Properties of Cultured Endothelium from Adult Human Vessels

Glenna Fry, Thomas Parsons, John Hoak, Helene Sage, Roger D. Gingrich, Louis Ercolani, Dai Nghiem, and Robert Czervionke

Endothelium was isolated from samples of aorta and vena cava obtained from cadaver donors at the time kidneys were harvested for transplantation. Digestion with collagenase and gentle swabbing were used to free the cells from the intimal surface. Low density seeding permitted isolation of individual colonies with typical endothelial morphology. Modified Medium 199 supplemented with 10%–20% human plasma-derived serum and an extract from the bovine hypothalamus (500 μg/ml) enabled subcultured colonies to grow to confluency when culture surfaces were coated with fibronectin (1 μg/cm²). The presence of Factor VIII antigen was demonstrated using an indirect immunofluorescence technique. A monoclonal antibody to cultured umbilical vein endothelium, specific for endothelium, reacted with the subcultured cells from the aorta and vena cava. Type IV procollagen, fibronectin, and thrombospondin were identified as labeled proteins secreted by cultures of adult endothelium that had been incubated with 3H-proline and 3H-glycine. When the cultured endothelium was used in a sodium-m-periodate stimulated T lymphocyte mitogenic culture system, the endothelium exhibited accessory cell function. Prostacyclin production stimulated by incubation with arachidonic acid and PGE₂ was variable from vessel to vessel. However, average values were lower than normally seen with cultured primary umbilical vein endothelium. (Arteriosclerosis 4:4-13, January/February 1984)

Many discoveries concerning endothelial function have been made in recent years following the development of methods for culturing endothelium. Umbilical vein endothelium has been used by many groups while others have studied endothelium from the bovine aorta, pulmonary artery, or vein. Both qualitative and quantitative differences have been observed in functional assays with endothelial cells from these different sources. We have examined adult endothelium under similar conditions to ascertain its functional characteristics in culture.

From the Cardiovascular Center and the Departments of Medicine and Surgery, University of Iowa, College of Medicine, Iowa City, Iowa and the Department of Biological Structure, University of Washington, Seattle, Washington.

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Address for reprints: John C. Hoak, M.D., Department of Medicine, Division of Hematology-Oncology, University of Iowa Hospitals, Iowa City, Iowa 52242.

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Materials

The hypothalamic extract was prepared by a modification of the method of Maciag et al. Minced bovine hypothalamus (Pel Freez Biologicals, Rogers, Arkansas) was blended with 0.1 M NaCl for 3 minutes in an ice-cooled blender chamber. The mixture was stirred for 2 hours on ice before centrifugation at 14,727 × g. The resulting supernatant was adjusted to 30% saturation with ammonium sulfate and stirred.
CULTURED ADULT HUMAN ENDOTHELIUM  Fry et al.  5

for 30 minutes on ice. The mixture was centrifuged 30 minutes at 14,727 \times g at 4°C. The supernatant was adjusted to 90% saturation with ammonium sulfate, stirred, and centrifuged as above. The resulting precipitate was dissolved in 0.05 M Tris-HCl, 0.5 mM ethylenediamine tetra-acetic acid (EDTA) pH 7.5. The solution was dialyzed against the same buffer at 4°C until no trace of ammonium sulfate was found in the dialysis fluid. The dialyzed material was lyophilized and stored at -20°C.

Human plasma-derived serum (HPDS) was prepared according to the method of Vogel, et al.4 through the recalcification of platelet-poor plasma. The resulting serum was dialyzed against Ringer’s solution, heated to 56°C for 30 minutes, centrifuged at 27,000 \times g for 20 minutes and filter-sterilized with 0.2 \mu m filters. The serum was stored at -20°C.

Human fibronectin was purchased from Collaborative Research, Incorporated, Waltham, Massachusetts. The source and preparation of other materials used in the culture procedures have been described elsewhere.5 Rabbit antiserum to 6-keto-prostaglandin F, (6-keto-PGF,) was prepared according to the method of Smith et al.6 IgGsorb was obtained from the Enzyme Center, Boston, Massachusetts. Unlabeled arachidonic acid was from NuChek Prep, Incorporated, Elysian, Minnesota. PGH2 was a gift from Robert Gorman, the Upjohn Company, Kalamazoo, Michigan. Authentic prostaglandin standards were obtained from the Upjohn Company or Sigma Chemical Company, St. Louis, Missouri. The calcium ionophore A23187 and rabbit antiserum to human Factor VIII antigen were purchased from Cappel Laboratories, Cochranville, Pennsylvania. Sodium ibuprofen was also a gift from Upjohn. Tyrode 3 microcarriers, Ficoll-Hypaque, and Percoll polyvinylpyrrolidone-coated colloidal silica particles were obtained from Pharmacia, Piscataway, New Jersey. Na-mperiodate was from the Sigma Chemical Company, St. Louis, Missouri. Medium 199 with 25 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), RPMI 1640 medium, phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were obtained from Grand Island Biological Company, Grand Island, New York. Nylon wool was a gift from the DuPont Nemours Company, Wilmington, Delaware. Barbitone buffer was from Oxoid Limited, England. 3H-thymidine (15.2 Ci/m mole), 2,3 3H-proline (16-35 Ci/m mole) and 2-3H-glycine (53.3 Ci/m mole) from New England Nuclear, Boston, Massachusetts. Other reagents used have been previously described.5,7

