Fibrinolytic Properties of a Human Endothelial Hybrid Cell Line (EA.hy 926)

By J.J. Emeis and C.-J.S. Edgell

The fibrinolytic characteristics of the endothelial hybrid cell line EA.hy 926, established by fusing a human umbilical vein endothelial cell with a human carcinoma cell line, were studied. The hybrid cell line produced large amounts of tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor type 1, and a small amount of urokinase. All plasminogen activator present in conditioned medium was complexed with inhibitor because the cells secreted plasminogen activator inhibitor in excess over plasminogen activator and no activator activity was detectable in conditioned media by direct activity assays. t-PA activator activity was, however, demonstrable in conditioned media after treatment with sodium dodecyl sulfate, in agreement with t-PA antigen determinations. Increased plasminogen activator inhibitor activity could be induced by incubating the cells in the presence of endotoxin or microtubule inhibitors, whereas increased t-PA activity could be induced by microtubule inhibitors. Interleukin-1 had no effect. The fibrinolytic characteristics of the hybrid cell line were stable for at least 30 passages. The perpetual human hybrid cell line EA.hy 926 therefore may be a useful tool for the study of fibrinolysis in cultured endothelial cells.

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plasminogen and S-2251. Preliminary experiments had shown that antihuman t-PA IgG did not inhibit urokinase, that antihuman urokinase IgG did not inhibit t-PA, and that the amounts of IgG added were sufficient to completely quench the activity of 3 μL of t-PA (100 IU/mL) or 3 μL of urokinase (40 IU/mL) and thus were sufficient to inhibit all PA activity present in EA.hy 926-conditioned medium.

**PAI activity.** The PAI activity was measured as described by Emeis et al. and Verheijen et al. Inhibition of urokinase was measured in the same way. Inhibitor activity will be expressed as international units of t-PA inhibited by 1 mL of medium. Inhibitor activities were corrected for inhibition present in nonincubated control culture medium. The apparent inhibition constant (K\textsuperscript{app}) of the inhibitor for t-PA was determined by Lineweaver-Burk analysis of the data obtained by the method of Verheijen et al. Stability of the inhibitor was determined by measuring the inhibitor activity of conditioned medium after incubation for one to 24 hours at 37°C. The type of inhibitor was determined by preincubation of samples, before the PAI activity assay, in the presence of a quenching mouse monoclonal antibody against PAI type-1 (20 μg/mL), for ten minutes at 37°C.

**Antigen determinations.** Concentrations of t-PA antigen\textsuperscript{11} and urokinase antigen\textsuperscript{9} were measured by enzyme immunoassay. Antigen determinations were kindly performed by Dr D.C. Rijken and J.J.L. Verheijen–van Lensel of the Gaubius Institute.

**Determination of relative molecular weights.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli\textsuperscript{4} or Weber and Osborn\textsuperscript{5} in 8% slab gels with 100-μL samples of conditioned media. After SDS-PAGE, the fibrin autography procedure for the detection of PAs was performed according to Laskutoff and Mussoni.\textsuperscript{35} Reverse fibrin autography was performed according to Erickson et al.\textsuperscript{34}

**Materials.** Human urokinase and bovine thrombin were obtained from Leo Pharmaceuticals, Emmen, The Netherlands; purified human melanoma cell-derived t-PA was provided by Dr J.H. Verheijen (Gaubius Institute); rabbit antihuman uterine t-PA IgG was a gift from Dr D.C. Rijken, and goat antihuman urokinase IgG was from Drs G. Dooijewaard and D. Binnema, all from the Gaubius Institute. Mouse monoclonal anti–PAI type-1 IgG1 was obtained from Monozyme (Charlottenlund, Denmark). The reagents used in the spectrophotometric PA assay and in the PAI assay have been detailed elsewhere.\textsuperscript{11} Escherichia coli lipopolysaccharide (serotype 0128:B12), colchicine, and polymyxin B were obtained from Sigma Chemical Co (St Louis); vinblastine sulfate (Velban) from Eli Lilly & Co (Indianapolis); and human interleukin-1 (IL-1) from Genzyme (Haverhill, UK). Mouse recombinant IL-1α was a gift from Dr P. Lomedico (Hoffman-La Roche, Nutley, NJ).

