Prostacyclin Expression by a Continuous Human Cell Line Derived From Vascular Endothelium

By Joseph E. Suggs, Michael C. Madden, Mitchell Friedman, and Cora-Jean S. Edgell

Prostacyclin is primarily an endothelial cell product. It contributes to the important role of endothelium in maintaining the fluidity of blood by inhibiting platelet aggregation and by promoting vasodilation. Endothelial cells in culture tend to senesce, and the level of prostacyclin expression decreases. A permanent human cell line, EA.hy 926, derived from a fusion of primary endothelial cells with cells of a less differentiated line, has been found to sustain basal and stimulated levels of prostacyclin synthesis.© 1986 by Grune & Stratton, Inc.

ARACHIDONIC ACID metabolites are autacoids that modulate cellular functions near the site at which they are produced. These structurally related metabolites have a wide range of physiological effects, some of which oppose others. Two arachidonic acid metabolites affect platelet aggregation at a site of vascular injury. Aggregation is promoted by platelet thromboxane A2 but it is limited by prostacyclin produced by endothelial cells. Thrombin induces prostacyclin production by endothelial cells, and these cells can also convert precursors released by aggregated platelets to prostacyclin. Thrombosis may thus be limited by prostacyclin from downstream endothelial cells. Prostacyclin is the major prostaglandin produced by endothelial cells; relatively, they produce much more of this eicosanoid than do other cell types. The cell type-specific synthesis and the short half-life of prostacyclin contribute to the locational control of its effects.

In addition to inhibiting platelet aggregation, prostacyclin acts as a potent vasodilator by causing a relaxation response in vascular smooth muscle. Agents such as angiotensin II, bradykinin, histamine, and thrombin can stimulate endothelial cells to produce prostacyclin. Thus, the endothelial cell lining of the vascular system facilitates blood flow by producing prostacyclin-mediated vasodilation as well as by prevention of excessive platelet aggregation.

The study of prostacyclin synthesis and regulation in isolated endothelial cells is hindered by the limited replication potential of these cells and their tendency to senescence in culture with the production of prostacyclin deteriorating in subcultures. Therefore, the potential for sustained expression of prostacyclin by a permanent cell line derived from human umbilical vein endothelial cells (HUV-EC) was investigated. This cell line, EA.hy 926, is a hybrid resulting from fusion of primary endothelial cells with cells selected from the continuous human line A549. The EA.hy 926 line represents cells that were clonally isolated following hybridization and subcloned again at passage 35. Since subcloning, the cells have been passaged at least 30 times more. The cells sustain expression of many differentiated functions of endothelium after exceeding 200 cumulative population doublings in culture. These functions include von Willebrand factor, thrombomodulin, and tissue plasminogen activator. Data presented here demonstrate that this cell line also produces basal levels of prostacyclin and responds to physiological stimulation to produce higher levels.

METHODS

Cell culture. EA.hy 926 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin at 100 U/mL, streptomycin at 100 μg/mL, and in the presence of HAT (100 μmol/L of hypoxanthine, 0.4 μmol/L of aminopterin, 16 μmol/L of thymidine). These cells were generally subcultured weekly at a relative cell density of 1:20. The EA.hy 926 cells used in these studies had been passaged more than 60 times. A549/8, one of the progenitor strains of the EA.hy 926 line, is a thioguanine-resistant strain selected from the human lung adenocarcinoma line established by Lieber and co-workers. It was grown in RPMI 1640 supplemented with 7% Nu-Serum (Collaborative Research, Bedford, Mass), penicillin at 100 U/mL, streptomycin at 100 μg/mL, and in the presence of 0.1 μmol/L of thioguanine.

HUV-EC were isolated as described by Gimbrone and grown in RPMI 1640 supplemented with 15% fetal bovine serum, bovine hypothalamus extract, penicillin at 100 U/mL and streptomycin at 10 μg/mL. Unpassaged cultures of HUV-EC were used in these studies for comparison with the EA.hy 926 cell line.

Sample preparation and radioimmunoassay. confluent cell layers in multiwell plates were rinsed twice with unsupplemented RPMI 1640 medium 48 to 72 hours after the previous change of serum-containing culture medium and were then incubated in RPMI 1640 at 0.2 mL/cm². After 1 hour, the medium samples were collected from each culture to represent its basal level of prostacyclin production. Bovine thrombin (Parke-Davis, Morris Plains, NJ, NDC 0071-1356-01) was then added to each culture at 0.5 U/mL in RPMI 1640, and samples were collected after a 5-minute incubation to represent the level of prostaglandins released by stimulated cells. All samples were centrifuged to remove cellular debris, and stored at −70 °C for immunological analysis.

