Development and characterisation of a rat brain capillary endothelial culture: towards an in vitro blood-brain barrier

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Summary

Primary culture of rat brain endothelial cells is described, based on the method of C. C. W. Hughes and P. L. Lantos. The cells have been characterised using morphological and immunocytochemical techniques, and systematic studies undertaken to determine the optimal culture medium and conditions required to grow the cells at high purity on a variety of substrata. The endothelial cells have a spindle-shaped morphology, and proliferate as plaques from small clusters of cells associated with capillary fragments in the starting material. Tight junction-like cell-cell appositions are seen at the electron-microscopic level. The cells show characteristic staining for antigens recognized by antibodies against von Willebrand factor (Factor VIII-related antigen), angiotensin-converting enzyme (ACE), the transferrin receptor (Ox-26), actin and vimentin. They also show binding of the lectin from Ulex europaeus (UEA I). Potential contaminating cells include smooth muscle, fibroblasts, pericytes and meningeal cells. Contaminants can be kept to < ca. 5% by careful removal of large vessels and meninges during dissection, by brief treatment with Ca2+ - and Mg2+-free saline, by growth in medium supplemented with plasma-derived serum treated for removal of platelet-derived growth factor (PDGF), and by occasional use of medium in which D-valine is substituted for L-valine. Cells attach well to collagen-coated plastic, less well to glass. Cells can be grown on transparent collagen filters (ICN, Cellagen and Costar, Transwell-Col), and on microparticle beads (Pharmacia, Cytodex-3). The culture has proved to be a useful preparation for studies of cellular physiology, pharmacology and biochemistry of the brain endothelium, and represents a first step in producing an in vitro model of the rat blood-brain barrier.

Key words: endothelium, brain, tissue culture, immunocytochemistry.

Introduction

A good in vitro model of the blood-brain barrier would have wide application in experimental, pharmaceutical and clinical studies. A great deal of information is now available for the in situ mammalian blood-brain barrier, principally from experiments in the rat (Davson et al. 1987). In vivo preparations preserve the normal anatomical arrangement of cells at the blood-brain interface, and give useful information about regional differences within the brain. However, they suffer from poor temporal and spatial resolution, the difficulty of detecting transfer of slowly penetrating substances, and poor access to the brain side of the endothelium. Isolated capillaries have proved useful for identification of membrane receptors and studies of carrier-mediated uptake (Pardridge, 1988), but as the medium has access to both apical and basal membranes, neither polarised features of uptake nor transcellular movement can be studied. The endothelial cells are also metabolically damaged during the isolation, and so are not ideal for the study of energy-dependent processes.

Culture of brain endothelial cells gives several advantages, in particular the preservation of cellular polarity and metabolism. Several methods have been described that are capable of producing confluent monolayers (reviewed by Jo6, 1992). Most studies have been done with bovine cells, in either primary or passaged culture (Bowman et al. 1981, 1983; Carson and Haudenschild, 1986; Méresse et al. 1989a; Audus et al. 1990). However, there is considerable variability in the starting material, and the absence of information on the in vivo bovine blood-brain barrier makes rigorous comparison difficult. While passaging has the advantage that it generates large stocks of cells for experimental study, it introduces additional complications due to de-differentiation, and selection of the most rapidly proliferating clones. There is considerable variability
between properties reported for bovine cultures from different laboratories. While most monolayers are relatively impermeable to horseradish peroxidase and albumin (reviewed by Audus and Borchardt, 1987), some cultures are permeable to lanthanum and sucrose, with a resistance of < 100 Q.cm² (Goldstein et al. 1986), while subculture of selected clones has achieved resistances of up to 800 Q.cm² (Rutten et al. 1987).

Several groups have studied transport of particular substances by the bovine endothelial monolayer (acetylated low density lipoprotein, Ac-LDL; Gaffney et al. 1985; Mèresse et al. 1989b; amino acids: Beck et al. 1984; proteins: Audus and Borchardt, 1987), but few have attempted a comprehensive characterisation of the culture. The extent to which tight junctions and enzymes are preserved is dependent on the time in culture and number of passages. Some properties that are lost in culture (expression of γ-glutamyl transpeptidase, γ-GTP; tight-junctional complexity and tightness) can be reinduced by co-culture with astrocytes or exposure to astrocytic conditioned medium (reviewed by Abbott et al. 1990a; Abbott and Revest, 1991; Greenwood, 1991; see also Debouck et al. 1990; Rubin et al. 1991a,b; Meyer et al., 1991). However, it has been necessary to use rat not bovine astrocytes, as the methods available for preparation and characterisation of pure astrocytes have so far not been available for bovine glia. Should components of the induction process prove to be species-specific, these cross-species co-cultures will lack important features of the interaction. All these factors provide a strong stimulus for the development of a culture model in which both endothelial cells and astrocytes can be prepared to high purity and characterised fully, so that their interactions can be studied under well-controlled and reproducible conditions.

