Production of Plasminogen Activators and Inhibitor by Serially Propagated Endothelial Cells from Adult Human Blood Vessels

Victor W.M. van Hinsbergh, Doede Binnema, Mariëlle A. Scheffer, Erik D. Sprengers, Teake Kooistra, and Dingeman C. Rijken

Endothelial cells were isolated from arteries and veins obtained from elderly people at autopsy and propagated for 37 to 69 population doublings. The cells secreted tissue-type plasminogen activator (t-PA) and PA inhibitor-1, and, after subculturing, urokinase-type PA (u-PA) antigen. The following differences between endothelial cells from adult arteries and veins were observed: 1) The cells had the potential to be propagated as a healthy monolayer. The diameter of aortic endothelial cells increased after 8 to 19 population doublings, while a homogeneous population of small diameter vena cava cells was retained for 35 population doublings. 2) The amount of secreted t-PA varied. Vena cava cells produced four times more t-PA than aorta cells, and 20-fold more than umbilical artery or vein endothelial cells. The t-PA mRNA content of vena cava cells did not exceed that of aorta cells, but was fourfold greater than that of umbilical cord endothelial cells. 3) The release of u-PA antigen varied. No u-PA antigen was detectable in conditioned medium of primary cultures of human aorta and vena cava endothelial cells or of early passage vena cava cells. After prolonged subculturing, vena cava cells started to secrete u-PA. Endothelial cells from aorta and other adult arteries, however, started secreting u-PA after one to four passages, parallel to the occurrence of enlarged endothelial cells. u-PA was present as a u-PA/Inhibitor complex and as a single-chain u-PA. These differences may be developmentally related to their artery or vein origin or may reflect differences acquired during the “life history” of these blood vessels in vivo. Our data suggest that the release of u-PA antigen by human macrovascular endothelial cells can be used as an indicator of cell senescence. (Arteriosclerosis 7:389-400, July/August 1987)

Plasminogen activators are serine proteases which convert plasminogen into plasmin. Urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) can be distinguished by their immunological differences and their interaction with fibrin. The mRNAs for both proteins have been cloned. Plasminogen activators are involved in many processes including tissue remodelling, development, tumor invasion, and fibrinolysis. Functional and immunological techniques have demonstrated t-PA in the endothelial lining of human blood vessels. It is generally assumed that fibrinolysis in human plasma is triggered by t-PA that is released from endothelial cells.

Cultured endothelial cells provide a powerful tool to study the production and regulation of plasminogen activators by endothelial cells. The production of t-PA has been demonstrated with endothelial cells cultured from umbilical cord vessels and from bovine aorta. In addition to t-PA, bovine aorta endothelial cells produce u-PA. Booyse et al. reported and characterized the presence of u-PA in the conditioned medium of endothelial cells from human umbilical vein. However, other authors were unable to detect any u-PA production by these cells. The plasminogen activators produced by human umbilical vein and bovine aorta endothelial cells are mainly found in a complex with a PA inhibitor, which is produced in relatively large amounts by endothelial cells in vitro.

Histological studies on biopsy and autopsy specimens of human arteries and veins suggest that regional differences may exist in the fibrinolytic activity of various blood vessels. Endothelial cells from adult human blood vessels have been isolated in vitro, but no information is available about the synthesis and secretion of fibrinolysis components by these cells. We describe here the serial propagation of endothelial cells from human aorta, iliac and carotid artery, and vena cava, and the production of two types of plasminogen activator and PA inhibitor by these cells.

Methods

Materials

A crude preparation of endothelial cell growth factor (ECGF) was extracted from bovine hypothalamus as de-
scribed by Maciag et al. Human fibronectin was a gift of J.A. van Mourik (Amsterdam). Pyrogen-free human serum albumin was purchased from the Central Red Cross Blood Transfusion Service (Amsterdam). Two-chain t-PA was purified from the supernatants of Bowes melanoma cells by Jaffe et al. urinokinase (a mixture of LMW and HMW urinokinese) was purchased from Leo Pharmaceuticals (Copenhagen). Human Lys-plasminogen was purified from Cohn fraction III by Syse Sepharose chromatography. Soluble CNBr-fibrinogen digest was prepared as previously described. H-D-Val-Leu-Lys-pNA (S-2251) was purchased from Kabi-Vitrum (Stockholm). M199 medium (methionine-free) and M199 medium, both supplemented with 20 mmol/l HEPES were obtained from Flow Laboratories (Irvine, Scotland). °S-methionine and °C-glycine-hippuryl-L-histidyl-L-leu-cine were purchased from New England Nuclear (Amherst, Massachusetts).

