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.\(^\text{13}\) and Verheijen et al.\(^\text{13}\) 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\(_{\text{app}}\)) of the inhibitor for t-PA was determined by Lineweaver-Burk analysis of the data obtained by the method of Verheijen et al.\(^\text{13}\) 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\(^\text{15}\) and urokinase antigen\(^\text{16}\) were measured by enzyme immunoassay. Antigen determinations were kindly performed by Dr D.C. Rijken and J.J.L. Verheijen–vanlersel of the Gaubius Institute.

**Determination of relative molecular weights.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli\(^\text{24}\) or Weber and Osborn\(^\text{25}\) in 8% slab gels with 10-μL samples of conditioned media. After SDS-PAGE, the fibrin autorgraphy procedure for the detection of PAs was performed according to Laskutoff and Mussoni.\(^\text{26}\) Reverse fibrin autorgraphy was performed according to Erickson et al.\(^\text{27}\)

**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.\(^\text{28}\) *Escherichia coli* lipopolysaccharide (serotype O128: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.

**Fig 1.** PAI activity in conditioned medium from EA.hy 926 cells after incubation for various periods of time in DME-BSA (B) or DME-FBS (A). Data shown are means ± SEM (n = 3 to 5). For assay procedure, see Verheijen et al.\(^\text{13}\)

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

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.\(^\text{44}\) The PAI activity in EA.hy 926–conditioned medium could be fully quenched by a monoclonal antibody against PAI type-1.\(^\text{45}\) It had a mol wt of approximately 50,000 as determined by reverse fibrin autorgraphy of EA.hy 926–conditioned DME-FBS, identi-
cal with that of PAI 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.

PAI. In agreement with the presence of PAI 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, SDS treatment of complexes between PA and PAI 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 PAI activity in conditioned media had remained unchanged with increasing passage number.

Effect of endotoxin and IL-1 on PAI activity. Incubation of EA.hy 926 cells in the presence of E. coli endotoxin...
Fig 4. Fibrin autography after SDS-PAGE (Laemmli system) of (A) 10 μL of 24-hour-conditioned DME-FBS medium from EA.hy 926 cells, (B) 10 μL of 24-hour-conditioned medium (medium 199 plus 20% human serum) of primary human umbilical vein endothelial cells, (C) human t-PA (6 mIU), and (D) human high-mol wt urokinase (140 mIU).

Table 1. Summary of Fibrinolytic Characteristics of Conditioned Media (24 Hours) From EA.hy 926 and A549/8 Cells

<table>
<thead>
<tr>
<th>Cell Medium</th>
<th>DME-FBS (n = 5)</th>
<th>DME-BSA (n = 5)</th>
<th>A549/8 DME-FBS or DME-BSA (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous PA activity</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>PA activity after SDS treatment (IU/mL)</td>
<td>42 ± 4*</td>
<td>22 ± 7*</td>
<td>None</td>
</tr>
<tr>
<td>Quenching of PA activity by anti-PA IgG (%)</td>
<td>100</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>NA</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.

Table 2. Fibrinolytic Characteristics of EA.hy 926–Conditioned Media at Various Passage Numbers

<table>
<thead>
<tr>
<th>Medium (24 hr)</th>
<th>DME-FBS</th>
<th>DME-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI activity (IU/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Passages 24-26 (n = 5)*</td>
<td>44 ± 11†</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>B. Passages 50-59 (n = 6)</td>
<td>36 ± 5</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>PA activity (IU/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Passages 24-26</td>
<td>42 ± 4</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>B. Passages 50-59</td>
<td>36 ± 6</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

*EA.hy 926 cells that had been cultured for 34 passages and subsequently cloned were used after a further 24 to 26 passages (A) or 50 to 59 passages (B). †Mean ± SD. None of the differences between A and B was statistically significant (Student’s t test).
followed by Bonferroni’s modified test (Wallenstein et al).