A successful brain capillary endothelial primary culture has been developed for the rat by Hughes and Lantos (1986; see also Hughes, 1988). The rat has the advantage that several monoclonal and polyclonal antibodies are available for the identification of endothelial cells, glia and other cell types, and can be used for assessment of culture purity. Results from the culture can be readily compared with results from in situ rat brain preparations. There is an extensive literature on the lineage and differentiation of rat glial cells in culture and in situ (Raff, 1989; Voight, 1989; Skoff, 1990), which makes the rat model particularly suitable for co-culture examination of the role of glia in induction of brain endothelial properties. The rat brain endothelial cell culture is well characterised, a suitable culture medium has been devised and, using inbred Lewis rats, the cultures have reproducible antigenic and transport properties. The culture has proved useful in the examination of membrane ion channels and receptors (Abbott and Revest, 1990; Revest et al. 1991), in studies of amino acid, peptide and protein uptake (Hughes and Lantos, 1989; Begley et al. 1990; Ramakahan, 1990; Revest et al. unpublished) and in investigating the effect of bile salts on the blood-brain barrier (Greenwood et al. 1991). The ability to use syngeneic combinations of endothelial and other cell types has proved particularly valuable in study of the immunological properties of the endothelial cells, and of their interactions with lymphocytes (Hughes and Lantos, 1986; Male et al. 1987; Hughes et al. 1988; Male et al. 1990; Risau et al., 1990). A modified method has proved useful in preparing microvascular cultures from rat retina (Greenwood, 1992).

This paper gives a full account of the method used to prepare the culture, and presents morphological and immunocytochemical evidence for the endothelial nature of the monolayer. It presents modifications of the method - for growth on glass, filters and microcarrier beads, and for subculturing cells - suitable for a variety of physiological and pharmacological applications. A preliminary account has been published (Abbott et al. 1990b).

Materials and methods

Preparation of primary culture

Brain capillary fragments were isolated, and endothelial cells cultured, using a modification of the method introduced by Hughes and Lantos (1986). Only grey matter was used, to reduce the amount of myelin in the digest. Two Lewis rats 2-3 months old gave sufficient capillary fragments for plating on 12/24-well or 60/96-well plates, or six 35 mm dishes. The first rat was anaesthetised in ether, the skin of the neck swabbed with 70% ethanol, and the animal decapitated. A triangular flap of cranium was cut to expose the brain, the brain was eased out of the skull with a fine spatula, and dropped into oxygenated ice-cold Buffer A (calcium- and magnesium-free Hanks' balanced saline solution (Ca,Mg-free HBSS, Gibco-BRL, Paisley), buffered with 10 mM HEPES (Sigma), pH 7.3, containing 100 i.u./ml penicillin and 100 μg/ml streptomycin (Sigma), with 0.5% bovine serum albumin (BSA, Sigma Fraction V, A9647) added after oxygenation.

The second brain was removed in the same way. Working in a laminar flow hood, the first brain was placed on a piece of sterile lint moistened with Buffer A, the cerebellum and brain stem were removed, and the brain sliced in half (sagittal section), one half being returned to the buffer. From the remaining half-brain, the meninges and choroid plexus were peeled off, the mid/hindbrain removed, and the cortex was rolled on dry lint to remove adherent surface cells, then returned to the wet lint. The white matter, striatum and optic nerves were removed. The remaining grey matter was transfused to fresh Buffer A, and chopped with a scalpel for < 1 min into uniform 2-3 mm pieces. The second half-brain was dissected in the same way, added to the suspension of the first, and chopped. The lint was replaced, the second brain was prepared in the same way, chopped and pooled with the first brain. The suspension was spun at 600 g, 4°C for 5 min.

The preparation involved two stages of enzymic dissociation to degrade extracellular matrix. Collagenase/dispease solution (0.1%, Boehringer, Mannheim, in Ca,Mg-free HBBS containing 10 mM HEPES, 100 i.u./ml penicillin and 100 μg/ml streptomycin), was oxygenated for 2-3 min, and pH adjusted to 7.3. 20 units/ml DNase 1 (deoxyribonuclease-5'oligonucleotide-hydrolyase, EC 3.1.21.1, Sigma Type I D4263) and 0.147 μg/ml TLCK (tosyl-lysine-chloromethyl-ketone, Sigma T7254) was added, the former to degrade DNA liberated from ruptured cells, the latter to inhibit clostripain, a non-specific proteinase contaminant of collagenase prep-
arations which damages cells (Helley et al. 1981). The supernatant from the spun cells was poured off, 15 ml of the enzyme mix was added, and the cells were digested for 1 h at 37°C to separate microvessels from other components. The suspension was agitated gently every 10 min.

The suspension was triturated with a Pasteur pipette until it had a creamy texture, then the trituration was repeated until a pipette with tip narrowed in a flame until all grey matter was dispersed. The suspension was centrifuged for 5 min at 600 g. The supernatant was removed, the cells were resuspended in 25% BSA in Buffer A, and the mixture was spun at 1000 g for 15 min (density-dependent centrifugation in BSA) to separate capillary fragments (heavier) from myelin, neurons, astrocytes and other single cell contaminants (lighter). The top layers were poured off, taking care to remove all last traces of myelin etc, and the capillary pellet was resuspended in Buffer A and spun at 600 g for 5 min. The supernatant was poured off, and the cells were resuspended in second collagenase/disperse solution (5 ml, concentration as above) and incubated for 3 h at 37°C, with occasional shaking.

