Virchows Arch (2000) 436:249–256

ORIGINAL ARTICLE

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Light and electron microscopic examination of endothelial cells from bovine retinal vessels in long-term cultures

Received: 19 May 1999 / Accepted: 4 August 1999

Abstract The aim of the present work was to examine and compare the ultrastructure of bovine retinal endothelial cells (BRECs) in vitro during several passages in a medium selective for endothelial cells. The identity of the endothelial cells was confirmed immunohistochemically, up to the tenth passage. Changes in their ultrastructure in comparison to endothelial cells in vivo occurred at the onset of culturing and not progressively with repeated passages. The cultured BRECs show high metabolic activity in all passages. While retaining their identity as endothelial cells, they modify their lipid metabolism, so that lipids are stored. This change in lipid metabolism was induced by the medium.

Key words Endothelial cells · Cell culture · Ultrastructure

Introduction

Endothelial cells in general control the exchange of substances between blood and tissue; in the retina they are an essential part of the blood–retina barrier. An important pathologic process in the ocular fundus is the formation of so-called epiretinal membranes (ERMs), which are characterized by cellular proliferations of new blood vessels into the vitreous or along the retinal surface. These proliferating vessels modify their tissue specificity and develop a substantial fibrovascular tissue, the epiretinal membrane.

Because endothelial cells are mainly involved in the formation of ERMs, it was the aim of the present study to investigate the immunological properties of endothelial cells in vitro in selective medium over successive passages. Bovine retinal vascular endothelial cells (BRECs) have been investigated with respect to potential changes in their immunreactivity towards endothelial markers and to changes in their fine structure, with the aim of making it possible to characterize the expected dedifferentiation in more detail.

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Materials and methods

Isolation and cultures of retinal endothelial cells

Bovine retinal endothelial cells (BRECs) were isolated and cultured by methods described by Bowman [2], Gitlin and d'Amore [11] and Wong et al. [33]. Bovine eyes from freshly slaughtered animals were kept in Betadine (providone-iodine solution) for 10 min, washed in sterile water and dissected under sterile conditions. After the connective tissue was dissected away, the eyeball was circumferentially incised about 6 mm behind the limbus and the anterior segment removed. The posterior globe was inverted and the vitreous gel was pulled out. The retina was gently peeled from the vitreous cavity and transferred into Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, N.Y., USA) containing 3% amphotericin B (Gibco), 600 IU/ml penicillin + 600 µg/ml streptomycin (pen-strep, Gibco). Four retinas were pooled and transferred onto a 53-µm nylon mesh, then rinsed thoroughly with DMEM containing 1% amphotericin B and 200 IU/ml penicillin + 200 µg/ml streptomycin (pen-strep). Tissue was scraped off and cut into pieces. To homogenize the retina, the tissue was pulled up several times through an injection syringe with a needle. The suspension was centrifuged at 1200 rpm for 10 min, after which the pellet was resuspended in 15 ml of an enzyme solution [500 µg/ml collagenase, 200 µg/ml pronase and 200 µg/ml DNAase I (Boehringer, Mannheim, Germany)]. Tubes containing the solution were shaken during incubation for 25 min at 37°C in a water bath. The resultant fragments were trapped on a 53-µm nylon mesh, washed with DMEM and centrifuged at 1200 rpm for 10 min. The pellet was finally resuspended in MCDB-131 medium supplemented with 10 mM sodium bicarbonate, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone and 20% platelet-poor horse serum (all chemicals: Sigma-Aldrich Chemie, Deisenhofen, Germany). The medium contained 1% amphotericin B (Gibco), 200 IU/ml penicillin +200 µg/ml streptomycin (pen-strep, Gibco) and the cells were seeded onto gelatin-coated (Gelantine: 300 Bloom, Sigma-Aldrich Chemie) 24-well tissue culture plates (Falcon, Heidelberg, Germany) or into 3.5-cm culture dishes. BRECs were cultured in a humidified atmosphere of 5% CO_2 and 20% O_2 at 37°C. Culture medium was subsequently changed twice per week. Confluent cultures were spliced at a ratio of 1:2.

Passages were performed by weak digestion after 4–8 days. Cells were washed twice with PBS, then incubated for 5–10 s with

0.5 ml trypsin (0.1% trypsin/0.02% EDTA, Biochrom, Berlin, Germany), and the trypsin was removed. When cells became spherical and detached, they were resuspended in MCDB-131 medium and inoculated into new culture dishes.

To examine the BRECs, cell smears were made on slides for each passage. Slides were stored at –20°C until immunostaining was performed.