Rabbit anti-von Willebrand factor antiserum was purchased from the Central Red Cross Blood Transfusion Laboratory (Amsterdam). Fluoresceine-conjugated swine antirabbit immunoglobulins (FITC swine antirabbit Ig) were obtained from DAKO Immunoglobulins (Denmark); rhodamine-labelled Ulex europeus lectin I were obtained from E.Y. Laboratories (San Mateo, California). Rabbit anti-t-PA IgG and anti-urokinase IgG were prepared as described previously. Afinitypurified goat anti-urokinase IgG was prepared by the use of purified Winkinase coupled to cyanogen bromide-activated Sepharose 4B. Anti-PA inhibitor IgG were raised in rabbits in our laboratory as described elsewhere.

**Isolation and Culture of Endothelial Cells**

Human aorta, carotid and iliac artery, and segments of vena cava superior (5 to 10 cm) were obtained at autopsy of five patients, who were 64 to 85 years old. The vessels were stored up to 6 hours in and rinsed with ice-cold buffer consisting of 140 mmol/l NaCl, 4 mmol/l KCl, 11 mmol/l D-glucose, 10 mmol/l HEPES (pH 7.3), 100 IU/ml penicillin, and 0.10 mg/ml streptomycin. Endothelial cells were detached from the vessels by a 20-minute incubation at 37° C in 0.10% in collagenase in M199 medium. After addition of human serum, the cells were collected by centrifugation and resuspended in M199 medium, supplemented with 20% human serum (not heat-inactivated), 200 μg/ml crude EGF, 20 mmol/l HEPES, 100 IU/ml penicillin, and 0.10 mg/ml streptomycin (growth medium) and seeded in 6 to 12 wells (16 mm diameter), which had been coated with 10 μg/cm² human fibronectin. At 3 to 4 hours after seeding, the endothelial cell spreading was clearly visible. The wells were then washed four times rather vigorously to remove the debris and the smooth muscle cells, while viable endothelial cells remained attached to the dish. The cells were propagated in growth medium renewed each 2 to 3 days. When the cells had become confluent, they were detached by treatment with trypsin/EDTA and were passaged with a split ratio of 1:5.

Endothelial cells from human umbilical cord artery and vein were isolated by the method of Jaffe et al. as previously described and were cultured in the same growth medium as given above.

**Preparation of Endothelial Cell-Conditioned Media**

Conditioned media of endothelial cells were obtained by incubating cells at 37° C for 24 hours with M199 medium (20 mmol/l HEPES and penicillin/streptomycin) supplemented with 10% human serum, or with medium supplemented with 3 mg/ml bovine serum albumin unless otherwise stated. The conditioned media were immediately centrifuged for 2 minutes in a Beckman Microfuge centrifuge to remove detached cells and cellular debris, and the supernatants were frozen at -20° C until use.

Incorporation of °S-methionine (20 to 40 μCi/ml) into endothelial cell proteins took place during incubation of endothelial cells with M199 medium (methionine-free), supplemented with 20 mmol/l HEPES, 0.3 mg/ml bovine serum albumin and penicillin/streptomycin. Radiolabelled conditioned media were centrifuged and frozen at -20° C as described above.

**Assay of Tissue-Type Plasminogen Activator Antigen**

Levels of t-PA antigen were measured by an enzyme immunonassay as previously described. In this assay, the t-PA inhibitor complex is detected with a 50% efficiency, when compared to uncomplexed t-PA. The data were not corrected for this efficiency. The detection limit of the assay was 0.01 to 0.02 pmol/ml. The intraassay variation of this assay is 15%; the interassay variation is 25%.

**Assay of Urokinase Antigen**

The assay of u-PA antigen was performed with an ELISA for urokinase as described by Binnema et al. A calibration curve obtained with urokinase from Leo Pharmaceuticals gives the assay a lower detection limit of 0.1 ng/ml and an inter- and intraassay variation of 20% and 6%, respectively. Free urokinase and urokinase in complex with PA inhibitor were detected with an equal efficiency.

**Assay of Plasminogen Activator Activity**

Plasminogen activator activity in the endothelial cell-conditioned medium was detected by a fibrin autograph technique after SDS-polyacrylamide gel electrophoresis (9% gels) of the endothelial cell-conditioned medium and removal of SDS. To allow discrimination between t-PA and urokinase-type plasminogen activator activity, parallel incubations were performed on fibrin gels containing either 120 μg/ml rabbit antihuman t-PA IgG or 80 μg/ml goat antihuman urokinase IgG.