Culture dishes were prepared by coating with collagen (see below). Culture medium was based on 80% Ham's F-10 medium (Gibco-BRL) with 16% bovine cell-free plasma-derived serum (PDS) and 4% supplement (see below). The PDS was prepared following the method of Vogel et al. (1978), except that cation-exchange CM-Sephadex (Sigma Type C50-120) was added as powder, not used as a column, so no concentrative step was necessary. The preparation of cell-free PDS without rupture of platelets, and with addition of CM-Sephadex, was designed to remove platelet-derived growth factor (PDGF), which is known to stimulate proliferation of potential contaminants of the endothelial culture, smooth muscle, fibroblasts and glial cells. The standard medium contained in addition (supplement, 4%, all chemicals from Sigma): 2 mM glutamine, 80 g/ml heparin (Grade I), 50 µg/ml gentamicin sulphate and 75 µg/ml endothelial cell growth supplement (ECGS, E2759). Variants of the culture medium and additional supplements tested are described below.

Plastic ultracentrifuge tubes were cleaned by sonication in Milton sterilising solution, then distilled water, and sterilised with 70% ethanol, washed with Buffer A, and shaken gently for 20 min to coat the walls with albumin to prevent cell adhesion. A Percoll gradient was prepared in the tubes by spinning isonitric Percoll (stock solution prepared from 50 ml Percoll plus 5.5 ml 10× strength HBSS + 45 ml HBSS, pH 7.3, the HBSS containing both Ca²⁺ and Mg²⁺ to aid clumping of capillaries) at 25,000 g at 4°C for 1 h.

The enzyme digest was spun at 600 g for 5 min, the supernatant was poured off, and Buffer A added to resuspend the pellet. The suspension was layered onto the top of the Percoll gradient, and spun at 1000 g for 10 min. The capillary fragments form a band about 4/5ths of the way down the gradient, below single cell contaminants. The capillary fragments were sucked into a Pasteur pipette, suspended in Buffer A, spun at 700 g for 5 min, and resuspended in 50:50 buffer A/culture medium. They were spun again at 600 g for 5 min, and finally resuspended in culture medium and plated out.

Variations of culture medium

Medium supplements tested included insulin, glutathione, selenium, vitamin C (ascorbic acid), and transferrin (all from Sigma). Growth could be measured using a DNA assay (see below). The effectiveness of PDS prepared by different methods, the effect of replacement of PDS by fetal calf serum (FCS), and the use of D-valine not L-valine medium was assessed. The effect of amphotericin B (Fungizone, 2.5 µg/ml, Sigma, occasionally used to prevent fungal growth) on cell growth was observed.

DNA assay

The method was based on that of Labarca and Paigen (1980). Hoechst reagent H33258 (Sigma) was made up in assay buffer (2 M NaCl, 20 mM HEPES, 2 mM EDTA, pH 7.4) at a concentration of 0.1 µg/ml. A series of DNA standards was made using salmon testis DNA (Sigma) ranging from 1 to 100 µg/ml. The standards were made up in Ca,Mg-free HBSS and sonicated. Cell samples were removed from the plates using trypsin (Sigma, 0.1 mg/ml) made up in Ca,Mg-free HBSS and sonicated; 200 µl of each cell sample, DNA standard, or blank was added to 3.3 ml of the Hoechst solution and sonicated. Samples were prepared in triplicate. After 10 min at room temperature, samples were read in a Perkin-Elmer Fluorescence Spectrophotometer, using 356 nm excitation wavelength and measuring emission at 458 nm. A calibration line was constructed from the standards, and the DNA content of the cell samples was calculated.

Attachment factors

The standard procedure used plastic dishes, coated for 1-2 h with collagen (either type I rat tail, Sigma type VII, 0.33 mg/ml, or prepared directly from fresh rat tail using the method of Freshney, 1986), fixed wet by exposure to ammonia vapour, and washed three times in HBSS before addition of cells. Other attachment factors tested included poly-L-ornithine (Sigma, 0.1 mg/ml) and poly-L-lysine (Sigma, 0.1 mg/ml), fibronectin (Sigma, 1 or 5 µg/cm²), laminin (Sigma, 2 µg/cm²), Matrigel (Collaborative Research, 1:5 and 1:10 dilution) and collagen either gelled with NaOH (Freshney, 1986) or made up in ethanol and allowed to dry on the plates/dishes. For gelatin coating, the solution (Sigma, 0.5 g/100 ml) was left on the dishes for 1 h, the liquid was poured off, and cells were added.

Light and electron microscopy

Cell and culture morphology was examined in a Nikon Diaphot microscope using phase-contrast optics. Monolayers were routinely photographed and cells counted to estimate cell number, density and percentage of contaminating cells. For scanning electron microscopy of capillary fragments, freshly isolated capillaries were allowed to attach to collagen-coated plastic coverslips (Tefranox, Miles) for 2 h. The coverslips were then gently immersized in buffered glutaraldehyde (2.5% glutaraldehyde, 5% sucrose in 0.1 mM sodium cacodylate, pH 7.4) and fixed for 45 min. After washing (3×) in buffer (0.1 M sodium cacodylate, pH 7.4), cells were post-fixed in 1% OsO₄ with or without 1.5% potassium ferricyanide to increase membrane contrast, and rinsed in buffer. Following dehydration in 70%, 80%, 95% and absolute ethanol, and propylene oxide, tissue was embedded in Spurr resin. Thin sections were cut with a diamond knife and floated onto copper grids with or without Formvar-coating, stained in 2% uranyl acetate in 50% ethanol, followed by 0.4% lead citrate,
washed with NaOH and distilled water and dried. Sections were examined in a Philips 300 or Hitachi H 600 transmission electron microscope at 60 kV.

