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MORPHOLOGIC PLASTICITY AND PERIODICITY: PORCINE CEREBRAL MICROVASCULAR CELLS IN CULTURE

DOUGLAS H. ROBINSON, YUAN-HSU KANG, STEVEN H. DESCHNER, AND THOR B. NIELSEN[1]

Diving Medicine (D. H. R., S. H. D.) and Casualty Care Research (Y.-H. K., T. B. N.) Departments, Naval Medical Research Institute, Bethesda, Maryland 20814

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SUMMARY

Porcine cerebral microvascular (PCMV) endothelial cell cultures and pericyte-endothelial cell cocultures were established and the self-organizational properties of the cells were examined in various culture conditions. Cultured PCMV endothelial cells were characterized by the capacity to produce prostacyclin in response to bradykinin. Cultured PCMV pericytes were identified with a smooth muscle actin-specific stain. PCMV endothelial cells organized into cord structures when left in culture for several weeks without passage. Lumina were observed in cross sections of these cords and appeared to form through a process of cell-selective autolysis. PCMV endothelial cells required three dimensions for self-organization, forming suspended cords in planes that either intersected or paralleled the culture vessel floor. After formation, suspended cords continued to exhibit a morphologic plasticity punctuated by the coordinated migrations of PCMV endothelial cells en masse. Sequential propagation of PCMV endothelial cell monolayers and development of suspended capillarylike cords recurred cyclically when cells were left in culture without passage for several weeks. Cord development was also observed in PCMV pericyte-endothelial cell cocultures with large proportions of pericytes. However, pericytes were not located in cross sections of suspended cords formed in coculture. Apparently, in some conditions of PCMV coculture, populations of PCMV endothelial cells and pericytes segregate. Retina-derived growth factor (RDGF) promoted this cell-type segregation and the subsequent formation of suspended cords in PCMV cocultures, although its exact mode of action is unclear. These results indicate that cultured cerebral microvascular endothelial cells and pericytes have capacities for complex, temporal self-organization that varies according to culture conditions.

Key words: capillary; endothelial cells; pericytes; cell-to-cell interactions; plasticity; periodicity.

INTRODUCTION

The formation of capillarylike structures by cultured endothelial cells has been described by several investigators (1,7,16,21,23,25). Angiogenesis in vitro requires diffusible factors, cell-matrix interaction, and endothelial cell interaction with other endothelial cells and accessory cells of the microvessel (8,22,31). Accordingly, the role of heterologous cell-to-cell interaction in modulating microvascular endothelial cell differentiation and organization in vitro is of interest and requires well-defined coculture techniques (27).

Current techniques for the primary isolation and culture of cerebral microvascular endothelial cells are designed to exclude the pericyte. Despite meticulous efforts to cull pericytes from isolated cerebral microvessels, inadvertent coculture of cerebral microvascular pericytes and endothelial cells does occur (2). Although most investigators have chosen to focus on the in vitro properties of brain capillary endothelial cells, relegating the pericyte to the role of a "contaminating" cell type, recent work has explored the properties of the pericyte in culture (14,27,33).

Here we describe the primary isolation of porcine cerebral microvascular (PCMV) endothelial cells and pericytes. PCMV endothelial cells periodically self-organize into cords that exhibit some morphologic similarities to microvessels. PCMV pericytes in coculture with PCMV endothelial cells modulate this temporal self-organization. The interaction of these two cerebral microvascular cell types in coculture is complex and seems to be regulated, in part, by supplemental retina-derived growth factor (RDGF).

MATERIALS AND METHODS

Materials. Dulbecco's phosphate buffered saline (DPBS), trypsin (0.05%)-EDTA (0.1%), gentamicin, Fungizone, and Medium 199 (M199) were obtained from

[1] To whom correspondence should be addressed at Metabolic Research Division, CCRD, Naval Medical Research Institute, Bethesda, MD 20814.

Quality Biological Inc. (Gaithersburg, MD). GIBCO, (Grand Island, NY) supplied L-glutamine. Heat-inactivated fetal bovine serum (FBS) was obtained from Hyclone, Logan UT. Collagenase (204 U/mg) was purchased from Worthington Biomedical (Freehold, NJ). Povidone-iodine 10% was obtained from Sherwood Pharmaceutical Co., Rahway, NJ. Ficoll-Paque lymphocyte isolation medium and Cytodex 1 beads were supplied by Pharmacia, Inc., Piscataway, NJ. RDGF was prepared by the method of Glaser et al. (15) and stored at −20° C. Gamma-glutamyl-4 methoxy-2-naphthylamide (GMNA), and fast blue BBN (diazotized 4′ amino diethoxybenzanilide), sodium heparin, 3-aminopropyltriethoxysilane, monoclonal antibody to a synthetic 8-amino acid sequence of human smooth muscle actin (A2547), α-naphthyl butyrate, and pararosaniline were obtained from Sigma Chemicals (St. Louis, MO). The lectins *Bandeiraea Simplicifolia* (BSA-1), *Ulex europaeus* (UEA-1), *Triticum vulgaris* (WGA-1), *Arachis hypogaea* (PNA-1), and *Solanum tuberosum* (STA-1) were purchased from Sigma. Fibronectin was obtained from Pierce Chemical, Rockport, IL, and Collaborative Research, Bedford, MA. Bradykinin was obtained from Calbiochem-Behring, La Jolla, CA. Rabbit antibody to human Factor VIII was purchased from DAKO (Copenhagen, Denmark).

Cell culture. PCMV cells were isolated by modifications of the method of Carson and Haudenschild (2) as follows: The brains of 4- to 6-mo. Yucatan minipigs (*Sus scrofa* Yucatan) were collected aseptically. The brains were obtained from pigs used in a septic shock study that included control animals and animals rendered septic with gentamicin-sensitive *Escherichia coli*. Talc-free gloves (Ace Scientific, New Brunswick, NJ) were used to avoid the inhibition of endothelial cell growth by talc (30). The brains were placed into Hanks' balanced salt solution (BSS) containing 25 m*M* HEPES, 100 μg/liter gentamicin and 2.5 μg/ml Fungizone (Hanks'). In the laboratory the brains were immersed in 10% Povidone-iodine for 2 min, and all subsequent treatments were carried out aseptically and at room temperature. The brains were then rinsed with 5 to 6 aliquots of Hanks' to remove the residual iodine. The cortices were dissected free of the hind brain and cerebellum; and the pia carefully removed. Subsequent steps were performed in containers rinsed with 1% bovine serum albumin (fraction V, USB) in DPBS. The gray matter of the exposed cortices were aspirated through a Pasteur pipette and collected. Aspirated material was centrifuged for 10 min at 500 *g* at room temperature. The pellets were resuspended in Hanks and homogenized with 16 strokes in a 40-ml Dounce homogenizer (Wheaton) using the tight-fitting pestle (25 to 75 μm). The homogenate was centrifuged, and the pellets were resuspended. The suspension was sieved sequentially through nylon fabric of 149-, 76-, and 20-μm mesh sizes, which had previously been sterilized with Povidone-iodine and rinsed in 70% alcohol. The microvessels retained by the 76- and 20-μm nylon fabrics were collected. The microvessels from each brain were resuspended in 6 ml of Hanks' and layered onto discontinuous gradients of Ficoll-Paque:DPBS. The gradients contained 2 ml each of 75% (vol/vol), 67% (vol/vol), and 33% (vol/vol) Ficoll-Paque, prepared in 15-ml conical tubes. The gradients were centrifuged for 10 min at 500 *g*. The 33 to 67% and 67 to 75% interface material were collected for examination, and the pellets were collected for culture. The pellets were diluted with Hanks', collected by centrifugation, and resuspended in 2 ml of Hanks' and diluted with an equal volume of collagenase (1 mg/ml). After 2 min the microvessels were diluted with Hanks' and collected by centrifugation. The purified microvessels were cultured in fibronectin-coated (Collaborative Research), 16-mm wells using the following medium: M199 containing 10% heat inactivated FBS, 500 μg/ml sodium heparin (Sigma), and 2 to 10 μl/ml of RDGF (13,15). The cultures were incubated at 37° C in air with 5% CO_2. After allowing 48 to 72 h for cell attachment, the medium was changed. The cells were subcultured no earlier than 7 d by treatment with trypsin-EDTA. The cells were subsequently cultured on gelatin-coated or uncoated plasticware. The medium was changed twice weekly. Growth requirements were investigated by selective deletion of nutrients and matrix and by supplementation with hormones and growth factors. Bovine retinal pericytes were isolated and cultured according to the method of Gitlin and D'Amore (14). Porcine aortic endothelial cells were isolated and cultured according to the method of Wu et al. (36).

