the cellular extracts, the increase in u-PA antigen was found completely in the nonactivatable fraction of u-PA antigen. The plasmin-activatable u-PA represents 66% and 59%, respectively, of the secreted and cellular u-PA of nonstimulated cells, whereas it was only 16% and 28% in the TNF-stimulated cells (Table 3). This may indicate that, together with the increase in u-PA production, a marked increase in the degree of activation occurred. A similar shift in the ratio between plasmin-activatable u-PA and total

Fig 4. Polarity of the secretion of u-PA, t-PA, PAI activity, and PAI-1 antigen was investigated with tight human umbilical vein endothelial cell monolayers cultured on fibronectin-coated polycarbonate filters (Transwell). The cells were incubated in M199 medium supplemented with 10% human serum or with 10% human serum and 500 U/mL TNF added to both compartments. After 24 hours' incubation at 37°C, u-PA antigen, t-PA antigen, PAI activity, and PAI-1 antigen were assayed in the upper (0.65 mL; apical side) and lower (2.35 mL; basolateral side) compartments. The data represent the mean ± range of duplicate filters.
Table 3. Tumor Necrosis Factor Changes the Ratio Between Plasmin-Activatable u-PA and Total u-PA Antigen Production by Human Endothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>Plasmin-Activatable u-PA (IU/well)</th>
<th>Total u-PA Antigen (IU/well)</th>
<th>Plasmin-Activatable u-PA/Total u-PA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECCM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.17 ± 0.04</td>
<td>0.26 ± 0.06</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>TNF (500 U/mL)</td>
<td>1.11 ± 0.65</td>
<td>7.32 ± 4.86</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.13 ± 0.08</td>
<td>0.22 ± 0.14</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>TNF (500 U/mL)</td>
<td>0.11 ± 0.08</td>
<td>0.42 ± 0.28</td>
<td>28 ± 16</td>
</tr>
</tbody>
</table>

Human umbilical vein endothelial cells (5 cm² wells) were incubated for 24 hours in M199 medium-10% human serum with or without 500 U/mL TNF. Immediately after incubation, 20 klU/mL trasylol was added. Conditioned media (ECCM) and 0.5% Triton X-100 extracts of the cells were assayed for their content of sc-u-PA by BIA and total u-PA antigen by ELISA. The data are the mean ± SD of three different cell cultures.

*In the ELISA, 1 IU u-PA corresponds with 10 ng u-PA.*

DISCUSSION

It is generally accepted that the fibrinolytic capacity of human endothelial cells is influenced by the relative production rates of t-PA and PAI-1. The cytokines TNF, lymphotixin, and IL-1 change this capacity by increasing the production of PAI-1, while the production of t-PA remains unaffected or even decreases. When the plasminogen activator activity in the conditioned medium of TNF-treated and untreated endothelial cells was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fibrin autography, only t-PA activity (and no u-PA activity) was detected. However, u-PA antigen has been demonstrated in the supernatant of subcultured human endothelial cells by immunoprecipitation of u-PA antigen and by ELISA. Specifically, cells with an enlarged diameter obtained at advanced in vitro age produced considerable amounts of u-PA. We have demonstrated by assay of u-PA antigen and u-PA mRNA that TNF induces the production of u-PA by primary and subcultured human endothelial cells. The relative increase in u-PA production was similar (5- to 30-fold) in primary cultures and in early and late passage subcultured cells. This suggests that different regulatory processes underlie the increase in u-PA production induced by in vitro senescence and the inflammatory mediator TNF.

The simultaneous induction of u-PA and PAI-1 by TNF is striking. The concentration dependency of the u-PA induction by TNF is identical to that of PAI-1, and the increases in PAI-1 mRNA (Fig 4) and in PAI activity span the whole period of increased u-PA production. Activation of plasminogen by u-PA results in a broadly acting proteolytic activity; it may be a spatially controlled process (see below). The increase in PAI activity may aim primarily to protect the endothelial cell and its environment against uncontrolled u-PA activity. A role of PAI-1 in the protection against PA-dependent proteolytic degradation of the extracellular matrix has been suggested by various recent studies on endothelial cells and other cell types.

An interesting finding was that the TNF-induced increase in u-PA production occurred in a polarized way. The u-PA was secreted mainly towards the abluminal side of the endothelium, rather than in the circulation. The induction of u-PA may be of importance in the destruction of matrix proteins by the generation of plasmin and by subsequent collagenase activation. In addition, u-PA has been suggested to play a role in cell migration. A correlation between u-PA activity and cell migration has been demonstrated in bovine adrenal capillary endothelial cells, cells that show spontaneous fibrinolytic activity. Because human artery and vein endothelial cells do not show spontaneous fibrinolytic activity, a presumed action of u-PA must occur immediately upon release or activation of the plasminogen activator, or at protected sites on the cell surface, eg, bound to the u-PA receptor. A spatially controlled action of u-PA has been suggested from several immunolocalization studies.
TNF INDUCES UROKINASE IN Endothelial Cells

The induction of u-PA in human endothelial cells by TNF reminds one of the stimulation of u-PA production by other angiogenic factors, such as b-FGF and phorbol myristate acetate (PMA), in bovine adrenal capillary endothelial cells.5.7.8 TNF also induces migration of bovine capillary endothelial cells in vitro9 and can induce reorganization of human endothelial cell monolayers from a cobblestone pattern into a layer of spindle-shaped cells.30 Although a role of TNF-induced u-PA production in cell shape alterations and cell migration is an attractive hypothesis, a causative relationship has not been established.13,60 Even if u-PA is involved in these processes, it is likely that the induction of u-PA is only a single violin in a whole orchestra ofconcertedly induced factors. The concomitant induction of u-PA and PAI-1 suggests that u-PA activity has to be tuned perfectly, and focuses attention on cell-bound and interstitial fibrinolysis in inflammation. The recent immunohistochemical demonstration of u-PA antigen in human endothelial cells of small blood vessels in inflamed appendices41 supports the in vivo relevance of our findings.

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