**Immunocytochemistry and fluorescence histochemistry**

Cells were grown to near-confluence on non-autofluorescent plastic 8-well chamber slides (Labtek, Gibco-BRL). Immunodetection of specific antigens used the biotin-streptavidin method. Primary antibodies used were: rabbit anti-human von Willebrand factor (Factor VIII-related antigen, Dako), mouse anti-cow angiotensin-converting enzyme (ACE, gift from Professor R. Auerbach, University of Wisconsin), mouse anti-rat transferrin receptor (OX-26, gift from Dr. D.W. Mason, Oxford University), mouse monoclonal OX-43 (marker for non-brain endothelium, gift from Dr. D.W. Mason), mouse anti-chicken actin (Amersham International), mouse anti-porcine vimentin (Amersham International), mouse anti-human fibronectin (Sera Lab), mouse anti-chicken tubulin (Amersham International), rabbit anti-cow glial fibrillary acidic protein (GFAP, Dako), and rabbit anti-mouse laminin (Gibco-BRL).

Cells were washed three times with HBSS. Surface antigen staining was carried out on unfixed cells. For the detection of internal epitopes the cells were permeabilized and fixed with acid/ethanol (one part 1% HCI:99 parts 70% ethanol, v/v) for 10 min, followed by a further wash with HBSS. Cells were exposed to 0.5% BSA in HBSS for 15-20 min to block non-specific binding sites. First-layer (primary) antibodies were diluted in HBSS containing 0.5% BSA, and were incubated with the cells at room temperature for 1 h. After washing three times with HBSS, the monolayer was incubated for 30 min with anti-species-specific biotinylated antibody in HBSS (anti-mouse Ig for monoclonals, and anti-rabbit Ig for polyclonals, Amersham International) for 30 min. This was then aspirated off, the cells washed in HBSS, and the third layer, either streptavidin-fluorescein or streptavidin-Texas red (Amersham International) placed on the cells for 15 min. After washing, cells not previously fixed were fixed in acid/ethanol for 10 min and then washed. In some cases cells were then counterstained with the nuclear fluorescent stain, propidium iodide, at a concentration of 10 µg/ml (Sigma). The chamber wells were then peeled off the slides and the cells mounted in Citifluor aqueous mountant and viewed on a Zeiss Universal microscope incorporating reflected light fluorescence and excitation, and barrier filters for fluorescein and Texas red. Negative controls used the following substitutes for the primary antibody: 10% rabbit serum in HBSS for polyclonals; 10% mouse serum in HBSS for monoclonals.

An additional marker used to detect endothelial cells was the lectin from *Ulex europaeus* (UEA I), which binds specifically to endothelial cells (Jackson et al. 1990). Cells were washed, fixed with acid/ethanol, and blocked with 2% normal horse serum (NHS) for 20 min. The fluorescein isothiocyanate (FITC)-lectin UEA 1 (Sigma, L9006) was incubated with the cells at room temperature for 3 h at a concentration of 20 µg/ml in HBSS. After washing in HBSS, cells were mounted in Citifluor, and viewed on a Zeiss microscope as above.

**Growth on different substrata**

Cells were routinely grown in plastic multiwell (24 and 96)
dishes and 35 mm Petri dishes. For particular experimental manipulations it was useful to grow cells on other substrata. Glass coverslips (BDH 13 mm diameter) were tested following different cleaning procedures, with either ethanol or chromic acid, followed by washes in water and HBSS. Porous filter multiwell inserts tested included Cellagen (ICN), Transwell-col and Transwell (Costar), Millicell (Millipore), and disks of dialysis tubing membrane. Microcarrier beads tested were Cytodex-3 (Pharmacia). Beads were prepared by swelling for 3 h in Ca,Mg-free phosphate-buffered saline (PBS). They were sterilised by washing in 70% ethanol, and left overnight in PBS. Microcarriers were either mixed with cells in culture medium at the time of plating (3 mg/ml), or added to subconfluent monolayers.

Subculturing cells
Uncontaminated confluent monolayers could be subcultured by very gentle trypsinisation. Cells were washed twice with HBSS to remove any serum, then incubated for 20 min, first with Ca,Mg-free HBSS and then with Ca,Mg-free HBSS containing 10 mM EDTA, which caused the cells to round up. The monolayers were then covered with as small a volume as possible of 0.05% trypsin in Ca,Mg-free HBSS plus 10 mM EDTA. After 10 s the dishes were filled with culture medium containing serum, to inhibit the action of the trypsin. After another 10 min, the medium was pipetted gently up and down a few times to loosen the cells, and the cell suspension was added to freshly collagen-coated dishes in a split ratio of approximately 1:5.

Results

Scanning electron microscopy of vessel fragments
The microvessel fragments are generally short, and occasionally branched, with a diameter around 10 µm (Fig. 1). The endothelial cells have rounded up, and present a smooth abluminal surface, with fragments of adherent basement membrane. There are relatively few additional adherent cells.

Identification of brain capillary endothelial cells: light microscopy
On first plating out, the culture showed clusters of rounded-up cells associated with the remains of small vessel (capillary) fragments, and scattered single cells and debris (Fig. 2). After 1-3 days, cells could be seen growing out of the clusters (Fig. 3); the single cells appeared less viable, and generally died. By 3-5 days the predominant cell type in the culture was a spindle-shaped cell approximately 10-20 µm across the broadest part, and around 100 µm long. Healthy cells showed a uniform phase-bright appearance with dark granular inclusions. The cells grew out from the clusters approximately radially, but with some swirling patterns and whorls. After several days of growth, clear alignments of the cells were visible, with neighbouring cells tightly packed against each other leaving few gaps. The cells became confluent at 7-10 days (Fig. 4).