Endothelial cell lines were obtained from the following sources: GM 7372 bovine aortic cells from the NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research, Camden, NJ; HUVEC, human umbilical vein cells from the American Type Culture Collection, Rockville, MD. L-929 mouse fibroblasts were obtained from the American Type Culture Collection, Rockville, MD. Bovine smooth muscle cells (AG2410) were obtained from the NIGMS Human Genetic Mutant Cell Repository.

Immunocytochemistry. Cells grown on glass cover slips were examined for characteristic markers by indirect immunofluorescence using previous methods (26). Some cells, which attached poorly to glass cover slips were seeded onto cover slips treated as follows: clean, dry cover slips were individually immersed for 30 s in 5% (vol/vol) 3-aminopropyltriethoxysilane in $CHCl_3$, washed in 50 ml $CHCl_3$, autoclaved, and coated with 1% sterile gelatin. Cells on cover slips were fixed for 10 min in 3.7% formaldehyde in DPBS or for 10 min in absolute methanol at −20° C. Cells in monolayer were examined for the presence of α-smooth muscle actin by incubation with a mouse monoclonal antihuman α-smooth muscle actin antibody (1:400) at 37° C for 2 to 4 h (20,32). Cells in a cord morphology were incubated with primary antibody from 4 to 6 h. Subsequently, primary antibody-treated cords were washed and incubated with rhodamine-labeled antimouse IgG (1:18) for 2 to 16 h. Cultured bovine retinal pericytes were used as controls. Cells were also stained with antibody to Factor VIII:rAg for 60 to 90 min and fluorescein or rhodamine-conjugated goat antirabbit antibody. HUVEC were used as positive controls. Cells were also examined for von Willebrand Factor according

to the method of Wu et al. (36). The primary antibody was a generous gift of Dr. Dominique Meyer, INSERM, Cedex, France.

Frozen sections. Cords were fixed in 3.7% formaldehyde and stained for the presence of α-smooth muscle actin in the manner described above. Subsequently, the cords were frozen with liquid nitrogen, and serial 15-μm cross sections were cut with a steel-knife microtome. The cross sections were mounted in glycerol on glass slides and examined for specific immunofluorescence.

Histochemistry. Cells from long-term cultures (> 6 passages) were examined for gamma-glutamyl transpeptidase (gamma-GTP) activity by the method of Dropulic and Masters (5). PCMV cells were also examined for the presence of Weibel-Palade body-related, nonspecific esterase (NSE) according to the method of Monahan-Earley et al. (24).

Lectin binding. Cells grown on glass cover slips were examined for lectin binding with a battery of fluorescein-labeled lectins. The method used was derived from that of Gerhart et al. (11). Serial dilutions of lectins in DPBS were made to determine optimal binding and staining patterns. Typically, concentrations of 20 to 30 μg/ml were used. Porcine aortic endothelial cells were used as controls.

DiI-Ac-LDL ingestion. PCMV cells were examined for the ability to ingest DiI-Ac-LDL according to the method of Voyta et al. (35). AG2410 smooth muscle and GM7372 aortic endothelial cells were used as negative and positive controls, respectively.

Prostacyclin determinations. Prostacyclin formation was determined according to a modified method of Forsberg et al. (9). PCMV cells were plated in 24-well plastic culture dishes without gelatin-coating at a concentration of 30 000 cells per well using M199 with RDGF (5 μl/ml) and 10% FBS. The cells were incubated at 37° C (5% CO_2:95% air) and allowed to grow to confluence (4 to 5 d). All subsequent steps were performed at room temperature. The cells were washed with M199 containing 1% FBS and 20 m*M* HEPES (pH 7.4). Bradykinin (1 μM) was dissolved in the same medium and the medium in each well was replaced with 0.30 ml of this solution. At 0, 1, 3, and 5 min, 0.25-ml samples of supernatant were removed and frozen on dry ice or put on ice for prompt assay. Four wells were assayed at each time point. A 100 μl sample of each was analyzed in duplicate for the stable prostacyclin metabolite 6-keto-$PGF_1\alpha$ with an assay system purchased from Amersham, Chicago, IL. The results obtained were calculated as the amount of 6-keto-$PGF_1\alpha$ released into the medium per well. Indomethacin (10 μM) in DPBS was used to suppress the formation of prostacyclin. Cells were treated with this solution for approximately 5 min, washed, exposed to the bradykinin solution, and 0 and 5 min 0.25-ml samples were removed for the 6-keto-$PGF_1\alpha$ assay. L-929 mouse fibroblasts were used as negative controls.

Morphogenic role of RDGF in PCMV coculture. In a study designed to investigate a possible morphogenic role for supplemental RDGF in PCMV cocultures, PCMV cocultures (Passage 6; 60% pericytes:40% endothelial

