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Cell Biology

Long-term culture of capillary endothelial cells

(bovine adrenal/human foreskin/human spleen/rat adrenal/clonal cell lines)

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ABSTRACT **Capillary endothelial cells from rats, calves, and humans, have been carried in long-term culture. Bovine capillary endothelial cells have been cloned and maintained by serial passage for longer than 8 months. This prolonged culture was accomplished by using tumor-conditioned medium, gelatin-coated plates, and a method of enriching cells in primary culture. Cultured bovine capillary endothelial cells produce Factor VIII antigen and angiotensin-converting enzyme, but do not have Weibel–Palade bodies. Human cells do contain Weibel–Palade bodies. Capillary endothelial cells are distinguished from aortic endothelial cells by their requirement for conditioned medium. Bovine capillary endothelial cells in regular medium grow slowly with a mean doubling time of 67 hr and eventually die. In tumor-conditioned medium, these cells grow rapidly with a doubling time of 28 hr and continue to proliferate for as long as the tumor-conditioned medium is present. In contrast, bovine aortic endothelial cells grow as rapidly in regular medium as in tumor-conditioned medium. This method allows the production of pure capillary endothelial cells that may prove useful for studies of tumor angiogenesis, metastatic mechanisms, and the role of capillary endothelium in other pathologic states.**

Recent advances in cell culture techniques have made it possible to obtain long-term cultures of vascular endothelial cells (1, 2). To date, most success has been achieved with endothelial cells derived from large vessels such as bovine or porcine aorta and the human umbilical vein (for review, see ref. 3). Most recently, bovine aortic endothelium has been cloned (4, 6).

However, long-term culture and cloning of *capillary* endothelial cells have not previously been possible. Del Vecchio *et al.* (5) reported the isolation of capillary endothelial cells from the adrenal cortex of rats. These cells, however, did not grow and could not be maintained *in vitro* for more than a few weeks. By modifying Del Vecchio's method and by using tumor-conditioned medium, gelatin-coated plates, and a method of enriching for capillary endothelial cells in primary culture, we have been able to culture capillary endothelial cells obtained from bovine adrenal glands, human adrenal glands, foreskin, and spleen, and rat heart, lung, kidney, and adrenal tissue. We now report long-term culture of human and bovine capillary endothelial cells and the cloning of bovine capillary endothelial cells.

METHODS

Tumor-Conditioned Medium. Solid tumors of sarcoma 180 were removed from C3H mice and were minced and trypsinized into T-75 Falcon flasks (no. 3024) containing 15 ml of Dulbecco's modified Eagle's medium supplemented with 10% calf serum. When the cells reached early confluence, the conditioned medium was collected and replaced with fresh medium containing 10% calf serum. Conditioned medium was collected from confluent cultures every 2 days thereafter. The cultures were split weekly at a ratio of 1:10. The conditioned medium was centrifuged (2000 rpm for 10 min) and passed through a Millipore filter (0.22 μm) to remove any floating tumor cells. The tumor-conditioned medium was stored at −30°C; it was thawed and centrifuged immediately before use.

Preparation of Gelatin-Coated Dishes. Falcon (no. 3002) 60-mm tissue culture dishes were flooded with 1% (wt/vol) gelatin (Difco) made up in magnesium- and calcium-free phosphate-buffered saline. The dishes were allowed to stand at 4°C overnight. Just before use, the gelatin was aspirated and the dishes were washed once with medium.

Isolation of Bovine Capillary Endothelial Cells. Six adrenal glands were obtained as cleanly as possible from slaughtered calves. Under sterile techniques, the glands were submerged in Betadine solution for 5 min and then washed repeatedly with about 3 liters of sterile saline solution. Each gland was bisected and the adrenal cortex was extricated from the capsule. The adrenal cortex was cut into 1-mm pieces. These were centrifuged in phosphate-buffered saline and then incubated in 0.5% collagenase (Worthington) at room temperature for 1 hr in a polyethylene centrifuge tube. This suspension was pipetted with a large-bore pipette against the wall of the centrifuge tube to break the tissue into smaller pieces. The suspension was then passed through a nylon-covered funnel (Nitex Mono Screen Cloth, HC3-110, Tetko, Elmsford, NY). Only capillary segments smaller than 110 μm passed through the filter. These were centrifuged at 650 rpm for 7 min at 4°C and the collagenase was discarded. The pellet was resuspended in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% calf serum and washed three times. The final pellet of cells was resuspended in 10 ml of Dulbecco's modified Eagle's medium and plated into four gelatinized dishes (60 mm). These plates were incubated at 37°C. Capillary segments and endothelial cell aggregates were the first to adhere to the substratum. Adrenal cortical cells and fibroblasts continued to float, and most were removed by aspirating the supernatant between 1 and 3 hr after plating. After the first aspiration of supernatant, tumor-conditioned medium was added and replaced every 2 days. Capillary fragments usually contained from one to four endothelial cells. As these capillary segments spread onto the coated plastic substratum, each segment became a microcolony with a characteristic appearance that persisted during the next 2–5 days (see Fig. 1 *a* and *b*).