Isolation and culture of retinal pigment epithelial cells

For preparation of bovine retinal pigment epithelial cells (RPE cells), the anterior segment, vitreous and neural retina were removed under sterile conditions. Adherent RPE cells were washed with DMEM containing 3% amphotericin B, 600 IU/ml penicillin +600 µg/ml streptomycin. RPE cells were removed from Bruch's membrane by gently brushing with a small hairbrush, and the eyecup was refilled with DMEM containing 1% amphotericin B, 200 IU/ml penicillin +200 µg/ml streptomycin (all chemicals: Gibco) and aspirated. Collection and refilling of the eyecup was repeated three times. The cell suspension was centrifuged at 900 rpm for 5 min. The pelleted RPE cells were resuspended in DMEM supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich Chemie) in 6-well tissue culture plates (Falcon). The cells were incubated in a humidified atmosphere of 5% CO_2 and 3% O_2 at 37°C.

The cultures of RPE cells maintained in DMEM remained in a lag phase for 2–4 weeks after their initiation. Then they started proliferating and with a splicing ratio of 1:2, cultures became confluent in about 10 days. In order to observe the effect of MCDB-131 medium on RPE cells, cells were grown in DMEM, and after the cells became subconfluent the medium was changed to MCDB-131 for at least 8 days.

Antibodies and immunohistochemical staining

A standard two-stage indirect immunohistochemical technique was applied using anti-von Willebrand factor (vWF)/factor VIII antibody (clone F8/86, DAKO, Glostrup, Denmark), anti-Pecam/CD31 antibody (clone 3 E 1D4, DAKO) and anti-smooth-muscle-actin antibody (smAc, clone 1A4, Neomarkers, Union City, USA) and diaminobenzidine as the chromagen.

For immunohistochemical labelling, acetone-fixed cell smears were thawed and incubated for 60 min with anti-vWF/factor VIII (diluted 1:40 in PBS/1% BSA, pH 7.4), anti-CD31/Pecam (diluted 1:20 in PBS/1% BSA, pH 7.4) or anti-smooth-muscle-actin (smAc) antibody (diluted 1:50 in PBS/15 BSA, pH 7.4) at 37°C in a humid chamber. After washing, samples were incubated with biotinylated species-specific secondary antibodies (diluted 1:50 in PBS/1% BSA, pH 7.4, RPN 1001, 1002, 1004 Amersham, Braunschweig) for 60 min at 37°C in a humid chamber. After washing slides were incubated with streptavidin-biotin-peroxidase complexes (diluted 1:100 in PBS/1% BSA, pH 7.4, RPN 1501, Amersham, Braunschweig). To enhance the staining of horseradish peroxidase reaction product, nickel–ammonium sulfate was added to the diaminobenzidine–H_2O_2 solution. This technique showed a strong bluish-black reaction product.

Human umbilical-vein endothelial cells (HUVEC, from Dr. Neuhaus, Bonn) were used to specify the reaction of antibodies. The specificity of the antisera employed was tested by omission of the primary antisera and substitution with normal PBS/1% BSA.

Electron microscopy

For standard transmission electron microscopy (TEM) confluent cell cultures were fixed within the polystyrene wells or as cell suspension with Karnovsky's solution in 0.1 M phosphate buffer (pH 7.3), postfixed in 2% OsO_4, dehydrated in ethanol, and embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 transmisson electron microscope.

Results

Habitus of isolated bovine retinal endothelial cells (BRECs) in vitro

With the present method of isolation and culture in MCDB-131, isolated BRECs were evident 4–8 days after the initial plating. Given a splicing ratio of 1:2, cultures normally become confluent in about 6–8 days. The morphology of cultured BRECs depended on the conditions: cobblestone phenotype especially in small colonies, and elongated phenotype in confluent contact-inhibiting monolayers (Fig. 1). The fibroblast-like structure was maintained through all passages up to passage 10 (1:2 split). Passages were performed by weak digestion to eliminate and suppress pericytes. BRECs were the first to detach from the wells, while cells remaining attached to the substratum were principally pericytes, stained by anti-smAc antibody and unstained with anti-vWF antibody and anti-CD31 antibody.

Immunohistochemistry of bovine retinal endothelial cells (BRECs) in vitro

The immunohistochemical staining revealed specific and strongly positive labelling of BRECs with anti-vWF (Fig. 2.). BRECs grown onto slide chambers or onto coverslips showed granular perinuclear staining with vWF even when they exhibited fibroblastoid morphology. Up to passages 7–10 staining with anti-vWF antibody was only slightly reduced. Labelling with anti-CD31 antibody resulted in nonhomogeneous staining. Cells in first passages were often unlabelled; in intermediate passages, a few cells were localized labelled, and most were unlabelled. With anti-smAc antibody, BRECs in cell suspension and in monolayers were not stained. Retinal pigment epithial cells as negative controls did not stain with anti-vWF antibody, anti-CD31 antibody, or anti-smActin antibody (Table 1).

