Characterization and Fibrinolytic Properties of Human Omental Tissue Mesothelial Cells. Comparison With Endothelial Cells

By Victor W.M. van Hinsbergh, Teake Kooistra, Mariëlle A. Scheffer, J. Hajo van Boeckel, and Goos N.P. van Muijen

It has been reported that omental fat tissue is a good source of human microvascular endothelial cells. By characterization we demonstrate that the epithelioid cells isolated from omental tissue are not endothelial cells, but mesothelial cells. They contain abundant cytokeratins 8 and 18, which are absent in endothelial cells, and vimentin. No staining with the endothelial-specific antibodies EN-4 and PAL-E is observed. A faint and diffuse staining of von Willebrand factor (vWF) is seen in mesothelial cells, whereas microvascular endothelial cells from subcutaneous fat display vWF in distinct granular structures. Human peritoneal mesothelium produces plasminogen activator-dependent fibrinolytic activity, which is essential in the resolution of fibrous exudates and may therefore be important in preventing the formation of fibrous peritoneal adhesions. This fibrinolytic activity is plasminogen activator-dependent, but has not been fully characterized. We report here that human omental tissue mesothelial cells in vitro produce large amounts of tissue-type plasminogen activator (t-PA), together with type 1 and 2 plasminogen activator inhibitor (PAI-1 and PAI-2). PAI-1 is predominantly secreted into the culture medium, whereas the major part of PAI-2 is found in the cells. No urokinase-type plasminogen activator is detected. On stimulation with the inflammatory mediator tumor necrosis factor (TNF), at least a threefold decrease in t-PA antigen is observed, together with an increase in both PAI-1 and PAI-2. TNF also induces a marked change in cell shape. Whereas TNF and bacterial lipopolysaccharide (LPS) have similar effects on the production of PA inhibitor by human endothelial cells, LPS has no or only a relatively small effect on the fibrinolytic properties of mesothelial cells. The decreased fibrinolytic activity induced by the cytokine TNF may impair the natural dissolution of fibrin deposits at the peritoneum in the presence of an inflammatory reaction.

MATERIALS AND METHODS

Materials. The specificities of our monoclonal antibodies (MoAbs) for cytokeratins 8 (M20) and 18 (M9), vimentin (V9), and desmin (D33) have been described previously.16-21 MoAb PAL-E22 was a gift of Dr R. Schlingemann (Department of Pathology, University of Nijmegen, The Netherlands). MoAb antibody EN-423 was obtained from Sanbio B.V. (Uden, The Netherlands); polyclonal antibodies against human vWF were from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Anti-tissue-type plasminogen activator (t-PA)44 immunoglobulin (Ig) G and anti-urokinase-type PA (u-PA) IgG25 were provided by Drs D.C. Rijken and G. Dooyewaard (Gaubius Institute TNO, Leiden, The Netherlands). FITC-labeled goat anti-mouse IgG was purchased from DAKO immunoglobulins (Glostrup, Denmark).

Human recombinant tumor necrosis factor (TNF)28 was a gift of Dr Jan Tavernier (Biogent, Gent, Belgium). The TNF preparation contained 2.45 x 10^7 U/mg protein and 40 ng LPS per milligram of protein. Bacterial LPS from Escherichia coli was purchased from Sigma (St Louis, MO).

Collagenase/dispose was purchased from Boehringer Mannheim (Mannheim, FRG); collagenase type II from Worthington (Freehold, NJ). Human serum was prepared from freshly collected blood of healthy donors and pooled (12 to 20 donors); newborn calf serum was purchased from Gibco (Grand Island, NY) and inactivated by treatment for 30 minutes at 56°C. A crude preparation of endothelial cell growth factor (EGGF) was prepared as described by Maciag et al.45 Heparin was from Leo Pharmaceuticals (Copenhagen, Denmark). M199 medium supplemented with 20 mmol/L HEPES was from Flow Laboratories (Irving, Scotland). [35S]methionine was from New England Nuclear (Amherst, MA).