Cloning. To enrich the primary cultures for bovine capillary endothelial cells and to remove contaminating nonendothelial

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cells, we used the following method. One or two colonies arising from a single capillary segment (i.e., usually two to four endothelial cells) were identified on the second or third day. They were circled with permanent ink on the bottom of the plate. A glass pasteur pipette was then drawn through a flame to produce a bead tip (approximately 0.1 mm). This was used to remove or crush any nonendothelial cells within the circle. In early experiments, the glass pipette was moved by a micromanipulator. However, with practice, the pipette could be held by hand. This technique was carried out on the stage of a Nikon inverted microscope (10× phase objective) in a laminar flow hood so that the culture dishes could remain open for 15–20 min during the procedure. Then, all of the residual cells in the dish outside the circle were swept away with a rubber policeman. The medium was changed twice to remove floating cells.

Occasionally the *cell sweeping* procedure was repeated once or twice during the week. The resulting lone endothelial cell colonies grew very slowly or not at all. Their growth could be facilitated by adding feeder cells (BALB/c 3T3 cells or bovine adrenal pericytes, treated for 2 hr with 10 μg of mitomycin per ml) at approximately 3400 cells per cm^2. At this density a few feeder cells usually settled near the tiny endothelial colony. When the endothelial cell colonies had grown to fill about 25–50% of the dish, they were removed by a brief exposure to trypsin/EDTA (1–3 min) and plated in a Costar Cuprak dish at a density of 200 cells per dish. The position of every microwell that contained one cell was recorded 12–18 hr later. As each cell became a colony, it was flooded with 5 μl of trypsin/EDTA and replated on a gelatin-coated 35-mm culture dish containing a feeder layer of subconfluent, mitomycin-treated bovine pericytes or BALB/c 3T3 cells (3400 cells per cm^2). Use of feeder cells was essential for successful transfer of the very small (5–20 cells) colonies isolated from the microwells of the Cuprak dish. Once the endothelial cells filled the spaces between the feeder cells, all cells were removed by trypsinization and transferred to new gelatin-coated plates that lacked feeder cells. Only the endothelial cells, but not the original feeder cells, survived this transfer. Subsequently, the cloned endothelial cells were carried in tumor-conditioned medium and passaged weekly at a ratio of 1:5.

Isolation of Capillary Endothelial Cells from Other Species. An adrenal gland obtained from a 50-year-old woman undergoing adrenalectomy for breast carcinoma was treated in the same manner as the bovine adrenal. A spleen was removed from a 14-year-old boy because of spherocytic hemolytic anemia, and 2 g of tissue from the periphery of the spleen was treated like the bovine adrenal. Human foreskin was obtained from a 3-month-old boy after elective circumcision. The skin was soaked in Betadine solution for 10 min and washed copiously with sterile saline. The subcutaneous tissue was removed from the skin and cut into 1-mm pieces. This tissue suspension was incubated in collagenase and carried through the same steps as used for the bovine adrenal. In order to obtain long-term cultures of the human capillary endothelial cells, the tumor-conditioned medium was diluted 1:1 with α minimal essential medium containing 20% human serum so that the final concentration of human serum was 10%. (The α minimal essential medium with human serum was passed through a 0.22-μm Millipore filter before use.) The final medium was supplemented with additional growth-stimulating factors. These included fibroblast growth factor, 250 ng/ml (6), human thrombin, 1 μg/ml (courtesy of John Fenton, Albany, NY), and endothelial cell growth supplement, 50 μg/ml (Collaborative Research, Waltham, MA). Nonendothelial cells were removed by the method used for bovine adrenal.