**Assay of Plasminogen Activator Inhibitor**

The PA inhibitor activity was measured by a modification of the quantitative assay for plasminogen activator activity as described previously. The PA inhibitor activity was determined by titration with increasing amounts of t-PA of a fixed volume of endothelial cell-conditioned medium or control medium (usually 5 or 10 μl). The amount of t-PA inhibition was calculated from the intersection of the ass-
ymptote of the titration curve with the X-axis. In all experiments this asymptote ran parallel to the control titration curve. The curves were determined in triplicate. The intraassay variation was less than 5%. The interassay variation coefficient was 10%. Determination of the dissociation constant Kd of the PA inhibitor for t-PA was performed as described by Verheijen et al. 38

The PA inhibitor was also demonstrated by reverse fibrin autolography essentially by the method of Loskutoff et al. 39 In brief, 30 mlU/ml of t-PA was added to a mixture of agarose and plasminogen-rich fibrinogen. To ensure homogeneous lysis of the fibrin plate, the mixture was passed through a 0.2 μm filter before the addition of thrombin. The gels were calibrated by use of the low molecular weight reference proteins.

**Immunoprecipitation**

Immunoprecipitation of radiolabelled PA inhibitor or t-PA/PA inhibitor complexes in 35S-methionine-labelled endothelial cell-conditioned medium was performed essentially according to the method of Kessler 40 with the modifications suggested by Bollen et al. 41 An aliquot of 100 μl of labelled medium was incubated with 10 μg of the appropriate antiserum IgG fraction for 18 hours at 4° C. The formed complexes were precipitated with formaldehyde-treated Staphylococcus aureus, were washed extensively, and were then dissolved in 50 μl of sample buffer for SDS-PAGE. The radioactivity in 5 to 10 μl samples of the dissolved immunoprecipitates was determined by liquid scintillation counting.

**Assay of Tissue-Type Plasminogen Activator mRNA**

Isolation of poly A-RNA from cell lysate of endothelial cells and quantitation of t-PA mRNA by hybridization with a t-PA cDNA probe was done as described by Van Zonneveld et al. 42

**Immunofluorescence Microscopy**

Immunofluorescence staining of endothelial cell-associated antigen was performed on cell monolayers that had been fixed in 80% (vol/vol) acetone for 10 minutes at 4° C and rinsed with phosphate-buffered saline (PBS). The cells were incubated with rabbit antihuman von Willebrand factor antiserum (1:80 dilution in 10% pig serum), with FITC-swine antirabbit Ig (1:50 in PBS), and finally with rhodamine-labelled Ulex europeus lectin I (1 mg/ml, 1:10 in PBS, 1 mmol/l MgCl2, 1 mmol/l CaCl2). The culture dishes were washed twice with PBS between each step. The cells were mounted with 1 mg/ml p-phenylenediamine in 10% (vol/vol) glycerol in PBS under glass cover slips and examined with a Leitz epifluorescence system.

**Other Assays**

Cellular angiotensin-converting enzyme activity was measured in Triton X-100 extracts with 14C-glycine-his-puromyl-L-histidyl-L-leucine as substrate, as previously described. 34 The protein content of the cells was assayed in duplicate by the method of Lowry et al. 43 by use of albumin as a standard. Cell counts were made in triplicate with a hemocytometer and the intraassay variation was 15%.

**Results**

**Serial Propagation of Endothelial Cells from Human Macrovessels**

Endothelial cells from human adult aorta, iliac and carotid artery, and vena cava were isolated by collagenase treatment and cultured on fibronectin-coated dishes in M199 medium supplemented with human serum and a crude ECGF preparation. Table 1 shows the maximal number of passages (split ratio 1:5) that were obtained with endothelial cells from five autopsies. Arterial cells from these macrovessels could be propagated for 15 to 24 passages (37 to 61 population doublings). They maintained a normal small diameter appearance (Figure 1A to 1C) for only two to seven passages (8 to 19 population doublings) (Table 1). Thereafter, the diameter of all cells became enlarged (Figure 1E and 1F), and the protein content per cell increased as shown for carotid artery endothelial cells in Figure 2. We compared the serial propagation of vena cava and aorta endothelial cells from two donors. In both cases vena cava endothelial cells grew for more

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**Table 1. Propagation of Endothelial Cells from Human Adult Macrovessels**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Vessel</th>
<th>Estimated seeding density (× 10⁶ cells/cm²)</th>
<th>Maximal number of passages with small diameter cells</th>
<th>Maximal number of passages with cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aorta</td>
<td>1.0</td>
<td>4–5</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Aorta</td>
<td>0.7</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Aorta</td>
<td>1.0</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>Vena cava</td>
<td>0.1</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>Aorta</td>
<td>0.7</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>Vena cava</td>
<td>0.7</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>Iliac artery</td>
<td>2.0</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>Carotid artery</td>
<td>2.0</td>
<td>7</td>
<td>15</td>
</tr>
</tbody>
</table>