Cultures of Endothelium

Aorta and vena cava samples were collected by the surgical transplant team when kidneys were removed for transplantation. The vessels were opened lengthwise, rinsed with Dulbecco’s phosphate buffered saline (PBS), and covered with a gauze sponge soaked in 0.1% collagenase in PBS. After a 20-minute incubation at 28–32°C, the sponge was removed and rinsed in modified Medium 199 (MM-199) with 20% fetal bovine serum (FBS). The vessel surface was wiped gently with a sterile swab moistened with the above medium. The swab was rinsed in a fresh tube of MM-199 with 20% FBS to remove the accumulated cellular material. The cell suspension was centrifuged at 300 \times g for 10 minutes. The cell pellets were resuspended in the MM-199 with 20% FBS and plated in 96 well tissue culture plates or 75 cm² tissue culture flasks (Falcon Plastics, Oxnard, California). After 24 hours the medium was removed and replaced with MM-199 with 10–20% human plasma-derived serum (HPDS) and 500 \mu g/ml hypothalamic extract (E). The medium was changed once a week. Conditions used to grow the adult endothelium were determined by testing the effect of FBS, HPDS, and hypothalamic extract on growth of primary umbilical vein endothelial cultures. The cell number in cultures containing the hypothalamic extract was 2–3 times that with serum alone within 96 hours. There was a slight, but significant, increase in the cell number when HPDS was used in place of FBS. Significantly fewer colonies were obtained when FBS and hypothalamic extract were used at the low seeding density. Attachment of cells transferred from primary to secondary cultures was enhanced by coating the surface with 1 \mu g fibronectin per cm² area. When clusters of adult cells with endothelial morphology reached 2–3 mm in diameter they were transferred to 12 well tissue culture plates (Linbro Plastics, Flow Laboratories, MacLean, Virginia) or 25 cm² flasks coated with fibronectin. The clusters were isolated with cloning rings when in flasks, and in the 96 well plates the only wells selected were those free of contamination by other cell types. Cells were removed with 0.25% trypsin-0.05% EDTA. The suspension was diluted with MM-199 with 20% FBS and seeded without centrifugation. Two hours later the medium was replaced by MM-199 with 10–20% HPDS and 500 \mu g/ml hypothalamic extract. Cultures were used for studies from the primary through the fifth passage. Other aliquots of the suspension were centrifuged and the pellets re-suspended in MM-199 with 20% FBS, 25 mM HEPES, and 7% methyl sulfoxide (DMSO) before freezing and storing in liquid nitrogen.

Endothelium from human umbilical veins was isolated as described previously.3 In our normal procedure, the cells were seeded at a density of 1 \times 10⁵ cells per cm² in MM-199 with 20% FBS. Twenty-four hours later the monolayer was rinsed with MM-199 without serum and fresh MM-199 with 20% FBS was added. Cultures were used for experiments within 72 to 120 hours after plating. When human umbilical vein endothelium was cultured under the same conditions as the adult endothelium, the cells were seeded at low density (<20 cells per cm²) and the medium was replaced at 24 hours with MM-199 with
10–20% HPDS and 500 μg/ml hypothalamic extract. (Some primary pools were used for passage and for experiments).

In some experiments the primary cultures and subsequent passages were treated with 50 μM ibuprofen (Motrin) continuously after 24 hours in culture. The medium was changed at least every 72 hours.

The influence of repeated proteolysis during subculture with trypsin was tested by subculturing parallel colonies with Cytodex 3 beads. The beads were added and incubated overnight at 37°C to allow migration of the cells onto the beads. The beads were gently resuspended and added to flasks containing MM-199 with 10% HPDS and 500 μg/ml hypothalamic extract, which had been coated with fibronectin. Flasks were left undisturbed for at least 24 hours. Subsequent passage was achieved by scraping the cells off the flask with a rubber policeman, pipetting to break up the clumps, and distributing the suspension onto fibronectin-coated culture surfaces.