Ultrastructure of the BRECs in vitro

So that the ultrastructure of the cultured endothelial cells could be visualized and examined for changes over time, cell samples were taken from several (2–6) separate cultures in each of passages 1–10 and analyzed in the transmission electron microscope. The spectrum of organelles in the endothelial cells was the same in all passages, and no differences in their ultrastructure were detected. Variations in the density of distribution of the organelles were present to the same extent in all passages (Figs. 3–6).

The cells usually lay side by side, forming a flat surface, although there was a slight degree of overlap. Interdigitations were rare. The exposed surfaces of the individual cells were also usually flat. Superficial differentiations such as microspikes, lamellipodia and occasionally individual microvilli were observed. The gap between adja-

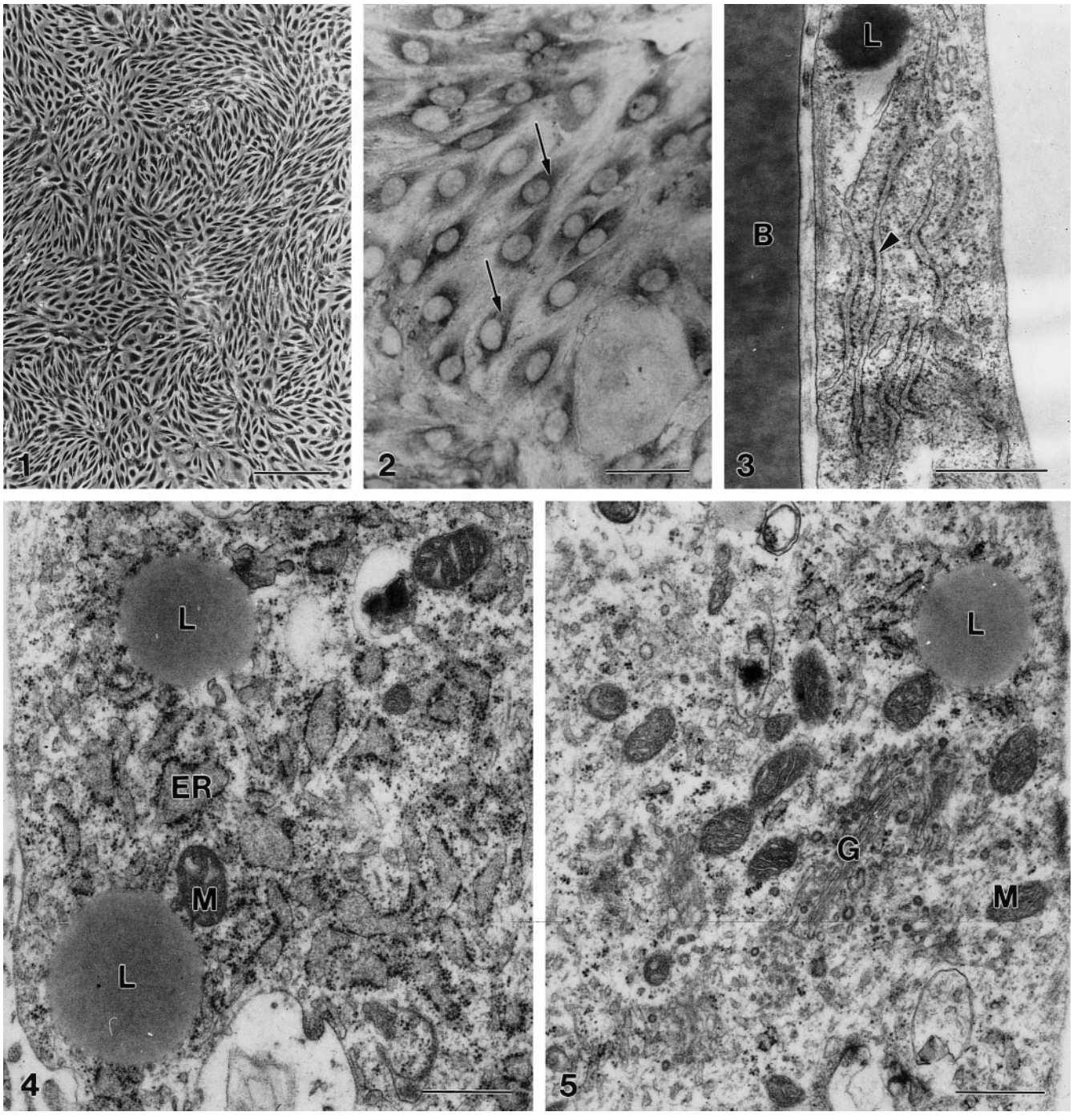


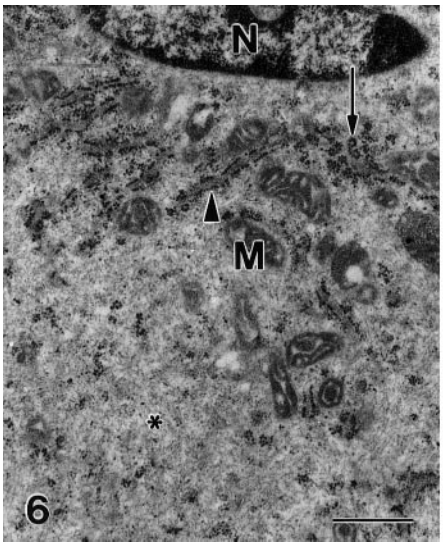
Fig. 1 Confluent culture of bovine retinal endothelial cells (BRECs) in MCDB-131 medium. The cells have a fibroblastoid habitus such as is typical of closely packed colonies of endothelial cells from the retina. *Bar* 1 mm

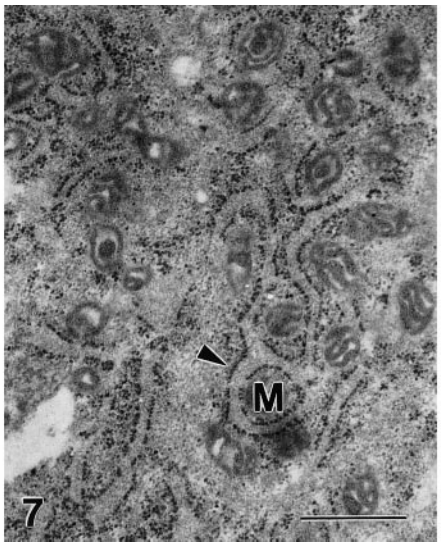
Fig. 2 BRECs of the 1st passage, grown in MCDB-131 medium. Enzymatic immunostaining with anti-von Willebrand factor (*arrows*). The reaction product of the immunostaining is mainly perinuclear. *Bar* 50 µm

Fig. 3 Confluent primary culture of BRECs (6 days). Part of an endothelial cell sectioned perpendicular to the plane of the culture dish. *Arrowhead* transitional endoplasmic reticulum, *B* base, *L* lipid droplet. *Bar* 0.5 µm