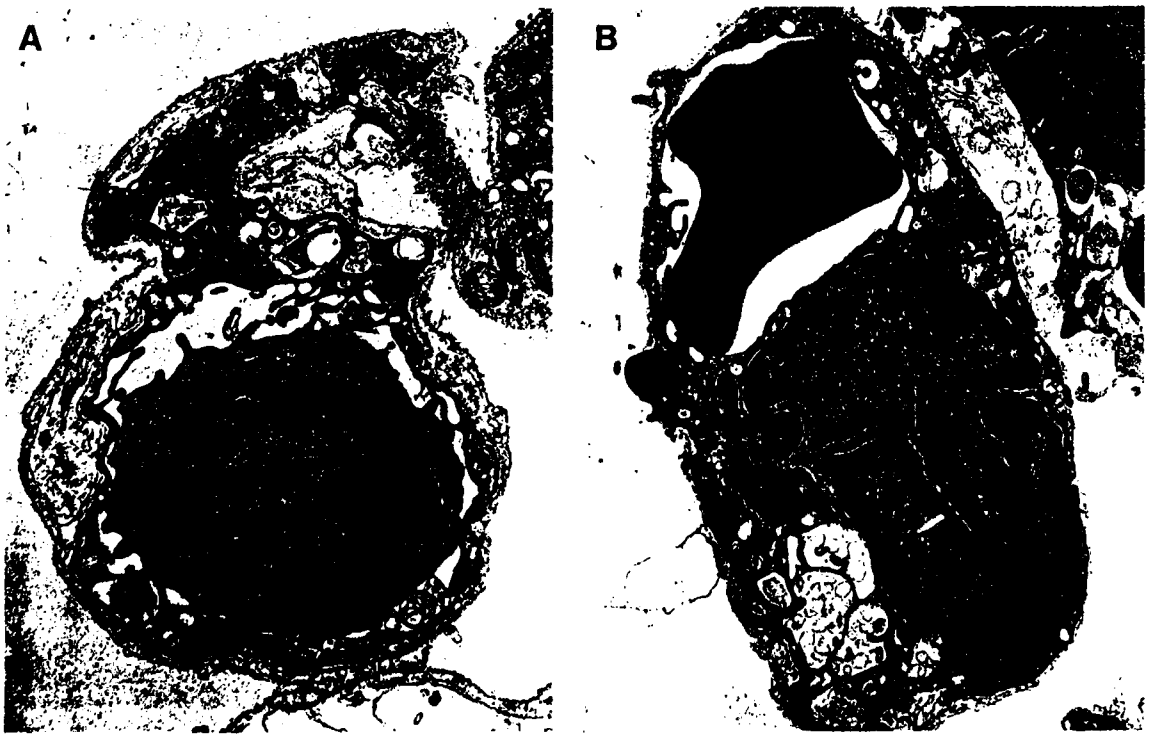

FIG. 1. Isolated porcine cerebral microvessels. Microvessels were isolated as described in Materials and Methods. The figures are transmission electron micrographs of untreated (*A* ×10 909) and collagenase-treated (*B* ×7636) microvessels.

cells) were subjected to continuous or intermittent supplementation with RDGF after plating. Cells that had been maintained in large, plastic culture flasks with RDGF-supplemented medium were passed onto gelatin-coated, 24-well plastic culture plates in growth medium consisting of M199, 10% FBS, and heparin. Half the plates were treated with 5 μl/ml RDGF; the other half received no supplemental RDGF. After 6 d in culture all cocultures were fed growth medium containing RDGF but no heparin. After 3 to 4 wk, large, floor-bound cords formed in cocultures treated continuously with RDGF. At the end of 5 to 6 wk, large, detached monolayers and complex networks of suspended cords and cell-aggregates were observed in these cocultures treated continuously with RDGF. Suspended cord formation did occur in the cocultures supplemented intermittently with RDGF, although the initiation of cord development was retarded as compared to that in cocultures supplemented continuously with RDGF. When examined at the end of 4 wk, these intermittently supplemented cocultures contained few large, floor-bound cords and few small cords attached to culture vessel walls. By 7 to 8 wk, intermittently supplemented cocultures displayed significant suspended cord development. However, cord structures observed in cocultures supplemented intermittently with RDGF were less numerous and less complex than those observed in cocultures supplemented continuously. In a parallel investigation, PCMV endothelial cell cultures (Passage 12; <5% pericytes) were exposed to continuous or intermittent supplementation with RDGF (5 μl/ml). One group of cells received RDGF continuously. The other group of cells received supplemental RDGF in cycles of 10 to 14 d with supplementation in the first 10 d of culture. Suspended cord formation was observed to be synchronous (initiation at 2 to 3 wk) and morphologically similar in PCMV endothelial cell cultures supplemented either continuously or intermittently with RDGF.

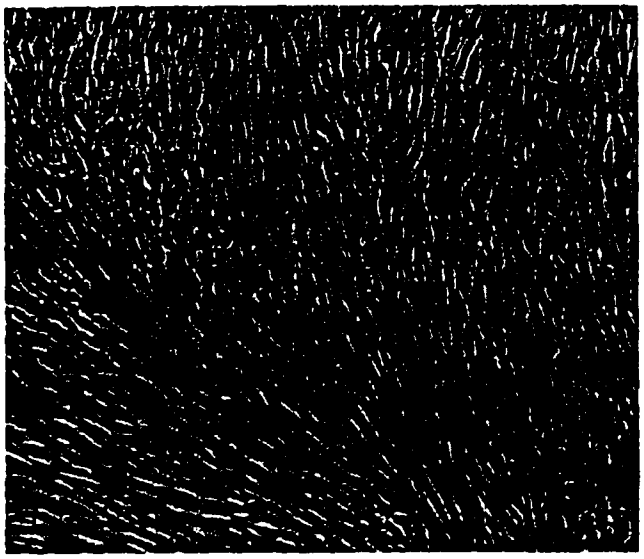

FIG. 2. Phase contrast photomicrograph of confluent PCMV endothelial cells. PCMV cells were cultured on a gelatin substratum in the presence of heparin and RDGF. ×28.

At 8 wk these PCMV endothelial cell cultures and cocultures were washed and fixed in formaldehyde. The cells were then stained in situ with monoclonal antibody to α-smooth muscle actin. The proportions of endothelial cell and pericyte populations in the PCMV endothelial cell cultures (<5% pericytes) seemed to be stable regardless of the RDGF supplementation schedule used. However, examination of PCMV cocultures supplemented with RDGF either continuously or intermittently revealed a decline, by half or more, in the pericyte population from that present in the coculture at the beginning of the investigation (approximately 60% pericytes).

Light and electron microscopy. Cells in culture were observed by phase contrast and Hoffman modulation contrast with an Olympus IM-2 fitted with an Olympus OM-2 camera. The cells were viewed for immunofluorescence with a Nikon Optiphot microscope equipped with fluorescence and phase contrast optics and photographed with a UFX II camera system using Tri-X film (Eastman Kodak, Rochester, NY) developed in ACU-1 (Acufine, Chicago, IL). In preparation for electron microscopy, samples were fixed for 30 min at 4° C in 1% paraformaldehyde, 2% glutaraldehyde, 0.1 *M* cacodylate, pH 7.5. The samples were postfixed in 1% osmium tetroxide, dehydrated in a series of graded ethanol solutions, and embedded in Epon (Polybed, Polysciences, Warrenton, PA). Thick sections were stained with toluidine blue. Ultrathin sections of 90 nm were cut with a diamond knife and the sections stained with uranium acetate and lead citrate. The sections were examined with a JEOL 100CX transmission electron microscope.