Rat adrenal was handled as the bovine adrenal was except that the Betadine wash was omitted. The adrenals were removed under sterile conditions from six rats. The capillaries from these glands were sufficient to seed two or three dishes (60 mm). Liver, spleen, heart, kidney, and lung were also removed from mice and rats. These organs were simply diced into 1-mm fragments and incubated in collagenase in the same manner as that used for the bovine adrenal. The chorioallantoic membranes of 10-day-old chicken embryos were removed and cut into 1-mm pieces and processed similarly.

Characterization. *Staining for Factor VIII antigen.* Cells were grown and stained on 12-mm gelatin-coated glass coverslips. At confluence, the coverslips were fixed in 100% methanol for 5 min at −20°C, washed in phosphate-buffered saline, and incubated for 60 min at 37°C with a 1:80 dilution of rabbit antiserum to human (Behring, Somerville, NJ) or bovine Factor VIII antigen (kindly provided by J. Brown, University of California, San Diego). The cultures were washed (three times for 15 min) in phosphate-buffered saline and incubated for an additional 60 min with a 1:20 dilution of fluorescein-conjugated anti-rabbit IgG. After additional washing in phosphate-buffered saline, the coverslips were mounted on microscope slides and photographs were taken with a Zeiss photomicroscope by using an epifluorescent illuminator and Kodak Tri-X film developed at 3200.

Angiotensin-converting enzyme. The activity of this enzyme in human capillary endothelial cells and bovine capillary endothelial cells was determined in the laboratory of Michael Gimbrone by the method of Friedland and Silverstein (7). In this sensitive fluorimetric method, hydrolysis of the synthetic substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu) is measured by detecting liberated L-histidyl-L-leucine (His-Leu) after its reaction with *o*-phthalaldehyde.

Electron microscopy. Endothelial cells were plated on gelatin-coated plastic coverslips and allowed to grow to various degrees of confluence. The cells were fixed *in situ* with 1% glutaraldehyde and 4% formaldehyde in phosphate buffer. They were postfixed with 1% osmium tetroxide. After they were stained with aqueous uranyl acetate (0.1%), they were dehydrated through graded alcohols and embedded in Epon 812. Subsequently, the cells were sectioned *en face* with a diamond knife on a Reichert ultramicrotome (OMU-2). They were stained with lead citrate and uranyl acetate and examined on a Philips 300 electron microscope (8).

RESULTS

Phase-Contrast Microscopy. Primary cultures of capillary endothelial cells have a characteristic appearance that distinguishes them from other cell types. On the first day after capillary segments are plated, one sees tiny cylindrical segments attached to the plastic, each containing two, three, or four endothelial cells (Fig. 1*a*). As each capillary segment flattens and spreads, the colony of endothelial cells assumes a circular or cylindrical form (Fig. 1*b*). Endothelial cells intimately contact each other and appear always to be circumscribed by a carpet of basement membrane. The dense nuclei often face toward a lumen. New cells grow out in circular rows (Fig. 1 *c–g*). The nuclei contain three or four nucleoli and the cytoplasm is thin and filmy. During the first 1–2 weeks the colonies of capillary endothelium from chicken, mouse, rat, bovine, and human tissues look nearly identical. By 3–4 weeks, sheets of endothelial cells fill the dish in bovine and human cultures, so that the original distinctive "capillary colony" outline disappears (Fig. 1 *h* and *i*). Now the cells all have the typical appearance of endothelium in a confluent monolayer—i.e., a hexagonal or cuboidal shape with a large round nucleus and very little overlapping of adjacent cells (Fig. 1 *g* and *h*). Early colonies

