HUMAN VASCULAR ENDOTHELIAL CELLS IN CULTURE

Growth and DNA Synthesis

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ABSTRACT

Human endothelial cells, obtained by collagenase treatment of term umbilical cord veins, were cultured using Medium 199 supplemented with 20% fetal calf serum. Small clusters of cells initially spread on plastic or glass, coalesced and grew to form confluent monolayers of polygonal cells by 7 days. Cells in primary and subcultures were identified as endothelium by the presence of Weibel-Palade bodies by electron microscopy. A morphologically distinct subpopulation of cells contaminating some primary endothelial cultures was selectively subcultured, and identified by ultrastructural criteria as vascular smooth muscle. Autoradiography of endothelial cells after exposure to [^{3}H]thymidine showed progressive increases in labeling in growing cultures beginning at 24 h. In recently confluent cultures, labeling indices were 2.4% in central closely packed regions, and 53.2% in peripheral growing regions. 3 days after confluence, labeling was uniform, being 3.5 and 3.9% in central and peripheral areas, respectively. When small areas of confluent cultures were experimentally "denuded," there were localized increases in [^{3}H]thymidine labeling and eventual reconstitution of the monolayer. Liquid scintillation measurements of [^{3}H]thymidine incorporation in primary and secondary endothelial cultures in microwell trays showed a similar correlation of DNA synthesis with cell density. These data indicate that endothelial cell cultures may provide a useful in vitro model for studying pathophysiologic factors in endothelial regeneration.

Normal vascular endothelium represents a slow renewal population of cells (see review in reference 1): endothelial mitoses are rarely seen in tissue sections, and autoradiographic studies with tritiated thymidine have consistently shown a low labeling index both in large vessels (1–4) and in the microcirculation (1–5). However, a variety of physiologic and pathologic stimuli can induce endothelial mitosis and new capillary growth (2–11) and these events play an important role in such processes as wound healing (7), the formation of granulation tissue, the reendothelialization of vessel-wall defects (6), and the growth of tumors (1, 11).

Recently, cell-free preparations derived from tumor cells have been shown to stimulate endothelial mitosis in vivo (11). However, the isolation and characterization of these and other possible mediators of endothelial regeneration and their mode of action have not been investigated in detail. Such studies have been hampered by the lack of a method for endothelial culture that would permit

analysis of physiologic and pathologic factors in endothelial growth.

A number of investigators have attempted to maintain endothelial cells in vitro by culturing isolated intimal cells (12–15) or full thickness segments of vessel walls (3). Such methods have not been used extensively because of uncertainty concerning the identity of cultured cells, or poor growth and maintenance of cell viability. Recently, Jaffe and his associates (16) reported primary culture of human umbilical vein endothelial cells which were identified by a variety of morphologic and immunologic criteria. Abstracts and brief accounts recording culture of endothelium from other groups, including ours, have also been reported (17–20). In the studies presented here, we have established primary and subcultures of umbilical vein endothelium and have characterized their population growth behavior using data derived from [^{3}H]thymidine incorporation studies. Our findings indicate that endothelium in culture behaves as a density-dependent population with respect to DNA synthesis and can be used for studies on the mechanism of endothelial growth and regeneration.

MATERIALS AND METHODS

Isolation of Endothelial Cells

Human umbilical vein endothelial cells were harvested from umbilical cords obtained at normal vaginal deliveries or Caesarean sections.[1] The method originally described by Maruyama (12) was modified as follows: (*a*) untraumatized umbilical cord segments, at least 20 cm in length, obtained under sterile conditions within 10–30 min of delivery time were used; (*b*) the umbilical vein was cannulated and perfused with 200–400 ml of lactated Ringer's (Hartmann's) solution to remove all traces of blood; (*c*) the vein lumen was filled with Dulbecco's phosphate-buffered saline (PBS, with calcium and magnesium) containing 1 mg/ml collagenase (*Cl. histolyticum*, grade B, Worthington Biochemical Corp., Freehold, N. J.); (*d*) after a 10–20-min incubation at 37°C, the contents of the vein were gently flushed out with an equal volume of Hanks' Balanced Salt Solution (BSS, calcium- and magnesium-free), and collected in a siliconized glass, conical centrifuge tube; (*e*) centrifugation (200 *g* for 5 min) yielded a small white pellet which was resuspended in culture medium.

[1] We are grateful to the staffs of the Boston Hospital for Women (Lying-In Division), and the Obstetrical Service of the Surburban Hospital, Bethesda, Md., for their kind cooperation in obtaining this material.

Culture Media

In preliminary experiments, the following observations were made: (*a*) comparatively rich media, such as Medium 199 (M199) or Dulbecco-Vogt (NIH Media Unit), supported growth, while minimal essential (Eagle's), or glutamine-deficient media did not; (*b*) for optimal growth, freshly explanted cells required medium supplemented with 20–30% serum (fetal calf, FCS; or calf, CS); (*c*) for maintenance of dense primary cultures or growth in subcultures, 10% serum was sufficient; (*d*) umbilical vein effluent cells did not attach to the substratum, nor would already established cultures continue to grow in serum-free medium. The addition of as little as 1–2% fresh serum restored these capabilities.

The standard culture medium in the studies reported here consisted of M199, with 15 mm/liter HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid Calbiochem, San Diego, Calif.) at pH 7.40, 60 mg/liter penicillin, 120 mg/liter streptomycin, supplemented with 20% FCS for primary cultures, or 10% FCS for subcultures. Each lot of bovine serum (Grand Island Biological Co., Grand Island, N.Y.) was screened for mycoplasma contamination before use (21).