The presence of such complexes with mol-PA cell-derived t-PA may be ascribed to dissociation of the assay (after SDS treatment) and by the immunoassay for t-PA antigen. The PA found in conditioned medium is further differentiated from urokinase by its dependence on soluble fibrin in the spectrophotometric assay system and by its quenching by anti-t-PA IgG but not by antiturokinase IgG. The absence of spontaneous PA activity in conditioned media can be ascribed to the secretion of excess PAI, which results in the formation of t-PA–PAI complexes devoid of PA activity. The presence of such complexes with mol ws of 100,000 and sensitivity to anti-t-PA IgG was demonstrated by fibrin autography. The presence of t-PA activity at lower amounts of t-PA produced because human endothelial cells in 24 hours, see Table 1 being within the range reported for human endothelial cells.

The amount of t-PA produced by human endothelial cells is highly variable and ranges from 4 to 8 to over 300 ng/10^6 cells in 24 hours, depending among other things on the source of the endothelial cells and the passage number. With respect to PA production, the EA.hy 926 cells thus resemble cultured human endothelial cells in that they secrete preponderantly t-PA, the amount of t-PA secreted (80 to 120 ng/10^6 cells in 24 hours, see Table 1) being within the range reported for human endothelial cells.

The PAI from EA.hy 926 cells closely resembled PAI secreted by primary human endothelial cells in culture, both with regard to its K_i, its instability in vitro at 37°C, its mol wt of approximately 50,000, and its quenching by a monoclonal antibody against PAI type-I. The PAI secreted by EA.hy 926 cells can thus be described as PAI type-1.

Endotoxin has been shown to induce increased synthesis of PAI in cultured human endothelial cells and is here shown to do so likewise in EA.hy 926. In contrast to other studies, however, IL-1 (either human or murine) had no effect on PAI activity in conditioned medium from EA.hy 926 cells.

The induction of PAI in endothelial cells in many ways resembles the induction in these cells of procoagulant activity, eg, in being inducible by endotoxin, IL-1, tumor necrosis factor, and phorbol ester. Because Nawroth et al. had shown that endotoxin-induced procoagulant synthesis in bovine aortic endothelial cells was decreased by the microtubule inhibitors colchicine and vinblastine and because Shepro et al. have shown that colchicine can decrease the u-PA activity in endothelial cells, the results suggest that the cell-bound u-PA activity in bovine aortic endothelial cells, abolished by colchicine, may be due to a difference in u-PA activity.

The differences seen in Fig 4 between the fibrin autographic patterns of PA activity produced by EA.hy 926 cells and umbilical vein endothelial cells might suggest a difference between cell types with regard to PA or PAI produced. The difference is, however, likely due to the difference in absolute amounts of t-PA produced because human endothelial cells that produce large amounts of t-PA have a similar pattern of activity on fibrin autography as do EA.hy 926 cells (see, eg, Fig 6 in Van Hinsbergh et al and Fig 2 in Van Hinsbergh).

The small amount of urokinase antigen in EA.hy 926–conditioned media is similar to the amount present in A549/8-conditioned medium, which suggests that it could be a property of the hybrid cell derived from the tumor cell parent. Reports on the secretion of urokinase by human endothelial cells in vitro suggest that production of urokinase can be either present or absent, presumably depending on the condition of the cells and the passage number. In most studies on primary cells, however, no urokinase secretion was detected. In conditioned media, urokinase was only detectable by immunoassay. Incubation of cells with thrombin, a procedure that has been shown to reduce cellular PA activity in bovine aortic endothelial cells, abolished cellular u-PA activity. This suggests that the cell-bound u-PA activity may be due to pro–u-PA, which is inactivated by thrombin.

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had demonstrated that colchicine inhibited PA activity in these cells, the effect of these compounds was also tested. The two compounds induced increased levels 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 different cultures are combined, which results in coefficients of variation similar to the interassay coefficients of variation of the activity assays (about 10%).

Because both the hybrid cell line and the parent cell line A 549/8 produced a small amount of u-PA (presumably pro-u-PA), this property of the hybrid cell line may have descended from the A 549/8 cell parent, although (see earlier) many cultured endothelial cells also secrete u-PA in vitro. The production of a small amount of latent PAI by A 549/8 cells, as shown by reverse fibrin autography, was unexpected in view of the absence of active PAI in conditioned media and should be kept in mind when interpreting drug effects on hybrid PAI production.

In most respects (secretion of t-PA; secretion of PAI type-1; increased synthesis of these components in the presence of serum; enhanced production of PAI in the presence of endotoxin), the EA.hy 926 hybrid cell line resembles cultured human vascular endothelial cells, as has also been shown 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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