Cells. Human mesothelial cells were isolated from freshly obtained omental fat tissue according to the method of Kern et al.46 with minor modifications. Omental tissue from patients was obtained...
according to the guidelines of the Institutional Review Board of the University Hospital Leiden, The Netherlands. The tissue was collected in ice-cold buffer (140 mmol/L NaCl, 4 mmol/L KCl, 11 mmol/L D-glucose, 1% pyrogen-free human serum albumin, 10 mmol/L HEPES (pH 7.3), 100 IU/mL penicillin, and 0.10 mg/mL streptomycin) and immediately transferred to the laboratory. The tissue was rinsed, cut into pieces, and incubated in 0.1% pyrogen-free albumin and penicillin/streptomycin for 20 minutes at 37°C. After incubation, large pieces of tissue were removed and gently washed with M199 medium. The incubation medium and the washing fluid were sieved through a 0.4% gauze. After subsequent centrifugation for 5 minutes at 600 g, the floating fat cells were carefully removed by aspiration, and subsequently the supernatant was removed. The pellet was resuspended in growth medium (M199 medium supplemented with 10% human serum, 10% heat-inactivated newborn calf serum, 20 mmol/L HEPES, and penicillin/streptomycin) and the cells were seeded on fibronectin-coated dishes. The cells were propagated at 37°C under 5% CO₂/95% air atmosphere in growth medium, which was renewed every 2 to 3 days. When indicated, the serum was replaced by 20% human serum or 20% heat-inactivated newborn calf serum. When the cells had become confluent, they were detached by treatment with trypsin/EDTA and were passaged with a split ratio of 1:4. Cells were used after 1 or 2 passages. Mesothelial cell-conditioned media were obtained by incubating confluent cells at 37°C for the indicated period of time (usually 24 hours) in M199 medium supplemented with 20% human serum, 20 mmol/L HEPES, and penicillin/streptomycin under 5% CO₂/95% air atmosphere, unless otherwise mentioned. The mesothelial cell-conditioned medium was immediately centrifuged and stored at −20°C until use. After two washes with phosphate-buffered saline (PBS), cell extracts were prepared in 0.5% Triton X-100 (Merck, Darmstadt, FRG) and stored frozen until use. Immunoprecipitation of radiolabeled PA inhibitor-1 from [³⁵S]methionine-labeled 24-hour mesothelial cell-conditioned medium was performed as previously described. A small number of capillaries was isolated from human subcutaneous fat by the same procedure as given for omental tissue mesothelial cells. In contrast to the mesothelial cell preparation, the preparation of subcutaneous fat capillaries also contained fibroblastoid cells. Endothelial cells from human umbilical artery and vein were isolated as previously described, and cultured in the same growth medium as described above.

**Immunofluorescence microscopy.** Immunofluorescence staining of mesothelial and endothelial cell antigens was performed on cell monolayers that had been grown on cover slips coated with crosslinked gelatin. The cells had been fixed in 80% (vol/vol) acetone for 30 minutes at 4°C and rinsed with PBS. The cells were incubated with various MoAbs for 30 minutes at room temperature, washed twice with PBS, and subsequently incubated for 30 minutes with FITC-labeled goat anti-mouse Ig (1:50 in PBS). The cells were mounted with 1 mg/mL p-phenylenediamine in 10% (vol/vol) glycerol in PBS under glass coverslips, and examined with a Leitz epillumination system. The endothelial-specific antibodies showed no reactivity with cultured human fibroblasts or smooth muscle cells.

**Characterization of human omental tissue mesothelial cells (HOMC).** A large number of epithelioid cells was obtained on isolation by collagenase treatment of omental tissue, most of them in small sheets of 10 to 200 cells (Fig 1a). The cells grew to confluency in 4 to 5 days; after reaching confluency, the diameter of the cells decreased by a continuation of cell division until a monolayer of closely apposed cells (Fig 1b) was formed. These cells could be propagated for several passages, usually 4 to 6 (split ratio 1:4). At the end of their life span the cells changed into multinucleated giant cells (Fig 1c). All further studies have been done with HOMC after 1 or 2 passages.

To establish the mesothelial nature of these cells, we examined whether they contain various types of intermediary filaments. With several MoAbs, the abundant presence of cytokeratins 8 and 18 was demonstrated in confluent and subconfluent HOMC (Fig 1, d and e). These cytokeratins were consistently found in these cells (cells from four different donors tested). Their presence strongly suggests that these cells are mesothelial cells (further indicated as HOMC). Human umbilical vein endothelial cells did not contain cytokeratins 8 and 18 (Fig 1f). Vimentin was present in both HOMC and umbilical vein endothelial cells. Desmin was detected in highly confluent HOMC by the use of the MoAb D33, but a considerable variation in the desmin content of the cell was observed (not shown). No immunofluorescent staining was observed when the MoAbs were replaced by nonproducer Sp 2/0 myeloma cell supernatants (not shown) or by an anti-fibrin monomer MoAb (Fig 1g).

