IN VITRO
Volume 15, No. 7, 1979

CEREBRAL MICROVESSELS AND DERIVED CELLS IN TISSUE CULTURE: ISOLATION AND PRELIMINARY CHARACTERIZATION

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(Received August 18, 1978; accepted February 5, 1979)

SUMMARY

Microvessels isolated from mouse forebrain were used as the source material for the derivation of cerebral vascular endothelium and smooth-muscle cells in culture. The microvessels were isolated by a mechanical dispersion and filtration technique, and were maintained in vitro as organoid cultures. A microvessel classification system was developed and proved to be useful as a tool in monitoring culture progress and in predicting the type(s) of microvessel(s) that would give rise to migrating and/or proliferating cells. The isolated cerebral microvessels were heterogeneous in diameter, size of individual vascular isolate, and proliferative potential. The isolated microvessels ranged in diameter from 4 μm to 25 μm and in size from a single microvascular segment to a large multibranched plexus with mural cells. The initial viability, determined by erythrosin B exclusion, was approximately 50% on a per cell basis. All microvessel classes had proliferative potential although the rate and extent of proliferation were both microvessel class- and density-dependent. The smaller microvessels gave rise to endothelial cells, whereas the large microvessels gave rise to endothelial and smooth-muscle cells. The viability and progress of a microvessel toward derived cell proliferation seemed to be directly proportional to the number of mural cells present.

Key words: cerebral microvessels; isolation procedure; tissue culture methods; morphology; endothelium.

INTRODUCTION

The use of blood vessels for tissue or organoid cultures in 1910 (1,2) was a first and was prompted by the pioneering culture work of Harrison in 1907 (3) on neural tissue. Carrel and Burrows (1) were probably the first to investigate the migration and proliferation of cells from vascular explants. From 1910 through 1973 little attention was given to this area until Fisher-Dzoga et al. (4) worked specifically on adventitial cell proliferation. Earlier studies are summarized in a review by Murray and Kopech in 1953 (5) and in a monograph by Pollak in 1969 (6). The early approaches to cultivating adventitial elements of the vasculature are not only of interest historically but they also serve as models for the cultivation of isolated microvessels. These organoid cultures of blood vessels usually produced a culture with a predominant cell type although multiple cell types were often noted during the early stages of outgrowth from the vascular explants. Since one of our long-term goals is to study the interaction of cell types in cerebral microvessels, it seemed appropriate to use a culture system that also would give rise to all cellular elements of the vessel.

Several investigators have reported methods for isolating brain capillaries from rat (7-9), rabbit (10), cow (11-13), and human (14), and have used them for metabolic, biochemical and/or histochemical studies. All these procedures used mechanical means to disperse the tissue; however, a variety of techniques were used to harvest and purify the disrupted capillaries. Many of these techniques are not well suited for in vitro culture studies because of the low viability of the purified

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capillaries. We have been able to isolate microvessels with sufficient viability to initiate primary cultures by combining a tissue culture system compatible with many cell types with a mechanical dispersion and filtration technique adapted to mouse brain from the method of Brendel, Megan and Carlson (11) for bovine brain. To date endothelium and smooth-muscle cell plaques from the same microvessel isolates have been obtained. This paper describes the isolation and preliminary characterization of cerebral microvessels and their derived cells in vitro.

MATERIALS AND METHODS

Source of cerebral microvessels. Central nervous system (CNS) tissue from weanling and young adult Swiss-Webster mice was used in these studies for the isolation of microvessels and the anatomical characterization of the vessels in vivo. In all, more than 300 isolations have been done and form the basis for this report.

Isolation of cerebral microvessels. For the in vitro studies reported herein, microvessels were isolated from mouse cerebrum by a mechanical dispersion and filtration technique similar to the one described by Brendel, Megan and Carlson (11) for bovine brain. The procedure, as adapted to mouse brain, differed in isolation salt composition, antibiotics and method of removing microvessels from the sieves for culture work. For each isolation, two forebrains were removed aseptically from young Swiss Webster mice anesthetized with 0.3 to 0.5 ml of a 3% aqueous solution of chloral hydrate and placed in a 60-mm petri dish (P-60) with 10 ml isolation salt solution [Hanks'-HEPES-Heparin (HHH), containing Hanks' salt base, 0.015 M HEPES (*N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1 U per ml sodium heparin, 100 U per ml penicillin-G, and 100 μg per ml streptomycin]. The forebrain tissue was washed and then transferred to an empty P-60 dish for chopping. The tissue was chopped with two scalpels (using a scissoring action) into approximately 1-mm cubes and transferred to a 7-ml Dounce homogenizer (Vitro or Bellco) containing 5 ml HHH isolation salt solution. The tissue was homogenized with 10 or more strokes of the loose-fitting pestle. Homogenization end point was determined by microscopic examination of the homogenate. The homogenate was filtered through a 210-μm mesh nylon screen supported in a 48-mm Millipore filter holder. The microvessels trapped by the screen were rinsed with approximately 35 ml HHH solution. The screen was removed from its holder and placed in an empty P-60 dish (dish A) upside down, 5 ml HHH solution added, and the microvessels washed free of the screen by agitation. The screen was transferred to another empty dish (dish B). A few microvessels and vessel fragments attached to dish A during this brief period; however, most could be resuspended. These suspended microvessels from dish A were rehomogenized with three or four strokes of the tight-fitting piston and the homogenate filtered through a 153-μm mesh filter and rinsed as above. This second screen was removed from the holder and placed in an empty dish (dish C) upside down.

Two to 3 ml modified Lewis medium (MLM) with 30% FBS (see below) was added to each dish (A–C), and the cultures then were incubated at 37° C in a CO_2 incubator for 1 to 2 hr. In some experiments, glass cover slips were placed in the P-60 dishes before the vessels were added. By the end of this period most of the microvessels that would attach had attached to the bottom of the dish (or glass). The screens, medium and unattached vessels were removed and 3 ml fresh MLM with 30% FBS was added. After 5 to 7 days, the medium was removed and replaced with 3 ml fresh MLM with 20% FBS. Subsequent feedings were with MLM with 20% FBS and were done once or twice per week depending upon the number of vessels and/or cells in the culture.

Medium. Medium used by Lewis et al. (15) for growing human endothelial cells derived from umbilical veins was employed in this study. However, salt, antibiotic and serum modifications were made so that the medium will be referred to as modified Lewis medium (MLM). MLM consisted of medium 199 with Hanks' salts (GIBCO E-12) supplemented with 20% or 30% heat-inactivated fetal bovine serum (FBS), 1% BME amino acid solution concentrate (GIBCO 105S), 1% BME vitamins concentrate (GIBCO 104), 60 mM dextrose, 0.05% Bacto-peptone (w/v), antibiotics as above, and 2 mM L-glutamine, and buffered with 0.015 M $NaHCO_3$ and 5% CO_2 in balanced air. The medium and supplements (when necessary) were filter-sterilized using 0.22-μm-pore-size Millipore filters.

Classification of isolated microvessels. Isolated microvessels were classified according to the number of branching points per microvessel isolate and whether or not the isolate had visible layers of

mural cells. Although this classification was arbitrary it proved to be a useful way to monitor culture progress and to evaluate the types of microvessels giving rise to migrating and/or proliferating cells. In this classification scheme isolated microvessels were placed into one of the six classes (I–VI) depicted in Fig. 1. Class I microvessels were small unbranched segments; class II were similar but with one to two branching points. Class III microvessels were slightly larger microvessels having three to six branching points; class IV microvessels were similar except that they had seven or more branching points and often took the form of a plexus. Class V microvessels were large, thick microvessels that contained continuous segments with mural cells. Class VI microvessels were complexes of vascular knots and/or cellular aggregates.