Human adult macrovessels were obtained from autopsy. Endothelial cells were cultured on fibronectin-coated dishes in M199 medium supplemented with 20% human serum and 200 μg/ml crude ECGF. When primary cultures had reached the postconfluent state, the cells were passaged every 7 days with a split ratio 1:5. At each passage level, part of the dishes with cells were split after 7 days, and from the remaining part the cell density and cell morphology were determined at Day 10 after seeding. Populations of small diameter endothelial cells are defined as containing more than 0.5 × 10⁶ cells/cm². Cell counts were made in triplicate. ND = not determined.
Figure 1. Phase contrast photomicrographs of human endothelial cells. Primary cultures of human endothelial cells from adult aorta (A), iliac artery (B), and vena cava (C) consist of small diameter endothelial cells. Enlarged endothelial cells are also present in primary cultures of aortic endothelial cells (D). E. Small and large diameter aortic endothelial cells after two passages. F. Vena cava endothelial cells after 24 passages show an increased diameter at confluency. Bar = 0.2 mm.

Population doublings (46 to 69) and maintained a healthy, small diameter appearance for many more passages (35 to 37 population doublings) than aorta endothelial cells. Endothelial cells from both umbilical artery and vein could be propagated for 50 to 60 population doublings and grew 10 passages (25 to 30 population doublings) in a cell density of 0.5 to 1.0 x 10^5 cells/cm^2 (not shown).

Characterization of Endothelial Cells

Confluent primary cultures of human endothelial cells from aorta, iliac and carotid artery, and vena cava consisted of small diameter endothelial cells (Figure 1A to 1C) comparable to those of umbilical artery and vein. The primary cultures of aortic endothelial cells also consistently contained a small proportion of endothelial cells with an enlarged diameter (Figure 1D). When endothelial cells were isolated from a very atherosclerotic part of a descending aorta, only enlarged cells were obtained (not shown). Sometimes a limited number of macrophages were observed, but these were lost from the endothelial cell cultures after one or two passages. Smooth muscle cell contamination in the endothelial cell cultures was always less than 2% as estimated by phase contrast microscopy. Only cultures without smooth muscle cells were used for serial passage studies.

The endothelial nature of early passage cells was established from the following criteria: 1) cobblestone morphology at confluency (Figure 1A to 1C); 2) the presence of von Willebrand factor in distinct and often tube-like granules (Figure 3A, C, D, F); 3) the presence of cell-associated angiotensin-converting enzyme (not shown); 4) the binding and endocytosis of acetylated low density lipoprotein (Figure 3E). The endothelial nature of the subpopulations
Figure 2. Effect of serial propagation of human carotid artery endothelial cells on cell density and cellular protein content. The cells were isolated and grown as indicated in Table 1. The cell protein was assayed after cells were washed three times with PBS. The phase contrast photomicrographs show the cells A after 6 passages and B after 12 passages. Bar = 0.2 mm.

was verified by immunofluorescence microscopy staining of von Willebrand factor. Von Willebrand factor was present in multinuclear and "senescent" endothelial cells from aorta and iliac artery (Figure 3C, D, F). Both small diameter and enlarged endothelial cells endocytosed Dil-labelled acetylated LDL (Figure 3E).

Table 2. Secretion of Tissue-Type Plasminogen Activator Antigen by Human Endothelial Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>t-PA antigen (ng/ml)</th>
<th>t-PA antigen production (ng/24 h/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-free</td>
<td>With serum</td>
<td></td>
</tr>
<tr>
<td>Aorta EC</td>
<td>29 ± 24 (4)</td>
<td>55 ± 47 (4)</td>
</tr>
<tr>
<td>Vena cava EC</td>
<td>122 ± 19 (2)</td>
<td>173 ± 19 (2)</td>
</tr>
<tr>
<td>Umbilical artery EC</td>
<td>6 ± 1 (3)</td>
<td>11 ± 6 (16)</td>
</tr>
<tr>
<td>Umbilical vein EC</td>
<td>6 ± 3 (5)</td>
<td>13 ± 7 (11)</td>
</tr>
</tbody>
</table>

Confluent cultures of human aorta, vena cava, and umbilical vessel endothelial cells (EC) (after one passage) were washed with M199 medium and incubated for 24 hours in 0.2 mCi/cm² M199 medium supplemented with 10% human serum or 0.03% albumin (serum-free). Tissue-type plasminogen activator (t-PA) antigen was assayed in triplicate by enzyme immunoassay. The values represent mean ± SD of the number of cell strains from different donors indicated in parentheses.