Confluent and subconfluent cultures of HOMC were examined for the presence of various endothelial cell mark-
Fig 1. Characterization of mesothelial cells from human omental fat tissue. (a through c) Phase contrast photomicrographs of mesothelial cells: (a) after isolation from the tissue; (b) confluent culture after one passage; (c) culture with senescent cells. (d through e) Immunofluorescence microscopy of cytokeratins 8 (d) and 18 (e) in mesothelial cells. (f) Absence of cytokeratin 18 in human umbilical vein endothelial cells. (g) Absence of fibrin monomer antigen in mesothelial cells. (h) vWF antigen in mesothelial cells. (i) EN4 antigen in human umbilical vein endothelial cells. (j) Endocytosis of Dil-acetylated LDL by human omental mesothelial cells. Magnification: a through c. x 70; d through j. x 175.

A faint and diffuse staining of vWF was observed in highly confluent HOMC by use of polyclonal anti-vWF antibodies (Fig 1h), whereas both confluent and subconfluent endothelial cells from umbilical vein and foreskin microvessels displayed vWF abundantly in granular structures. The MoAbs PAL-E and EN4 recognized endothelial cell-specific antigens on human umbilical vein and foreskin microvascular endothelial cells, as shown for EN4 in Fig 1i. No staining for EN4 and PAL-E was found in various HOMC cultures from four different donors (not shown). In only one of these cultures a few PAL-E positive cells were observed (less than 1 per 200 cells). A minute contamination of endothelial cells cannot be excluded in this culture. The uptake of acetylated-LDL has also been used to recognize endothelial cells and macrophages. Mesothelial cells from omental tissue ingested large amounts of Dil-labeled acetylated-LDL (Fig 1j). The immunofluorescence in HOMC after 4 hours of incubation with 20 µg/mL Dil-acetylated-LDL was significantly higher than in identically treated human umbilical vein endothelial cells (three independent experiments).

When subcutaneous fat was used to isolate endothelial cells, no sheets of mesothelial cells, but a limited number of small capillaries (Fig 2a), were obtained together with fibroblastoid cells. The endothelial cells of the capillaries adhered to the culture dish and spread. The cells were examined daily by phase contrast microscopy (Fig 2b) and their location was indicated on the culture dish. After 5 to 7 days the cells were fixed and examined. They displayed the presence of vWF in discrete granules (Fig 2c), similar to umbilical vein and foreskin microvascular endothelial cells.
FIBRINOLYTIC PROPERTIES OF MESOTHELIAL CELLS

Fig 2. Characterization of microvascular endothelial cells obtained from human subcutaneous fat. (a and b) Phase contrast microscopy of endothelial cells. (a) Capillary after isolation; (b) capillary endothelial cells after 4 days in culture. (c) Indirect immunofluorescent staining of vWF in cells that have been maintained in culture for 7 days. Magnification: a and c, ×200; b, ×80.

Production of PA and PAI by HOMC. HOMC produced large amounts of t-PA. During a 24-hour incubation period in the presence of 10% human serum, they secreted 148 ± 98 ng t-PA antigen/10^6 cells (mean ± SD of six cultures from different donors). As is shown in Fig 3, the amounts of t-PA produced by HOMC markedly exceeded the amount produced by human umbilical vein endothelial cells. No u-PA activity was found in HOMC-conditioned medium by fibrin autography (Fig 3). Also, no u-PA antigen was found in the 24-hour conditioned media and the cell extracts of HOMC from three different donors (detection limit 0.5 ng/mg cellular protein).

Despite the production of large amounts of t-PA antigen, most HOMC cultures did not show spontaneous fibrinolytic activity due to the concomitant production of excess of PAI. In only 1 of 10 cell strains of HOMC we detected a fibrinolytic activity of 12 IU/mL in the 24-hour conditioned medium. In the other cultures an excess of PAI was found in the 24-hour conditioned medium; PAI activity ranged from 2 to 180 IU/mL. Both PAI-1 antigen and PAI-2 antigen could be demonstrated in the conditioned medium and the cellular extracts (see below).