Viability. Viability was determined by a dye exclusion test using 0.04% erythrosin B in Hanks' balanced salts solution (HBSS) (16) on both freshly isolated microvessels and microvessels in cultures of varying ages. Viable cells were unstained, whereas nonviable cells had diffuse staining of the cytoplasm and nucleus. In those studies in which the various classes of microvessels were evaluated for their proliferative potential, and in which addition of dye was undesirable, phase-contrast microscopy was used. In these cases, the microvessel was considered dead when no clearly visible cells presented with a phase bright perinuclear area and a well defined nuclear membrane.

Fixation. If the cultures were to be used for SEM they were washed 3 times before fixation with 0.16 M sucrose in 0.1 M cacodylate buffer (17), pH 7.4, to remove serum and any debris. The osmolality of the solution was approximately 360 mOsM. Cultures for TEM were not prewashed. Cultures to be used for either SEM (on glass cover slips) or TEM (in plastic petri dishes) were fixed according to a modification of Haudenschild et al. (18) in 2.5% glutaraldehyde in sucrose/cacodylate buffer at room temperature for 30 min. The cultures then were washed at room temperature with, and stored in, sucrose/cacodylate buffer at 4° C until further processing.

Microvessels of intact brain to be used as control tissue for morphology were fixed in situ by perfusion in chloral hydrate anesthetized mice. The perfusion was through the left ventricle of the heart; this consisted of a 5-min perfusion with a dilute fixative (1% formaldehyde, 1.25% glutaraldehyde) followed by a 5- to 10-min perfusion with a more concentrated solution (4% formaldehyde, 5% glutaraldehyde), both in 0.1 M cacodylate buffer. After the in situ fixation the brain was removed and stored in fixative until processed for TEM.

Scanning electron microscopy (SEM). After fixation the cultures were postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer for 15 min, rinsed in distilled water, dehydrated in an ethanol (ETOH) series (30%, 50%, 70%, 95% and 100% ETOH; 5 min each), and ETOH-CO_2 critical point dried (19) in a Polaron bomb. The dried specimens were attached to aluminum specimen stubs with copper tapes. The specimens first were given a conductive coating of carbon and then a coating of 40% palladium-60% gold alloy. The coated specimens were stored in a desiccator at ambient temperature and pressure until examined. A Kent Cambridge Stereoscope SEM (Model S-4) operated at 10 or 20 kV was used to study and photograph the specimens.

Transmission electron microscopy (TEM). After fixation the cultures were postfixed with 1% to 2% osmium tetroxide in water or cacodylate (no sucrose) buffer for 15 to 30 min at room temperature followed by 0.5% to 2% uranyl acetate in 0.05 M maleate buffer (pH 5.2) for 30 min in the dark at 4° C (20,21). The final pH of the uranyl acetate-buffer mix was approximately 4.6 to 4.9. The specimens were rinsed with distilled water, dehydrated in an ethanol series (three changes of 75%, 95% and 100% ETOH; 5 min each), and infiltrated with epoxy resin mixture (48 ml Epon 812, 24 ml DDSA, 28 ml NMA and 2 ml DMP-30 or 50 ml Epon 812, 12.5 ml DDSA, 37.5 ml NMA, and 2 ml DMP-30 which was found to have better cutting characteristics in our hands) first as a 1:1 mix with ethanol for 1 hr and then with resin [three changes in 2 to 3 hr (18)]. The resin was partially polymerized at 55° C for 12 to 24 hr, the plastic dish cracked, and the resin casting peeled free. Polymerization of the casting was completed at 60° to 65° C for 48 to 72 hr.

The cured resin castings (approximately 2-mm thick) were examined on the inverted phase microscope, and the area of interest was identified and circumscribed (usually 1 mm^2). When necessary, the scribed areas were mapped photographically for future reference. When an area was prepared for sectioning it was first cut out of the casting with a jeweler's saw and mounted either cell side up or on edge on an empty epoxy resin block with a drop of unpolymerized epoxy resin. The

mounted specimens were incubated at 60° to 65° C for 12 to 24 hr to polymerize the bonding epoxy resin. Silver to gold sections were stained with bismuth subnitrate (21), examined and photographed in a Philips EM 300 electron microscope operated at 60 kV. Brain tissue was processed similarly except toluene and toluene-resin mixture steps were added before embedding in resin, and polymerization was performed at a single temperature (60° to 65° C).

RESULTS

Morphology of the Isolated Microvessel

The isolation procedure used in this study selected for those microvessels that survived homogenization and sieving and also attached to the substratum (plastic petri-dish or glass cover-slip surfaces) during a 1- to 2-hr incubation at 37° C. Many microvessels never attached to the substratum even if the incubation time was extended to 24 hr. The microvessel density in culture ranged from $\leqslant 6$ to 13 per cm^2. In a typical isolation the attached microvessel had a number of attachment sites which were seen as smooth, thick filopodia or lamellipodia (22). Fig. 2 illustrates a class IV microvessel attached to the substratum by such a lamellipodia at the end of a vascular segment.

The ultrastructure of the isolated microvessel (Fig. 3*b*) was remarkably similar to that of the intact, unaltered microvessel in vivo (Fig. 3*a*). The freshly isolated microvessel in culture retained its histological organization. The endothelial cells were easily distinguished from mural cells such as pericytes and smooth-muscle cells. Sometimes, red blood cells were present in the lumen; when absent, however, the lumen tended to be collapsed. When collapsed, the luminal surface of the endothelium had numerous microvillar-like folds. Pinocytotic vesicles were prominent. Preserved elements of the endothelium included tight junctions, junctional folds, cytoplasmic organelles and nuclei with nuclear pores and dispersed chromatin. The basement membrane was swollen and electronlucent, and was often irregular if the vessel was collapsed. Pericytes and smooth-muscle cells were present and were identified by their relationship to the basement membrane or the presence of contractile filaments, caveoli and hemidesmosomes. Disrupted cells with swollen mitochondria were present in the vessel wall or in a position normally occupied by astrocyte foot processes in vivo.

Class Distribution of the Isolated Microvessels

A typical isolation of microvessels from mouse brain contained a variety of microvessel classes (Table 1). When the plated microvessels in dish B (a single homogenization and sieving) were compared with microvessels from dish C (two cycles of homogenization), there were only two significant differences: (a) there were fewer vessels in dish C; and (b) dish C had no class VI vessels. Classes I–III were presumed to be predominantly capillaries and together constituted 73% and 86% of the microvessels in dishes B and C, respectively. The remainder of the microvessels were larger than "capillaries" and fell into classes IV–VI.

Viability of the Isolated and Cultured Microvessels

The viability of microvessels was determined by the exclusion of erythrosin B. In classes I–IV microvessels, in which nuclei were clearly visible and cell (or nuclear) counts possible, the viability was determined on a per cell basis. The composite viability of a freshly isolated microvessel (classes I–IV) culture was usually about 50%, with one or more live cells in each microvessel isolate (class I being an exception). We considered these isolates useful because a single live segment or even a single cell could give rise to a cell plaque.

Characteristics and Fate of Microvessels in Culture

The cultured microvessel has a number of noteworthy characteristics in addition to its class which can be seen by phase-contrast microscopy in the living culture. These are generally related to the physical appearance of the microvessel and

TABLE 1

CLASS DISTRIBUTION OF ISOLATED VESSELS[a]

	Dish B[b]	Dish C
Vessel Class	No. (%)	No. (%)
I	28 (37)	20 (36)
II	19 (25)	18 (34)
III	8 (11)	9 (16)
IV	2 (3)	3 (5)
V	9 (12)	5 (9)
VI	9 (12)	0 (0)
Total Vessels Sampled	75 (100)	55 (100)

[a] Total area sampled is one-seventh of total P-60 area.
[b] Two B and two C dishes were used for this sampling.