Primary and Subcultures

For primary stock cultures, 1–2 $\times$ 10^6 umbilical vein effluent cells in 4 ml of medium were plated in Falcon T-25 (25 cm^2) plastic flasks (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.). After 12 h, supernatant medium was aspirated, the flask rinsed three times with Hanks' BSS, and fresh medium added. Thereafter, medium was exchanged at 48-h intervals. All cultures were incubated at 37°C in a humidified 5% CO_2-air atmosphere.

After 6–10 days, primary cultures which had formed uniform monolayers were selected for subculturing. Very brief exposures (2–3 min) to 0.25% trypsin-0.05% EDTA in saline were sufficient to release 60–80% of the monolayer. Harvested cells were collected in 10 vol of 30% FCS-M199, centrifuged, and resuspended at known concentrations in 10% FCS-M199 for subculturing.

Replicate Microwell Cultures for DNA-Synthesis Studies

Freshly isolated cells or cells harvested from primary stock cultures were replicate plated in flat-bottomed, Falcon MicroTest-II tissue culture plates (2.0 $\times$ 10^4 cells per microwell in 0.200 ml medium). Medium was exchanged daily.

At various stages of growth, cultures were exposed to [*methyl*-^{3}*H*]thymidine (6.0 Ci/mM sp act, Schwarz/

Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) 1.0 μCi/ml in culture medium for 12 h. Radioactive medium was aspirated and each well treated as follows: PBS rinse twice, acetic acid-ethanol (1:3) fixative for 10 min, distilled water rinse, 10% trichloroacetic acid (TCA) at 4°C for 15 min, 5% cold TCA rinse, dried at 50°C for 30 min.

Each microwell bottom, with its adherent cell monolayer, was punched out of the plate into a scintillation vial, using a blunt-ended stainless steel rod.[2] 10 ml of a scintillation mixture, consisting of six parts Toluene-Liquifluor (New England Nuclear, Boston, Mass.) and four parts ethylene glycol monomethyl ether, were added to each vial. After 4 h at room temperature, the resulting clear solution was counted in a Packard Tri-Carb Liquid Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Measured activities per vial usually varied from 10 to 100 times background levels and agreed within ±10% among replicate microwells.

For each interval, data (counts per minute per microwell) from 10 to 20 replicate microwells were averaged and related to the number of cells per microwell. Hemocytometer cell counts were obtained from trypsin-EDTA resuspended cells pooled from 10 nonradioactive microwells on each plate.

Cytologic and Autoradiographic Techniques

For cytology, cells were fixed with glacial acetic acid-ethanol (1:3) or 10% Formalin, and stained with toluidine blue or Wright's stain.

For autoradiography, cover slip cultures were exposed to [*methyl*-^{3}H]thymidine in culture medium for 3 h (2 μCi/ml) or 12 h (0.1 μCi/ml). At the end of the labeling period, the cultures were washed with PBS. After fixation, cover slips were immersed in 5% TCA at 4°C for 15 min, rinsed with distilled water, and mounted on glass microslides for dipping in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.). After 48 h exposure at 4°C, the autoradiographs were developed and lightly stained.

The percentage of labeled cells (thymidine index) in growing cultures was determined, after a 3-h terminal exposure, at 9, 24, 48, and 72 h. A minimum of 20,000 cells on duplicate cover slips were counted for each period. In separate experiments, the distribution of labeled cells as a function of cell density was determined in: (*a*) recently confluent monolayers; and (*b*) monolayers which had attained confluence 3 days previously (postconfluent), after a 12-h terminal exposure. Triplicate cover slip preparations were scanned and two types of sampling areas selected: (*a*) confluent, central areas where cells were in close-packed array, and (*b*) less dense, peripheral areas where cells were in incomplete contact with their neighbors. The percentage of labeled cells was determined in 20 rectangular areas (0.25 × 0.35 mm) of each type, using an ocular reticle. 3,000–4,000 cells were counted on each cover slip.

In each experiment cultures were provided with fresh media daily, up to the time of the labeling period.

Electron Microscopy

Cultured cells were processed for electron microscopy in the Falcon plastic dishes *in situ*. They were fixed for 1 h in 2.5% glutaraldehyde-0.1 M cacodylate buffer (pH 7.4), postfixed in 2% OsO_4 for 1 h, stained en bloc with 0.1% aqueous uranyl acetate, dehydrated with ethanol, stained with toluidine blue while in the 100% ethanol, infiltrated with ethanol-Epon for 1 h, and embedded in Epon. After curing of the Epon at 55°C for not more than 12–14 h, the brittle plastic dish was cracked and the partially polymerized, but still elastic Epon layer was peeled off and cured upside down at 60°C for 24 h.[3] Selected areas were then cut either parallel or perpendicular to the plane of the cell monolayer. They were stained with uranyl acetate and lead citrate and examined in a Philips EM 200 electron microscope.