Fig. 3. At 3 days after plating, spindle-shaped cells are growing out of the cell clusters that represent the capillary fragments isolated from rat brain grey matter. Bar, 100 µm.
Fig. 4. Relatively pure confluent endothelial monolayers at 10 days after plating. The cells show the typical elongated spindle-shaped morphology, aligned longitudinally, and form a uniform monolayer with few gaps. Bar, 100 µm.
Small flaws in the monolayer could sometimes be detected at the meeting point between cells proliferating from different clusters, or within a single cluster where cells changed orientation (Fig. 5). From photographs of uncontaminated monolayers it was estimated that there were about $4 \times 10^4$ cells/well in the 96-well plates, i.e. approximately 1000 cells/mm$^2$. From 3-5 days of culture, cells with other morphologies were sometimes visible. These included bipolar cells with long fine processes, fatter spindle-shaped cells forming...
Fig. 5. A relatively uniform monolayer, but with a small gap (arrow) occupied by flattened contaminating cells, probably pericytes (see Fig. 8). Such flaws are relatively rare in monolayers seeded at high density and growing rapidly without contaminants. Bar, 100 μm.

Figs 6-10. Contaminating cell types growing in regions devoid of endothelial cells, or on the surface of the monolayer. A complete cytochemical characterisation has not been attempted, but on morphological criteria (see text), the elongated cells (Fig. 6) are probably fibroblasts, the plaque of fatter cells with 'ridge and valley' morphology (Fig. 7) is probably smooth muscle, the polygonal cells with fine processes (arrow Fig. 8) are probably pericytes or leptomeningeal cells, and the multipolar cells growing on the surface of the monolayer (arrows, Figs 9 and 10) are probably either pericytes or glial cells. Bars, 50 μm.

Table 1. Immunocytochemistry of cultured rat brain endothelial cells

<table>
<thead>
<tr>
<th>Antibody to:</th>
<th>Immunodetection in vitro</th>
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<tbody>
<tr>
<td>von Willebrand factor (F-VIII related antigen)</td>
<td>+++</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme</td>
<td>+</td>
</tr>
<tr>
<td>Transferrin receptor (OX-26)</td>
<td>++</td>
</tr>
<tr>
<td>Non-brain endothelium (OX-43)</td>
<td>-</td>
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<tr>
<td>Glial fibrillary acidic protein</td>
<td>-</td>
</tr>
<tr>
<td>Actin</td>
<td>+++</td>
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<tr>
<td>Vimentin</td>
<td>+++</td>
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<tr>
<td>Fibronectin</td>
<td>-</td>
</tr>
<tr>
<td>Tubulin</td>
<td>-</td>
</tr>
<tr>
<td>Laminin (tight junctions open)</td>
<td>++</td>
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Thin-section electron microscopy
In electron-microscopic thin sections, cells showed a well-fixed morphology, with good preservation of membranes and organelles including mitochondria (Figs 11-13). Spots resembling tight junctions could be detected in most regions of membrane apposition between cells of the predominant type, usually within a region of wider membrane spacing resembling a zona adhaerens (Fig. 13). Regions of cytoplasmic density could occasionally be seen adjacent to the junctional zone. There was a prominent basal lamina under the cells. Occasional membrane specialisations (coated pits, omega profiles) could be seen (Figs 11 and 12). There were relatively few 'vesicular' profiles.

Immunocytochemistry and fluorescence histochemistry
The results of the light-microscope immunocytochemistry are summarised in Table 1. The cultured monolayers were positive for the endothelial specific markers, von Willebrand factor (Factor VIII-related antigen) and ACE (angiotensin-converting enzyme), and for the transferrin receptor known to be expressed at high concentrations on brain but not peripheral
Figs 11 and 12. Electron micrographs of endothelial cells grown on a collagen filter (ICN Cellagen). There is good preservation of cell membranes and organelles, and occasional coated pits can be seen (*). The arrows mark close membrane appositions resembling zonulae occludentes. Note the paucity of ‘vesicular’ profiles. A prominent basal lamina can be seen on the under-surface of the cells. Bars: Fig. 11, 1 μm; Fig. 12, 0.5 μm.

Fig. 13. Electron micrograph of the contact zone between adjacent endothelial cells, grown on plastic, and treated with potassium ferricyanide to increase membrane contrast. The arrows mark a region of close cell:cell apposition resembling a zonula occludens, within a longer region of cleft and associated cytoplasmic density resembling a zonula adhaerens. Bar, 0.2 μm.

endothelium (Jefferies et al. 1984). The expression of von Willebrand factor (Fig. 14), ACE (Fig. 15) and the transferrin receptor (detected with OX-26, Fig. 16) was punctate and multifocal, with a concentration in the perinuclear zone. The FITC-labelled lectin from UEA I showed a strong uniform staining of the complete monolayer (Fig. 17). The antibodies to GFAP, fibronectin and tubulin gave negative staining, as did the monoclonal antibody against non-brain endothelium OX-43 (Robinson et al. 1986). However, there were positive reactions against actin (Fig. 18) and vimentin (Fig. 19), the latter demonstrating prominent intracellular filaments. A detailed immunocytochemical characterisation of contaminating cells was not attempted, but cells identified as contaminants on the basis of cell shape (see above) were negative for von Willebrand factor and did not bind the lectin from Ulex europaeus.