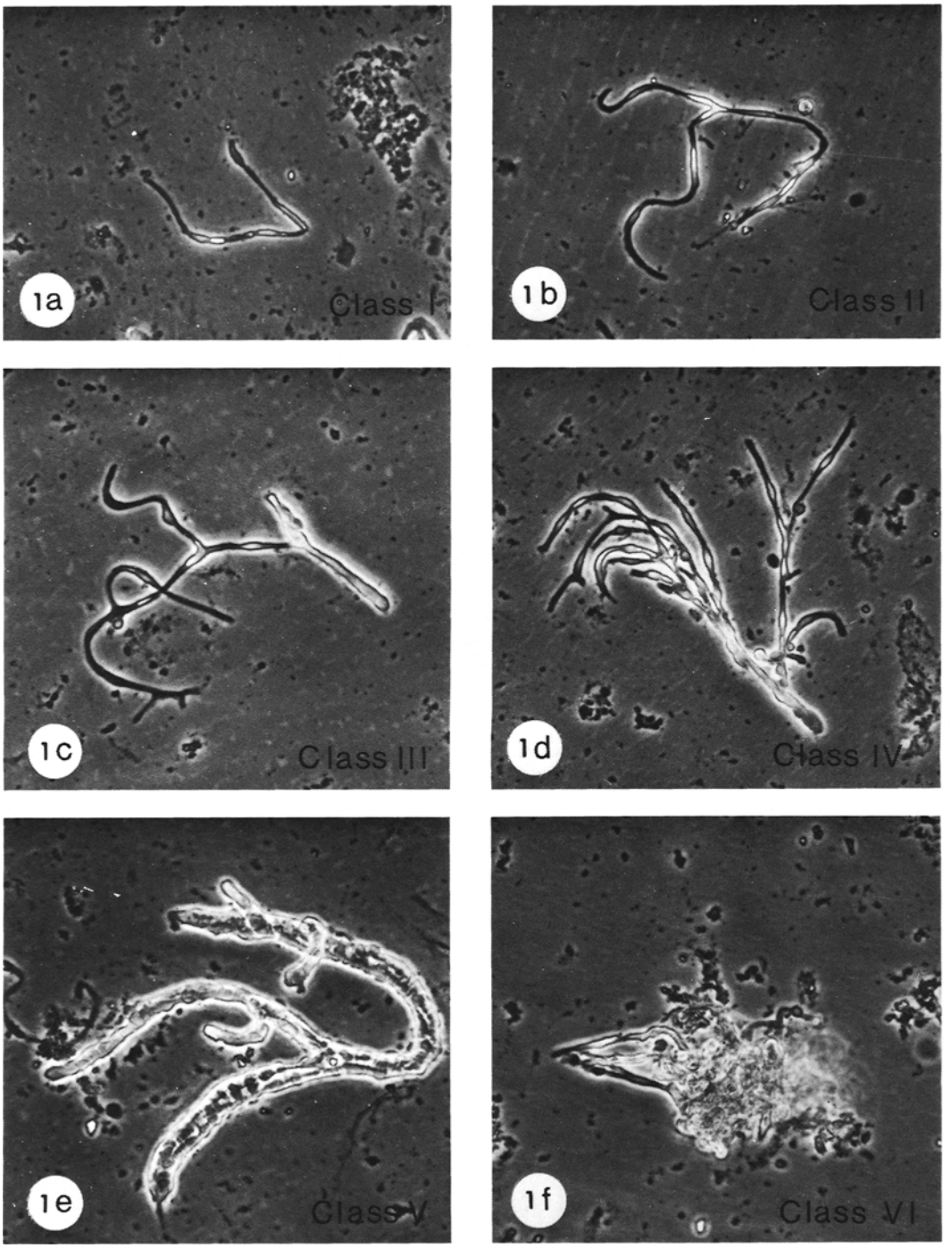

FIG. 1. Microvessel classification based on the number of branching points and whether or not the microvessel isolate had a layer of mural cells. *a*, Class I, small unbranched segments; *b*, class II, one to two branching points; *c*, class III, three to six branching points; *d*, class IV, seven or more branching points and often a plexus; *e*, class V, two or more layers of mural cells; *f*, class VI, complexes of vascular knots and/or cellular aggregates. ×200.

TABLE 2

HISTORY OF 15-DAY CULTURES[a]

Description or Event	% Microvessel in Each Class					
	I	II	III	IV	V	VI
Vessels followed (n)	37[b] (n = 48)	29 (n = 38)	13 (n = 17)	4 (n = 5)	10 (n = 14)	7 (n = 9)
Vessels with mural cells	4[c]	47	71	100	100	N.O.[d]
Vessels dying without proliferation	100[e]	100[e]	82	33	21	0
Vessels swelling or thickening	13	8	59	50	71	N.O.
Vessels curling into knots	6	11	6	17	43	N.A.[f]
Vessels with migrating and/or proliferating cells	0[e]	0[e]	18	67	78	78
Vessels dying after migration and/or proliferation	0[e]	0[e]	12	17	14	22

[a] Cultures of isolated microvessels were observed during the first 15 days in vitro. Pool of dishes B and C from Table 1.

[b] Percent of the total microvessel sequentially observed for 15-day period.

[c] Percent within each class that fit the description or event.

[d] Not observable; specimen too thick.

[e] The dead of 100% classes I and II microvessels without proliferation is believed to be artificially high due to the repeated handling during culture observations and photomicrography. This belief is borne out by the observation that similar microvessels in other cultures have given rise to cell plaques.

[f] Not applicable because by definition class VI are knotted on day 0.

are of interest because they change as the microvessel progresses and gives rise to cells or regresses and then dies. Table 2 describes the fate of each class of microvessel during the first 15 days of culture. For convenience the data from dishes B and C were pooled since there were not significant differences between dishes in the fate of microvessels within a given class.

Only 4% of the class I microvessels had visible or identifiable mural cells at the LM level. These were most likely pericytes. In contrast all classes IV and V microvessels had visible mural cells, whereas classes II and III were intermediate (47% and 71%, respectively) with regard to the presence of mural cells. During the 15-day observation of this sampling, all class I microvessels

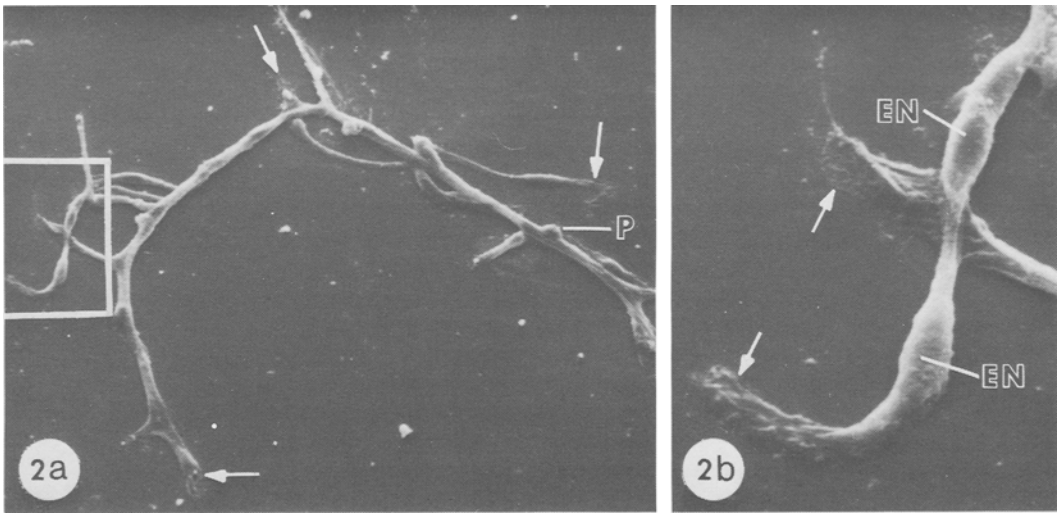

FIG. 2. SEM of a class IV microvessel 4 hr after isolation. Attachment sites (*arrows*) occur most often at the ends of the microvessel segments and appear to involve the endothelial cells. EN, endothelial nucleus; P, pericyte. *a*, ×400; *b*, enlargement of outlined area in *a*, ×1700.