Fig. 4 BRECs of the 2nd passage, grown in MCDB-131 medium. Region of the cytoplasm in which an expanded rough endoplasmic reticulum (*ER*) dominates. *L* lipid droplet, *M* mitochondrion. *Bar* 0.5 µm

Fig. 5 BRECs of the 2nd passage, grown in MCDB-131 medium. Region of the cytoplasm in which mitochondria (*M*) are particularly numerous. *G* Golgi apparatus, *L*, lipid droplet *Bar* 0,5 µm





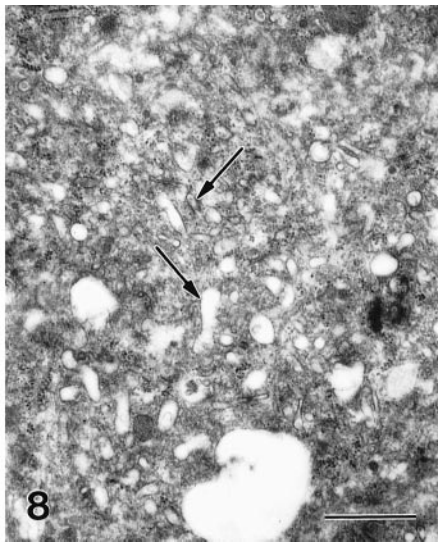


Fig. 6 BRECs of the 4th passage, grown in MCDB-131 medium. The cell organelles are inhomogeneously distributed and predominantly occupy perinuclear positions. An adjacent cytoplasmic region (*asterisk*) contains few organelles. *Arrow*, polysome, *arrowhead*, rough endoplasmic reticulum, *M* mitochondrion, *N* nucleus. *Bar* 0.5 µm

Fig. 7 BRECs of the 4th passage, grown in MCDB-131 medium. Perinuclear cytoplasm region, in which mitochondria (*M*) and rough endoplasmic reticulum (*arrowhead*) are associated. *Bar* 0.5 µm

Fig. 8 BRECs of the 8th passage, grown in MCDB-131 medium. A single sample with dominant smooth endoplasmic reticulum (*arrows*) in the region near the nucleus. *Bar* 0.5 µm

Table 1 Immunohistological staining of bovine retinal endothelial cells (*BRECs*) and bovine retinal pigment endothelial cells (*RPEs*) with various antibodies, in different passages (*HUVEC* human umbilical vein endothelial cells, *P* passages, *vWF* von Willebrand factor, ++ strong positive labelling, + positive labelling, +/– punctate labelling, – no labelling)