Antibody against laminin, a basement membrane component, gave little reaction in control cultures, apart from small patches due to flaws in the monolayer (Fig. 20A, contrast with vimentin pattern Fig. 20B), but showed punctate staining along the cell margins when cells were pretreated for 5 min with hypertonic solution, 1.4 M mannitol in HBSS (Fig. 21A-C). Hypertonic solutions are known to open brain endothelial tight junctions both in vitro (Dorovini-Zis et al. 1984) and in vivo (Rapoport and Robinson, 1986).

Culture purity

In the initial stages of establishing the culture, there was some variability in the percentage of non-spindle-shaped cells (Figs 6-10). Several treatments were successful in removing these contaminating cells. Thorough removal of large vessels, meninges and choroid plexus tissue during the primary dissection proved to be necessary. Careful attention to the preparation of plasma-derived serum to remove platelet-derived growth factor, PDGF (mitogenic for fibroblasts and smooth muscle), was important. Brief (10-20 min) treatment with Ca,Mg-free HBSS on day 2 or 3, and/or growth for 2-3 days in medium made from Ham’s F-10 containing no L-valine, supplemented with D-valine (0.69 mg/ml), were found to reduce the percentage of contaminating cells. D-valine can be metabolised by endothelial cells but not by contaminating cell types (Picciano et al. 1984).

Fig. 22A shows an experiment to assess the effect of culture medium and attachment factors on the percent-
Rat brain endothelial culture

Fig. 14. Antibody against Factor VIII-related antigen (von Willebrand factor, FITC) gives a relatively punctate staining, although brighter and more aggregated in some cells than others, and concentrated in the perinuclear zone. Cells fixed/permeabilised in acid/ethanol prior to application of antibody. Bar, 20 μm.

Fig. 15. Antibody against angiotensin-converting enzyme (ACE, Texas red) shows a relatively uniform granular staining over the surface of the endothelial cells. Nuclei stained with propidium iodide. Cells fixed/permeabilised prior to application of antibody. Bar, 20 μm.

Fig. 16. Antibody against Ox-26 (FITC, cells not fixed), specific for the transferrin receptor, shows punctate staining over the cell surface, with some clustering in the perinuclear region. Cells fixed prior to mounting. Bar, 50 μm.

Fig. 17. The FITC-labelled lectin from *Ulex europaeus* stains all cells within a plaque, evidence that in spite of some variation in morphology, they are all endothelial cells. Cells fixed in acid/ethanol. Bar, 100 μm.

age of contaminating cells, identified by cell shape. For this series, no Ca,Mg-free wash or D-valine treatment was used, so the percentage of contaminating cells in the control culture (PDS medium, plated on rat tail collagen) was greater than normal (10% cf. <5%). Growth on purified type I collagen (Sigma), rat tail collagen with fibronectin, laminin or both, did not change the percentage of contaminants. However, substitution of FCS for PDS caused an increase in contaminants to 30%.

**Growth rate and effects of medium, substrata and medium supplements**

There was some variability in growth rate in different batches of plasma-derived serum, prepared fresh from bovine blood under similar conditions. The major variable appeared to be abattoir practice, PDS prepared from blood collected from a Kosher abattoir being consistently less effective than that collected from animals stunned before exsanguination. There appeared to be greater red cell lysis in the former.

Serum prepared in a manner designed to induce platelet lysis (and hence liberation of platelet-derived endothelial cell growth factor, PD-ECGF; Ishikawa et al. 1989) had no additional growth-promoting effect. Different batches of FCS produced variable growth rates, which could be faster or slower than in PDS (Fig. 22B). A reasonable compromise used FCS with D-valine for days 1-3, and PDS in normal Ham’s F-10 (no D-valine) thereafter (Fig. 23). Culture medium prepared from good batches of PDS, and used from the day of plating, was the medium of choice.

In experiments comparing the basic medium and medium containing supplements, it was found that vitamin C (5 μg/ml), glutathione (325 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml) and selenium (0.005 μg/ml) gave significantly enhanced cell growth, and the
addition of all supplements was more effective than each alone.

Cell attachment factors: effects on attachment and growth

Cell attachment was assessed by examining the density of cell clusters after the first medium change. Rat tail collagen-coated tissue-culture plastic was the preferred substrate for the brain endothelial cells, attachment and growth being equally good on wet collagen fixed in NH$_3$ vapour and on collagen 'gelled' using NaOH (Freshney, 1986) (Fig. 24). Attachment was impaired if the collagen was allowed to dry out before plating. No significant improvement in cell attachment was observed in multiwells treated with 0.1 mg/ml poly-L-lysine or poly-L-ornithine, although the former improved attachment to glass. The addition of fibronectin (5 µg cm$^{-2}$) and/or laminin (2 µg cm$^{-2}$) to the collagen matrix before initial plating did not significantly improve cell attachment or growth (Fig. 22B). Cells grew well on thin layers of gelatin (5 mg/ml), with a morphology similar to that on collagen. However, the monolayers tended to detach from the dish when disturbed, for example during feeding. Cells grown on Matrigel (1:5 or 1:10 dilution) showed a different morphology compared to those grown on collagen. They were more irregular, with a number of processes, and did not form smooth monolayers. This may have been due to the difficulty of obtaining a very thin layer of Matrigel, as the cells grew into the gel, not on its surface (cf. Minakawa et al. 1991).