RESULTS

Growth Behavior and Cytology

Fresh umbilical vein effluents contained small clumps of rounded cells which attached to the substratum and spread to form small epithelioid clusters within the first 2–6 h in culture (Fig. 1 *a*). These clusters increased in size and gradually coalesced to form incomplete monolayers by 3–5 days. Cells within these early monolayers were uniform in appearance: elongated (30–40 μm wide, 60–95 μm long), with single ovoid nuclei containing two to three prominent nucleoli surrounded by a perinuclear granular region, and a broad, thin peripheral cytoplasm with indistinct borders (Fig. 1 *b*). By 1 wk, single cell-thick confluent monolayers of densely packed polygonal cells (35–50 μm wide) had formed (Fig. 2). The tendency for overgrowth into multicell-thick aggregates was not observed, and cells retained their

[2] We thank Dr. C. F. McKhann, Department of Surgery, University of Minnesota Hospital, Minneapolis, Minn., for this useful suggestion.

[3] We are indebted to Dr. Christian Haudenschild, Childrens' Hospital Medical Center, Boston, Mass., for this method.

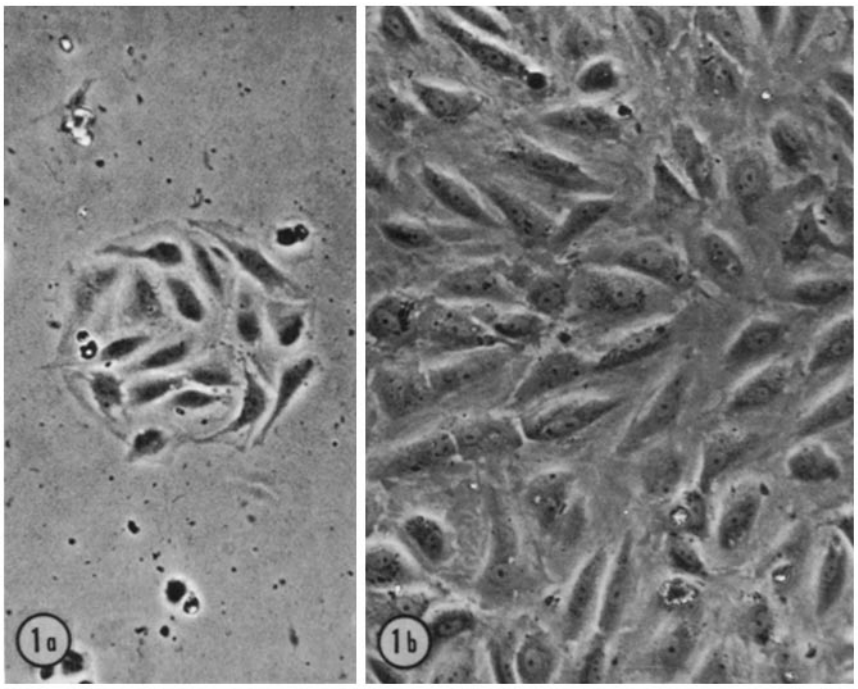


FIGURE 1 *a* 6 h in culture, a small cluster of endothelial cells attaches to the bottom of dish and spreads. Phase contrast. × 200.

FIGURE 1 *b* Early monolayer, 3 days in culture. The cells are elongated and have broad, thin peripheral cytoplasm with indistinct borders. Phase contrast. × 200.

epithelioid appearance, closely packed array, and attachment to the substratum for as long as 4–5 wk in primary culture.

Although 90% of cells in umbilical vein effluents were viable by trypan blue exclusion, cell counts after 12 h in culture showed that only 20–50% had attached to the substratum. In successful primary cultures, initial inocula of 5–6 × 10^5 viable cells per T-25 flask yielded 2–3 × 10^6 cells after 10–12 days. Population doubling times of approximately 48 h were observed during the logarithmic phase of growth. At confluence, cell density stabilized at approximately 1 × 10^5 cells per cm^2.

In our experience with over 200 freshly obtained umbilical cords during a 16-mo period, about one-third of primary cultures did not reach confluent densities. Primary inocula of less than 3 × 10^5 viable cells (dye exclusion) usually failed to become established in 25-cm^2 flasks. Initiation of growth in culture also appeared to be related to the presence of multicellular aggregates in the venous effluent. Comparable numbers of dispersed cells yielded significantly fewer colonies.

First passage subcultures consisted of homogeneous populations of cells that appeared similar to primary cultures by phase-contrast and light microscopy: individual cells remained mononucleate and polygonal and formed a closely packed monolayer. Subsequent subcultures exhibited increasing pleomorphism of cells and the tendency to form confluent monolayers was decreased.

Electron Microscopy

The ultrastructure of normal human umbilical vein endothelium has been described recently by Parry and Abramovich (22). At full-term pregnancy it generally resembles other large vessel endothelia, but is characterized by the presence of a large number of Weibel-Palade bodies (WPBs) (23). Also designated "specific endothelial organelles" (24), WPBs are membrane-bound, rod-shaped ovoid granules containing parallel arrays of 6–26 tubular structures, each approximately 150 Å in width.