**RESULTS**

**PAI.** Because endothelial cells are often known to secrete in vitro more PAI than PA, thus masking the detection of PA in most functional activator assays, we first measured the PAI activity of conditioned media. As shown in Fig 1, all EA.hy 926-conditioned media tested contained PAI activity. In DME-FBS, the inhibitor activity increased with incubation time up to 12 hours. In DME-BSA the inhibitor remained at a constant, much lower level.

EA.hy 926-conditioned DME-FBS inhibited both t-PA and urokinase; the correlation coefficient for t-PA inhibitor and urokinase inhibitor activities was .83 (n = 14). No urokinase inhibition was detectable in serum-free conditioned media. No inhibition of either t-PA or urokinase was detected in 24-hour–conditioned media from A549/8 cells.

By Lineweaver-Burke analysis (Fig 2), the K\textsuperscript{app} for the inhibitor of t-PA was 0.9 ± 0.4 × 10\textsuperscript{-12} mol/L (mean ± SD, n = 7), similar to the K\textsuperscript{app} measured in conditioned medium of human umbilical vein endothelial cells (0.8 ± 0.5 × 10\textsuperscript{-12} mol/L, n = 4) in parallel experiments.\textsuperscript{38}

The PAI present in EA.hy 926–conditioned media had a half-life in vitro of 2.5 hours during incubation at 37°C—similar to the half-life of 2.3 hours determined for PAI from human umbilical vein endothelial cells.\textsuperscript{44} The PAI activity in EA.hy 926–conditioned medium could be fully quenched by a monoclonal antibody against PAI type-1.\textsuperscript{45} It had a mol wt of approximately 50,000 as determined by reverse fibrin autography\textsuperscript{43} of EA.hy 926–conditioned DME-FBS, identi-
cal with that of PAl in human umbilical vein endothelial cell–conditioned medium. In conditioned medium from A549/8 cells a band of inhibitory activity was also detected at the same position; the intensity of this band was, however, much weaker.

PAl. In agreement with the presence of PAl activity in conditioned media, no PA activity was detected when conditioned media were tested directly in the spectrophotometric assay. After preincubation with SDS (final concentration, 21 mmol/L), the EA.hy 926–conditioned media did show PA activity. As shown elsewhere,35 SDS treatment of complexes between PA and PAl results in the reappearance of PA activity. The amount of PA activity detected in EA.hy 926–conditioned media after SDS treatment increased with time, both in DME-FBS and in DME-BSA (Fig 3A). The PA activity in all SDS-treated conditioned media could be quenched completely by preincubation of SDS-treated samples with anti–t-PA IgG (Table 1), although not by nonimmune IgG or antiurokinase IgG. Control assays demonstrated that the measured activity was plasminogen dependent and strongly potentiated by soluble fibrin. No PA activity was detected in A549/8-conditioned media.

By enzyme immunoassay, t-PA antigen was detected in EA.hy 926–conditioned media. The t-PA antigen concentrations increased with time, both in DME-FBS and in DME-BSA (Fig 3B). No t-PA antigen was found in A549/8-conditioned medium.

Small amounts of urokinase antigen (2 ng/mL) were detected in 24-hour–conditioned media from the EA.hy 926 cells and A549/8 cells (Table 1).

After SDS-PAGE (Laemmli system) and fibrin autography, conditioned media from EA.hy 926 cells showed a broad zone of PA activity of plasminogen-rich fibrin plates that extended from a mol wt of 100,000 down to a mol wt of 70,000 (Fig 4). After electrophoresis in Weber Osborn gels, PA activity occurred in two discrete regions corresponding to mol wts of 100,000 and 70,000 (not shown). Purified human t-PA showed a lysis zone at a mol wt of 70,000 and urokinase at 55,000 in both types of gel. EA.hy 926–conditioned media showed no lysis zone at a mol wt of 55,000; A549/8-conditioned media showed no lysis zones whatsoever. No lysis zones were present when plasminogen-free fibrin gels were used instead of plasminogen-containing fibrin gels. Incorporation of anti–t-PA IgG into the fibrin gel completely suppressed the formation of lysis zones by EA.hy 926–conditioned medium.