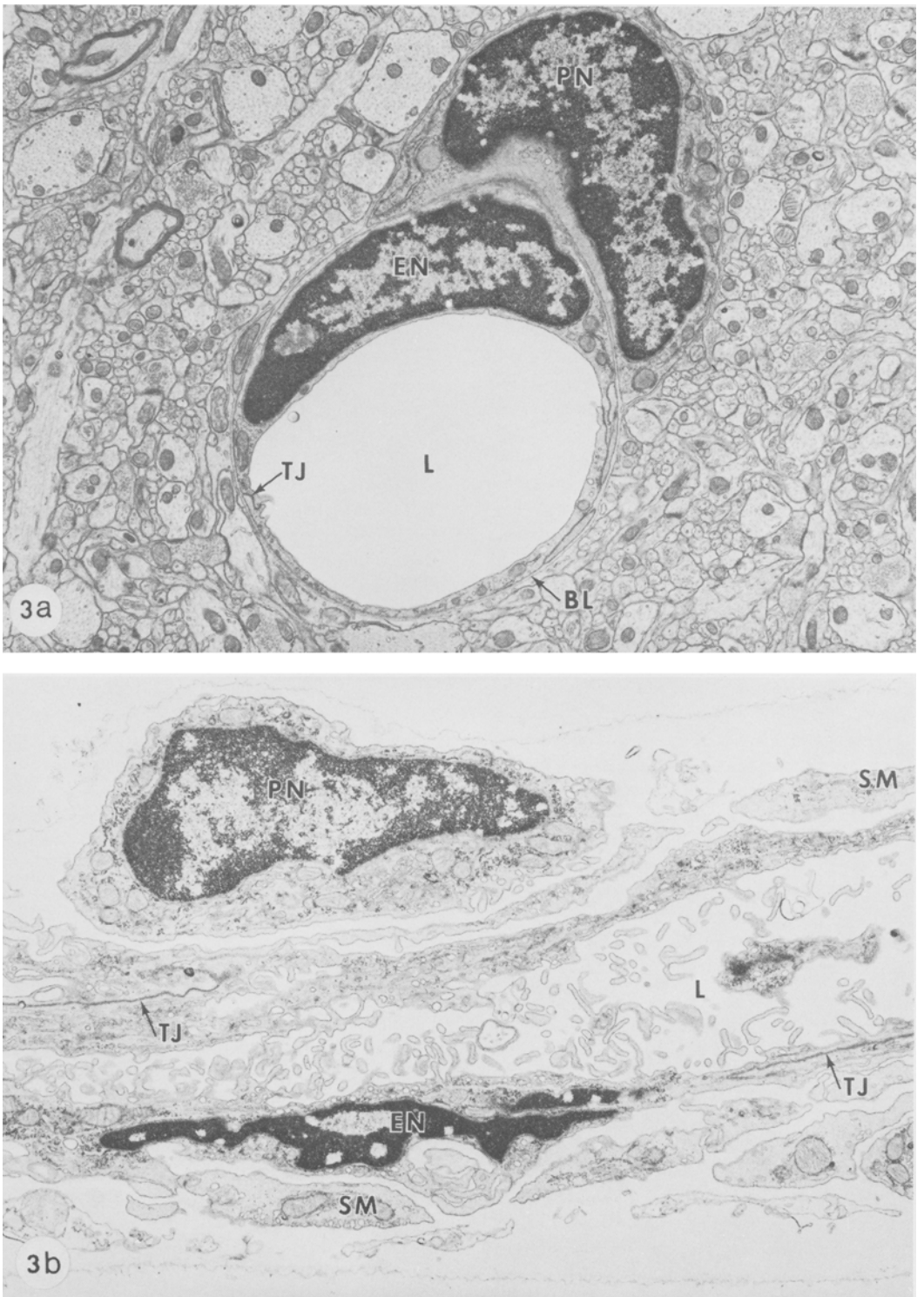

FIG. 3. TEM showing the ultrastructure of a microvessel (cross section) in vivo (*a*) and a microvessel (longitudinal section) in vitro (*b*) 1 hr after isolation. BL, basal lamina; EN, endothelial nucleus; L, lumen; PN, pericyte nucleus; SM, smooth muscle; TJ, tight junction. *a*, ×7200; *b*, ×9200.

died without giving rise to proliferating cells even though 13% of the microvessels of this class showed early signs of blastogenesis with nuclear swelling and vessel thickening. Six percent of the microvessels retracted and changed in form from elongated vessels to vascular knots. Class II microvessels had a similar fate.

Although 82% of class III microvessels failed to proliferate and died, they still survived better in culture than did class I or II. The majority (59%) of class III microvessels showed early signs of blastogenesis and 18% actually gave rise to migrating and/or proliferating cells. About 67% (12:18, class III, Table 2) of the microvessels died after giving rise to cells, thus leaving approximately 6% of the class III microvessels surviving after 15 days in culture.

Class IV microvessels did markedly better in culture than any of the lower three classes. Only 33% of the microvessels died without first proliferating. Although only 50% of the microvessels in this class showed signs of blastogenesis, 67% of the vessels did give rise to migrating and/or proliferating cells. This initial response was followed by a 25% loss of microvessels, thus leaving 50% survival of the vessels. Class V microvessels had a fate similar to class IV except class V microvessels knotted at about 2.5-fold more frequently than did class IV. This high frequency of knotting in class V microvessels is thought to be due to the contraction of the smooth-muscle elements in the isolated microvessel wall.

Class VI microvessels were knotted by definition and were difficult to observe live in phase-contrast microscopy. However, none of the vascular knots died during the 15-day observation period without first giving rise to cells. The number of proliferating and surviving microvessels in this class was very similar to those in classes IV and V.

Emergence of Cells from the Cultured Microvessel

Migrating cells emerged from the isolated microvessel as early as 1 to 2 days after isolation. Migration continued and/or proliferation began 5 to 7 days after isolation and continued for up to 15 to 25 days depending upon microvessel class and the density (isolates per cm^2) of microvessels in the culture. The emergence of cells from all classes of vessels was seen earlier in cultures with a relatively high microvessel density (approximately 20 per cm^2) than in cultures with a low microvessel density (≤ 6 cm^2).

In microvessel classes I–IV, cells emerged most often from the ends of the vascular segments (Fig. 4*a–d*); however, in classes III and IV, cells also emerged from the sides of the vascular segments, usually from segments with visible mural cells. In class V microvessels, cell emergence was predominantly from the sides of the vascular segments but a few cells did not emerge from the segment ends. In class VI microvessel knots, it was usually not possible to determine the exact source of the emerging cells. Cells generally emerged from the base of the knot where it made contact with the substratum (Figs. 5*c*, 8*b*).

Morphology of Emerging and Derived Cells from Cultured Microvessels

Presumptive endothelial and smooth-muscle cells. The type of cell that emerged from the isolated microvessel was microvessel-class-dependent. The cells that emerged from classes I and II microvessels with no mural cells were presumed to be endothelial cells and had characteristic cytomorphologies (Figs. 4*b,c*, 6*a*). Migration was usually first manifested at the ends of the segments by the emergence of a cell process with filopodia and lamellipodia on its advancing edge. As migration proceeded and the nucleus became clearly visible, the cells often appeared as elongated fusiform cells partly in and partly out of the vessel. As the cell emerged more fully, its advancing edge broadened and the cell took on a flat polygonal shape more typical of an endothelial cell (Fig. 4*c*). However, criteria for identification of cell type at this stage were not well defined since these presumptive endothelial cells as well as smooth-muscle cells and pericytes may be pleomorphic in sparse cultures such as these. The emerging cells from the side of segments from class V microvessels (Figs. 5*a*, 6*d*) or from the base of class VI microvascular knots were presumed to be smooth-muscle cells; however, other cell types including endothelium have been noted. These cells emerged rapidly and, as free cells, took on fusiform or stellar shapes (Figs. 4*e*, 6*d*). With time these cells became flat and broad with a clearly visible cytoskeleton ordered in lines parallel to the long axis of the cells (Fig. 6*e*). These were smooth-muscle cells with their contractile filaments (see TEM below). Classes III and IV microvessels gave rise to cell types reflecting their composition of endothelial and mural cells when the latter were present. Fig. 6*d* is an example of a