**Determination of Culture Purity**

The presence of Factor VIII antigen in subcultured endothelium was verified by indirect immunofluorescence.

A monoclonal antibody was produced by fusion of NS-1 mouse myeloma cells with spleen cells from Balb/c mice which had been hyperimmunized with cells from primary cultures of human umbilical vein endothelium.5 Five antibodies with significant binding to human umbilical vein endothelium were obtained. One of these antibodies appears to bind only to endothelium. Cultures of fibroblasts from the umbilical artery and smooth muscle from the umbilical vein as well as cellular components from peripheral blood were tested to determine the amount of antibody bound. Binding to suspended cells was measured by a radioimmunoassay.6 When compared to nonspecific binding of mouse IgM, only endothelium demonstrated a significant increase in radioactivity.

(Endothelium 9× nonspecific binding, other cell types <0.5× nonspecific binding). This monoclonal antibody was used to test cultures from several samples of the adult vessels. Cells were detached with 0.2M urea and 0.1% EDTA in PBS. After washing the suspension was incubated 60 minutes on ice with the monoclonal antibody. The cells were pelleted and washed twice before incubating with a fluorescent-conjugated rabbit antimmune immunoglobulin for 60 minutes on ice. After washing three times the cells were resuspended in PBS with 30% glycerol and 0.1% paraphenylenediamine and examined by fluorescence microscopy.

**Protein Secretion**

To examine protein secretion by the subcultured adult endothelium, one 75 cm² flask of nearly confluent cells was preincubated for 1 hour in 5 ml of glycine-free Dulbecco’s Modified Eagle’s Medium containing sodium ascorbate (50 μg/ml) and β-aminopropionitrile (64 μg/ml). The cells were then incubated for 20 hours in fresh preincubation medium containing 25 μCi/ml, 2,3 3H-proline and 2-3H-glycine.

The culture medium was initially processed in the presence of protease inhibitors as described previously.9 It was subsequently dialyzed directly against chromatography buffer (6M urea, 50 mM Tris-HCl pH 8.0 containing 0.2 mM phenylmethylsulfonyl fluoride and 2.5 mM EDTA) at 4°C. After centrifugation the sample was chromatographed on DEAE-cellulose as previously described.9 Gradient elution of a sample of culture medium proteins had shown a single peak; therefore bound proteins were stripped from the column with 0.5M NaCl in chromatography buffer as an alternative to gradient elution.

Collagenase digestion and radiimmune precipitation was performed as described previously.10 Affinity-purified rabbit antibodies to human Types I and IV collagen, human fibronectin, bovine Type III procollagen, and bovine thrombospondin were isolated and utilized according to published procedures.11

Proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels containing 0.5 M urea and were visualized by fluorescence autoradiography.9

**Accessory Cell Function**

Mononuclear cells (MC) were isolated from cadaveric donor splenic tissue by gentle teasing of the tissue with forceps. The cells were expressed into the surrounding RPMI 1640 medium, diluted with PBS, and centrifuged over a Ficoll-Hypaque density gradient.12

Mononuclear phagocytes (MC) were obtained by a modification of the method of Pertoff et al.12 MC at 1 × 10⁶ cells/ml of RPMI 1640 with 25 mM HEPES buffer were sedimented with an equal volume of the RPMI 1640 solution containing 2% sheep erythrocytes (Microbiological Associates, Incorporated, Bethesda, Maryland) at 150 g at 4°C, in nunc tubes (Vanguard International, Neptune, New Jersey). After a 1-hour incubation at 4°C, the pelleted cells were gently resuspended and centrifuged at 450 × g for 30 minutes over a Ficoll-Hypaque gradient at 4°C. Nonpelled MC were recovered and washed in RPMI 1640 solution as above and subsequently centrifuged at 300 × g for 30 minutes over an 80% gradient of Percoll at 27°C. Mononuclear cells ~90% enriched for mononuclear phagocytes, determined by positive esterase stain,14 were recovered from the 1.060 specific gravity density band, washed three times in PBS, and maintained in PBS at 4°C until the initiation of experiments.