Growth on different substrata

In 96-well plastic plates, cells routinely grew to

![Fig. 22](image.png)
Fig. 20. Fluorescence micrographs of the same field, a control monolayer (HBSS) photographed with different filters to demonstrate staining with antibodies against: (A) laminin (FITC); and (B) vimentin (Texas red) + laminin (FITC). Comparison of the images shows that the bright green patches in (A) and (B) correspond to imperfections in the monolayer, where the underlying basement membrane is exposed. There is little anti-laminin staining in the areas of uniform endothelial plaque. Cells were fixed/permeabilised with acid/ethanol between application of the anti-laminin and anti-vimentin antibodies. Bar, 50 μm.

Fig. 21. A-C. Fluorescence micrographs of the same field in a monolayer treated with 1.4 M mannitol to open the tight junctions, photographed with different filters to demonstrate staining with antibodies against: (A) laminin (FITC); (B) vimentin (Texas red); and (C) both. Laminin is now exposed in a punctate pattern, forming beaded rows along the endothelial cell margins (contrast Fig. 20). This confirms that the hypertonic treatment has opened the endothelial junctions. Cells were fixed/permeabilised in acid/ethanol between application of the anti-laminin and anti-vimentin antibodies. Bar, 50 μm.
Rat brain endothelial culture

Fig. 23. Cells grown in fetal calf serum medium with D-valine show a similar morphology to those grown in the usual PDS medium with L-valine, but with relatively irregular cell outlines and more frequent gaps in the monolayer (cf. Fig. 4). Bar, 50 μm.

Fig. 24. Cells grown on 'gelled' collagen show a similar morphology to cells grown on collagen fixed with ammonia vapour (cf. Fig. 4). Bar, 100 μm.

Fig. 25. Endothelial cell growth curve. The DNA content of the culture shows a peak at 7 days and subsequent decline. Values are means ± s.e.m., determinations from 5 wells of a 60/96-well plate per time point.

confluence within 7-10 days, then started to detach and die off. This is shown in Fig. 25 where the amount of DNA is seen to peak at 7 days. In 24-well plates, plated at similar density, growth was generally slower, and confluence took 15-20 days. There was poor attachment of cells to glass; the most successful treatment involved pre-cleaning in chromic acid, washing under running tap water, sterilising with 70% ethanol, and coating with poly-L-lysine (0.1 mg/ml) before collagen treatment. Cells on glass tended to have a more stringy morphology, with apparent gaps between cells along their long edges.

There was poor cell attachment to opaque polycarbonate filters, which were also inconvenient to use, as cells could only be visualised by treating the filters with agents to render them transparent (e.g. toluene), but which also killed the cells. Good cell attachment and growth was observed on ICN Cellagen (Fig. 26) and Costar Transwell-Col filters, although the poorer transparency of the latter made assessment of the culture and cell identification difficult. Microcarrier beads added to the cell suspensions at the time of plating showed rather poor growth, only 10-20% of the beads being covered after 2-3 weeks, even when the dish was gently agitated to improve mixing of cells and beads. However, microcarriers that were colonised were covered with a relatively complete monolayer (Figs 27 and 28). Cells did grow up and over microcarriers seeded onto established endothelial monolayers.

Subcultured cells

Subcultured cells, passaged once, showed a morphology similar to that of the primary cultures (Fig. 29). Relatively pure subcultures could be produced, provided the original monolayer was low in contaminants, and only gentle trypsinisation was used.
Fig. 26. Uniform growth of endothelial cells on ICN Collagen filter. The optical distortion is due to the sag of the flexible collagen filter. Bar, 100 μm.

Figs 27 and 28. Growth of endothelial cells on Cytodex-3 microcarrier beads, seeded into the wells at the time of plating. Only relatively few of the beads are colonised, but those that are generally show complete coverage. Bars: Fig. 27, 100 μm; Fig. 28, 50 μm.

Fig. 29. Endothelial cells sub-cultured (passaged once) following mild trypsinisation, preserve the spindle-shaped morphology of the primary cultures, and form uniform monolayers (cf. Fig. 4). Bar, 100 μm.
Reproducibility

With care in preparing plasma-derived serum (PDS), and standardisation of dissection and trituration procedures, the method for culture preparation is very reproducible, producing usable cultures in 90-100% of preparations. Although it is advisable to include internal controls in experiments on multiwell places, interplate comparisons show good consistency in transport kinetics (Ramlakhan, 1990; Revest et al. unpublished), indicating reproducibility of cell physiology.

Discussion

Identification and characterisation of brain capillary endothelial cells in primary culture

The spindle-shaped morphology of the predominant cell type in culture, the presence of apparent tight junctions, the positive staining for von Willebrand factor, ACE and the transferrin receptor, and the binding of the lectin UEA 1, show that these cultured cells are genuinely endothelial and retain at least some properties of in situ brain endothelium (Jefferies et al. 1984; Hughes and Lantos, 1986; Jackson et al. 1990). Cultures of rat brain microvessels prepared by this method also express the P-glycoprotein drug-resistance transporter (Greenwood, 1992). As the meninges and most large surface vessels are removed in the brain dissection and by density centrifugation, as the vessel endothelial tight junctions, as previously described (Dorovini-Zis et al. 1984; Rapoport and Robinson, 1986). The cytoplasmic density associated with the junctional zone is more characteristic of the zonula occludens of developing than of mature brain endothelium (Simonescu et al. 1988). The fact that anti-actin antibody gives uniform cytoplasmic staining (Fig. 20) rather than the concentration at cell margins characteristic of tight brain endothelium (Rubin et al. 1991b) suggests that the cells grown on plastic do not express the full cytoskeletal organisation associated with mature blood-brain barrier tight junctions.