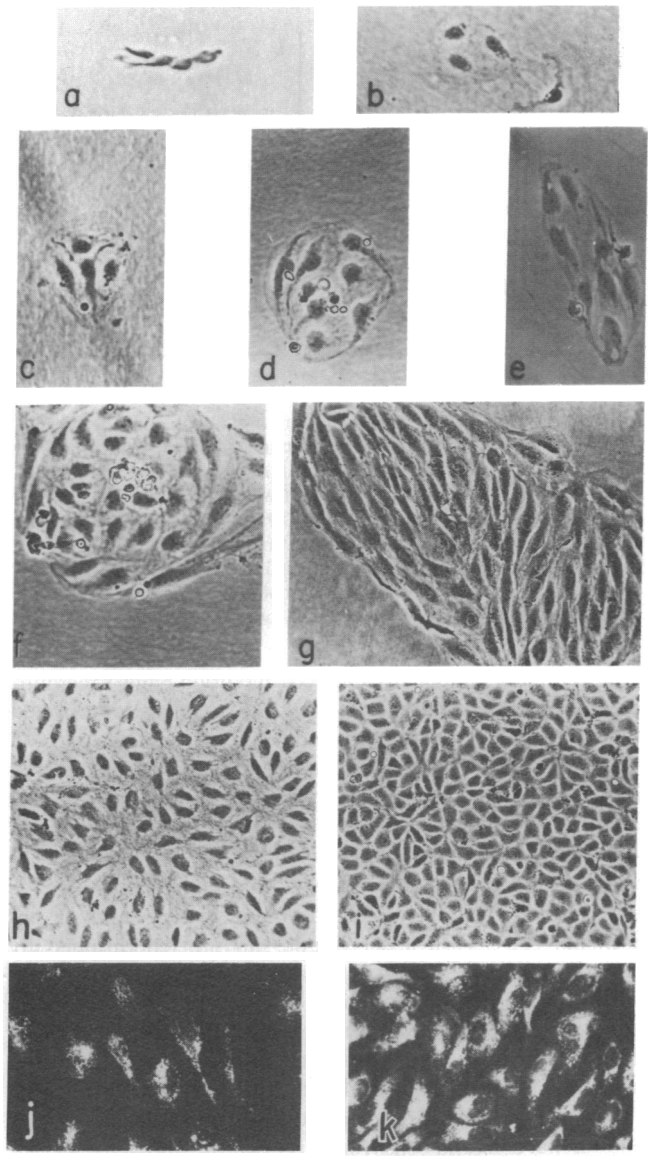

FIG. 1. Capillary endothelial cells in culture viewed by phase-contrast microscopy. (*a*) Human foreskin capillary segment after 2 hr in culture. (×180.) (*b*) Human foreskin capillary segment after 8 hr in culture. (×180.) (*c*) Bovine adrenal capillary endothelium after 18 hr in culture. (×180.) (*d*) Human foreskin capillary endothelium at 5 days in culture. (×180.) (*e*) Rat adrenal capillary endothelium at 5 days. (×180.) (*f*) Colony of human foreskin capillary endothelium, day 7. (×180.) (*g*) Colony of adrenal capillary endothelium, day 7. (×180.) (*h*) Confluent human foreskin capillary endothelium at 7 weeks, second passage. (×90.) (*i*) Cloned, confluent bovine adrenal capillary endothelium, sixth passage. (×90.) (*j*) Human foreskin capillary endothelial cells, third passage. Stained with fluorescent antibody against Factor VIII antigen. (×440.) (*k*) Cloned bovine adrenal capillary endothelium, eighth passage. Stained with fluorescent antibody against Factor VIII. (×440.)

of capillary endothelium have a remarkably similar appearance, whether the cells are obtained from the same organ in different species (Fig. 1) or from different organs of the same species (data not shown). If the cells are allowed to remain confluent for extended periods of time, occasional cells undergrow the monolayer. Such cells appear more elongated than do the cuboidal cells in the monolayer. They appear in cloned as well as uncloned cultures and are therefore not a contaminating cell type. The cells that undergrow the monolayer are of endothelial origin, as determined by the methods described below.

Electron Microscopy. Under electron microscopy, the cultured capillary endothelial cells had the general characteristics of vascular endothelium and, when they were compared to the capillaries within the donor organ (i.e., bovine adrenal cortex), they showed many similar ultrastructural features (Fig. 2). The bovine capillary endothelial cells showed abundant organelles in the cytoplasm together with large numbers of 100-Å filaments, as noted in cultured endothelial cells from other origins (8, 9). Sparse, 60-Å filaments were distributed primarily over the ventral (basal) cytoplasm both *in vivo* and *in vitro*. This internal polarity distinguished them morphologically from one possible contaminant cell, the vascular smooth muscle cell, which has been described by others to be nonpolar in culture, with abundant, evenly distributed 60-Å filaments (10). Cultured capillary endothelial cells had elongated mitochondria with parallel cristae. In contrast, round mitochondria with rounded cristae or abundant lipid droplets as seen in cortical adrenal cells were absent. The specific endothelial organelles (Weibel–Palade bodies) were present in cultured human capillary endothelial cells, but were absent in both cultured and *in vivo* bovine capillary endothelium. Fenestrae were not noted in the cultured cells. The peripheral cytoplasm showed complex interdigitations with adjacent cells, often forming small spaces empty in cross section (Fig. 2*d*).