Cell extracts. In contrast to conditioned medium, Triton X-100 extracts of EA.hy 926 cells did show spontaneous PA activity (3 to 5 IU/mL for cells cultured in DME-BSA and about 0.5 IU/mg for DME-FBS). Extracts of A549/8 cells showed a similar spontaneous PA activity. The cellular PA activity was fully quenched by antihuman urokinase IgG but not by antihuman t-PA IgG, which is in contrast to the situation in conditioned media. SDS treatment of cell extracts did not induce any further PA activity, again in contrast to conditioned media. Incubation of cells with thrombin (5 National Institutes of Health Units/mL in DME-BSA) for ten minutes followed by extraction of cells completely suppressed PA activity in cell extracts.

Stability of the EA.hy 926 fibrinolytic properties. To judge the stability of the fibrinolytic properties of the cells with passage, EA.hy 926–conditioned media were again studied after a further 30 passages. As can be seen in Table 2, both the PA activity and the PAl activity in conditioned media had remained unchanged with increasing passage number.

Effect of endotoxin and IL-1 on PAl activity. Incubation of EA.hy 926 cells in the presence of E. coli endotoxin
Table 1. Summary of Fibrinolytic Characteristics of Conditioned Media (24 Hours) From EA.hy 926 and A549/8 Cells

<table>
<thead>
<tr>
<th>Spontaneous PA activity</th>
<th>EA.hy 926 DME-FBS (n = 5)</th>
<th>EA.hy 926 DME-BSA (n = 5)</th>
<th>A 549/8 DME-FBS or DME-BSA (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA activity after SDS treatment (IU/mL)</td>
<td>None</td>
<td>42 ± 4*</td>
<td>None</td>
</tr>
<tr>
<td>Quenching of PA activity by anti-PA IgG (%)</td>
<td>None</td>
<td>97 ± 4*</td>
<td>NA</td>
</tr>
<tr>
<td>t-PA antigen (ng/mL)</td>
<td>91 ± 30*</td>
<td>56 ± 8*</td>
<td>None</td>
</tr>
<tr>
<td>u-PA antigen (ng/mL)</td>
<td>1.9 ± 0.9*</td>
<td>1.0 ± 0.7*</td>
<td>3.6 ± 2.8*</td>
</tr>
<tr>
<td>PAI activity (IU/mL)</td>
<td>44 ± 11*</td>
<td>6 ± 2*</td>
<td>None</td>
</tr>
<tr>
<td>Mol wt of PA</td>
<td>70,000-100,000</td>
<td>70,000-100,000</td>
<td>50,000</td>
</tr>
<tr>
<td>Mol wt of PAI</td>
<td>50,000</td>
<td>50,000</td>
<td>50,000</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

*Mean ± SD.

0.1 to 10.0 µg/mL resulted in significantly increased PAI activity in conditioned DME-FBS (Table 3). Only an insignificant increase in PAI activity was found when EA.hy 926 cells were cultured in DME-BSA in the presence of endotoxin. A similar effect of endotoxin on PAI production has been described for human umbilical vein endothelial cells. In contrast to umbilical vein endothelial cells, EA.hy 926 cells did not respond to human IL-1 (0.1 to 10.0 U/mL) with increased PAI synthesis or mouse recombinant IL-1α (1,000 U/mL). Neither endotoxin nor IL-1 influenced PA activity.

Effect of microtubule inhibitors. The microtubule inhibitor colchicine (50 and 100 nmol/L) induced significant increases in both PAI activity and t-PA activity in conditioned media (Table 4). Vinblastine (25 nmol/L) had a similar effect as colchicine on t-PA activity but did not affect PAI activity (Table 4). The addition of polymyxin B (10 µg/mL) did not influence baseline or inhibitor-induced levels of PAI and t-PA activity, which demonstrates that the effects were not due to endotoxin contamination of the microtubule inhibitors used.

**DISCUSSION**

The study of the differentiated properties of endothelial cells in vitro is hampered by the difficulty of reproducibly obtaining adequate numbers of cells necessary for such studies. These difficulties are partly due to the relatively small numbers of cells obtainable from a single source of endothelial cells (e.g., umbilical cord) and to the limited life span of endothelial cells in culture, although the use of endothelial cell growth factors has reduced some of these problems. Other difficulties stem from changes in cell characteristics during long-term culture including changes with regard to fibrinolytic characteristics. Stable endothelial cell lines could help to solve some of these problems, provided that such cell lines retain the differentiated endothelial characteristics being studied. The hybrid human endothelial cell line EA.hy 926 has previously been shown to synthesize and secrete von Willebrand factor and has a normal intracellular distribution of this protein as demonstrated by immunofluorescence. The cell line also expresses thrombomodulin activity and antigen, sustains basal and stimulated levels of prostacyclin production, and produces platelet-activating factor.