Cells marker	BREC 1.P	BREC 2.P	BREC 3.P	BREC 4.P	BREC 5.P	BREC 6.P	BREC 7.P	BREC 8.P	BREC 10.P	HUVEC	RPE 2./3.P
Anti-vWF-AB	++	++	++	++	++	++	++	+	+	++	–
Anti-smAc-AB	–	–	–	–	–	–	–	–	–	–	–
Anti-Pecam-AB	–	–	+/-	+/-	+/-	–	–	–		+	–

cent cells was of a constant width, ca. 15 nm, over large regions of the culture. Junctions in the form of focal contacts (adhesion plaques) were present between endothelial cell and polystyrene substrate and between neighboring cells. In the region of these focal contacts, the intercellular gap measured about 15 nm in width or was reduced.

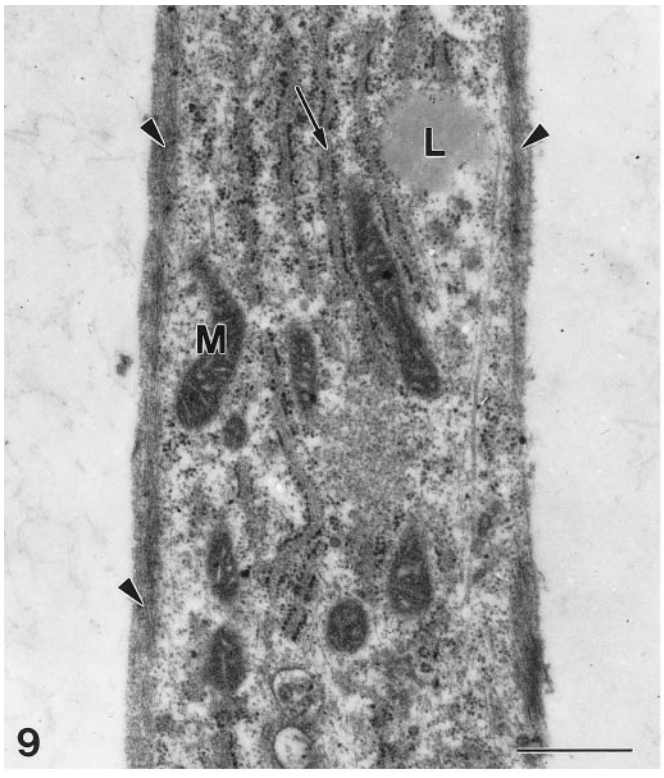
In all passages the cells generally contained numerous organelles. The rough endoplasmic reticulum (ER) was often dilated to form broad cisternae (Fig. 4) and in some cases was spread out so that it had an ergastoplasmic appearance. Smooth and rough ER was usually integrated as transitional ER. In a few cells smooth ER was the predominant organelle in parts of the cytoplasm. A striking feature was the presence of insular aggregations of mitochondria, some of which were closely associated with rough ER (Fig. 7). Golgi apparatus, polysomes, individual vesicles and groups of vesicles (coated and not coated) were abundant (Fig. 5).The spectrum of more or less common organelles in the cytoplasm of the BRECs included vacuoles, phagosomes, multivesicular bodies and membrane fragments arranged in twisted stacks.

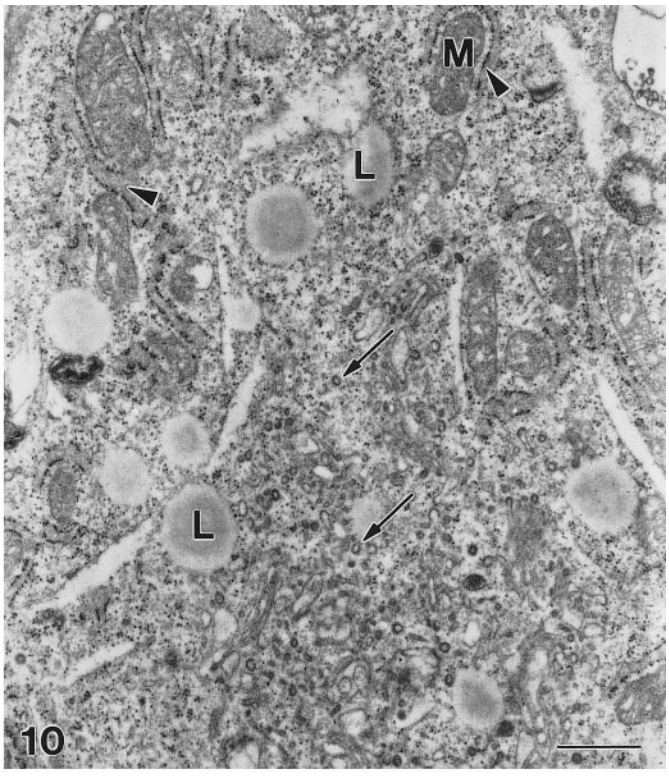
Certain changes in the cytoplasm of these cells, in comparison with endothelial cells in vivo, were particularly striking: lipid droplets stored as cytoplasmic inclusions, an expansion of the smooth ER and a denser structure of the cytoskeleton. Lipid droplets were present in the endothelial cells of all passages (Figs. 3, 4, 9, 10). The distribution density of lipids in the cytoplasm differed within passages, some cells being nearly full of lipid droplets and others containing only small amounts of them. Smooth ER, a site of lipid synthesis, mainly took the form of transitional ER. In some cells of an 8th passage, part of the cytoplasm near the nucleus was dominated by smooth ER (Fig. 8). The intermediate filaments were more or less tightly packed in the cells of all passages, as was a terminal network just below the plasmalemma on all sides of the cell (Fig. 9).