Factor VIII Antigen. Indirect immunofluorescent staining with antiserum to bovine Factor VIII antigen revealed the presence of the antigen in primary cultures of bovine capillary endothelial cells as well as in clonal lines of these cells after 75 population doublings (Fig. 1*k*). Staining was localized in cytoplasmic granules that were frequently, but not exclusively, concentrated in the perinuclear space. A similar distribution of the antigen was seen when human foreskin capillary endothelial cells were stained with antiserum to human Factor VIII antigen (Fig. 1*j*). When immunofluorescent staining was performed on primary cultures, the endothelial cells could be seen as colonies of fluorescent cells surrounded by unstained cells. After elimination of nonendothelial cells and subsequent cloning, staining could be observed in nearly every cell in the culture. Several nonendothelial cell lines, including bovine smooth muscle cells, human foreskin fibroblasts, and Swiss mouse 3T3 cells, all failed to stain with the Factor VIII antisera used for these experiments.

Angiotensin-Converting Enzyme. Two different clones of bovine capillary endothelium that demonstrated Factor VIII antigen also displayed significant activity of angiotensin-converting enzyme. Bovine aortic endothelium and human umbilical vein endothelium had angiotensin-converting enzyme activity equivalent to capillary endothelium. Activity of this enzyme was absent in bovine smooth muscle cells.

Behavior of Capillary Endothelial Cells in Culture. Several features distinguished capillary endothelial cells from aortic endothelial cells. Perhaps the most striking difference was in the response to tumor-conditioned medium. Bovine aortic endothelial cells proliferated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and could be passaged for up to 6 months. In contrast, early-passage bovine capillary endothelial cells failed to proliferate and could not be maintained in this medium for longer than approximately 7–10 days before they began to die. However, when the same medium was first "conditioned" by incubation with tumor cells for 24–48 hr, the capillary endothelial cells proliferated rapidly and formed an organized monolayer. When late-passage bovine adrenal capillary endothelial cells were seeded at low density in Dulbecco's modified Eagle's medium containing 10% calf serum, they grew slowly with a mean population doubling time of 67 hr. However, when the same cells were grown in tumor-