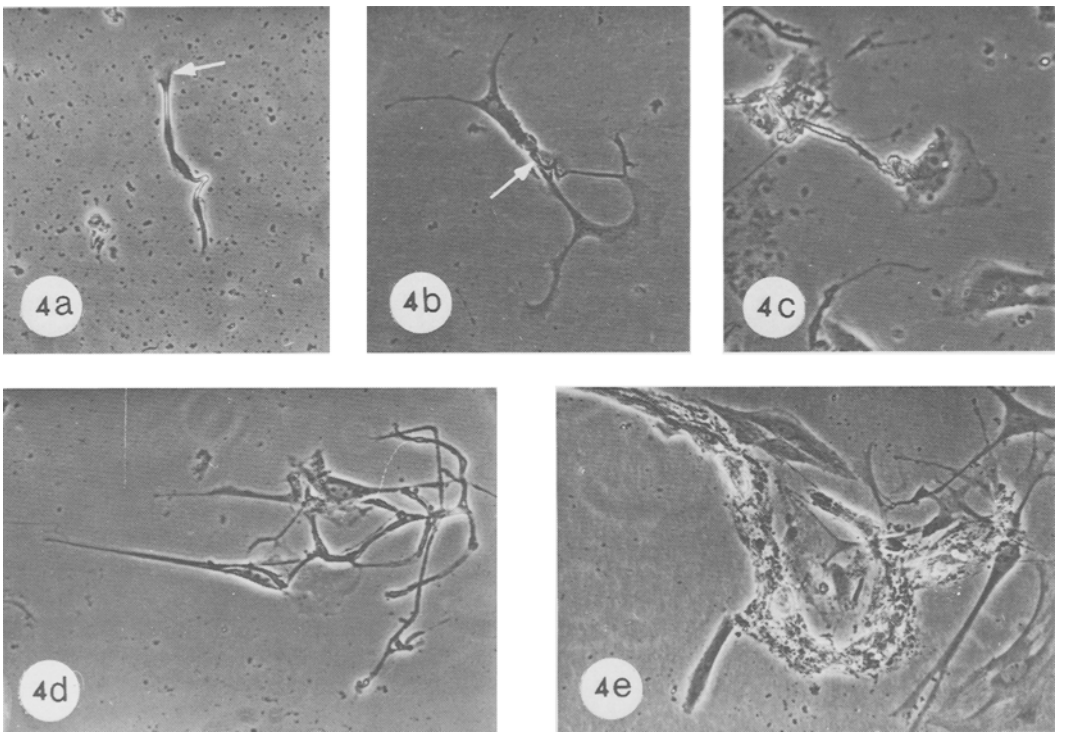

FIG. 4. Phase-contrast photomicrographs of emerging cells from various microvessel classes in a 6-day-old culture. *a*, Class I microvessel showing the earliest signs of cell migration with emerging filopodia (*arrow*). *b*, Newly emerged cell with its class I microvessel still attached (*arrow*, point of attachment). This cell is presumed to be endothelial because it is emerging from a segment free of mural cells. This cell shape is transitory and usually changes to the form seen in *c*. *c*, Newly emerged cells have taken on a flat polygonal shape more typical of endothelial cells. *d*, Emerging cells from the segment ends of a class IV microvessel. Note the elongated form of these cells. This cell shape is also transitory. *e*, Emerging cells from the sides and end of a class V microvessel. These cells were presumed to be a mixture of endothelial and mural cells; however, the pleomorphism of the emerging cell at this stage made positive identification uncertain. ×170.

class III microvessel that appeared to be mural-cell-free and gave rise to presumptive endothelial cells from segment ends.

A cell plaque (or colony) is formed when a focus of cells begins to proliferate. Plaques of endothelial-like (Fig. 6*b*) and smooth-muscle-like cells (Fig. 6*e*) have been followed and ultimately have given rise to typical monolayers of flat polygonal, contact-inhibited endothelial cells (Fig. 6*c*) or to fusiform, multilayered smooth-muscle cells with characteristic mounding of cells into transverse ridges (Fig. 6*f*). It should be pointed out that many cell plaques grew to a limited size at which point proliferation ceased. Endothelial plaques grew more slowly and ceased proliferation earlier than did smooth-muscle plaques. Sometimes the endothelial plaque was overgrown by a neighboring smooth-muscle plaque.

The cells in the early endothelial plaques often had irregular cytoplasmic outlines with processes when the cells were separated but more regular cytoplasmic outlines when the cells were in close contact. The smooth-muscle cells showed a similar change in morphology depending on the cell density in the plaque. Transmission electron microscopy was done on cells selected by light microscopy from endothelial plaques or groups of smooth-muscle cells in which the cytoskeleton was clearly visible. Depending on the level of the cell sampled, there was variation in the appearance of the cytoplasmic organelles. Thus when sections were taken near the points of attachment, bundles

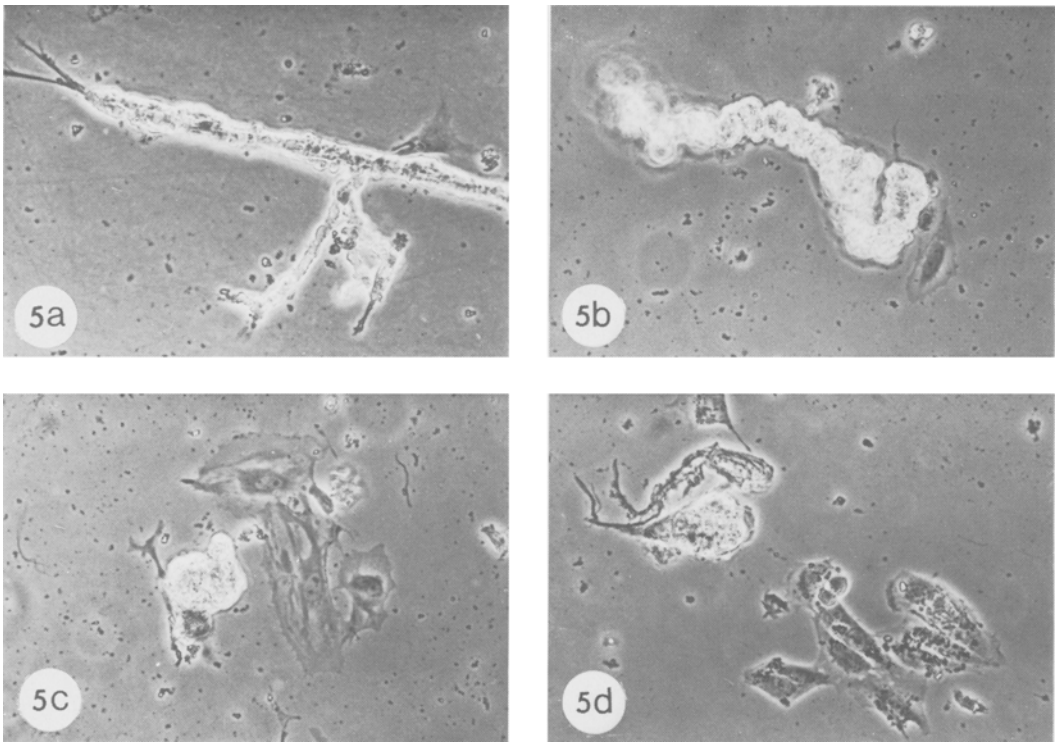

FIG. 5. Phase-contrast photomicrographs of the various stages of early endothelial plaque formation in vitro—in this case from the ends of class V microvessels in a 6-day-old culture. *a*, Filopodia of an emerging cell are seen at the end of the vessel, whereas the broad advancing edge of another cell is seen emerging from the side of this microvessel. *b*, Fully emerged endothelial-like cell is seen at the end of a partially contracted microvessel. *c*, Group of three cells beside a retracted vascular knot. Cell proliferation usually begins at this stage. *d*, Initial endothelial cell plaque of proliferating cells beside a retracted vascular knot, the presumed origin of the cells. ×170.

of filaments predominated, whereas at the level of the nucleus, rough endoplasmic reticulum, membrane-bound dense bodies and irregular mitochondria predominated (Fig. 7*a*). Weibel-Palade bodies (23) were not seen nor were junctional complexes observed. The nuclei were generally round and nuclear pores were demonstrable in oblique sections. In the smooth-muscle cells fibrillar bundles were prominent, and, at the level of the nucleus, the Golgi complex was well developed (Fig. 7*b*).