Cell purity and source of contaminating cell types

By careful attention to the steps in the dissection that remove meninges and large vessels, with their associated fibroblasts, leptomeningeal and smooth muscle cells, these contaminants were kept to a minimum. Smooth muscle cells when present had a characteristic 'ridge and valley' morphology (cf. Hughes, 1988), and tended to overgrow the endothelium at longer times, but this was not a serious problem on days 7-15 in the bulk of the cultures. The bipolar cells with fine processes are probably fibroblasts, and the flattened cells that tend to grow enclosed by whorls within endothelial plaques are probably pericyte (Schor and Schor, 1986) or leptomeningeal - these are generally minor contaminants. The majority of contaminating cells could be removed by treatment on day 3 with Ca²⁺- and Mg²⁺-free saline, and/or by growth from days 1-3 in D-valine. Careful preparation of the plasma-derived serum used in the medium is necessary to ensure that PDGF (mitogenic for contaminating cells) is removed (Vogel et al. 1978).

Growth rate and effects of medium supplements

The most effective medium, now adopted as standard, had a fixed supplementation with (µg/ml): ECGS (75), heparin (80), vitamin C (5), glutathione (325), insulin (5), transferrin (5) and selenium (0.005). Hughes (1988) found a synergistic effect between heparin and ECGS, the percentage increase in growth with heparin, with ECGS, and with both, being 17, 63 and 200%, respectively. Efforts to increase the amount of platelet-derived endothelial cell growth factor (PD-ECGF) in the serum by stimulating platelet lysis during serum preparation were unsuccessful, possibly because the amount liberated was small, and the PDGF released at the same time promoted competing non-endothelial cell growth.

The variability in culture growth between different batches of plasma-derived serum is inconvenient, and switching to a defined medium would be preferable in the long term. However, culture growth in 96-well plates is sufficiently uniform to permit study of kinetics of amino acid, peptide and protein transport (Hughes and Lantos, 1989; Begley et al. 1990; Ramlakhan, 1990; Revest et al. unpublished), and when combined with micro-DNA assay to correct for small variations in cell growth.

It was sometimes necessary to reach a compromise between culture purity and growth rate. Cultures that started with a high percentage of endothelial cells tended to grow slowly initially, presumably because of the absence of endothelial growth factors from contaminating cell types. Paradoxically, these cultures sometimes showed greater contamination by non-endothelial cells at later times, apparently because the small percentage of contaminants proliferated faster than the endothelial cells. The best compromise was a high density of endothelial cells, with growth stimulated by a smaller population of non-endothelial cells, yet with the endothelium able to proliferate fast enough to confluence to exclude contaminants. Flaws or gaps in the culture tended to be occupied by non-endothelial cells, but it was not clear whether this was cause or effect.
Cells grew less well in Fungizone, so this treatment was used when necessary rather than routinely.

**Cell attachment factors**

Treatment of plastic with poly-L-ornithine and poly-L-lysine or addition of other cell attachment factors (laminin, fibronectin) did not give significant advantage compared with a coating of natural rat tail collagen. The normal protocol therefore used only collagen-coated substrata.

**Growth on different substrata**

Of the substrata tested, collagen-coated tissue culture plastic appeared to be the preferred surface for attachment and proliferation. This may reflect the ability of collagen to form the appropriate bonding structure to the substratum, or some direct charge interaction with the cells. Growth on glass was less successful in producing large monolayers, but small plaques could be achieved. This has proved sufficient for experimental investigations where access to single cells and small clusters is required and high-resolution optics are needed, as for patch clamp and fluorescence imaging (Abbott and Revest, 1990; Revest et al. 1991). Culture on porous filters is much easier on the transparent ICN Cellagen filters than on opaque filters, partly because visual inspection of the developing cultures and periodic treatment (e.g. to remove contaminants) is important in producing good monolayers. The ICN filters have a relatively low molecular mass cut-off (<4,000 Da) so are suitable for studies of the small ion and molecular permeability of the monolayers, but not for studies requiring flux of macromolecules. For the latter, the Costar filters (pore size 0.4 μm) may be preferable. Growth on microcarrier beads is possible, but further work needs to be done to establish the optimal timing for seeding onto monolayers, and to develop methods for loading the beads on to flow-through columns for experimental manipulation.

The studies reported here show that the method is capable of producing a confluent monolayer of cultured rat brain endothelial cells retaining antigenic and other properties resembling the brain endothelium in vivo. The culture has proved sufficiently pure to permit characterisation of transport kinetics for amino acids, peptides and proteins, and to study membrane ion channels, receptors and intracellular second messengers. The use of primary culture gives reproducible results, and the use of microcarrier beads makes it possible to develop tight monolayers by using relatively pure 'clones' of endothelial cells, as for bovine cultures (Dehouck et al. 1990), and by exposing them to astrocytic conditioned medium and agents to elevate intracellular cAMP (Rubin et al. 1991a, b). The ability to grow the endothelial cells on a range of substrata opens the way to a variety of physiological, biochemical and pharmacological studies, and to improved understanding of the blood-brain barrier in vivo.

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**References**