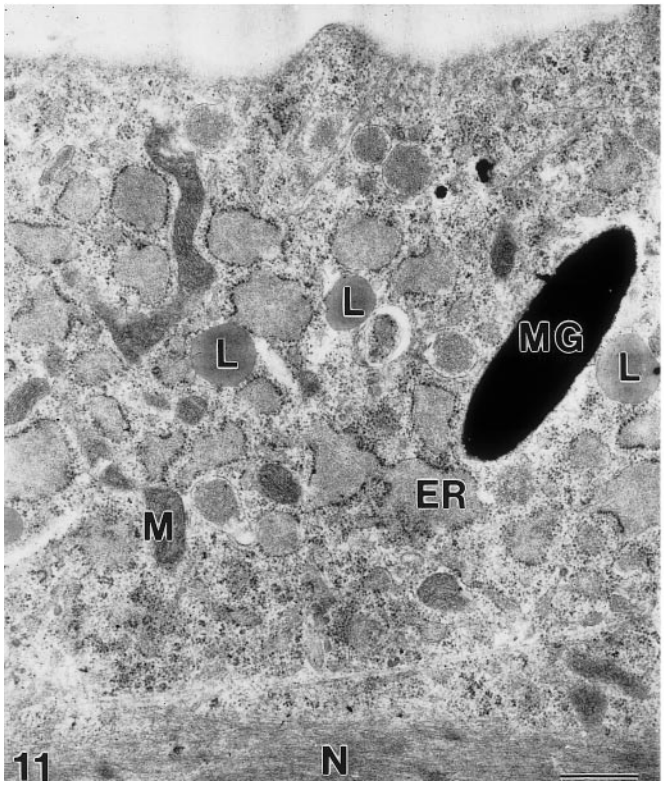
Pericytes were distinguishable from endothelial cells on the basis of their intracytoplasmic myofibrils with focal densities.

Cells used as controls

Freshly isolated RPE cells did not grow in MCDB-131 medium. RPE cells that had first been cultured in







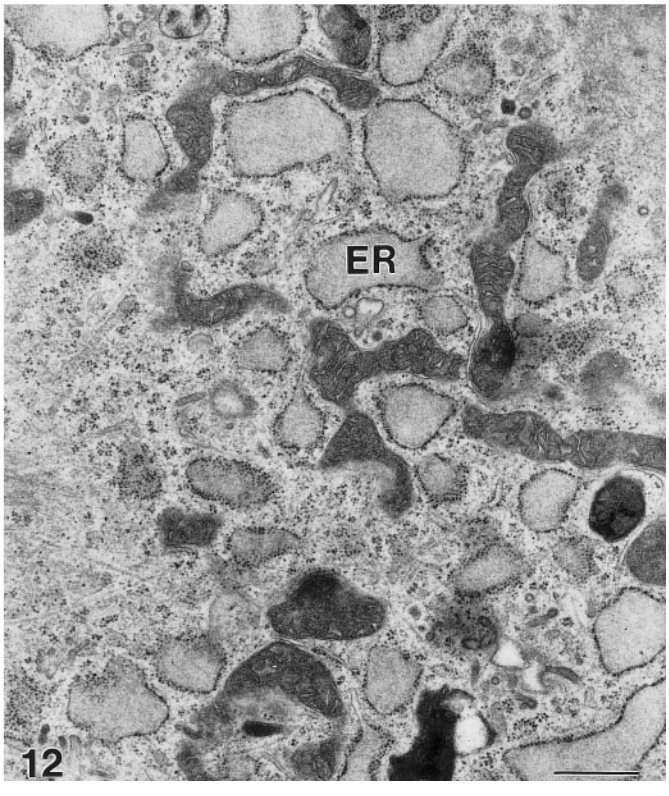


Fig. 9 BRECs of the 8th passage, grown in MCDB-131 medium, with distinctly developed subplasmalemmal network (*arrowheads*). *Arrow*, rough endoplasmic reticulum, *L* lipid droplet, *M* mitochondrion. *Bar* 0.5 µm