In the present study, we demonstrate that this hybrid cell line secretes t-PA, as shown by the plasminogen activation.
Table 3. Effect of Incubation with E coli Lipopolysaccharide on PAI Activity in EA.hy 926–Conditioned Medium

<table>
<thead>
<tr>
<th>E. coli Lipopolysaccharide (μg/mL)</th>
<th>PAI Activity (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>45 ± 17*</td>
</tr>
<tr>
<td>0.1</td>
<td>70 ± 13</td>
</tr>
<tr>
<td>0.5</td>
<td>112 ± 16†</td>
</tr>
<tr>
<td>1.0</td>
<td>116 ± 15†</td>
</tr>
<tr>
<td>5.0</td>
<td>138 ± 35†</td>
</tr>
<tr>
<td>10.0</td>
<td>139 ± 41†</td>
</tr>
</tbody>
</table>

Cells were incubated for 24 hours in DME-FBS containing the indicated concentration of lipopolysaccharide.

*Mean ± SD. n = 5.
†Significantly different from control (P < .05) by analysis of variance followed by Bonferroni's modified t test (Wallenstein et al46).

Table 4. Effect of Vinblastine and Colchicine on Fibrinolytic Characteristics of EA.hy 926–Conditioned Medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>t-PA Activity (IU/mL)</th>
<th>PAI Activity (IU/mL)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DME-FBS only)</td>
<td>34 ± 4†</td>
<td>29 ± 4</td>
<td>8</td>
</tr>
<tr>
<td>+ Colchicine (50 nmol/L)</td>
<td>40 ± 5†</td>
<td>40 ± 5†</td>
<td>4</td>
</tr>
<tr>
<td>+ Colchicine (100 nmol/L)</td>
<td>56 ± 6†</td>
<td>67 ± 9†</td>
<td>6</td>
</tr>
<tr>
<td>+ Vinblastine (25 nmol/L)</td>
<td>40 ± 1†</td>
<td>32 ± 3</td>
<td>4</td>
</tr>
<tr>
<td>+ Endotoxin (1 μg/mL)</td>
<td>30 ± 3</td>
<td>59 ± 10†</td>
<td>6</td>
</tr>
<tr>
<td>+ Endotoxin and colchicine (50 nmol/L)</td>
<td>43 ± 3‡§</td>
<td>80 ± 11‡</td>
<td>4</td>
</tr>
<tr>
<td>+ Endotoxin and colchicine (100 nmol/L)</td>
<td>53 ± 5‡§</td>
<td>119 ± 19‡§</td>
<td>4</td>
</tr>
<tr>
<td>+ Endotoxin and vinblastine (25 nmol/L)</td>
<td>49 ± 14‡§</td>
<td>81 ± 16‡§</td>
<td>4</td>
</tr>
</tbody>
</table>

Cells (two passages, two to four cups per experimental variable) were cultured for 24 hours in DME-FBS containing the indicated concentrations of additives.
†Mean ± SD.
‡Significantly different from control (P < .05) by analysis of variance for two-way factorial design followed by Bonferroni’s modified t test.46
§Significantly different from control plus endotoxin (P < .05) by analysis of variance.
endotoxin, thus showing that the high level of t-PA and PAI activity in EA.hy 926 cells, both in the presence and absence of endotoxin, thus showing that the high level of t-PA and PAI production by EA.hy 926 cells can still be increased further.

As is evident from the SDs in Tables 1 to 3, the values found for t-PA and PAI varied from one cell culture to another. Still (Table 2), no change in the mean level of these parameters was seen with increasing passage numbers, thus showing that these parameters do not change with passage number. Within one culture, the levels of t-PA and PAI showed little variability, as can be seen in Table 4 in which data from two cultures are combined, which results in showing that these parameters do not change with passage numbers, thus showing for other endothelial cell-specific functions. The hybrid cell line differs from endothelial cells in not being responsive to IL-1. We suggest that the hybrid cell line EA.hy 926 may prove to be a useful additional tool for the study of endothelial cell fibrinolysis in vivo.

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