RESULTS

Cell isolation and culture. Isolated porcine brain microvessels were substantially free of other organized tissue components as observed by phase contrast microscopy. Examination of purified microvessels by transmission electron microscopy revealed endothelium with adherent pericyte or smooth muscle cells and afibrillar matrix ablumenally (Fig. 1 *A*) and occasional leukocytes or red blood cells in the vascular lumen. A short treatment with collagenase resulted in removal of substantial amounts of the adherent matrix and nonendothelial cells (Fig. 1 *B*). When the microvessels were incubated with growth medium in matrix-coated dishes, a proportion of the vessels attached and the cells propagated, usually in tightly adherent clusters. Cells could be propagated for more than 18 passages and yielded nearly uniform monolayers of compact, phase-dense polygonal cells (Fig. 2). Typically, two culture conditions were used: a) RDGF and heparin supplementation for cells grown on coated plasticware and b) RDGF supplementation for cells grown on uncoated plasticware.

Cell identification. PCMV pericytes or pericytelike cells were identified by indirect immunofluorescent staining with a monoclonal antibody to α-smooth muscle actin. Cells were considered to be pericytes if two conditions were fulfilled: a) cells were isolated from microvessels without a continuous muscular coat and b) cells so isolated stained with antibody to α-smooth muscle

actin. Cells staining positively with this antibody exhibited a microfilament pattern (Fig. 3 *B*). Endothelial cells exhibited a relatively dim and diffuse fluorescence due to a nonspecific binding of the rhodamine-labeled secondary antibody. Bovine retinal pericytes served as a positive control and exhibited a characteristic microfilament pattern in 95 to 100% of cells stained (Fig. 3 *C*).

Bradykinin-stimulated prostacyclin formation was used as a biochemical marker for endothelial cells.

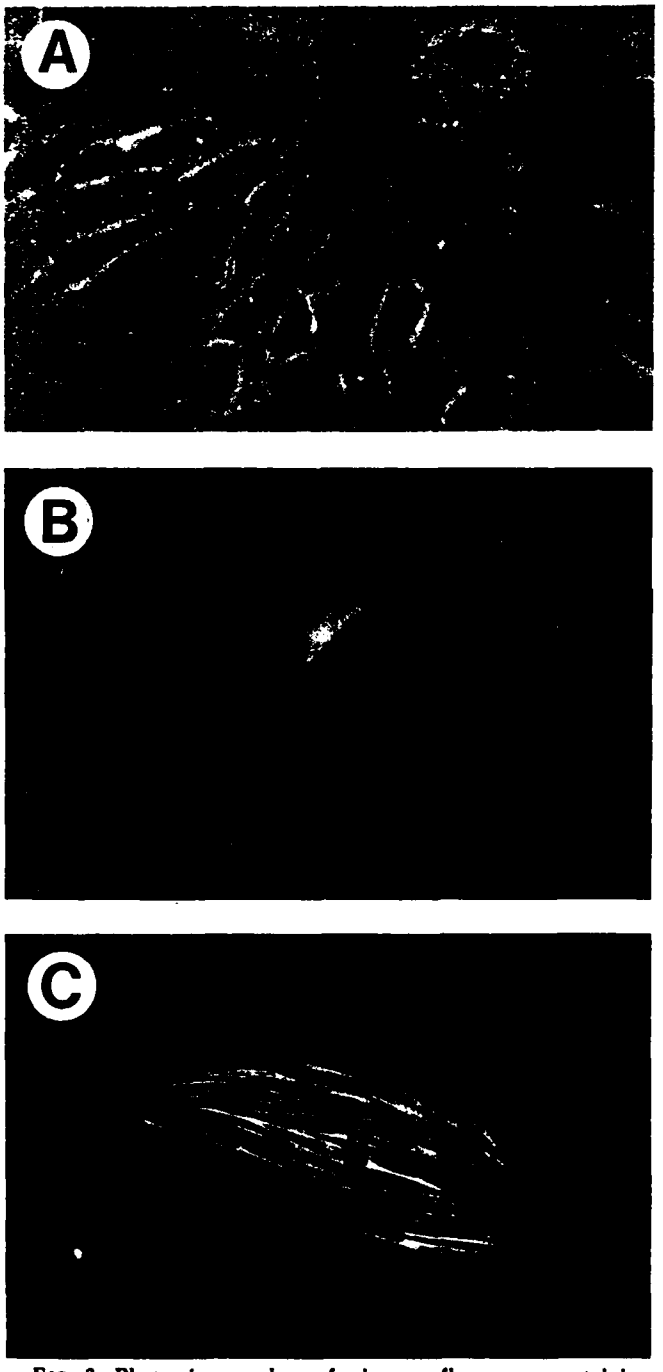

FIG. 3. Photomicrographs of immunofluorescent staining with antibody to α-smooth muscle actin. The same field of PCMV cells were observed with phase contrast (*A* ×280) or epi-fluorescence (*B* ×280). Retinal pericytes were observed with epi-fluorescence (*C* ×168).

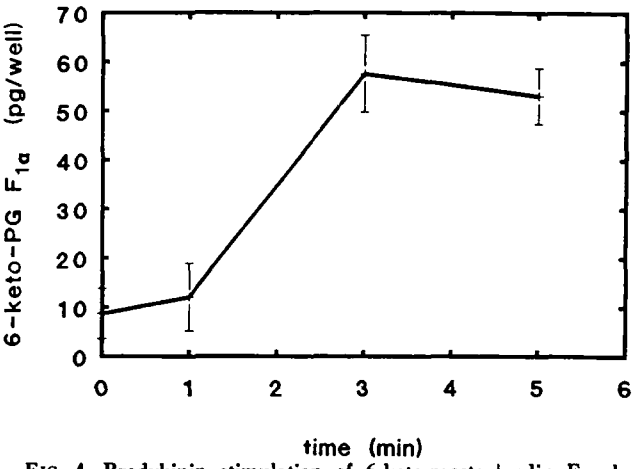

FIG. 4. Bradykinin stimulation of 6-keto-prostaglandin F$_{1\alpha}$ by PCMV endothelial cells. PCMV endothelial cells were stimulated with 1 μM bradykinin for the times indicated, and the levels of 6-keto-prostaglandin F$_{1}\alpha$ were determined by radioimmunoassay. After pretreatment with indomethacin (10 μM) for 5 min, cells produced 3.72 ± 0.3 pg/0.1 ml 6-keto-prostaglandin F$_{1}\alpha$ in the absence of bradykinin, and 3.55 ± 0.15 when stimulated with bradykinin for 5 min.

Treatment of the cells with a 1 μM solution of bradykinin produced a statistically significant ($P < 0.001$) rise in prostacyclin formation at 3 min. (Fig. 4). The maximal response was greater than fourfold at 5 min. This response was suppressed by pretreatment of the cells with a 10 μM solution of indomethacin.