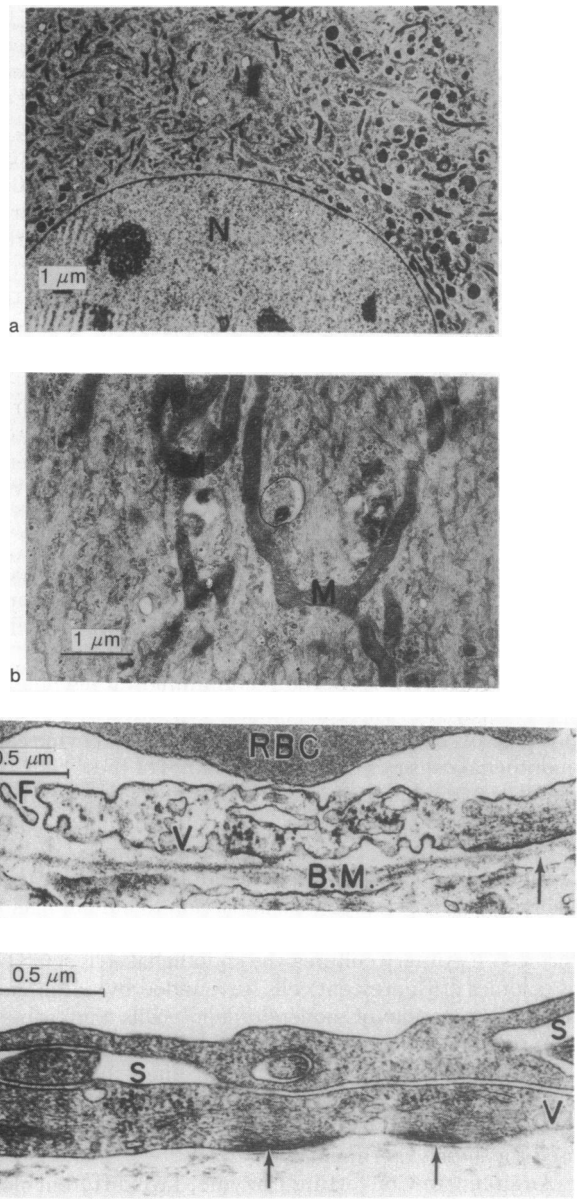

FIG. 2. Electron micrographs of cloned bovine adrenal capillary endothelial cells in culture. (*a*) Section cut *en face* through the nucleus (N) and perinuclear cytoplasm. There are numerous organelles, including elongated mitrochondria. This mitrochondrial configuration is characteristic for capillary endothelial cells in the adrenal; by contrast, adrenal cortical cells show round mitochondria. (*b*) An area in the cytoplasm. One-hundred-angstrom filaments are abundant. Note the elongated shape of the mitochondria (M) containing interdigitating elongated cristae, which are not found in adrenal parenchymal cells. Note the absence of lipid droplets. (*c*) Flat cytoplasmic extension of an individual bovine capillary endothelial cell *in vivo*, showing the adhesion site with microfilaments at the base (arrow). The cytoplasm is fenestrated (F) and shows coated and noncoated vesicles (V), basement membrane (BM), and erythrocytes (RBC). (*d*) Cross section through bovine capillary endothelial cells *in vitro*. Extensions of the cells show complex interdigitations with formation of spaces (S). Microfilaments are also in the basal portion of the cellular extension (arrows). Fenestrae are not seen *in vitro*, and vesicles (V) are less frequent in this section.

conditioned medium (mixed 1:1 with fresh Dulbecco's modified Eagle's medium containing 10% calf serum), the cells grew rapidly with a doubling time of 28 hr (Fig. 3*a*). In contrast, bovine aortic endothelial cells, whether early or late passage,

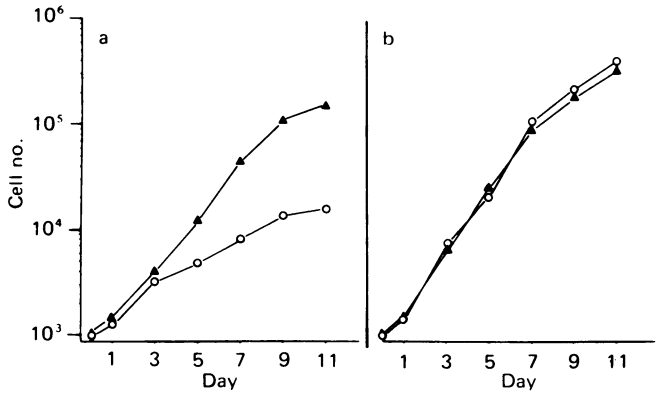

FIG. 3. Response of bovine capillary and aortic endothelial cells to tumor-conditioned and nonconditioned medium. One thousand bovine capillary endothelial cells (*a*) or bovine aortic endothelial cells (*b*) were seeded in Falcon no. 3008 multiwell chambers in Dulbecco's modified Eagle's medium containing 10% calf serum (○) or in the modified medium that had been conditioned by 48 hr of incubation with sarcoma-180 cells and then mixed 1:1 with fresh medium containing 10% calf serum (▲). The medium was changed every third day. Cell numbers represent the average of four experimental samples.

grew as rapidly in regular Dulbecco's modified Eagle's medium containing 10% calf serum as they did in tumor-conditioned medium (Fig. 3*b*). Although we routinely used cells derived from mouse sarcoma 180 to prepare tumor-conditioned medium, the growth of capillary endothelial cells was also stimulated by medium that had been conditioned by other types of tumor, including B16 melanoma, and by an established line of human bladder carcinoma (11). Medium conditioned by bovine aortic endothelial cells also supported growth of capillary endothelial cells. To a lesser extent, medium conditioned by smooth muscle cells and by foreskin fibroblast cells also supported capillary endothelium. This indicated that facilitated growth of capillary endothelium is not specific to tumor-conditioned medium. We used medium conditioned by tumor because large quantities could be easily prepared.