A radioimmunoassay (RIA) method (New England Nuclear, Boston) was used to assess levels of prostacyclin in these samples as 6-keto-PGF1α, the stable derivative. The RIA uses 1H-labeled 6-keto-PGF1α, and was capable of distinguishing between standards of 5 pg/100 mL and 10 pg/100 mL. After the culture supernatant samples were removed, the protein contents of cell layer were determined so that results from cell lines that grow to different cell densities could be normalized in terms of protein mass. Trypsinized cells were lysed in water, and the protein content was measured by the Bradford assay.

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of the extract dissolved dissolved in methanol 200 mL/25 cm² of confluent cells. Recovery of evaporated in a stream of nitrogen at 45 °C, and the residue was identified by comparing their elution volumes with those of metabolites were detected by scintillation counting. The components were purified standards chromatographed separately and detected by radioimmunoassay (RIA) data represent the amounts of prostacyclin produced in 1 hour under basal conditions divided by 12 (or amounts per 5 minutes) and the amounts produced within 5 minutes after adding thrombin. The mean level ± the SD for a number (n) of parallel cultures is presented for each cell type. The human umbilical cord endothelial cells (HUV-EC) were primary cultures derived from a single umbilical cord, the EA.hy 926 cultures were from passage 67.

High-performance liquid chromatography (HPLC) analysis. To radiolabel arachidonic acid metabolites for detection following HPLC fractionation, rinsed 25-cm² monolayers were incubated in the presence of 5 μCi of ³H-arachidonic acid at 50 Ci/mmol (New England Nuclear) in 5 mL of RPMI 1640. After 19 hours, the exogenous arachidonic acid solution was removed, the cultures were rinsed twice, and fresh RPMI 1640 was added. This medium was collected after 1 hour to represent the basal production of arachidonic acid metabolites. It was replaced with 5 mL of RPMI 1640 containing 0.5 U/mL of thrombin, which was collected after 5 minutes to represent the arachidonic acid metabolites produced by stimulated cells.

For HPLC fractionation, the arachidonic acid metabolites were extracted from RPMI 1640 into organic solvents. The RPMI 1640 samples were first adjusted to pH 3.5 with 9% formic acid, then extracted with 2 vol cyclohexane/ethyl acetate (1:1), followed by 2 vol of ethyl acetate. The combine extracts of each sample were evaporated in a stream of nitrogen at 45 °C, and the residue was dissolved in methanol 200 μL/25 cm² of confluent cells. Recovery of radiolabeled 6-keto-PGF₁α through this extraction procedure was 94%.

Arachidonic acid metabolites were fractionated by the HPLC method of VanRollins and colleagues using a column of Altex 5-μm Ultrasphere ODS particles and a gradient of acetonitrile in aqueous phosphoric acid pH 2 for elution. Each injected sample was 150 μL of the extract dissolved in methanol. Fractions containing ³H-labeled metabolites were detected by scintillation counting. The components were identified by comparing their elution volumes with those of purified standards chromatographed separately and detected by ultraviolet (UV) absorbance.

RESULTS

EA.hy 926 cells were found to produce relatively high levels of prostacyclin as determined by the RIA data shown in Table 1. The results per mass of cell protein are presented for the purpose of comparing the hybrid cell line with its two progenitor cell types, all of which have different cell sizes and culture densities at confluence. Under basal conditions, the hybrid cells produced much more prostacyclin than A549/8 cells, although less than primary cultures of HUV-EC. The EA.hy 926 cells also responded strongly to thrombin stimulation to produce 63-fold more prostacyclin. The response of HUV-EC was even stronger than that of the hybrid cells, but the response of A549/8 cells was much weaker.

The HPLC method also demonstrated that the 6-keto-PGF₁α prostacyclin derivative was a product of EA.hy 926 cells, as shown in Fig 1. The amount produced under basal conditions was substantially increased by thrombin stimulation. Low levels of certain other products of the cyclooxygenase pathway were also detectable in samples from EA.hy 926 cells and from HUV-EC cells, but the stable prostacyclin derivative was the major component. As expected, primary cultures of HUV-EC produced even more prostacyclin, but even with stimulation A549/8 cells did not produce levels detectable by this method.