Porcine cerebral microvascular endothelial cells did stain with antibody to Factor VIII:rAg (not shown). However, Factor VIII staining varied from cell to cell in the same PCMV endothelial cell population. Because of this apparent differential expression of Factor VIII:rAg in PCMV endothelial cells, Factor VIII immunocytochemistry could not be used as a reliable cell marker. PCMV endothelial cells did not stain with antibody to von Willebrand Factor. Differential expression of von Willebrand Factor in porcine endothelial cells from various organs has been noted although, to our knowledge, no reports exist concerning the presence or the differential expression of Factor VIII:rAg in PCMV endothelial cells (12,36). Weibel-Palade bodies were not found when porcine cerebral apillary isolates and cultured PCMV endothelial cells were examined ultrastructurally. In addition, cultured PCMV endothelial cells were NSE-positive. Transmission electron micrographs revealed the presence of NSE ectoenzyme activity on the surface of cultured PCMV endothelial cells (not shown). However, NSE-positivity could not be used as a cell marker as similar enzyme activity has been observed in a variety of cell types (24). Dropulic and Masters (5) report that cultured mouse brain capillary endothelial cells express gamma-glutamyl transferase activity after several serial passages and in the absence of a conditioned medium. However, PCMV endothelial cells did not express stainable gamma-glutamyl transferase activity after long-term cultivation (> 6 passages) and in the absence of supplemental RDGF. Accordingly, several investigations have indicated that the expression of

FIG. 5. Ring cell formation in PCMV endothelial cells. A modulation contrast photomicrograph was made of PCMV endothelial cells grown on gelatin-coated, silanated glass cover slips. ×220.

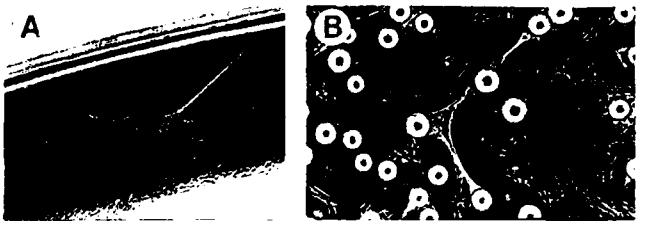

FIG. 6. Three-dimensional support for PCMV cells. *A* is a phase contrast photomicrograph of capillarylike cords attached to the culture vessel wall and floor. ×75. The illustration of the cords is hampered by the occurrence of cells on different optical planes and the optical distortion induced by the rim of the culture vessels. These limitations could be partially overcome by the use of Hoffman modulation contrast or of low magnification (deep plane of focus) phase contrast objectives. *B* depicts Cytodex beads as a three-dimensional support. Tissue culture wells containing Cytodex beads were coated for 1 h with 1% gelatin, rinsed with DPBS, and seeded with PCMV cells. PCMV endothelial cells can be observed both on the plastic surface (as phase-dense cells) and organized into small refractile cords suspended between beads. This figure is representative of three PCMV cultures. ×17.

gamma-glutamyl transferase activity in cultured cerebral microvascular endothelial cells depends on other culture conditions, including the use of astrocyte-conditioned medium and time in culture i.e. evaluating activity in freshly isolated microvessels or cultured cells in early passage (4,16). PCMV endothelial cells did not ingest DiI-Ac-LDL during a 4-h incubation period. Similar findings were made by Gaffney et al. (10), who observed that bovine cerebral capillary endothelial cells did not take up DiI-Ac-LDL. Both PCMV endothelial cells and PCMV pericytes were positive for lectin binding with *Triticum vulgaris* (WGA-1) (not shown). Control porcine aortic endothelial cells did not bind this lectin. PCMV endothelial cells and pericytes did not bind the other lectins tested.

Long-term PCMV cultures varied significantly in cellular composition. For example, two long-term cultures were compared by performing differential counts on cells grown on cover slips and subsequently stained with the α-smooth muscle actin antibody. One culture, in which PCMV endothelial cells were selected for in early passage by use of RDGF, heparin and gelatin, was stained in Passage 12 and found to be virtually free of pericytes (< 5% pericytes). The other long-term culture, propagated after primary culture on uncoated plasticware without supplemental heparin was stained in Passage 6 and found to be composed of 60% pericytes and 40% endothelial cells. Such PCMV cultures with large proportions of pericytes were defined as PCMV cocultures.

Cultured cerebral microvascular endothelial cells have been observed to display phenotypic diversity (19,28). Accordingly, various cellular morphologies were observed in early passage PCMV endothelial cell cultures (< 5% pericytes) precluding identification by a standard, distinct cellular morphology. Endothelial cells and pericytes in PCMV cocultures were usually morphologically indistinguishable at low densities under phase contrast microscopy. However, PCMV endothelial cells plated at low density on silane-treated cover slips were observed to form "rings" (Fig. 5). These rings were composed of one or two cells and were similar to those

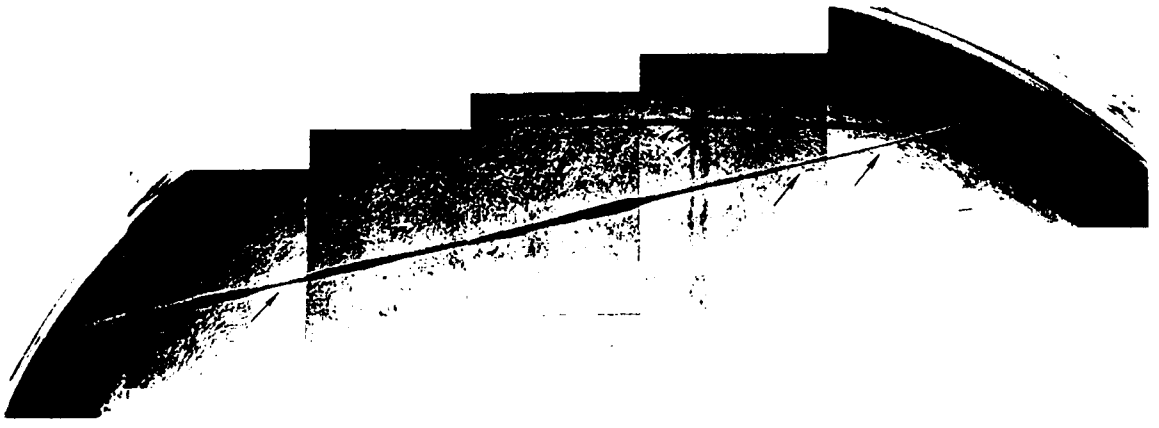

FIG. 7. A suspended PCMV endothelial cell cord. *Arrows* point to the cord suspended between opposite points on the wall of a 16-mm well, and the *arrowheads* point to scribe marks on the bottom surface of the flask. This figure is representative of eight PCMV cultures. ×14.

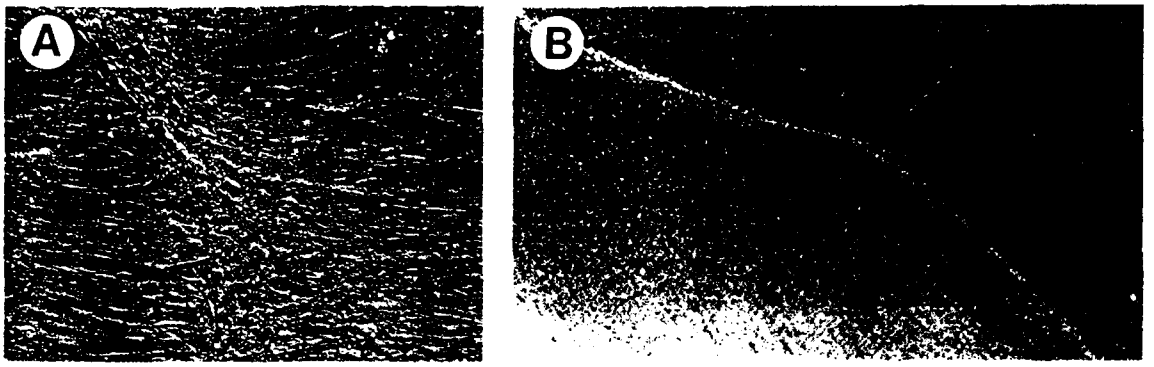

FIG. 8. Cocultured PCMV endothelial cells and PCMV pericytes. *A* is a modulation contrast photomicrograph of the "hill-and-valley" morphology observed in PCMV cocultures. ×46. *B* is a large, floor-bound cord photographed using Hoffman modulation optics. ×28.

formed by cultured adrenal cortical capillary endothelial cells (8).