Secretion of Plasminogen Activators

A linear secretion of t-PA antigen was observed with human endothelial cells as illustrated for aorta endothelial cells (Figure 4). In the presence of 10% human serum, the cells secreted 1.2 to 2.0 times more t-PA antigen than in the absence of serum. This increase in t-PA antigen secretion paralleled the increase in the rate of 3S-methionine incorporation in endothelial cells proteins (not shown). A marked difference in the amount of t-PA antigen produced was observed between endothelial cells from aorta, vena cava, and umbilical cord artery and vein (Table 2). No t-PA antigen could be detected in the lysates of these cells (not shown). The level of t-PA mRNA found in endothelial cells from adult human macrovessels was four- to sixfold higher than in cells from umbilical cord vessels (Table 3).

No urokinase antigen could be detected in the conditioned medium of primary cultures of human macrovascular endothelial cells as shown for carotid artery endothelial cells in Table 4. During serial propagation of the arterial endothelial cells particularly, a gradual increase in the secretion of urokinase antigen was observed. This increase was not accompanied by a change in the secretion of t-PA antigen or in the release of lactate dehydrogenase (Table 4).

Table 3. Cellular Tissue-Type Plasminogen Activator mRNA Content of Human Endothelial Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>t-PA mRNA (µg cDNA hybridized/10⁶ cells)</th>
<th>t-PA antigen production (ng/24 h/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta EC</td>
<td>13.7 ± 1.7</td>
<td>66 ± 9</td>
</tr>
<tr>
<td>Iliac artery EC</td>
<td>8.8 ± 1.7</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>Vena cava EC</td>
<td>8.1 ± 1.2</td>
<td>165 ± 2</td>
</tr>
<tr>
<td>Umbilical artery EC</td>
<td>1.9 ± 0.4</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Umbilical vein EC</td>
<td>2.4 ± 0.9</td>
<td>9 ± 3</td>
</tr>
</tbody>
</table>

Endothelial cells (EC) from aorta, iliac artery, and vena cava (all from donor 4, Table 1) and from umbilical artery and vein were used after three passages. Confluent cultures in T25 flasks were incubated in M199 supplemented with 20% human serum for 24 hours, after which the medium was taken for assay of tissue-type plasminogen activator (t-PA) antigen (mean ± range of duplicate assay is given). The t-PA mRNA was quantified by hybridization with a 32P-labelled t-PA cDNA clone in a dot blot test. The values represent the mean ± SD of five estimations made at serial dilutions of a total RNA preparation.
We compared the secretion of plasminogen activators by endothelial cells from aorta, iliac artery, and vena cava of one donor (Figure 5). The aorta was moderately atherosclerotic, the iliac artery, severely atherosclerotic, and the vena cava appeared unaffected at autopsy. During propagation of aorta endothelial cells, we observed a gradual increase in urokinase antigen secretion; t-PA antigen remained rather constant (Figure 5). In contrast to t-PA release and overall protein synthesis, urokinase antigen secretion was mostly higher in serum-free medium than in the presence of 10% human serum. After one passage, the endothelial cells from the iliac artery secreted considerable plasminogen activators.

Table 4. Release of Plasminogen Activators by Human Carotid Artery Endothelial Cells During Subculturing

<table>
<thead>
<tr>
<th>Cells</th>
<th>t-PA antigen (ng/ml)</th>
<th>u-PA antigen (ng/ml)</th>
<th>LDH release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>6.9 ±1.7</td>
<td>&lt;0.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>After 3 passages</td>
<td>5.0 ±0.8</td>
<td>4.0 ±0.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>After 5 passages</td>
<td>ND</td>
<td>4.1 ±0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>After 10 passages</td>
<td>6.0 ±1.7</td>
<td>7.8 ±1.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>After 11 passages</td>
<td>5.3 ±1.0</td>
<td>15.1 ±2.5</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Human carotid artery endothelial cells were cultured as described in Table 1. Confluent cells were incubated for 24 hours with 0.2 ml/cm² M199 medium supplemented with 0.03% albumin, Tissue-type plasminogen activator (t-PA) antigen, urokinase-type PA (u-PA) antigen, and lactate dehydrogenase (LDH) were assayed in the endothelial cell-conditioned medium as described in the Methods section. The LDH values are expressed as the percent of the amount of LDH present in the cells. The values of t-PA antigen and u-PA antigen represent the mean ± range of duplicate determinations. ND = not determined.