Another striking difference between early-passage capillary endothelial cells and cells from large arteries and veins was the inability of capillary cells to grow on tissue culture plastic unless it had first been coated with a gelatin matrix. When plated on uncoated dishes, these cells became vacuolated and failed to proliferate. In contrast, bovine aortic endothelial cells grew equally well on tissue culture dishes with or without a gelatin substratum.

There was also a difference in the viability of capillary endothelial cells from several different species. For example, bovine capillary endothelial cells grew the most rapidly. They also survived repeated passage, maintained a high plating efficiency, and could be cloned. Human capillary endothelial cells were less hardy. They grew more slowly and required additional growth factors (fibroblast growth factor, thrombin, and endothelial cell growth supplement) in the tumor-conditioned medium. Although they could be passaged every 3 weeks for up to 5 months, and although confluent monolayers have been established from a single capillary (i.e., four cells), we have not as yet succeeded in cloning human lines. Rat, mouse, and chicken embryo cells were the most sensitive to manipulation. They failed to survive one passage.

Since the bovine capillary endothelial cells have been cloned, they have been carried to date for as long as 8 months without a significant change in growth rate or morphology. These cells have also survived freezing, storage, and thawing.

DISCUSSION

The short-term culture of endothelial cells derived from capillaries has been achieved in several laboratories. Such cultures have been reported from the rat epididymal fat pad (12), rat adrenal (5), human foreskin (13), and rabbit retina (14,¶,‖). We now report that capillary endothelial cells can be cultured from diverse organs of several mammalian species, grown as long-term cultures free from contaminating capillary pericytes, and, for endothelium from bovine adrenal, cloned. These techniques allow production of pure capillary endothelial cells necessary for metabolic studies. To date, only bovine capillary endothelial cells have been grown in large quantities. Although it is tedious to clone bovine capillary endothelial cells from primary culture, they are not difficult to maintain thereafter.

Primary cultures of capillary endothelial cells are exceedingly fragile and do not survive the first passage from mixed cultures by the conventional cloning ring technique. For this reason we reversed the method and removed all of the contaminating cells first so that the endothelial cells remained in place untouched. When the colonies grew to fill most of the dish, passage was possible.

Factor VIII antigen has been found only in endothelial cells, platelets, and megakaryocytes (15). It is considered the most reliable marker for endothelial cells in culture. Angiotensin-converting enzyme has also been shown to be a marker for endothelial cells (16, 17). Although this enzyme has also been found in brush border cells of the gut and in the proximal tubules of the kidney, only endothelial cells are known to have *both* Factor VIII antigen and the angiotensin-converting enzyme. We have found both markers in late-passage cultures of cloned capillary endothelial cells as well as in early-passage, uncloned cultures.

There are several potential applications of these capillary endothelial cultures. Tumor angiogenesis takes place at the level of *capillary* endothelium, not aortic endothelium. Therefore, it will be important to compare the reactivity of these two kinds of endothelium to a challenge by fractions containing tumor angiogenesis activity (18–20). If capillary endothelial cells are indeed more sensitive to the action of tumor angiogenesis factors than aortic or venous endothelium, then these new cloned capillary endothelial cells may serve as a quantitative *in vitro* assay system to detect stimulators as well as inhibitors of angiogenesis (21).

Capillary endothelial cells may also be valuable for the study of metastatic mechanisms. The metastatic process operates mainly at the level of capillary endothelium, not aortic or large vein endothelium. Fidler (22) and Nicolson and Brunson (23) have shown that target site specificity of metastatic implants may be guided by information residing in the tumor cell itself. Whether certain tumor cells *in vitro* will adhere differentially to capillary endothelial cells from different organs is now susceptible to test.

Capillary proliferation appears also to play a central role in three other diseases: diabetic retinopathy, rheumatoid arthritis (24), and psoriasis (25). It is possible that cultures of capillary endothelium may be more useful than aortic endothelium for studying stimulators of neovascularization or deficient inhibitors of neovascularization that could be associated with these pathologic states.

Finally, cultures of capillary endothelium may help to elucidate important functional differences between the capillaries of one organ from those of another that have not been previously detectable. For example, the general phenomenon of the "homing" of lymphocytes, so important to a variety of immunological reactions, may possibly be amenable to *in vitro* analysis.

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