Cell-to-cell interactions: plasticity of PCMV cultures. Cultures in which the proportion of pericytes was less than 5% typically formed cords 3 to 4 wk after plating (Fig. 6 *A*). This phenomenon was observed after as many as 17 passages when cells were plated in 24-well plastic culture plates (well diameter: 16 mm). Similar cord formation was observed 3 to 4 wk after plating in large flasks (T75) but only with frequent medium changes i.e. every 2 d. PCMV endothelial cells could be plated and propagated in the absence of RDGF, heparin, and matrix-coating of culture plasticware. Under these conditions, cord formation was occasionally observed at 1.5 to 2 wk, approximately 2 wk earlier than in conditions with heparin, RDGF, and matrix-coating.

The in vitro formation of cords by PCMV endothelial cells required three-dimensional space. Cords routinely formed on the walls of 16-mm wells and occasionally on the walls of large culture flasks, often extending to an attachment site in the cell monolayer. Characteristically, cells in monolayer partially detached from the culture vessel surface and migrated up the vertical surface of the culture vessel wall, forming small cords suspended at an angle between the culture vessel wall and the culture vessel floor (Fig. 6 *A*). In some instances, Cytodex 1 beads (Fig. 6 *B*) subserved the three-dimensional condition necessary for cord development. By way of a series of discrete, monolayer detachments and migrations, PCMV endothelial cells often formed cords in which both attachment sites were *above* the surface of the culture plate. Figure 7 depicts one such suspended, "tightrope" cord that spans a 16-mm well.

Differences were observed in the morphology of late postconfluent culture depending on cell composition. PCMV cocultures with large numbers of pericytes exhibited various endothelial cord morphologies in addition to the ridges or "hill and valley" structures that

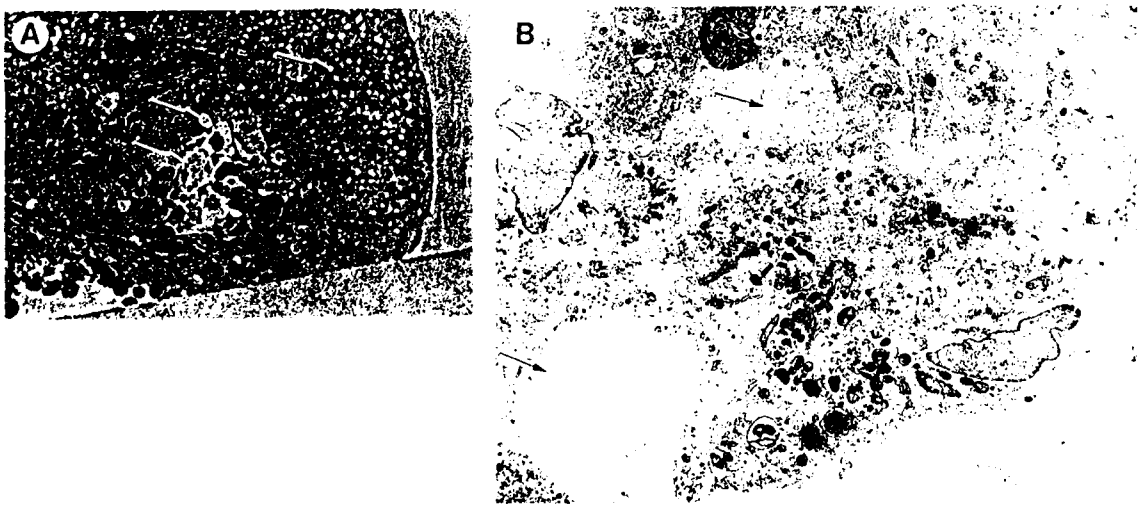

FIG. 9. Crosssections of a PCMV cord. A cord formed in a T-75 flask was gently rinsed with DPBS and fixed for electron microscopy as described in Materials and Methods. Toluidine blue-stained thick sections (*A* ×2380) were examined by phase microscopy, and ultrathin sections were examined by transmission electron microscopy (*B* ×2333). *Arrows* mark areas of partial or complete cell autolysis.

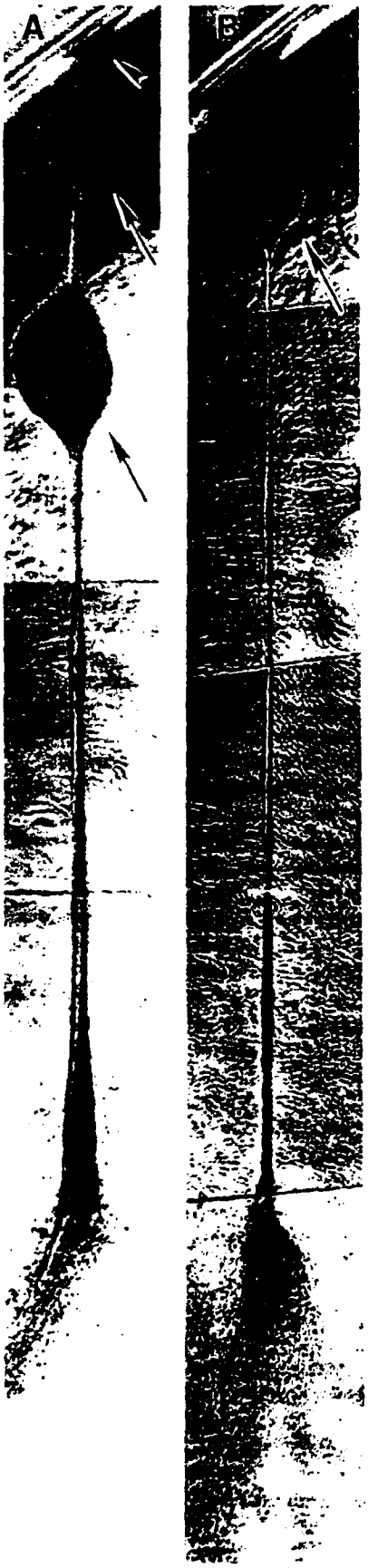

FIG. 10. Movement of PCMV endothelial cell aggregates. The same field containing a PCMV cell cord was photographed at 5 wk. (*A*). Twenty-four hours later movement and fusion of cell-aggregates had occurred (*B*). *Arrows* point to mobile cell aggregates. ×60.

are typical of pericytes in culture (Fig. 8 *A*). Cell aggregates and suspended cords and cell aggregates were also observed in PCMV cocultures. In addition, these cultures contained numerous, large floor-bound cords (Fig. 8 *B*), suspended cords (Fig. 8 *B*), cell aggregates and often exhibited complex self-organization, with elaborate networks of intersecting cords and cell aggregates (not shown). PCMV endothelial cell cultures also contained complex networks of cords but less numerous and less complex than those in PCMV cocultures. The coordinated migration and detachment of endothelial cells and the subsequent formation of an array of large floor-bound cords and suspended cords in PCMV cocultures seemed to require both continuous supplementation with RDGF and culture in *small* vessels, i.e. 16-mm wells. In no other coculture conditions did we observe similar complex morphogenesis. In contrast, in PCMV endothelial cell cultures neither intermittent nor continuous RDGF supplementation affected suspended cord formation.

