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 immunoreactivity 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.

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, 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, then rinsed thoroughly 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% CO2 and 20% O2 at 37°C. Culture medium was subsequently changed twice per week. Confluent cultures were split 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

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

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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% CO₂ and 3% O₂ 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 complex (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–H₂O₂ 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% OsO₄, 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 transmission 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 epithelial cells as negative controls did not stain with anti-vWF antibody, anti-CD31 antibody, or anti-smActin antibody (Table 1).

Ultrastucture 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-