Fig. 10 BRECs of the 10th passage, grown in MCDB-131 medium. Organelle-rich region of cytoplasm with many coated vesicles (*arrows*) and partial associations between mitochondria (*M*) and rough endoplasmic reticulum (*arrowheads*). *L* lipid droplet. *Bar* 0,5 µm;

Fig. 11 Bovine retinal pigment epithelial cells (RPE cells), which as a control were similarly cultured in MCDB-131 medium, likewise produced lipid droplets (*L*). *ER* rough endoplasmic reticulum, *MG* melanin granule, *M* mitochondrion, *N* subplasmalemmal network. *Bar* 0.5 µm

Fig. 12 RPE cells cultured in DMEM medium. Only a few or (as shown here) no lipid droplets could be found. *ER* rough endoplasmic reticulum. *Bar* 0.5 µm

DMEM medium and then grown on in MCDB-131 medium contained many organelles. Their endoplasmic reticulum and mitochondria were particularly well developed (Fig. 11). Lipid droplets were present in abundance (Fig. 11). In contrast, there were very few or no lipid droplets in RPE cells that had grown exclusively in DMEM medium (Fig. 12). Retinal endothelial cells grew poorly in DMEM, with a large number of contaminations by other cells.

Discussion

Under the isolation and culture conditions used in this study, BRECs formed confluent contact-inhibiting monolayers. The population of the cultured BRECs was of the noncloned, mixed type. The exact vascular origin of the retinal endothelial cells is not known; they may have come from arterioles, capillaries, or veins. The morphology of these microvascular endothelial cells ranged from cobblestone to elongated phenotype. In early colonies the cells were almost cobblestoned, whereas confluent cultures were mainly composed of elongated, closely associated cells with a fibroblast-like morphology. This phenomenon is normal for cultures of bovine aortic endothelial cells [16] and is also found for cultures of bovine retinal capillary endothelial cells maintained at confluence [4, 28, 29]. In our studies the fibroblast-like morphology was exhibited in all cultures and was independent of the number of passages and isolation.

The major problem with microvascular cultures from the retina is contamination of endothelial cells by pericytes or by other cells such as glial and smooth muscle cells. Several techniques have been used to select endothelial cells. A common technique is to provide nutritional enviroments that selectively promote growth of endothelial cells – for example, MCDB-131 medium. MCDB-131 medium was formulated by Knedler and Ham [15]; supplemented with epidermal growth factor and hydrocortisone, it is described as a medium that can be used to support clonal growth of human microvascular endothelial cells [20, 31]. In our study, the use of this medium allowed suppression of the growth of other cells, especially that of pericytes, which are equally present in the retina in vivo in a ratio of 1:1 compared to endothelial cells [17].

As mentioned above, it is known that the endothelial line is not a homogeneous population of cells [5, 8, 23]. Endothelial cells of different organs and especially of venules, arteries and capillaries have different physiological functions. This heterogeneity has been documented both morphologically [8, 16, 30, 34, 35] and histochemically [10, 21] in vivo.

Endothelial cells obtained from different tissues and grown in vitro also exhibit immunochemical and enzymatic differences [12, 14]. Previously, however, endothelial cells of differing origins had been found to differ at the ultrastructural level [32]. In cultured BRECs, phenotypic diversity is likewise evident in both habitus and function of the cell [1, 26]. The morphology of endothelial cells is also dependent on the composition of culture medium and on the presence or absence of a polystyrene-coated substrate [1, 9, 18].

The BRECs in vitro were characterized by phase-contrast microscopy, electron microscopy and specific immunohistochemistry. For the latter, the endothelium-specific vWF was the most important marker. This is a carrier protein for coagulation factor VIII. The protein is stored in Weibel-Palade bodies in the endothelium of large vessels and is found in cytoplasmic vesicles associated with the rough endoplasmic reticulum and Golgi apparatus in microvessel endothelium [6]. It has been reported that the intensity of the labelling with anti-vWF antibodies can depend on whether the preparation has been digested enzymatically and on the enzyme concentration and its duration [5]. We have observed no difference in vWF labelling between cells that were enzymatically treated, mechanically detached from one another or not treated at all. A second important specific marker for endothelial cells is platelet endothelial cell adhesion molecule 1 (Pecam/CD31). Pecam1 is a 130-kDa protein that has dual functional roles. It is expressed on the surface of various circulating cells and is a major constituent of the endothelial cell intercelluar junction. It is not present on nonvascular cells such as fibroblasts, epithelium or muscle cells. It has been suggested that Pecam promotes the release of neutrophil granule proteases that dissolve the basement membrane and assist the transmigration process of leukocytes (for review see [19]). In our experiments labelling of the BRECs grown in vitro with anti-Pecam antibodies (CD31) gave no convincing results, which is consistent with the findings of other authors [1]. It seems that endothelial cells in vitro do not express the adhesion molecule Pecam to the same extent as they do in vivo, as part of the natural tissue.