Thick cross sections of cords revealed multiple cell layers and regions of cell loss or lumina (Fig. 9 *A*). In some instances this cell loss seemed to be cell-selective, extending through serial cross sections and forming capillarylike lumina. Transmission electron micrographs of cord structures with lumina revealed occasional tight junctions and cellular debris indicating regions of apparent complete or partial cell autolysis or both (Fig. 9 *B*). Frozen cross sections of coculture cords suspended above the culture vessel floor were examined for α-smooth muscle actin (not shown). Only nonspecific immunofluorescence was observed, suggesting the absence of pericytes in these cord structures. However, small numbers of pericytes in or on these cords may have gone unnoticed because this immunocytochemical technique was limited by both the thickness of the cross sections and the number of samplings. On the other hand, anti-α-smooth muscle actin staining of PCMV coculture cells in situ clearly demonstrated the presence of overlapping layers of both PCMV pericytes and endothelial cells surrounding nonspecifically stained *floor-bound* endothelial cords such as that depicted in Figure 8 *B*.

Morphologic plasticity was observed not only in the formation of cords but in the movement of cell aggregates along the cords. Figure 10 depicts the time course of cell-aggregate movement along a suspended cord. In Figure 10 *A* two large cell-aggregates (*arrows*) are observed along the cord and a footplate cell aggregate (*arrowhead*) is attached to the wall. Twenty-four hours later (Figure 10 *B*) the two masses (*arrows*) have migrated and merged with the footplate at the edge of the well. In other instances, the footplate was observed to migrate along the wall of the culture vessel (not shown).

On occasion, PCMV endothelial monolayers detached partially or completely after fluid agitation in culture

vessels. Subsequently, partially detached monolayers contracted to form stationary endothelial cell aggregates that were also observed at the time of PCMV endothelial cord development.

Periodicity of cultures. The formation of cords exhibited a distinct periodic or temporal self-organization. Two to three weeks after formation, suspended cords often broke at one attachment site. The cord cells then aggregated, forming stationary cell aggregates that rested on the vessel floor or, more commonly, the vessel wall, as seen in Figure 10 *B*. Concurrent with the formation of suspended cords, new monolayers of endothelial cells propagated on the culture vessel floor. These new monolayers spread from either viable, stationary cell aggregate(s) or from cells in sparse monolayer left behind at the periphery of the vessel floor after endothelial monolayer detachments. After several weeks, cell aggregates disintegrated into cellular debris and amorphous material (not shown). Several cycles of cord formation with a period of 3 to 4 wk were observed in PCMV endothelial cultures maintained without passage for 16 wk. Cocultures of PCMV endothelial cells and pericytes exhibited similar periodic self-organization when supplemented continuously or intermittently with RDGF. The periodicity observed in PCMV endothelial cell cultures did not require supplemental RDGF.

Discussion

The techniques for the primary isolation and culture of cerebral microvascular cells have been developed over the last few years (2,16,27,33). Our isolation procedure is derived from that of Carson and Haudenschild (2), but differed substantially in the addition of a density centrifugation step and the substitution of a 2-min enzyme digestion step for a 16-h enzyme digestion step. This modified procedure yielded a capillary isolate that was significantly free of extracapillary debris.

We approached the identification of PCMV endothelial cells and pericytes in a stepwise manner. As pericytes may be identified immunocytochemically by monoclonal antibody staining of α-smooth muscle actin (17,20,32), we characterized PCMV cells using this method. While positive staining established direct evidence for the presence of pericytes, lack of staining with anti α-smooth muscle actin antibody served as indirect evidence that PCMV cells were endothelial, assuming that the cultures contained only pericytes and endothelial cells. Through manipulations of culture conditions we were able to obtain PCMV cultures virtually free of pericytes as determined by α-smooth muscle actin staining. Subsequent analysis of these cells for prostacyclin formation provided biochemical evidence of their endothelial origin (9,16). The variability of Factor VIII immunocytochemistry in PCMV endothelial cells precluded its use as a reliable cell marker.

In the first report of long-term, adrenal capillary endothelial cell culture, gelatin-coated substrata and special growth factors found in tumor-conditioned medium were considered necessary for cell proliferation (7). In the following years, however, in vitro growth requirements for capillary endothelial cells from different tissues were found to be less demanding (1,16). In particular, Goetz and colleagues (16) observed that capillary endothelial cells from adult bovine brain did not require tumor-conditioned medium or matrix-coated surfaces for long-term culture (16). Accordingly, we found that PCMV endothelial cells could be cultured on plastic surfaces, eventually forming capillarylike cords without the use of supplemental RDGF. However, we did observe a possible morphogenic role of supplemental RDGF in the capillarylike cord development of some PCMV cocultures.

Although capillarylike cord formation has been observed by other investigators in cultured capillary and venous endothelium, the capillarylike cord formation in PCMV cultures exhibits distinct differences. Cord formation in PCMV cell cultures has the following features: a) RDGF was required for cord formation *only* in PCMV cocultures containing large numbers of pericytes and the addition of RDGF did not seem to inhibit cord formation in PCMV endothelial cell cultures. b) The requirement for three-dimensional surface attachments was satisfied by the presence of culture vessel walls, endothelial cell aggregates, and Cytodex beads. In cocultures, floor-bound pericytes seemed to serve as tethering sites for cord attachment on the culture vessel floor. c) The time required for cord formation depended on other culture conditions e.g., the deletion of a gelatin matrix and heparin in the growth medium. d) The use of small culture vessels seemed to enhance PCMV endothelial cell monolayer detachments and subsequent cord formation in both PCMV endothelial cell cultures and PCMV cocultures. In contradistinction to these observations of PCMV cellular morphogenesis in vitro, Maciag et al. (21) reported that preconfluent cultures of human umbilical vein endothelial cells organize into tubular structures after 4 to 6 wk in culture. However, depletion of the growth factor was essential to the development of these tubular structures. Montesano and colleagues (25) established a three-dimensional condition for endothelial capillarylike tube formation by sandwiching cell monolayers between layers of collagen matrix. After approximately 2 d in this matrix, bovine adrenal cortical endothelial cells supplemented with tumor-conditioned medium formed a network of anastomosing cords. Goetz et al. (16) described the organization of adult bovine brain capillary cells into small, tubelike structures when cultured for 1 wk in plasma clots. No supplemental growth factor was required for cord formation in their system, although it is probable that platelet-derived growth factor or another serum-derived growth factor or both were present in the plasma clots. The role of culture system geometry in endothelial cell self-organization has not been extensively explored.