T lymphocytes were prepared from MC by a modification of the method of Greaves and Brown.15 MC were resuspended in Barbitone buffer pH 7.2 containing 5% FBS at 5 × 10⁶ cells/ml. Platelet contaminants and phagocytic cells were removed first by addition of 50 units/ml bovine thrombin to MC with
continuous rocking for 5 minutes at 27°C. After sedimentation of platelet thrombi at unit gravity for an additional 5 minutes, the lymphocyte-enriched supernatant was recovered, supplemented with an equal volume of FBS, and warmed to 37°C. These cells were then loaded on a nylon wool column prewetted with Barbitone buffer supplemented with 5% FBS. After incubation at 37°C for 30 minutes, the column was then eluted with Barbitone buffer containing 5% FBS. Eluted cells were adjusted to 5 × 10⁶ cells/ml and mixed with an equal volume of 2% sheep erythrocytes in nunc tubes. The cell suspension was sedimented by centrifugation at 150 × g at 4°C. After 1 hour at 4°C, pelleted cells were gently resuspended and sedimented over a Ficoll-Hypaque gradient at 350 × g. Nonpelleted cells were discarded. Adherent erythrocytes were lysed by 0.17 M ammonium chloride buffered to pH 7.2 with Tris. Cells recovered were >99% T lymphocytes as assessed by esterase staining. Purified T lymphocytes were resuspended at 5 × 10⁶ cells/ml in PBS containing 1 mM Na-m-periodate and incubated at 0°C for 30 minutes. ¹⁷ Less than 0.1% of the cells were mononuclear phagocytes as assessed by esterase staining. Purified T lymphocytes were washed in PBS three times and maintained in PBS at 4°C prior to initiation of experiments.

Endothelial cells were removed from the culture flasks by incubating 5 minutes with 5 ml 0.05% EDTA and subsequent scraping with a rubber policeman. After centrifugation for 10 minutes at 300 × g, the pellet was washed with the HEPES buffered RPMI 1640 with 20% FBS and pipetted gently to obtain a single cell suspension. The cell number was adjusted to 1 × 10⁹/ml.

T lymphocytes were resuspended at 5 × 10⁶ cells/ml in PBS containing 1 mM Na-m-periodate and incubated at 4°C for 30 minutes.¹⁷ Mononuclear phagocytes and endothelial cells were irradiated with 2400 rads from a cesium source (Mark 1 Model 22 Irradiator, JLS Shepherd, Glendale, California) to prevent replication during the culture.

Limiting dilution analysis with periodate-treated T lymphocytes revealed that at a concentration of 1 × 10⁴ cells/ml mitogenic proliferation was absolutely dependent upon addition of accessory cells. Therefore 0.2 ml of T lymphocytes at 1 × 10⁴ cells/ml in RPMI 1640 with 25 mM HEPES and 10% FBS were distributed into flat bottom microwells (Microtest II 3040, Falcon Plastics). We added 50 μl medium or irradiated MP or endothelial cells (syngeneic with T lymphocytes) to wells containing unstimulated or periodate-treated T lymphocytes. Quadruplicate wells were incubated in a 95% humidified air/5% CO₂ atmosphere at 37°C for 96 hours. During the last 18 hours of culture, 2 μCi of ³H-thymidine were added to each microwell. Cells were harvested onto glass filter fibers with a multiple sample automated harvester (MASH II, Microbiological Associates).¹⁸ Tritiated thymidine incorporation was determined by liquid scintillation spectrometry. Results are expressed as mean total counts per minute from quadruplicate cultures.

**Prostacyclin Production**

Endothelial cultures in 4.5 cm² wells of Linbro multiwell plates or 2 cm² wells (Falcon No. 3047 24-well plates) were rinsed twice with Hanks balanced salt solution (without NaHCO₃) containing 15 mM HEPES pH 7.4. Agents used to stimulate prostaglandin production were 0.5 U/ml bovine thrombin in incubation medium pH 7.4 (IM),⁵ 10 μM A23187 in IM, 20 μM arachidonic acid in tris-buffered saline pH 8.6 (TBS), 0.93 μM PGH₃ in TBS, and the appropriate buffer controls. The monolayers were incubated with 0.5 ml (0.22/2 cm² well) of each solution for 15 minutes at 37°C. The wells containing arachidonic acid, or PGH₃ and their respective buffers were scraped with a rubber policeman before incubation. At the end of the incubation, the supernatant was removed and centrifuged at 1100 × g for 10 minutes. It was frozen at −20°C. Prostacyclin (PGI₂) production was determined by a radioimmunoassay for its stable degradation product 6-keto-PGF₁α.¹⁹ Appropriate solution controls were tested for cross reactivity. Corrections for cross reactivity were made when indicated. Unscraped monolayers were rinsed with PBS and the cells removed with trypsin-EDTA. Cell counts were performed with a hemocytometer.

**Cyclooxygenase Antigen Determination**

Monolayers (75 cm² flasks) of primary umbilical vein endothelium, passed vena cava, and aortic endothelium were rinsed with PBS and removed by scraping with a rubber policeman. The resulting suspension was centrifuged at 15,000 × g, the supernatant removed, and the pellet frozen at −80°C. A radioimmunoassay for cyclooxygenase antigen twenty was performed in the laboratory of Gerald Roth.