Table 5. Effect of Bacterial Lipopolysaccharide on Production of Plasminogen Activator Inhibitor Activity by Human Artery Endothelial Cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>PA inhibitor activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum-free</td>
</tr>
<tr>
<td>Without addition</td>
<td>8.4</td>
</tr>
<tr>
<td>LPS</td>
<td>16.2</td>
</tr>
<tr>
<td>LPS and Polymyxin B</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Iliac artery endothelial cells (after three passages) were incubated in 0.2 ml/cm² M199 medium supplemented with 150 µg/ml ECGF and 0.03% pyrogen-free human serum albumin (serum-free) or 1% human serum, and incubated for 24 hours with or without 10 µg/ml E. coli lipopolysaccharide (LPS). Polymyxin B (10 µg/ml) was mixed with lipopolysaccharide immediately before addition to the cells. The experimental variation in the assay of PA inhibitor activity was less than 5%.
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Figure 4. Time-dependence of t-PA antigen secretion by human aorta endothelial cells. Confluent first passage cells were washed and incubated in M199 medium supplemented with 10% human serum or 0.03% pyrogen-free human serum albumin for various time intervals. The data represent the means ± SD of triplicate determinations.

amounts of urokinase antigen. Early passage vena cava endothelial cells produced very small amounts of urokinase antigen together with large amounts of t-PA antigen. However, when vena cava cells were propagated for 18 passages, large amounts of urokinase antigen were secreted by these cells (Figure 5). Similarly, primary cultures of human umbilical vein or artery endothelial cells secreted no urokinase antigen, but with subcultured cells urokinase antigen could be demonstrated in the serum-free conditioned medium of these cells (not shown).

Plasminogen activator activity was demonstrated after SDS polyacrylamide gel electrophoresis and fibrin autography and was characterized by the use of specific anti-t-PA and anti-u-PA IgG (Figure 6). In primary cultures of aorta and vena cava endothelial cells, t-PA activity was detected in cell supernatants with molecular weights of 60 kD and 100 kD. The 100 kD form represents a complex of t-PA with PA inhibitor (see below), which during SDS-polyacrylamide gel electrophoresis partly dissociated, resulting in a smear of activity from 100 kD to 60 kD. After 18 passages, a small amount of urokinase-type plasminogen activator activity at a molecular mass of 55 kD was observed in serum-free conditioned medium of vena cava cells in addition to large amounts of t-PA activity with 100 kD and 60 kD molecular weights.

The presence of actinomycin D (20 ng/ml) prevented the production of urokinase antigen by aorta endothelial cells (Figure 7). Furthermore, the synthesis of urokinase-type plasminogen activator by human endothelial cells could be established by immunoprecipitation with anti-urokinase IgG of radiolabelled conditioned medium. The urokinase-type plasminogen activator secreted by aorta and vena cava endothelial cells has molecular masses of 55 and 95 kD (Figure 8). Similar molecular masses of urokinase-type plasminogen activator were secreted by serially passaged endothelial cells from umbilical cord vein and artery. The 95 kD u-PA and the 100 kD t-PA were also precipitated with anti-PA inhibitor IgG, indicating that they represent PA/PA inhibitor complexes.

Production of Plasminogen Activator Inhibitor Activity by Aorta and Vena Cava Endothelial Cells

Human macrovascular endothelial cells produce PA inhibitor activity during incubation in the presence of human

Figure 5. Production of t-PA antigen and u-PA antigen by endothelial cells obtained from various macrovessels of one donor (patient 4 in Table 1). Confluent cells were incubated with 0.2 ml/cm² M199 medium supplemented with 0.03% albumin (hatched bars) or 10% human serum (open bars) for 24 hours. The conditioned medium was subsequently centrifuged and frozen. t-PA antigen and u-PA antigen were assayed by enzyme Immunoassay of the same conditioned media.
serum as shown for human aorta endothelial cells in Figure 9. The characteristics are identical to those of the PA inhibitor activity secreted by umbilical cord endothelial cells; the Kd value of complex formation with t-PA was 3 to 5 pM. The PA inhibitor activity in endothelial cell-conditioned medium is relatively unstable at 37°C; it has a half-life time of 2 to 3 hours. In serum-free medium containing pyrogen-free human serum albumin, only small amounts of PA inhibitor activity were found after incubation with the various types of human endothelial cells (Figure 9).

The production of PA inhibitor activity increases two- to fivefold after the first passage of endothelial cells from adult artery and vein, and remains at a constant high rate in both early and late passage subcultures of these cells. It can be enhanced twofold by addition of bacterial lipopolysaccharide (Table 5). Bacterial lipopolysaccharide had no effect on the production of t-PA or the overall protein synthesis. The effect of lipopolysaccharide on PA inhibitor activity was prevented by polymyxin B.