Incubation of HOMC with TNF resulted in a dramatic change in cell morphology. The cells became spindle-shaped within 24 hours after addition of 500 U/mL TNF, and part of them detached (Fig 4). Cell counts showed that after this incubation period the cell number decreased to 82% ± 15% of that in control incubations (mean ± SD, n = 8). No change in cell morphology was observed in mesothelial cells treated for 24 hours with 10 µg/mL LPS (cell number 94% ± 8% of control, n = 8). The addition of TNF also resulted in at least a threefold decrease in the production of t-PA antigen, as is shown for two representative cultures in Table 1. This decrease was found in cells that had been cultured either in human serum or in newborn calf serum, notwithstanding the fact that large differences in the basal rate of t-PA production were observed under these conditions (Table 1). When TNF was inactivated by heating at 90°C for 15 minutes, it had lost its ability to alter the properties of HOMC (not shown). The addition of LPS (10 µg/mL) reduced t-PA production by only 20% or less. No production of u-PA was induced by incubation with either TNF or LPS. Concomitantly a decrease in t-PA antigen production, the PAI activity

Fig 3. Plasminogen activator activity was visualized by fibrin autography after SDS-PAGE of the 24-hour conditioned medium of three different cultures of human umbilical vein endothelial cells (lanes 1 through 3), and three cultures of human omental mesothelial cells (lanes 4 through 6). Bowes melanoma t-PA (lane 7) and u-PA (lane 8) were used as standards. To allow further discrimination between t-PA and u-PA activity, parallel incubations were done on fibrin gels containing either 120 µg/mL rabbit anti-human t-PA IgG (B) or 80 µg/mL goat anti-human urokinase IgG (C). No antibodies were added to fibrin gel A.
Fig 4. Phase contrast photomicrographs of human omental fat mesothelial cells incubated for 24 hours in M199 medium containing 20% human serum, 150 μg/mL ECGF, and 5 U/mL heparin (A), and supplemented with 10 μg/mL bacterial LPS (B), or with 500 U/mL TNF. The values of culture 1 represent the mean ± SD of three parallel cultures of cells from one donor grown on dishes coated with fibronectin, gelatin, or without coating. The data of culture 2 are the mean of duplicate fibronectin-coated dishes with HOMC from another donor. The experimental variation of duplicate dishes was less than 10%. Cellular protein content was determined in parallel wells at the start of the incubation period.

Table 1. Effect of LPS and TNF on the Production of t-PA and u-PA Antigens and PA Inhibitors by Human Omental Tissue Mesothelial Cells

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Addition</th>
<th>t-PA Antigen (ng/mg cell protein)</th>
<th>u-PA Antigen (ng/mg cell protein)</th>
<th>PAI Activity (IU/mg cell protein)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Culture 1</td>
<td>Culture 2</td>
<td>Culture 1 and 2</td>
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<tr>
<td>A</td>
<td>None</td>
<td>590 ± 48</td>
<td>154</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>505 ± 83</td>
<td>165</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>TNF</td>
<td>198 ± 28</td>
<td>77</td>
<td>≤0.5</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>449 ± 40</td>
<td>129</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>384 ± 7</td>
<td>96</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>TNF</td>
<td>108 ± 12</td>
<td>44</td>
<td>≤0.5</td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>113 ± 13</td>
<td>25</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>120 ± 16</td>
<td>21</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>TNF</td>
<td>47 ± 1</td>
<td>3</td>
<td>≤0.5</td>
</tr>
<tr>
<td>D</td>
<td>None</td>
<td>116 ± 2</td>
<td>22</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>114 ± 18</td>
<td>25</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>TNF</td>
<td>35 ± 7</td>
<td>4</td>
<td>≤0.5</td>
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</table>

PAases and PA inhibitor activity were assayed in the 24-hour conditioned medium of confluent mesothelial cells (cell density 1.4 to 1.5 × 10⁵ cells/cm²). The values of culture 1 represent the mean ± SD of three parallel cultures of cells from one donor grown on dishes coated with fibronectin, gelatin, or without coating. The data of culture 2 are the mean of duplicate fibronectin-coated dishes with HOMC from another donor. The experimental variation of duplicate dishes was less than 10%. Cellular protein content was determined in parallel wells at the start of the incubation period.
addition of 10 pg/mL ECGF, and 5 U/mL heparin without (control) or with the addition of 10 pg/mL LPS or 500 U/mL TNF. Open bars represent the amount of PAI antigen in the conditioned medium (mean ± SD, five experiments); hatched bars represent the amount of PAI in the cell extracts after the incubation period (2 to 3 experiments). Significance from the t-test for paired data: (*) P = .05; *P < .02; **P < .01; ***P < .001.

Various serine proteases, like thrombin, activated protein C, and plasmin, can react with PAI-1 and subsequently degrade it into a 42-Kd degradation product. To evaluate whether consumption of PAI-1 occurred during incubation of HOMC with TNF, S-methionine was added to the incubation medium and metabolically labeled PAI-1 was immunoprecipitated after incubation. No 42-Kd PAI-1 degradation product was found in the conditioned medium of TNF-treated and untreated HOMC (not shown).