**Results**

In the presence of HPDS and the extract from the bovine hypothalamus, cultures of endothelium from adult vessels grew from very low density. Efforts to grow these isolates in a primary culture without low density plating produced some cultures which appeared to be endothelial and others which were overgrown by other cell types. Because of this variability, we chose the low density plating method to achieve minimal contamination. Cells from individual wells of 96 well plates were successfully transferred when fibronectin-coated surfaces were used. The resulting cultures could be subcultured at a 1:3 to a 1:5 split ratio and reached confluence in 4 to 10 days. After serial passage the adult endothelium could be frozen and stored in liquid nitrogen. It was not possible to maintain the cultures beyond fifth passage because the rate of replication decreased.

**Culture Purity**

Figure 1 shows a small colony with epithelioid morphology 3–5 days after primary isolation on the left.
Figure 1. The phase contrast photomicrograph on the left is a colony of adult aortic endothelium typically seen at 3 to 5 days after primary isolation. On the right is a confluent monolayer of adult aortic endothelium in second passage. No stain was used with either sample. Magnification × 200. The bar represents 50 μM; the magnification is the same for both photographs.

Figure 2. Fluorescent photomicrograph of a monolayer of adult aortic endothelium (third passage) treated with a rabbit antibody to human Factor VIII antigen and fluorescein conjugated goat antirabbit immunoglobulin. Magnification × 540. The bar represents 20 μM.
On the right is a phase-contrast micrograph of a confluent monolayer of aortic endothelium in second passage. The presence of Factor VIII antigen is demonstrated by immunofluorescence as shown in Figure 2. Note that all the cells were positive in this third passage culture of adult aortic endothelium. Figure 3 shows a suspension of adult aortic endothelium that was reacted with the monoclonal antibody to umbilical vein endothelium. All of the cells display membrane fluorescence. The failure of this antibody to bind to cell types that might be found as contaminants indicates that all these cells contain the endothelial antigen recognized by the antibody.

**Protein Secretion**

Quantitation of radiolabeled protein secreted by adult endothelial cells revealed similar levels of incorporation as had been observed for human umbilical vein endothelial cells (unpublished results). Approximately $5 \times 10^6$ dpm were recovered in total culture medium protein per $10^6$ cells. When these proteins were chromatographed on DEAE-cellulose, 36% of the applied dpm were recovered in the unbound fraction, while 46% were subsequently eluted with 0.5 M NaCl, based on a total recovery of 82%. These fractions are shown in Figures 4 and 5 after resolution by SDS-PAGE under both reducing and nonreducing conditions.

The principal collagenous component secreted by adult endothelial cells was Type IV procollagen, shown in Lane A after reduction as a doublet consisting of two pro-$\alpha$ chains and as a disulfide-bonded molecule of approximately $0.5 \times 10^6$ molecular weight before reduction (Lane C, Dithiothreitol; DTT). This component was specifically digested by bacterial collagenase and comprised approximately 4% of the total protein-associated dpm in the culture medium (data not shown). In addition, it was specifically precipitated by affinity-purified antibodies to human Type IV collagen, but did not react with antibodies to several other collagen types (data not shown). The characteristic lack of binding to DEAE-cellulose at pH 8.0 and the mobility on SDS-PAGE, both before and after reduction, provided further evidence for identification of the adult endothelial cell collagenous protein as Type IV procollagen.

Other proteins secreted by adult endothelial cells were recovered in the fraction eluted by 0.5 M NaCl (Figures 4 and 5, Lane B). Included in this group were fibronectin (M, 240,000–250,000 after reduction) and thrombospondin (M, 190,000 after reduction). Both of these glycoproteins are secreted by a wide variety of endothelial cells and, collectively, constitute 40%–60% of the total protein-associated radioactivity in the culture media. Fibronectin was selectively precipitated by affinity-purified antibodies to human plasma fibronectin (cold-insoluble globulin), as revealed by SDS-PAGE of the radioimmune precipitate (data not shown). Antibovine thrombospondin IgG, which exhibits extensive crossreac-
Figure 4. Characterization of proteins secreted into the culture medium by adult vena cava endothelial cells in vitro. Cells were metabolically labeled with \(^{3}H\)-proline and \(^{3}H\)-glycine as described in Methods, and culture medium proteins were chromatographed on DEAE-cellulose at 4°C. Unbound proteins (A) and proteins eluted using 0.5 M NaCl (B) were subsequently analyzed by SDS-PAGE (3%/6%/10% composite gel) under both reducing (+ DTT) and nonreducing (− DTT) conditions. Visualization was by fluorescence autoradiography. The positions of migration of fibronectin (FN), thrombospondin (TS), and standard (std) Types I and IV procollagens are indicated.