In the present study, the presence of WPBs was

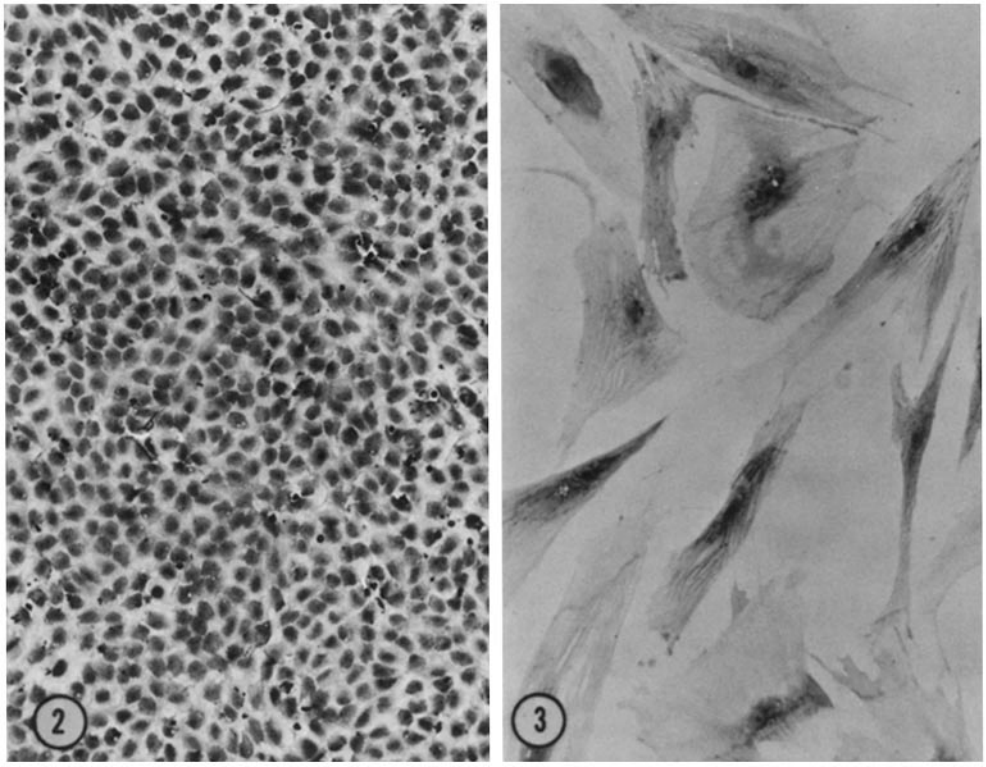


FIGURE 2 Low power micrograph of a confluent monolayer of endothelial cells 7 days in culture. Note the closely packed, single cell-thick monolayer of uniform epithelioid cells. Toluidine blue stain. × 70.

FIGURE 3 Micrograph of a selective subculture of the second cell type, identified as smooth muscle (see text). Cells are irregular in shape, much larger than the endothelial cells, and occasionally binucleate. Toluidine blue stain. × 90.

used for positive identification of cultured cells as endothelium, since these organelles have not been reported in cultures of either fibroblasts (25, 26) or smooth muscle cells (27). In single thin sections cut parallel to the cell surface, WPBs were found in 30–70% of cells from primary cultures (Fig. 4 *a*) and a subculture in its eighth passage. Sections perpendicular to the cell surface confirmed that primary cultures were composed of a single layer of cells with intercellular junctions (Fig. 4 *b*). Other ultrastructural characteristics of cultured endothelial cells included: prominent nucleoli, a perinuclear region rich in mitochondria, endoplasmic reticulum, and Golgi complexes, and attenuated peripheral cytoplasm containing free ribosomes and micropinocytosis vesicles. The peripheral cytoplasm often contained abundant numbers of microfilaments. A detailed ultrastructural description of cultured endothelium in primary cultures and subcultures is the subject of a separate paper (C. Haudenschild, R. S. Cotran, and J. Folkman, manuscript submitted).

Other Cell Types in Umbilical Vein Cultures

Nucleated blood cells were not seen in Wright's stained primary cultures after the first 24 h. Although selective cloning techniques were not employed, overgrowth by fibroblast-like cells did not occur in primary stock cultures or subcultures of endothelial cells. However, morphologically distinct, very large (50 × 400 μm) cells were occasionally observed among the usual endothelial cell colonies in about 10% of primary cultures. Cells of this type have been selectively subcultured (Fig. 3, compare with Fig. 2). They lack Weibel-Palade granules, and contained prominent dense bundles of myofilaments with "fusiform condensations" (Fig. 5) similar to those described in cul-