Prostacyclin release by EA.hy 926 cells could be stimulated by melittin and by arachidonic acid as well as by thrombin. When cells that had been stimulated with 20 mmol/L of arachidonic acid were washed and then restimulated within 15 minutes, the amount of prostacyclin released was only 55% of that released at the initial stimulation. Dejana and colleagues have shown that cells are refractory to restimulation for several hours and have suggested that cyclooxygenase may be irreversibly inactivated by arachidonic acid metabolism. The Table 2. Prostacyclin Production by Different Cell Types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>6-keto-PGF₁α (ng/10⁶ cells/h)</th>
<th>Species</th>
<th>Anatomic Source</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Endothelium</td>
<td>1.47ng/10⁶ cells/h</td>
<td>Cow</td>
<td>Pulmonary artery</td>
<td>30</td>
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<tr>
<td>Endothelium</td>
<td>20 ng/10⁶ cells/h</td>
<td>Cow</td>
<td>Pulmonary artery</td>
<td>31</td>
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<tr>
<td>Smooth muscle</td>
<td>0.175 ng/10⁶ cells/h</td>
<td>Rabbit</td>
<td>Pulmonary artery</td>
<td></td>
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<tr>
<td>Fibroblastic</td>
<td>0.085 ng/10⁶ cells/h</td>
<td>Human</td>
<td>Embryonic lung</td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>0.175 ng/10⁶ cells/h</td>
<td>Rat</td>
<td>Prostate</td>
<td></td>
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<tr>
<td>Endothelium</td>
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<td>Umbilical vein</td>
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<tr>
<td>Endothelium</td>
<td>14.4 ng/10⁶ cells/h</td>
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<td>32</td>
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<td>Endothelium</td>
<td>29.2 ng/10⁶ cells/h</td>
<td>Human</td>
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<tr>
<td>Endothelium</td>
<td>21.6 ng/10⁶ cells/h</td>
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<td>Pulmonary artery</td>
<td></td>
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<tr>
<td>Endothelium</td>
<td>27 ng/10⁶ cells/h</td>
<td>Pig</td>
<td>Aorta</td>
<td>33</td>
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<tr>
<td>Endothelium</td>
<td>8.4 ng/10⁶ cells/h</td>
<td>Cow</td>
<td>Aorta</td>
<td>34</td>
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<tr>
<td>Smooth muscle</td>
<td>0.5 ng/10⁶ cells/h</td>
<td>Rabbit</td>
<td>Aorta</td>
<td>35</td>
</tr>
<tr>
<td>Macrophage</td>
<td>0.035 ng/10⁶ cells/h</td>
<td>Mouse</td>
<td>Peritoneum</td>
<td></td>
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</tbody>
</table>

*Published values for the amount of prostacyclin derivative measured in medium from several cell types isolated from various species.

*Data from this reference were converted to comparable units by assuming the use of 5 mL of medium per 20-cm² culture dish.
Fig 1. Chromatographic identification of arachidonic acid metabolites generated by three different cell types under basal and thrombin-stimulated conditions. Culture supernatants were extracted and analyzed by high-pressure liquid chromatography (HPLC) following incorporation of \(^{3}H\)-arachidonic acid by confluent cultures of each cell line. The elution positions of purified standards are indicated.

**DISCUSSION**

It is clear that the premanent EA.hy 926 cells have retained the capacity to produce prostacyclin in a range close to that produced by HUV-EC in primary cultures. This trait is presumably inherited from the endothelial cell progenitor of the hybrid cell line; thus, prostacyclin expression has been sustained in the EA.hy 926 line for hundreds of cell generations. Although the levels of prostacyclin fluctuated when measured on different occasions, no consistent trend was detectable in the results of various experiments performed with the hybrid line over a 6-month interval. The amount of prostacyclin released by HUV-EC also varied from one primary culture to another, and other researchers have noted variability among parallel cultures of the same HUV-EC preparation. The EAhy 926 cells are like HUV-EC in this respect. The variability may reflect conditions that are not well controlled by conventional cell culture methods. In contrast to EA.hy 926 cells and HUV-EC, the A549/8 cells
produced relatively very little prostacyclin under basal or stimulated conditions, as expected for most nonendothelial cell types. Data from several investigators have indicated that substantial levels of prostacyclin production is a relatively specialized property of endothelial cells, although there is variability in the results from different laboratories and from different species. Some of these data are summarized in Table 2. In comparable units, the EA.hy 926 cells produce the prostacyclin derivative at a basal level of 1 to 4 ng/10⁶ cells per hour.

In addition to prostacyclin, endothelial cells produce other arachidonic acid metabolites, the spectrum of which is relatively specific for this cell type. Some of these metabolites can be identified in the HPLC analyses, as indicated in Fig 1. PGF₂α and HHT are common to stimulated EA.hy 926 cells and HUV-EC, but not detectable in medium from nonendothelial cell type A549/8. Additional similarities were observed between primary HUV-EC and EA.hy 926 cells in contrast to A549/8 cells in the regions of the chromatograms where the hydroxyeicosatetraenoic acids (HETEs) elute (data not shown).

The vigorous growth, unlimited replication potential, and clonal purity of the human endothelial cell-derived EA.hy 926 line could facilitate studies of the pathways and regulation of prostacyclin biosynthesis.

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