Other cell types. Occasionally, cell types other than endothelium or smooth muscle were seen in these microvessel cultures. A rare astroglial cell (Fig. 8*a*) was seen in some cultures and was clearly recognizable by its processes. However, there were other cell types that have not been identified (Fig. 8*b*). The pericyte was present in most of the isolated microvessels, but without morphological or other criteria for positive identification in vitro, we can not determine the fate of these cells in culture.

DISCUSSION

We have been able to isolate microvessels with sufficient viability to initiate primary cultures by combining a tissue culture system compatible with many cell types with a mechanical dispersion and filtration technique adapted to mouse brain from the method of Brendel, Megan and Carlson (11). To date, endothelium and smooth-muscle plaques from the same microvessel isolates have been obtained. Morphologically, the isolated microvessels from mouse brain were more heterogeneous than those isolated from beef brain by Brendel, Megan and Carlson (11), and the yield was lower from the mouse material. There are a number of reasons for these differences. First, the system that was developed selected for those

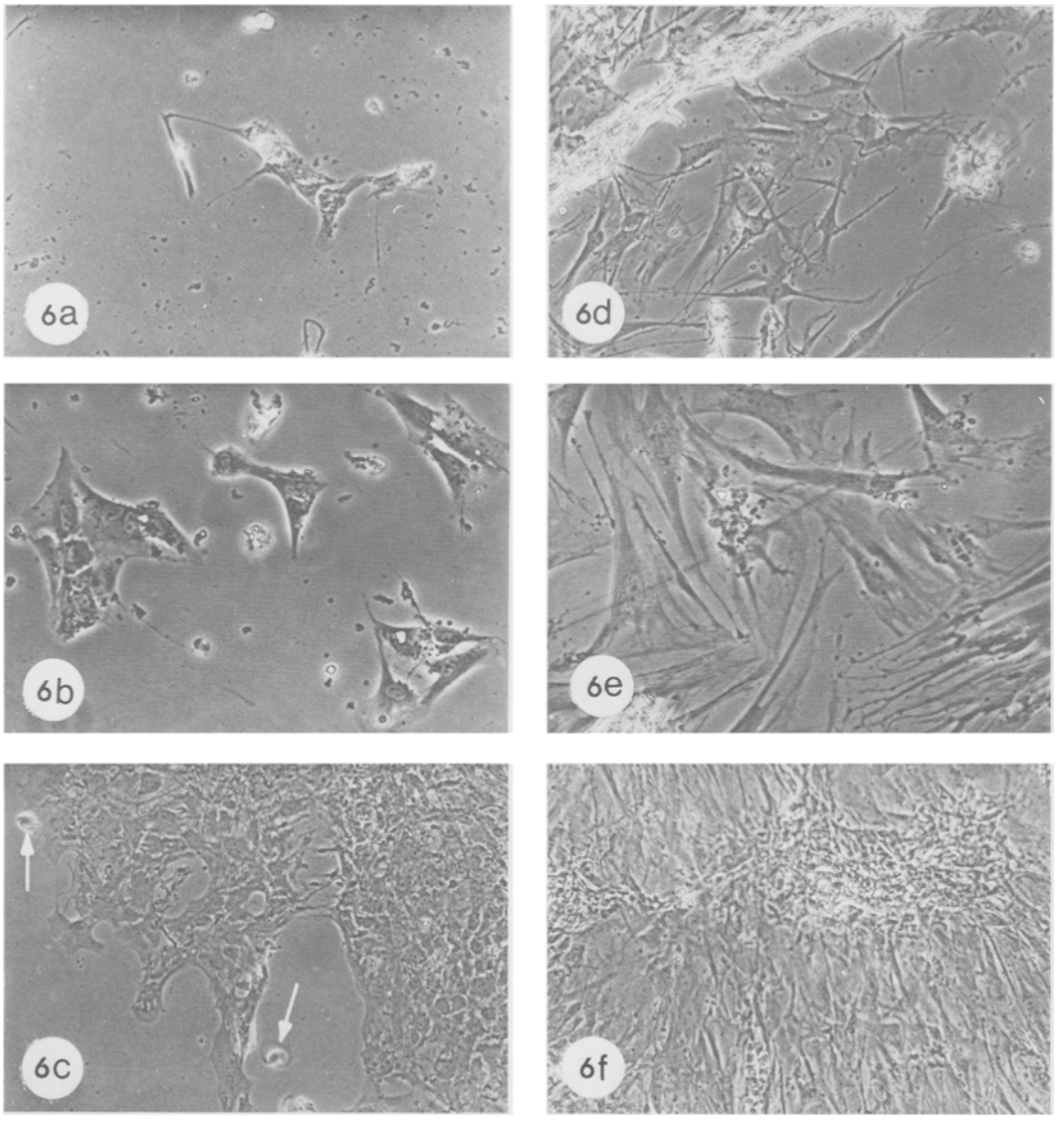

FIG. 6. Phase-contrast photomicrographs of the in vitro stages to the formation of full endothelial plaques (*a–c*) or to advanced smooth-muscle multilayers (*d–f*). *a*, Class II microvessel showing another form of cell emergence. When the confinement of the basement membrane is lost, endothelial-like cells emerge from all points along the vascular segment. Five-day culture. ×100. *b*, Fully emerged and proliferating endothelial-like cells. Seven-day culture. ×85. *c*, Endothelial cell plaque from a 21-day culture. Proliferation is reduced and localized to the periphery. Mitotic cells are indicated (*arrows*). ×85. *d*, Class V microvessel with side emerging and proliferating cells presumed to be predominantly smooth-muscle. Five-day culture. ×100. *e*, Subconfluent monolayer of pleomorphic smooth-muscle cells. Twelve-day culture. ×170. *f*, Multilayered smooth-muscle cells with characteristic mounding of cells into transverse ridges. Twenty-five-day culture. ×85.

microvessels surviving homogenization and sieving and attaching to the plastic or glass substratum. Many microvessels never attached and could be seen as free-floating microvessels. These were larger classes of microvessels for the most part, but small arteries and veins were also included. Second, there was variation in the technique used to disperse the mouse brain based on the number of strokes required to homogenize the tissue and the differences of the pestle fit from homogenizer to homogenizer. These last two variables not only affected the number of vessels