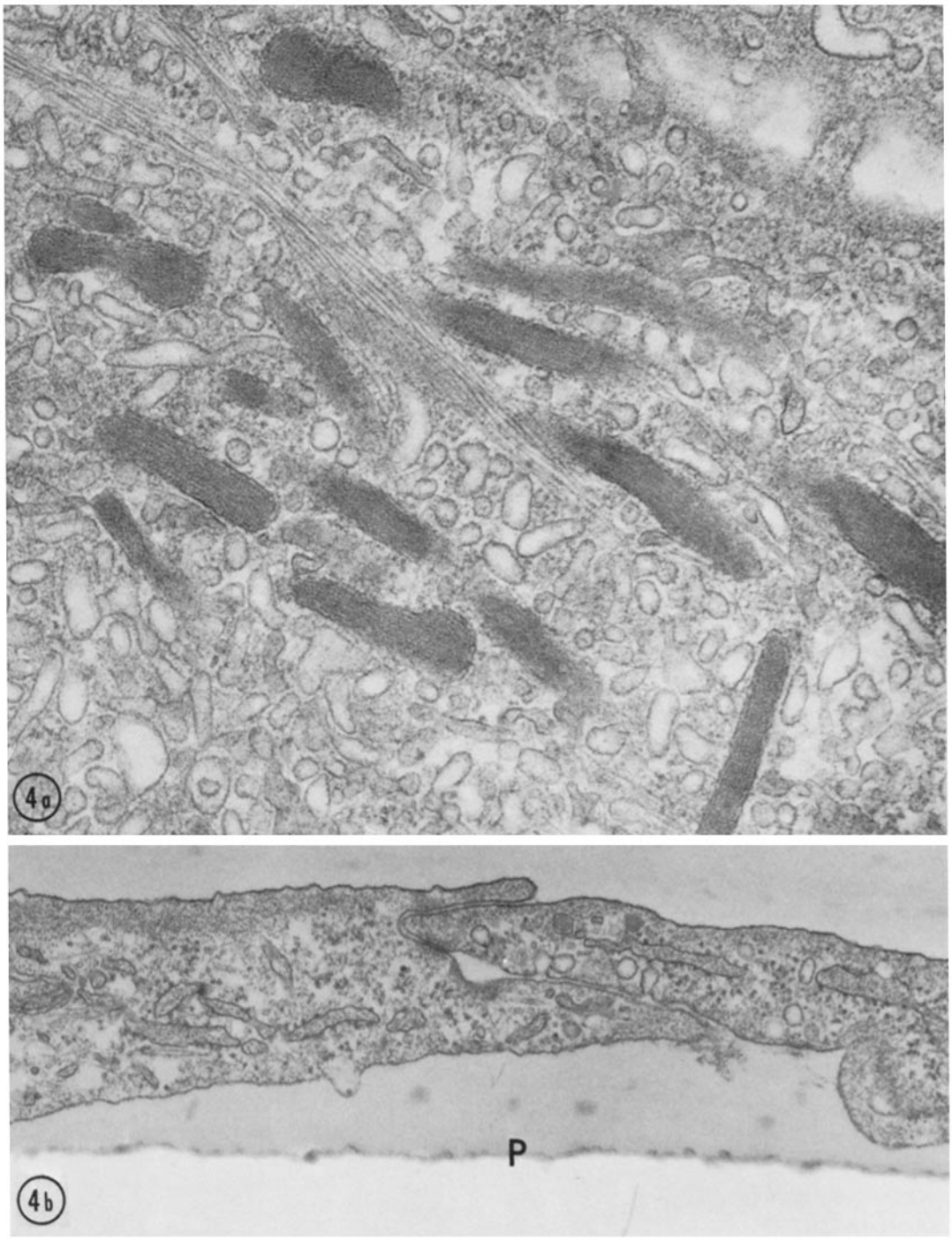


FIGURE 4 *a* Electron micrograph of a portion of an endothelial cell 7 days in culture. The section has been cut parallel to the cell surface. Note abundant pinocytotic vesicles and the typical rod-shaped WPBs. × 50,000.

FIGURE 4 *b* Peripheral portions of two endothelial cells 7 days in culture. The section was cut perpendicular to the cell surface (*P* is the surface of the plastic culture dish). Note the single layer of cells, pinocytotic vesicles, and intercellular junction. × 30,000.