After SDS polyacrylamide gel electrophoresis of conditioned medium from aorta, vena cava, and iliac artery endothelial cells, a PA inhibitor with a molecular mass of about 46 kD could be visualized by reverse fibrin autography (Figure 10). This PA inhibitor and complexes of PA inhibitor with t-PA or u-PA can be precipitated by anti-PA inhibitor-1 IgG. In primary culture the production of PA inhibitor by human macrovascular endothelial cells increases progressively. The PA inhibitor is present in large quantities in the conditioned medium of both early and late passage subcultures of various types of human macrovascular endothelial cells. In the presence of serum it represents 8% to 12% of the secreted protein, as estimated by incorporation of 35S-methionine in proteins.

Discussion

We have described the production of plasminogen activators by serially propagated endothelial cells from adult
human arteries and veins. During subculturing, the secretion of t-PA antigen remained rather constant, but the production of urokinase antigen markedly increased, parallel with a loss of the small diameter morphology of the cells. The amount of t-PA secreted by aorta and particularly vena cava endothelial cells was many times higher than that produced by umbilical cord vessel endothelial cells.

Long-term propagation of human macrovascular endothelial cells was realized by growing these cells on fibronectin coated dishes and by the addition to the culture medium of human serum and a crude hypothalamus extract, which contains ECGF as active component. The life span of vena cava endothelial cells was only a few passages longer than that of aorta cells (Table 1). However, when we compared the number of passages during which the cells maintained a normal, small diameter morphology, a marked difference between arterial and venous cells was observed. After four passages, the number of aorta endothelial cells per surface area rapidly decreased, concomitant with an increase in cell diameter and in the protein content of the cell. Their venous counterparts, vena cava endothelial cells from the same donors, could be maintained for 12 to 14 passages in a small diameter morphology. Endothelial cells from human umbilical artery also maintained a normal morphology for a large number of passages. An increase in cell diameter was often observed at the end of the life span of various cells, including endothelial cells.

Although it is tempting to speculate that the endothelial cells from adult arteries may have made more population doublings in vivo during their lifetimes, we cannot exclude the possibility that the differences between aorta and vena cava endothelial cells are due to differences intrinsically related to the vessel type from which they were isolated. A comparative study of the characteristics and serial propagation of aortic and venous endothelial cells from young human individuals will be necessary to solve this question. Suggestive of a change in endothelial cell function in human arteries over a lifetime is the observation reported by Repin et al. that the diameter of endothelial cells in human arteries as estimated by scanning electron microscopy increases with age, and that markedly enlarged endothelial cells cover arteriosclerotic lesions. Antonov et al. also recently reported the occurrence of both small and large diameter endothelial cells in primary isolates from human aorta.

Interestingly, the difference in passage number at which the cell diameter of endothelial cells from adult arteries and veins increases, runs more or less parallel with the increase in urokinase antigen production by these cells (compare Table 1 and Figure 5). No significant secretion of urokinase antigen was observed in serum-free or serum-containing conditioned media of primary endothelial cell cultures from adult macrovessels or from umbilical artery or vein. This is consistent with histological observations on human blood vessels showing that only t-PA activity or t-PA antigen is associated with the endothelium. However, urokinase antigen was found in the conditioned medium of early passage arterial cells, particularly when the cells were incubated under serum-free conditions. Many authors, including ourselves, have previously reported that human endothelial cells do not produce urokinase activity in contrast to bovine aorta endothelial cells. However, Booyse et al. recently demonstrated that (pro)urokinase was produced by human umbilical vein endothelial cells. After incubation of their cells for 4 days in serum-free

**Figure 9.** Time-dependence of the accumulation of PA Inhibitor activity secreted by human aorta endothelial cells. The cells were incubated with 0.2 ml/cm² M199 medium supplemented with 10% human serum (•) or 0.03% pyrogen-free human serum albumin (○). PA inhibitor activity was assayed by titration with t-PA of the endothelial cell-conditioned medium. The data represent the means ± SD of triplicate determinations.

**Figure 10.** PA inhibitor in conditioned medium of human aorta endothelial cells (after two passages) was demonstrated by reverse fibrin autography (A) and immunoprecipitation from 35S-methionine-labelled endothelial cell-conditioned medium with rabbit anti-PA inhibitor-1 IgG (B) as indicated in Methods. B shows the fluororadiogram obtained after SDS-polyacrylamide gel electrophoresis of the immunoprecipitate. No 46-kD radioactivity was precipitated with nonimmune rabbit IgG.
medium, they found two molecular species, a 54 kD protein and a 100 kD protein. The same molecular species were demonstrated with endothelial cells from adult human arteries in this study. They represent an urokinase/PA inhibitor complex and probably pro-urokinase. We also confirmed the production of urokinase antigen by umbilical cord artery and vein endothelial cells when we used cells under suboptimal conditions, such as prolonged incubation in serum-free medium or cells at the end of their lifespan. Similarly, late passage vena cava endothelial cells produced large amounts of urokinase antigen.