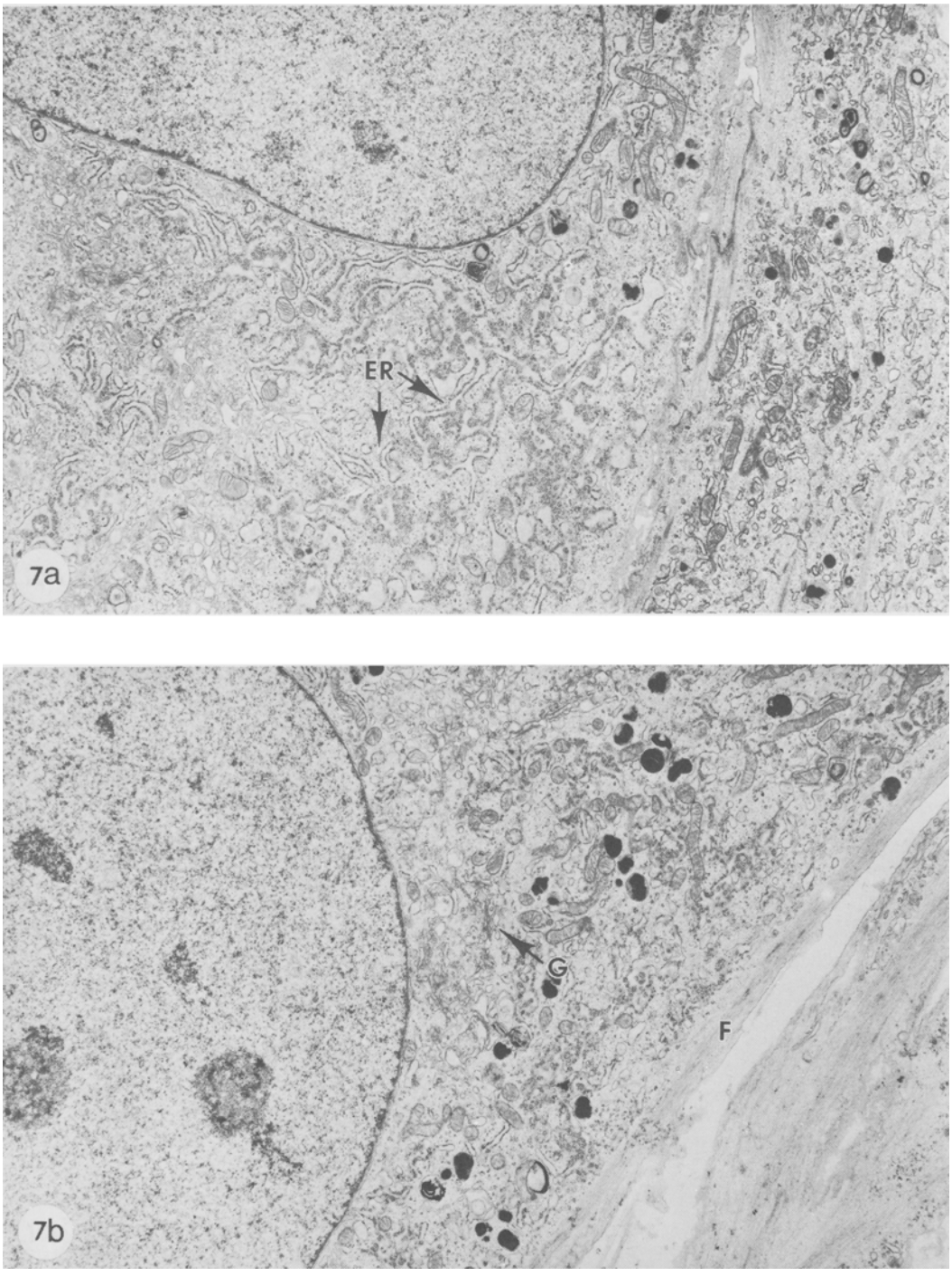

FIG. 7. TEM of cell plaques derived from isolated cerebral microvessels. *a*, Section of plaque identified as endothelial cells at the LM level revealed this cell with prominent rough endoplasmic reticulum (ER). Twenty-five-day culture. ×6400. *b*, Section of plaque identified as smooth-muscle cells at the LM level. The Golgi (G) apparatus is prominent and fibrillar bundles lie beneath the cytoplasmic membrane. Thirty-day culture. ×6400.

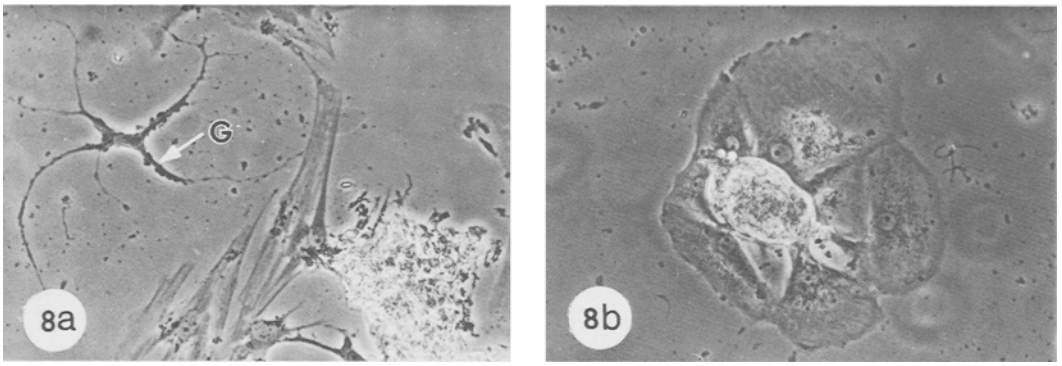

FIG. 8. Phase-contrast photomicrographs of cell types that are occasionally seen in microvessel cultures. *a*, Astroglial cell with its characteristic cytomorphology (G) and smooth-muscle cells next to a vascular knot. Six-day culture. ×170. *b*, Unknown cell type emerging from the base of a vascular knot. These could be epithelial cells from a choroid plexus knot. Ten-day culture. ×170.

available for attachment but also their viability. Third, younger animals, 21 to 35 days old, gave better results than older ones 45 days old or more. This may be related to the state of maturation of both the vessels and the brain tissue. Fourth, the yield of attached microvessels was always higher when the nylon-mesh material used for sieving was pliable enough to allow the capillary action of a few drops of medium to draw the mesh into intimate contact with the culture surface when plating the microvessels. Stiff mesh tended to warp and wrinkle and made few contacts with the surface. Finally, more microvessels attached to plastic (Falcon) than to glass although the outgrowth in terms of number of cells produced per isolate was very similar on the two substrates.

Since the microvessels isolated from mouse cerebellum are heterogeneous in size and morphological characteristics and since vessel diameter is a poor index of the specific type of microvessel isolated (24), we developed a microvessel classification system that simplified culture monitoring and had some predictive value. This enabled us to identify and study isolated microvessel types over time. Although the classification of the microvessels into six classes (I–VI) is obviously arbitrary and is not intended to allow for the accurate identification of microvessel types [Rhodin (25,26)] with their expected mural anatomies, it proved to be a very useful tool that allowed the evaluation of the efficiency of the isolation technique and the quality of the microvessel isolates and generalization about the fate of microvessels in a given class.

The viability of both the freshly isolated and the cultured microvessel as measured by the exclusion of erythrosin B was lower than anticipated. Our viability data show that none of the class I or II microvessels survived to proliferate. Although proliferation failed to occur in this specific study, observation of other samples showed that vessels of these classes often gave rise to cell plaques. It seems likely that the daily handling during culture observation and photomicrography may have led to this effect. Better results are obtained when the isolates are left undisturbed for periods up to 1 week in the protective atmosphere of the incubator. The large vessel classes do not seem to be as fragile, and increasing survival rates are found with increasing vessel class.

One of the early signs that a microvessel will give rise to proliferating cells is the swelling of the cell nucleus and the thickening of the microvessel in the perinuclear region of the cell cytoplasm (blastogenesis). This is not an invariable finding since some cells seem to migrate before blastogenesis and proliferation. For example, in classes IV and V microvessels, the number of microvessels showing migration and/or proliferating cells was 67% and 78%, respectively. However, the early signs of blastogenesis seen in classes I–III result in very few (in the case of class III) to no vessels with migrating or proliferating cells (classes I and II). This effect, too, is likely an artifact related to the trauma of repeated examination of the cultures during the experimental period.