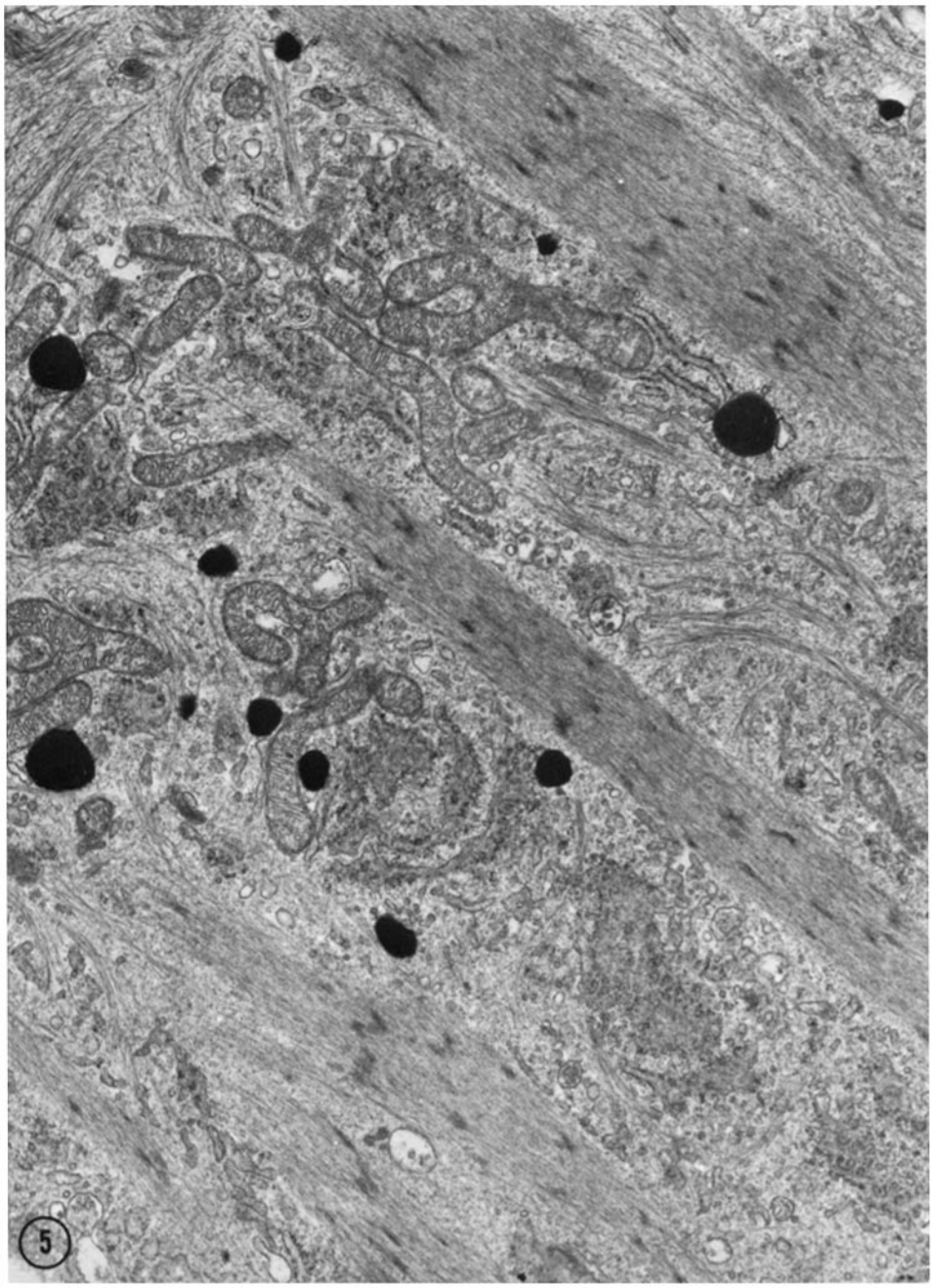


FIGURE 5 Electron micrograph of a portion of the larger, smooth muscle cell type exhibiting myofibrils with fusiform densities, abundant endoplasmic reticulum, and numerous mitochondria. × 14,000.

tured aortic smooth muscle cells (27). In the cultures selected for DNA synthesis experiments, this "contaminant" was absent or occurred at a frequency of less than one in 10,000 cells by phase-contrast examination.

DNA Synthesis in Endothelial Cultures: Autoradiographic Studies

GROWING CULTURES: Primary cultures exposed to [^{3}H]thymidine for 3 h after 6, 21, 45, and

TABLE I

*Regional [^3H]Thymidine Labeling in Human Endothelial Cultures**

	Percent labeled nuclei (± SD)‡	
	Central areas	Peripheral areas
Recently confluent cultures	2.4 (± 0.4)	53.2 (± 4.8)
Postconfluent cultures	3.5 (± 1.5)	3.9 (± 2.3)

* Triplicate cover slip cultures were exposed to [*methyl*-^{3}H]thymidine for 12 h before fixation.
‡ See text for sampling method.

69 h of growth. Labeled nuclei were rarely observed during the first 24-h interval. At 48 h, when increases in viable cell numbers were evident, 5% of nuclei had incorporated radioactive thymidine. At 72 h, when a patchy monolayer was forming by coalescence of larger colonies, 15% of nuclei were labeled.

CONFLUENT AND POSTCONFLUENT CULTURES: In recently confluent cultures, marked regional variations in labeling were observed after exposure to [^{3}H]thymidine for 12 h, as seen in Table I. In central areas of the monolayer, where cells were in contact on all sides, the density of labeled nuclei was low (Fig. 6). Around the periphery of the culture, where cells were in incomplete contact and growth was still occurring, more than 20-fold greater labeling was observed (Fig. 7).

In postconfluent cultures, maintained with daily media changes, these regional differences were not observed. The overall labeling index averaged less than 4% after a 12-h exposure to [^{3}H]thymidine.

REGENERATION AFTER "WOUNDING": During routine media changes, elongated scrapes in confluent primary cultures were occasionally created by the aspirating pipette. These denuded areas filled in completely within 12–24 h, reconstituting the monolayer. When standardized lesions (1 × 5 mm) were made and the cultures exposed to [^{3}H]thymidine for 12 h, marked increases in labeling were observed in the traumatized areas compared to the adjacent intact monolayer (Fig. 8).

DNA Synthesis: Replicate Microwell Cultures

Radioactive thymidine incorporation, assayed by liquid scintillation, was compared in primary and secondary endothelial cultures at various stages of culture growth (Fig. 9). Both populations showed minimal incorporation on day 1, five to sevenfold increases during the growth phase (days 2–3), and gradual decline as cultures reached confluent density (days 4–8). At each stage of growth, subcultured cells showed higher levels of incorporation.

Radioactive thymidine incorporation in postconfluent cultures remained at low levels despite daily exchanges of fresh medium and did not vary significantly in response to media containing 10, 20, or 30% FCS.