In PCMV cocultures with large numbers of pericytes, RDGF seems to modulate the interaction of the two cell types permitting monolayers of endothelial cells to detach and migrate en masse. Consequently, a variety of endothelial structures developed including floor-bound cords, suspended cords, complex cord networks, and motile cell aggregates. PCMV endothelial-pericyte cell

segregation discrete endothelial cell monolayer detachments in the suspended cord formation of some PCMV cocultures may require two important conditions for initiation or maintenance or both: a) the presence of a biochemical promoter such as RDGF and b) physical factor(s) including the turbulent fluid sheer stress induced by fluid agitation; culture system geometry that affects spatially-specific patterns of cell-to-cell interaction necessary for cord formation; the relative volume of medium to cell number affecting the concentration of exogenous or endogenous PCMV endothelial cell growth factors and/or; fluid pressure gradients due to differences in the depth of the medium in different culture vessels.

Although the concept of biophysically induced cell differentiation is speculative, turbulent fluid sheer stress has been reported to induce bovine aortic endothelial cell proliferation in vitro (3). RDGF-mediated, flow-sensitive pericyte-endothelial cell segregation and pericyte growth inhibition may involve: a) direct enhancement of endothelial cell proliferation, b) inhibition of pericyte proliferation, or c) uncoupling of pericyte inhibition of endothelial cell proliferation. Specifically, this process may be mediated by changes in cell adhesion molecules, extracellular matrix or both (6,23,37). Culture system geometries or the other physical variables listed above may have similar mechanisms of cell-to-cell interaction. Interestingly, in PCMV cocultures supplemented with RDGF, pericytes could not be located in portions of capillarylike cords suspended above the floor emphasizing that pericyte modulation of PCMV endothelial cell differentiation in PCMV cocultures may be only on or near the culture vessel floor.

Recent evidence suggests that capillary endothelial cells express basic fibroblast growth factor which might be auto-stimulatory (29). If a growth factor is necessary for capillary proliferation in vitro, PCMV endothelial cell expression of this or another mitogen might precede the morphologic differentiation we observed in cultures containing few pericytes (endothelial cell:pericyte ratio of 20:1 or greater) and no supplemental RDGF. In contrast, PCMV cocultures with large numbers of pericytes, supplementation with exogenous RDGF seemed to be necessary for optimal endothelial proliferation preceding differentiation. In this instance, the endogenous endothelial production of a growth factor might be insufficient to overcome pericyte inhibition. These observations are in keeping with those of Orlidge and D'Amore (27) who suggest that in vitro cell contact-mediated growth inhibition of bovine adrenal cortical endothelial cells by bovine retinal pericytes begins to decline at an endothelial:pericyte ratio of 20:1 (27). Once the pericyte:endothelial cell ratio has reached this critical threshold in our culture system, supplemental RDGF seems to be unnecessary for or inhibitory to PCMV endothelial cell cord formation. Comparisons of the relative volume of medium in confluent populations of PCMV endothelial cells suggest that the concentration of an endogenous endothelial cell growth factor in a T75 flask is two- to threefold greater than in a 16-mm well. The depletion of a putative endogenous growth factor due to frequent medium repletion could have initiated cord formation in 16-mm culture vessels. This observation of growth factor depletion as a signal for PCMV endothelial cell differentiation in vitro agrees with that of Maciag et al. (21). However, the relationships of growth factor levels and PCMV endothelial cell morphogenesis in our culture system are complex and suggest that physical and other biochemical factors may have ancillary roles in PCMV endothelial cell cord development.

The motility of PCMV endothelial cell-aggregates in cords indicates a dynamic level of coordinated plasticity that continues well beyond cord formation as an endpoint of differentiation. It is possible that if the physical constraints of culture vessel size were abolished, suspended endothelial cell cords would lengthen and narrow using cell aggregates as cellular repositories. In experiments culturing PCMV endothelial cells with Cytodex beads bound to the culture vessel floor in gelatin, small, threadlike cords composed of as few as two endothelial cells developed without the detachment of monolayers. Cytodex beads apparently subserved spatial conditions necessary for a more discrete PCMV endothelial migration, permitting a more capillarylike cord formation in vitro.

Lumen formation in PCMV endothelial cords may involve two stages: formation of a solid cord of cells followed by the autolysis of specific cells in the interior. Electron micrographs revealed significant amounts of cellular debris in the lumina of some cords. Several investigators have found intraluminal debris in their models of angiogenesis (8,21,23,25). Montesano et al. (25) have suggested that intraluminal debris results from the death of cells that are surrounded by other cells and therefore excluded from interaction with the collagen matrix used in their model. We cannot apply this supposition to our model of angiogenesis because PCMV endothelial cells did not require the use of a supplemental collagen matrix for cord development, although we cannot discount the possibility that the cells were excluded from an endothelial-secreted matrix. Maciag and colleagues (21) postulated that the cultured HUVEC of their angiogenesis model used such debris and other cells as "scaffolding," although they offered no theory as to the mechanism of lumen formation. In stained cross sections of cords, lumen formation occasionally seemed to be cell-selective where cell loss was monocellular. The endothelial cells bordering such lumina were unaffected by autolysis. The mechanism of this putative cell-selective process may be similar to that operative in the programmed cell death observed in nematode development (18,34). In the case of PCMV endothelial cell plasticity, the organizational selectivity of programmed endothelial cell death may be expressed in the formation of microvascular channels. Tunneling or hollowing out of lumina through hydrostatic forces seems to play no role in lumen development, because lumina were observed in cords where flow was impossible. We cannot discount the possibility that a limitation in nutrient diffusion across several concentric cell layers may have contributed to cell death and lumen formation. However, programmed cell

death is not necessarily exclusive of limitations in nutrient diffusion.

The self-organization in our PCMV culture systems is periodic. As suspended cord formation progressed, new waves of endothelial cells propagated in monolayers that in time detached and formed new cords. In cultures that were maintained 16 wk without passage, several cycles of 3 to 4 wk each were observed. Temporal changes in cell density, relative pericyte-endothelial cell populations, accumulation or depletion of extracellular matrix components, coordinated cell-to-cell interaction, and physical factors may be responsible for the cycling of monolayers and cord development. To our knowledge, periodic endothelial cell self-organization has not been previously reported. Furthermore, we know of no reports concerning similar periodic plasticity in mammalian cell culture.

The dynamic morphologic plasticity and periodicity we observed in cultured PCMV cells may provide new insights into the mechanisms of angiogenesis. Corresponding in vitro investigations of cerebral capillary differentiation, the role of the pericyte in microvascular development, and the mechanisms of growth factor-mediated cell-to-cell interaction may facilitate the understanding of microvascular growth, injury, regeneration, and repair. In particular, the putative role of physical promoters for angiogenesis requires elucidation through appropriate biophysical and molecular studies. And finally, validation of the concept of programmed endothelial cell death as an integral step in the development of microvessels may improve our understanding of the derangement of normal cellular processes preceding cell death in various microvascular pathophysiologies.

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