The endothelium of the isolated microvessels appears to play a role in the initial attachment of the microvessel as shown in Fig. 2 and is often the first cell type to emerge and migrate from the microvessel isolate (Figs. 5*a*,*c*, 6*a*,*b*). This early emergence usually occurs first from the ends of

vessel segments in the lower classes of microvessels. Emergence of cells from all other points on the small microvessels (classes I-III) occurs later. In larger classes of microvessels, cells appear from the sides as well as the ends of vessels and include smooth-muscle cells as well as endothelium. Proliferation is more prominent from the sides of these larger microvessels. Other cell types have been seen and one of them may be similar to the pericytes described by Buzney, Frank and Robinson (27), which they derived from retinal capillaries from bovine, monkey and man. However, without morphological or other criteria for positive identification of these cells in vitro, cell type identification is not possible.

The TEM revealed that in classes I-III microvessels, the predominant mural cells, although few in number, were pericytes and these cells increased in number in parallel with increasing class number. An occasional smooth-muscle cell was seen in class III microvessels in contrast to class V vessels which had two or more layers of smooth-muscle cells. In class V microvessels, pericytes were difficult to distinguish from smooth-muscle cells because of the swelling and loss of detail of basal lamina. In class VI microvessels (vascular knots and/or cellular aggregates) it was not possible to identify mural cells as separate and apart from cellular aggregates at the LM level; however, at the TEM level the vascular knots appeared to be made up predominantly of class V microvessels containing the usual mural cell compliment. Vascular knots develop at different times in the culture. With the exception of day 0, when even knots form from classes I-IV microvessels, it is usually a sign of microvessel degeneration and death. When class V microvessels knot in culture, they are indistinguishable from a class VI microvessel from an initial isolate.

There are a number of conditions that can adversely affect both the isolated microvessels and the derived cells, especially the newly emerging endothelial cell. Simple feeding and microscopic observation can prove deleterious to the microvessels and cells. A rise in pH and a drop in temperature during a 15- to 30-min observation and/or photomicrography session may have led to the preferential loss of classes I and II microvessels in the sampling of microvessels presented in Table 2.

The data in Table 2 as well as data from other parallel experiments indicate that small microvessels of classes I-III do not fare as well in vitro as their larger counterparts. The viability and progress of a microvessel toward derived cell proliferation seem to be directly proportional to the number of mural cells. The possibility exists that the mural cells or even the remnants of glial foot processes on the isolated microvessel elaborate a substance(s) that is necessary for the maintenance and/or the proliferation of endothelial cells in vitro. We make the assumption that this factor is mainly a requirement for endothelial and/or pericyte growth because smooth-muscle cells, when present, proliferate and often dominate a culture regardless of the status of the endothelial cell.

REFERENCES

1. Carrel, A., and M. T. Burrows. 1910. Cultivation of adult tissues and organs outside of the body. J. Am. Med. Assoc. 55: 1379-1381.
2. Fleig, C. 1910. Sur la survie d'elements et de systemes cellulaires, en particulair des vaisseaux apres conservation prolongie hors de l'organisme. C. R. Soc. Biol. 69: 504-506.
3. Harrison, R. G. 1907. Observations on the living developing nerve fiber. Proc. Soc. Exp. Biol. Med. 4: 140-143.
4. Fisher-Dzoga, K., R. M. Jones, D. Vesselinovitch, and R. M. Wissler. 1973. Ultrastructural and immunohistochemical studies of primary cultures of aortic medial cells. Exp. Mol. Pathol. 18: 162-176.
5. Murray, M. R., and G. Kopech. 1953. *A Bibliography of the Research in Tissue Culture 1884 to 1950.* Academic Press, New York.
6. Pollak, O. J. 1969. Tissue Cultures. In: H. S. Simons, J. E. Kirk, and O. J. Pollak (Eds.), *Monographs in Atherosclerosis.* Vol. 1. Williams and Wilkins, Baltimore, pp. 1-143.
7. Goldstein, G. W., J. S. Wolinsky, and J. Csejtey. 1977. Isolated brain capillaries: A model for the study of lead encephalopathy. Ann. Neurol. 1: 235-239.
8. Goldstein, G. W., J. S. Wolinsky, J. Csejtey, and I. Diamond. 1975. Isolation of metabolically active capillaries from rat brain. J. Neurochem. 25: 715-717.
9. Goo, J., and I. Karnushina. 1973. A procedure for the isolation of capillaries from rat brain. Cytobios 8: 41-48.
10. Mrsulja, B. B., B. J. Mrsulja, T. Fujimoto, I. Klatzo, and M. Spatz. 1976. Isolation of brain capillaries: A simplified technique. Brain Res. 110: 361-365.
11. Brendel, K., E. Megan, and E. C. Carlson. 1974. Isolated brain microvessels: A purified, metabolically active preparation from bovine cerebral cortex. Science 185: 953-955.
12. Orlowski, M., G. Sessa, and J. P. Green. 1974. γ-Glutamyl transpeptidase in brain capillaries: Possible site of a blood brain barrier for amino acids. Science 184: 66-68.

13. Sessa, G., M. Orlowski, and J. P. Green. 1976. Isolation from bovine brain of a fraction containing capillaries and a fraction containing membrane fragments of the choroid plexus. J. Neurobiol. 7: 51-61.
14. Siakotos, A. N., G. Rouser, and S. Fleischer. 1969. Isolation of highly purified human and bovine brain endothelial cells and nuclei and their phospholipid composition. Lipids 4: 234-239.
15. Lewis, L. J., J. C. Hoak, R. D. Maca, and G. L. Fry. 1973. Replication of human endothelial cells in culture. Science 181: 453-454.
16. Bhurgan, B. K., B. E. Loughman, T. J. Fraser, and K. J. Day. 1976. Comparison of different methods of determining cell viability after exposure to cytotoxic compounds. Exp. Cell Res. 97: 275-280.
17. Dawson, R. M. C., and W. H. Elliott. 1962. Buffers and physiological media. In: R. M. C. Dawson, S. C. Elliott, W. H. Elliott, and K. M. Jones (Eds.), *Data for Biochemical Research.* Clarendon Press, Oxford, pp. 192-209.
18. Haudenschild, C. C., R. S. Cotran, M. J. Gimbrone, Jr., and J. Folkman. 1975. Fine structure of vascular endothelium in culture. J. Ultrastruct. Res. 50: 22-32.
19. DeBault, L. E. 1973. A critical point drying technique for SEM of tissue culture cells grown on plastic substratum. *SEM Symposium/1973.* IIT Research Institute, Chicago, pp. 317-324.
20. Karnovsky, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. J. Cell Biol. 35: 213-236.
21. Riva, A. 1974. A simple and rapid staining method for enhancing the contrast of tissue previously treated with uranyl acetate. J. Microsc. (Paris) 19: 105-108.
22. Kessel, R. G., and C. Y. Shih. 1974. Cells in culture. In: *Scanning Electron Microscopy in Biology.* Springer-Verlag. New York, pp. 63-85.
23. Weibel, E. R., and G. E. Palade. 1964. New cytoplasmic components in arterial endothelia. J. Cell. Biol. 23: 101-112.
24. Wieman, M. P. 1973. Anatomy. In: R. Wells (Ed.), *The Microcirculation in Clinical Medicine.* Academic Press, New York, pp. 1-11.
25. Rhodin, J. A. G. 1968. Ultrastructure of mammalian venous capillaries, venules, and small collecting veins. J. Ultrastruct. Res. 25: 452-500.
26. Rhodin, J. A. G. 1976. The ultrastructure of mammalian arterioles and precapillary sphincters. J. Ultrastruct. Res. 18: 181-223.
27. Buzney, S. M., R. N. Frank, and W. G. Robinson, Jr. 1975. Retinal capillaries: Proliferation of mural cells *in vitro*. Science 190: 985-986.

The authors gratefully acknowledge Mrs. Yumi Wu for her technical assistance in many of the microvessel isolations.

This work was supported in part by an Arteriosclerosis Specialized Center of Research grant from the National Heart, Lung and Blood Institute, National Institutes of Health (HL-14230) and Grant 584-127703 from the Veterans Administration.