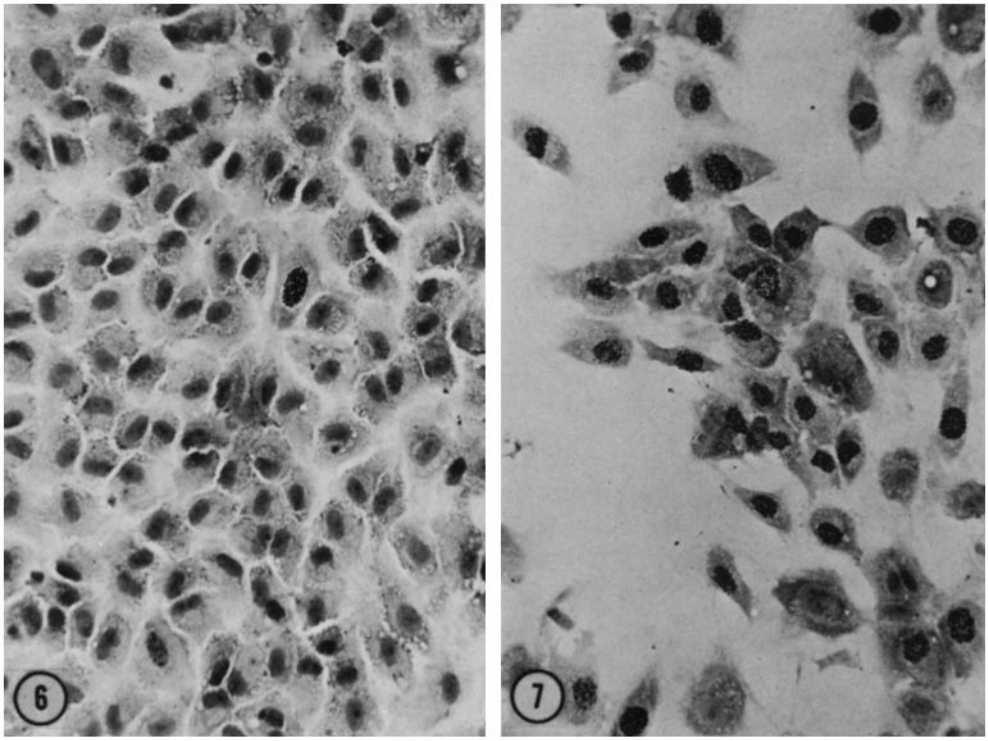
DISCUSSION

The techniques detailed in this report allowed us to isolate a population of human large vessel endothelial cells that were capable of replicating in vitro. Cells in both primary cultures and subcultures retained ultrastructural features characteristic of endothelial cells. Sequential phase-contrast observations and autoradiography showed the development of a confluent monolayer of endothelium, with a low index of cell replication which could be stimulated by wounding. These properties are similar to those of vascular endothelium in vivo (1–6), suggesting that confluent endothelial cell cultures could be useful in studying the pathophysiology of endothelial growth.

Isolation and Identification of Cultured Endothelial Cells

The human umbilical vein has several advantages as a source of endothelial cells. It is a nonbranching, large vessel with a broad intimal surface. Cannulation, flushing, and recovery of effluents are technically uncomplicated. However, although this material is readily available "premortem," many variables such as fetal distress, maternal anesthesia, and anoxic interval during transportation to the laboratory, can affect the viability of isolated cells.

Maruyama (12) and subsequently Fryer et al. (13) used trypsin digestion to isolate umbilical vein endothelium. Their primary cultures failed to grow and exhibited progressive degenerative changes. By using collagenase rather than trypsin, Jaffe and co-workers (16) were able to obtain endothelial cells which apparently grew to confluence in primary cultures. In the present study, control of digestion time and concentration of collagenase were found to be critical for successful



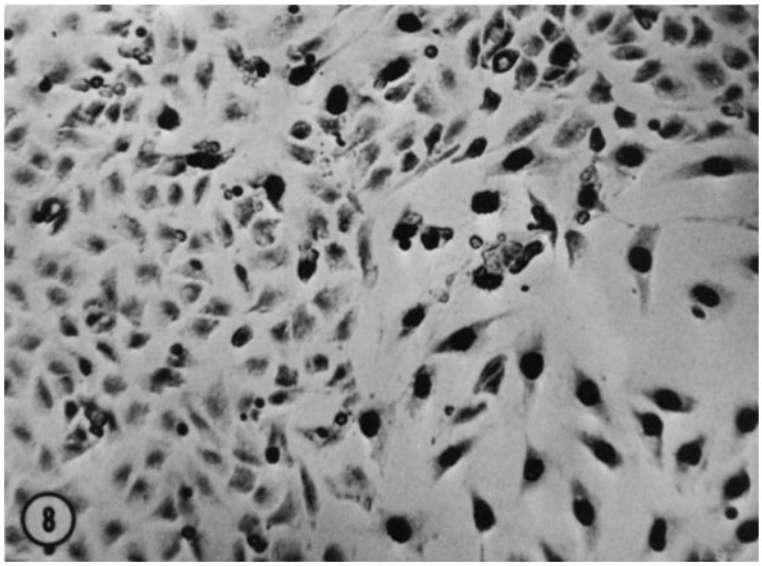


FIGURE 6 Autoradiograph from the central region of a confluent primary endothelial culture after exposure to [^{3}H]thymidine. There is one heavily labeled cell and two lightly labeled cells. Compare with Fig. 7, which is a peripheral region from the same culture. × 200.

FIGURE 7 Peripheral area of an endothelial culture showing heavy labeling after exposure to tritium-labeled thymidine. Compare with Fig. 6, which is the central region of the same culture. × 200.

FIGURE 8 Portion of confluent endothelial culture in which a small area was denuded experimentally. Cells were exposed to [^{3}H]thymidine for 12 h after denudation. Note heavy labeling in the vicinity of the denuded area (right side of photograph). × 100.