The relatively high levels of u-PA in serum-free conditioned medium in comparison to serum-containing medium contrasted to the levels of t-PA in these media and to the overall protein synthesis of the cells. Levin and Loskutoff demonstrated that intracellular and secreted u-PA in bovine endothelial cells decreased dramatically within 30 minutes after addition of fetal bovine serum. Concomitant with this decrease, an increase in 100 kD PA activity was observed by these authors, suggesting that the decrease in u-PA activity was at least partly due to an increase in PA inhibitor activity. Our assay of u-PA antigen recognizes pro-urokinase, urokinase, and the urokinase-PA inhibitor complex with the same efficiency, which indicates that the increase in u-PA antigen in serum-free medium reflects an increase in the production of u-PA itself. Several mechanisms may underlie the increase in u-PA secretion during serial propagation of endothelial cells. First, the secretion of u-PA may reflect a state of cell dysfunction, during which cellular contents are released in the medium. Leakage of cytoplasmic proteins appear unlikely, because no parallel accumulation of lactate dehydrogenase was observed. A second explanation may be that under serum-containing conditions, endothelial cells are able to bind u-PA effectively, similar to monocytes and U-937 cells. A disproportional decrease of such receptors to the secretion of u-PA would result in the accumulation of u-PA antigen in the medium. Thirdly, u-PA secretion may be induced by growth-limiting circumstances (or repressed in growing cells). In this respect it is noteworthy that under growth-limiting conditions human endothelial cells can reorganize spontaneously to form tube-like structures. The generation of these structures proceeds much faster when the endothelial cells are grown on a fibronectin matrix that is degraded by plasmin or u-PA.

Tissue-type plasminogen activator is considered as the physiological trigger for the extrinsic fibrinolysis route, and t-PA was produced by primary cultures of all the types of human endothelial cells we tested. In contrast to urokinase-type plasminogen activator, t-PA secretion remained constant during serial propagation of endothelial cells. The amount of t-PA secreted by aorta and particularly vena cava cells was considerably larger than that produced by umbilical cord artery or vein endothelial cells. Interestingly, iliac artery endothelial cells secreted less t-PA antigen than these from aorta and vena cava of the same donor. The difference in t-PA antigen secreted by cultured aorta and umbilical cord vein endothelial cells was paralleled by a similar difference in the amount of t-PA mRNA in the cells. However, the difference in the production of t-PA by vena cava, aorta, and iliac artery endothelial cells was not related to different levels of t-PA mRNA. Therefore, the difference in t-PA secretion by these cells may reflect a difference in other factors, such as the cellular processing of t-PA, intracellular degradation, the efficiency of translation, or a combination of these.

We have previously observed that umbilical cord endothelial cells produce a two- to 20-fold excess of a fast acting PA inhibitor in comparison to t-PA antigen. Although considerably more t-PA is produced by endothelial cells from adult human macrovessels, there is always at least an equivalent amount of PA inhibitor produced by these cells, so that no PA activity can be detected by a direct spectrophotometric assay. The large production of PA inhibitor by human endothelial cells in culture is remarkable and may represent an in vitro adaptation of the cells. Still, cultured endothelial cells from adult artery are able to increase their PA inhibitor production upon stimulation by LPS, similar to umbilical vein endothelial cells. The physiologic importance of this effect is stressed by the observation that endotoxin treatment also enhances the systemic level of PA inhibitor activity in rabbits and rats in vivo.

In conclusion: endothelial cells from adult human arteries and veins secrete t-PA and PA inhibitor and, after subculturing, u-PA antigen. The differences between these types of cells and umbilical artery and vein endothelial cells are: 1) the amount of secreted t-PA and t-PA mRNA content, 2) the possibility of being propagated as a healthy monolayer, and 3) the release of u-PA antigen. These differences may be developmentally related to vascular origin or may reflect differences acquired by exposure to environmental influences, e.g., injury, during the in vivo "life history" of the blood vessels from which the cells were obtained. Our data suggest that the release of u-PA antigen by human macrovascular endothelial cells is related to the exhaustion to proliferate as a normal monolayer. In this respect human endothelial cells distinctly differ from bovine aorta endothelial cells.

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V W van Hinsbergh, D Binnema, M A Scheffer, E D Sprengers, T Kooistra and D C Rijken

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