Activity with human thrombospondin precipitated a radiolabeled component of Mr 190,000 which comigrated on SDS-PAGE after reduction with a thrombospondin standard (data not shown).

The secretory phenotype of adult vena cava and aortic endothelial cells in vitro was therefore found to resemble very closely that of human umbilical vein endothelial cells, in that three major glycoproteins in the molecules, weight range of 150,000–250,000 after reduction, were recovered from the culture medium: fibronectin, type IV procollagen, and thrombospondin. In the secretion of fibronectin and thrombospondin, adult endothelial cells resemble most other endothelial cells in vitro. However, in contrast to bovine endothelial cells from aorta, cornea, and vena cava, which secrete primarily the interstitial Type III procollagen into the culture medium, human adult endothelial cells secreted the basement membrane procollagen, Type IV.

Accessory Cell Function

A comparison of accessory cell function of adult human endothelial cells and mononuclear phagocytes is seen in Table 1. The addition of equal numbers (5 × 10⁴) of endothelial cells or MP to periodate-treated T lymphocyte cultures resulted in a twofold greater stimulation of \(^{3}H\)-thymidine incorporation by endothelial cells. These effects could not be attributed to overgrowth of accessory cells during culture as their addition to nonstimulated T cell cultures did not result in significantly greater incorporation of \(^{3}H\)-thymidine. Nor could they be attributed to contamination from culture supernatants, as the supernatants were ineffective in promoting mitogenesis of periodate-stimulated T cells at these low density culture conditions.

Table 1. \(^{3}H\)-Thymidine Incorporation of T Lymphocyte Cultures

<table>
<thead>
<tr>
<th>X-irradiated accessory cells</th>
<th>T lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-stimulated</td>
<td>Periodate-stimulated</td>
</tr>
<tr>
<td>Control</td>
<td>217*</td>
</tr>
<tr>
<td>Mononuclear phagocytes</td>
<td>200</td>
</tr>
<tr>
<td>Adult endothelium</td>
<td>197</td>
</tr>
</tbody>
</table>

Primary cultures of purified T lymphocytes from a cadaveric donor were treated with or without Na-m-periodate as described in methods. Nonperiodate-treated syngeneic purified mononuclear phagocytes or cultured human adult endothelial cells were added to T lymphocyte cultures. An equivalent volume of medium was added to control cultures.

*Counts per minute.
pmol/10^6 cells. Primary aortic endothelial cultures produced a range of 0 to 71 pmol/10^6 cells. The range for subcultured vena cava and aortic endothelium was from 13 to 168 pmol/10^6 cells and 0 to 33 pmol/10^6 cells respectively. All values represent the amount released with the stimulus minus the amount released with the respective buffer.

The results of prostacyclin production with 20 μM arachidonic acid, 0.93 μM PGH₂, 0.5 U/ml bovine thrombin, and 10 μM calcium ionophore A23187 are shown in Table 2. The primary umbilical vein was grown in the usual manner (1 x 10^5 cells/cm² in MM-199 with 20% FBS) or with the medium used for adult cultures (1 x 10^5 cells/cm² in MM-199 with 10–20% HPDS + 500 μg/ml bovine hypothalamic extract). The adult endothelium was plated at low density and subcultured as described. Significantly lower values were seen with the passed adult cells than with the primary umbilical vein endothelium (Columns 1 and 2). When portions of these primary umbilical vein cultures were subcultured (without low density seeding) there was a steady decline in prostacyclin production produced by all of the stimuli (data not shown).

The possibility that repeated proteolytic digestion or depletion of cyclooxygenase activity could be the cause for decreased prostacyclin production prompted us to test the following modifications. Parallel samples of umbilical vein endothelium that had been seeded at low density (<20 cells/cm²) were cultured with or without 50 μM ibuprofen (a reversible inhibitor of cyclooxygenase). The medium containing ibuprofen was added 24 hours after the initiation of the primary culture, and fresh medium was added at least every 72 hours. To verify that the concentration of ibuprofen used was sufficient to inhibit prostaglandin production, the medium from primary cultures of umbilical vein endothelium (1 x 10^5 cells/cm²) with or without ibuprofen was tested to determine the amount of 6-keto-PGF, accumulated in 72 hours. The medium with and without 50 μM ibuprofen contained 37 ± 9 and 1216 ± 371 pmol/ml respectively. Some of the cultures grown in each type of medium were subcultured with Cytodex 3 microcarriers as described or with the usual trypsin-EDTA method to rule out an effect of proteolytic digestion.

Arachidonic acid-stimulated production of prostacyclin by cultures of umbilical vein endothelium grown and subcultured under these conditions is shown in Table 3. All values were less than 7% of the amount seen in primary cultures of umbilical vein endothelium shown in Table 2. When the cyclooxygenase was protected, values were slightly but significantly higher in the trypsin-EDTA subcultured cells.