We used anti-smooth-muscle-actin (smAc) antibodies, which are negative for endothelial cells, to detect contamination with pericytes and smooth muscle cells. The anti-smooth-muscle-actin antibodies recognize the alpha smooth muscle isoform actin. The antibodies show no cross-reaction with actin from fibroblasts (according to the manufacturers). In the electron microscope the pericytes can be distinguished from endothelial cells by their actin filaments [2].

The observed aggregation of organelles in the cytoplasm of cultured cells is a manifestation of subdivision into various metabolic centres. Its significance for fine-structural analysis is that individual sections do not reflect the cytoarchitecture of the cell as a whole. In this paper we have allowed for that by examining a correspondingly large number of samples.

The amounts and kinds of organelles in the endothelial cells of all passages in particular, the abundance of ER and mitochondria – indicate that these cells are very active metabolically. It is notable that the character of the cells changed abruptly soon after they had become established. Unlike endothelial cells in vivo, which normally do not store lipids, the cultured cells of all passages in

the present study contain lipid droplets as an extra organelle. These droplets are the morphological correlate of an altered lipid metabolism, which is bound to the smooth endoplasmic reticulum. In rare cases a dramatic increase in the amount of smooth ER was observed. Otherwise, it was impossible to evaluate the smooth ER quantitatively in the samples examined here, because in endothelial cells it does not exist as a separate organelle system; rather, it is present as transitional ER, forming a functional unit with the rough ER, a switch that depends on metabolic requirements. A conversion of the metabolism towards lipid storage can be regarded as a degenerative process, which possibly produces the foam cells found in cultures that have become too old. Whether degeneration actually occurs ultimately depends on what special cell functions are being affected or impaired by the changes it has undergone. For instance, Stolz and Jacobson [32] noted a maintenance of biochemical heterogeneity despite loss of ultrastructural charcteristics. Roux et al. [24, 25] have shown in cultures of brain microvessel endothelial cells, that lipid synthesis is influenced by extracellular serum lipoproteins, and lipids within the cell are provided from lipid metabolism and from the serum. There is an inverse relationship between lipid synthesis and the serum lipoprotein concentration. The authors conclude that there is a negative feedback control by lipoproteins, as has been demonstrated in endothelial cells by others [13, 22]. In these circumstances, it would be less probable that lipids from the medium caused lipid storage in the retinal endothelial cells in our study, but more likely that this is due to a defect in cellular feedback sensitivity and/or other cellular control mechanisms, caused by a hitherto unknown factor. The extracellular milieu, however, exerts a strong pressure on the cells, so that even cerebral endothelial cells can be stimulated to synthesize collagen [27].

In the present case, although the cells are in an active metabolic situation, they show distinct features of dedifferentiation. This dedifferentiation, however, does not proceed step by step, as has been observed in other cell cultures, but rather occurs soon after introduction of the cells into an in vitro milieu consisting of medium selective for endothelial cells. Even the RPE cells that were "deceived" into growing in this medium as a control stored unusually large amounts of lipids. From these results we deduce that the MCDB-131 medium or the supplements influence lipid metabolism. The endothelial cells in our experiments thus appear in two lights. On the one hand, the cytoarchitecture of the cells has changed conspicuously, and on the other a positive immune response to the specific antigen of endothelial cells (vWF) persists through all passages. This discrepancy may not be an exception and should therefore be kept in mind when cells are being used as an experimental model.

The increase in density of the cytoskeleton – in particular the subplasmalemmal network, which consists partly of contractile proteins [7] – may cause an increase in the ability of the cells in culture to move about. The focal contacts also say something about motility, because they are always associated with actin filaments of the subplasmalemmal network by way of certain cytoplasmic attachment proteins, and the cell cortex is connected by transmembrane proteins to components of the extracellular matrix that has been adsorbed to the petri dish. Hence, bundles of actin filaments can bind to the plasma membrane and produce cell locomotion by pulling on the extracellular matrix or on another cell [3].

These experiments have shown that BRECs cultured in selective medium (MCDB-131) are extremely active metabolically and are immunologically identifiable as endothelial cells until the last passage tested. At the same time, from the onset of culture conditions there is a dramatic rise in lipid production, which indicates a change in lipid metabolism induced by the medium.

Acknowledgements We thank Claudine Strack, Parand Pour Nouroz and Ursula Rau-Schneider, whose technical expertise made this work successful. We wish to thank PD Dr. A. Wegener for assistance with the translation. This study was supported by *Aktion Kampf der Erblindung*.

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