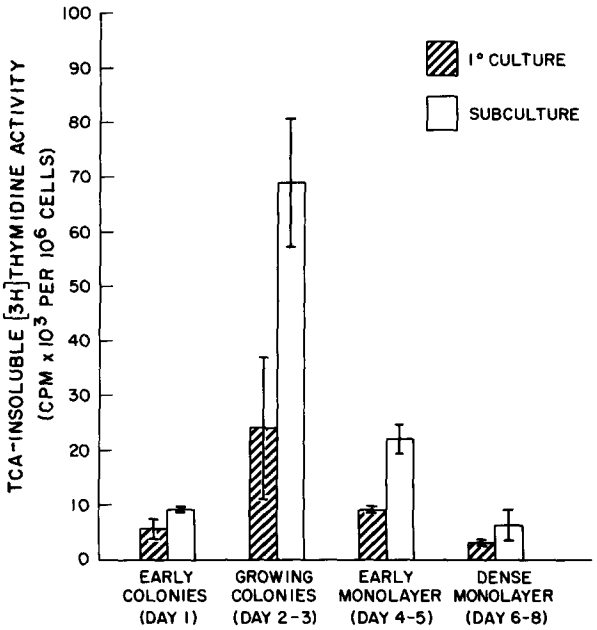


FIGURE 9 [³H]Thymidine incorporation into TCA-insoluble activity in primary and subcultured endothelial cells at successive stages of culture growth. Medium was exchanged daily and the stage of growth determined by phase-contrast examination. Each bar represents the average incorporation (± SD) of three separate cultures (10–20 replicate microwells per culture), after exposure to [*methyl*-³H]thymidine for 12 h.

cell isolation and culture. Exposure for 10–20 min to 1 mg/ml collagenase in PBS (pH 7.2) yielded a uniform population of rounded cells in small clumps, 90% of which were viable by dye exclusion, and 20–50% of which formed epithelioid colonies within 12 h in culture.

The usefulness of collagenase may be due to its action on the subendothelial basement membrane (28), loosening patches of the intimal lining which can then be washed free. In addition, unlike trypsin, collagenase does not tend to digest the cell membrane (29); increase in viable cell number in primary cultures and our success with subculturing may be related to this fact. In harvesting endothelial cells for subculture, it was necessary to limit exposure to trypsin to 2–3 min in order to maintain viability.

Early studies of endothelium in tissue culture (12–15) lacked definite criteria of identity. Maruyama (12) felt that there were no characteristic light microscope markers for this cell, and therefore sought to obtain only intimal cells for his cultures. Recently, Jaffe et al. (16) have used fluorescent antibody staining of thrombosthenin, mixed cell agglutination reactions for ABO blood group antigens, and electron microscope criteria to identify cultured umbilical vein endothelium.

In the present study, cells in primary and subcultures retained an epithelioid morphology and tended to form uniformly confluent monolayers. On electron microscope examination, both primary and subcultured cells contained characteristic rod-shaped cytoplasmic inclusions, first described by Weibel and Palade (23), which have been observed in high density in normal umbilical vein endothelium (22). To our knowledge these bodies have not been reported in other cell types.

The existence of a second cell type in endothelial cultures of umbilical vein origin has also been reported by early workers (12, 13). These very large, pleomorphic cells were interpreted as atypical endothelial cells or macrophages. In the current study, an infrequent second cell type with distinctive phase-contrast features was also noted. By limiting exposure time to trypsin in subculturing, endothelial cells were harvested essentially free of this contaminant. Detailed studies on selectively subcultured contaminant cells have shown that they are smooth muscle cells, derived from the vessel wall (30).

Growth and DNA Synthesis in Endothelial Cell Cultures

Although some observations on cell growth in cultures of human umbilical vein endothelium have been reported by previous workers (12–20), no detailed data are available. While increases in the size of endothelial colonies during the first day in culture probably were the result of spreading of rounded cells on the flask surface, autoradiographic studies confirmed that DNA synthesis was involved in the subsequent formation of confluent monolayers.

Primary endothelial cultures reached and maintained a stable postconfluent density of about 1×10^5 cells per cm^2 of flask surface. As confluent density was approached, DNA synthesis, by autoradiographic and liquid scintillation analysis, became sharply decreased. Denudation of small areas in postconfluent monolayers resulted in localized increases in DNA synthesis. These three observations satisfy the operational definition of postconfluent inhibition of cell division as described by Martz and Steinberg (31). Certain other cells also exhibit these characteristics under conditions of in vitro culture. However, adult endothelium in vivo is one of the few cell types which exists as a single cell layer with a low mitotic index capable of being stimulated by wounding. In this respect, postconfluent endothelial cultures do seem to provide an in vitro analogue to naturally occurring populations of vascular endothelium.

When radioactive thymidine incorporation was quantitated by a liquid scintillation technique (Fig. 9), growing cultures showed the highest values, while cultures approaching confluent densities had lower values. Higher activities seen in subcultures, compared to primary cultures, at each stage of growth may reflect their better adaptation to culture conditions. The low levels in postconfluent cultures were not significantly influenced by daily media changes or serum concentrations. Thus, postconfluent microwell cultures provide a stable low background of DNA synthesis against which small amounts of possible mediators of endothelial mitosis could be tested.

We thank Ms. Christine E. Keller for her excellent technical assistance and Dr. Pietro M. Gullino for his encouragement and helpful discussions.

This work was supported in part by grants from the National Cancer Institute, (5R01 CA 08185), National Heart and Lung Institute (HL 08251), the American Cancer Society, National Chapter (IC-28), and gifts from the Merck & Company and the Alza Corporation. M. A. Gimbrone is a recipient of Surgical Scientist Training Grant from the U. S. Public Health Service and R. S. Cotran is a recipient of Career Development Award from the National Heart and Lung Institute.

Received for publication 12 September 1973, and in revised form 12 November 1973.

Note Added in Proof: The study by Jaffe et al. (16) describing endothelial cultures of human umbilical veins has since been published. (Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphological and immunologic criteria. *J. Clin. Invest.* **52**:2745.)

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