Cultures from adult vessel samples were also treated with 50 μM ibuprofen continuously after the first 24 hours. One-half of them were subcultured with the microcarrier method and half with trypsin-EDTA.

The values for the subcultured umbilical vein endothelium (Table 4) and the adult vena cava endothelial cultures (Table 5), with arachidonic acid, thrombin, or ionophore stimulation, were nearly equivalent. Those seen with aortic endothelium and arachidonic acid (Table 5) were substantially decreased.

When the 6-keto-PGF, value produced by each stimulus was divided by the amount produced by its respective buffer control, a ratio of 8.2, 3.8, and 12.6

### Table 2. 6-Keto-PGF, (pmol/10⁶ Cells)

<table>
<thead>
<tr>
<th>Endothelium</th>
<th>Umbilical vein (primary)</th>
<th>Adult (passed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FBS</td>
<td>HPDS + E</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>481 ± 47</td>
<td>410 ± 43</td>
</tr>
<tr>
<td>PGH₂</td>
<td>169 ± 15</td>
<td>107 ± 22</td>
</tr>
<tr>
<td>Thrombin</td>
<td>32 ± 3</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>A23187</td>
<td>212 ± 12</td>
<td>91 ± 9</td>
</tr>
</tbody>
</table>

Primary cultures of umbilical vein endothelium were grown in MM-199 with 20% FBS or MM-199 with 20% HPDS and 500 μg/ml hypothalamic extract (E). Adult endothelium was grown in the MM-199 HPDS + E combination. Release of 6-keto-PGF, was stimulated with 20 μM arachidonic acid, 0.93 μM PGH₂, 0.5 U/ml bovine thrombin, and 10 μM A23187. Values equal the difference between the amount released by the stimulus and that released by buffer alone.

### Table 3. Umbilical Vein 6-Keto-PGF, Production (pmol/10⁶ Cells)

<table>
<thead>
<tr>
<th>Arachidonic acid</th>
<th>Cyclooxygenase unprotected</th>
<th>Cyclooxygenase protected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-EDTA</td>
<td>19 ± 1</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Beads — no enzyme</td>
<td>14 ± 1</td>
<td>17 ± 3</td>
</tr>
</tbody>
</table>

Umbilical vein endothelium was initially seeded at less than 20 cells/cm² and grown in MM-199 with 10% HPDS and 500 μg/ml E with (cyclooxygenase protected) or without 50 μM ibuprofen. Parallel colonies were subcultured with 0.25% trypsin-0.05% EDTA or with Cytodex 3 beads and scraping. 6-keto-PGF, was stimulated with 20 μM arachidonic acid.
Table 2) 31 and <10 ng/mg protein respectively.

That umbilical vein endothelium could be propagated and aortic endothelium (cultured as described in colonies were transferred. Maciag, et al.23 reported the corresponding values for primary umbilical vein endothelium.

The amount of cyclooxygenase antigen in these cultured endothelial cells has also been determined. Primary umbilical vein endothelium (1 x 10⁵ cells/cm²) cultured in MM-199 with 20% FBS had 190 ng of cyclooxygenase antigen/mg protein, vena cava, 3.4, 1.2, and 1.1; with passed aortic endothelium, 1.1, 1.1, and 1.1; for arachidonic acid, thrombin, and A23187 respectively. The values for all the passed endothelial cultures were significantly lower with each of the three stimuli (p < 0.01) than the corresponding values for primary umbilical vein endothelium.

The pattern of proteins which included basement membrane (Type IV) collagen, thrombospondin, and fibronectin secreted by the cultures of adult endothelium was very similar to that described by Sage et al.11 for umbilical vein endothelium. This result and the ability of the endothelial cells to serve an accessory cell function in the T lymphocyte culture system similar to that reported by Ashida et al.24 are other characteristics which have been maintained after propagation.

Prostacyclin production, another characteristic of endothelium, was decreased in cultured adult endothelium when compared to primary umbilical vein endothelium. The effect seen with umbilical vein endothelium using the same culture conditions as those used for the adult endothelial cultures represented a steady decline in prostacyclin production with serial subculture (unpublished observation). The umbilical vein endothelial cultures which were started at a low density gave results similar to the adult vena cava cultures. Glassberg et al.25 reported results similar to our arterial endothelial cultures when thrombin at 5 U/ml was used to stimulate cultures of iliac artery endothelium and umbilical vein endothelium. The increase shown by thrombin-treated cultures over control cultures was very small and was below the 3 pmol/ml detection limit of our radioimmunoassay. However, the fact that Glassberg et al.25 used culture conditions similar to ours (except for the addition of thrombin) with a similar decreased responsiveness to thrombin-stimulated prostacyclin production suggests that culture conditions can greatly affect prostacyclin production.