DISCUSSION

Omental fat tissue has been advocated as a convenient source of human microvascular endothelial cells. A marked contribution of mesothelial cells in these preparations might be anticipated, since the omentum contains four folded layers of mesothelial cells. We have demonstrated that the cell monolayers that are easily obtained from human omental fat adipose tissue are predominantly, if not all, mesothelial cells (HOMC). We also demonstrated that the inflammatory mediator TNF, but not bacterial LPS, markedly decreased the production of t-PA and hence the fibrinolytic activity of the mesothelium reported by various investigators. Our data show that large amounts of two PA inhibitors, PAI-1 and PAI-2, are synthesized by cultured human mesothelial cells. Enhanced production of these inhibitors probably underlies the lack of detectable free

Table 2. Characteristics of Human Endothelial Cells and Omental Tissue Mesothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>HUVEC</th>
<th>FMVEC</th>
<th>HOMC</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intermediary filaments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>Cytokeratin 18</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>Desmin</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td><strong>Endothelial markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1,3,6,7,8,A</td>
</tr>
<tr>
<td>Weibel Palade bodies</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>1,3</td>
</tr>
<tr>
<td>Angiotensin converting enzyme</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>1,3</td>
</tr>
<tr>
<td>Uptake Dil-acetylated LDL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3,7,8,A</td>
</tr>
<tr>
<td>MoAb BMA 120</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>2,8</td>
</tr>
<tr>
<td>MoAb EN-4</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>A</td>
</tr>
<tr>
<td>MoAb Pal-E</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>A</td>
</tr>
<tr>
<td>t-PA production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6,4</td>
</tr>
<tr>
<td>Protein C activation</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>2,6</td>
</tr>
<tr>
<td>Prostacyclin production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8</td>
</tr>
</tbody>
</table>

The presence of intermediary filaments and endothelial markers in human umbilical vein endothelial cells (HUVEC), human foreskin microvascular endothelial cells (FMVEC), and human omental tissue mesothelial cells (HOMC) is given. References for studies on HOMC are indicated.

Abbreviations: ND, not determined; +++, abundantly present; +, present; −, absent/below detection level; A, this report.
In addition to PAI-1, which is predominantly secreted, HOMC also synthesize a relatively large amount of PAI-2, which remains mainly within the cells. It has to be established whether the fibrinolytic inhibitor extracted from mesothelial cells by Pugatch and Poole represents this type of PA inhibitor. If a large amount of PAI-2 is also present in mesothelial cells in vivo, it may contribute to fibrinolysis inhibition on damage of these cells. Addition of TNF causes an increase in the PAI-2 level both in the cells and in the conditioned medium. Because TNF markedly affects the morphology of HOMC and even induces cell detachment, we cannot exclude that leakage of PAI-2 from the cells contributes to the increase in PAI-2 in the supernatant fluid of TNF-treated cells.

We have previously demonstrated that the inflammatory mediator TNF also increases PAI-1 production in human endothelial cells. However, in contrast to endothelial cells, which respond as potent to LPS as to TNF, HOMC responded much less to LPS than to TNF. HOMC also differed from human endothelial cells by the excessive production of PAI-2. The production of t-PA by HOMC, on the other hand, was in the same order of magnitude as that by large vein endothelial cells, which agrees well with a recent observation on tissue extracts of peritoneum and saphenous vein biopsies. The rate of t-PA production by HOMC markedly exceeds that by human umbilical vein endothelial cells.

In summary, our studies show that the epitheloid cells obtained from omental tissue are mesothelial in character and produce various regulators of fibrinolysis: t-PA, PAI-1, and PAI-2. The inflammatory mediator TNF can significantly decrease the fibrinolytic activity both by decreasing t-PA production and increasing PAI inhibition.

ACKNOWLEDGMENT

We thank Walter Fiers and Jan Tavernier (Biogent, Ghent, Belgium) for providing us with human recombinant TNF, and Reinier Schlingeman for a gift of PAL-E antibody. We thank Gerard Dooijewaard for assay of u-PA antigen, Jeanette OpdenBerg and Petra Turion for excellent technical assistance, and Clara and Marisa Horsting for typing of the manuscript.

REFERENCES

10. Benzer H, Blümel G, Prza F: Über zusammenhänge zwischen...
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VW van Hinsbergh, T Kooistra, MA Scheffer, J Hajo van Bockel and GN van Muijen