Our efforts to determine the cause for the decreased prostacyclin production have not produced definitive results. Protection from cyclooxygenase depletion by culturing cells in the presence of 50 μM ibuprofen did not prevent the decreased production. Similar results were also obtained when cells were subcultured with microcarriers and transferred by scraping instead of by exposure to trypsin-EDTA, suggesting proteolysis is not the cause. A recent report by Ody et al.26 with endothelium cultured from

Table 5. 6-Keto-PGF₁α Production by Cultured Adult Endothelium (Cyclooxygenase Protected) (pmol/10⁶ Cells)

<table>
<thead>
<tr>
<th>Method of subculture</th>
<th>Arachidonic acid</th>
<th>Bovine thrombin</th>
<th>A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>2.0 ± 0.7</td>
<td>0.9 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Beads — no enzyme</td>
<td>1.0 ± 0.7</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Vena Cava</td>
<td>21 ± 3.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Beads — no enzyme</td>
<td>17 ± 2.4</td>
<td>0.8 ± 0.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Cell suspensions from adult vena cava or aorta samples were seeded as described in Table 2. Release of 6-keto-PGF₁α was also described in Table 2.

Discussion

In contrast to our previous experience with standard approaches, we have shown that adult human endothelium can be grown in culture from isolates plated at very low density. An extract from the bovine hypothalamus was an essential component of the medium. While human plasma-derived serum was not essential, it greatly increased the number of colonies produced. Fibronectin-coated surfaces were necessary to increase the plating efficiency when colonies were transferred. Maciag, et al.23 reported that umbilical vein endothelium could be propagated in a similar manner, but that colonies formed from low density seeding could not be transferred more than once. Perhaps the human serum used in our system enhances growth. The purpose of the low density isolation is to separate the colonies with epithelioid morphology from those manifesting fibroblastic characteristics. It has been our experience that many primary isolates seeded at higher density appear to contain more than one cell type. All cells stained positively for Factor VIII antigen, a specific marker for endothelium (Figure 2). A monoclonal antibody to human umbilical vein endothelium shown to react only with the endothelium of sections from a surgical specimen of normal adult human vein8 also gave a positive reaction with the cultured adult endothelium. These results are further proof of minimal contamination with other cell types and retention of this specific antigen in culture.

The umbilical vein endothelial cultures which were started at a low density gave results similar to the adult vena cava cultures. Glassberg et al.25 reported results similar to our arterial endothelial cultures when thrombin at 5 U/ml was used to stimulate cultures of iliac artery endothelium and umbilical vein endothelium. The increase shown by thrombin-treated cultures over control cultures was very small and was below the 3 pmol/ml detection limit of our radioimmunoassay. However, the fact that Glassberg et al.25 used culture conditions similar to ours (except for the addition of thrombin) with a similar decreased responsiveness to thrombin-stimulated prostacyclin production suggests that culture conditions can greatly affect prostacyclin production.

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the piglet aorta indicated a decline in total prostanoioid secretion at confluence in early passages of endothelium compared to primary cultures. This reduction could be overcome by the addition of fibroblast growth factor.

The preliminary results from the determination of the quantity of cyclooxygenase by Dr. Roth would lead us to believe that the amount of the antigen is declining in culture. Further studies must be done to determine if this is the case. It is unlikely that this decrease is due to depletion of the enzyme as a consequence of production of prostaglandins or their derivatives in the culture, because the effect was not prevented by ibuprofen. Further studies are in progress to correlate antigenic activity with functional activity.

The cultured endothelial cells from adult vessels retain many of the functions and characteristics typical of endothelium. Production of prostaglandins from both subcultured umbilical vein and adult endothelium appears to be modified. Final assessment of the factors affecting prostaglandin production by endothelial cultures beyond the primary stage is not complete at present, but results in the current study are sufficient to alert investigators of a potential problem. The demonstration that adult human vascular endothelium can now be grown in culture removes an obstacle for many investigators who thought it impossible. Nevertheless, with present techniques, alterations in the functions of these cells may make comparative assessments hazardous if the effects of the culture conditions are not considered.

Acknowledgments

We thank Gerald Roth for performing the radioimmunooassay for cyclooxygenase antigen and Charles Johnson for the radiolabeling experiments. We also thank Donna Haycraft, Constance Schroeder, John Pirro, Christopher Helfner, and James Kisthard for expert technical assistance and Barbara Freiburg for typing the manuscript.

References


Index Terms: endothelium • prostacyclin • tissue culture
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