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Establishment of a human *in vitro* model of the outer blood–retinal barrier

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Abstract

The outer blood–retinal barrier is composed of a monolayer of retinal pigment epithelium, Bruch's membrane and the choriocapillaris which is fenestrated. Endothelial proliferation and breaching of Bruch's membrane leads to the neovascular form of age-related macula degeneration (ARMD). The aim of this study was to generate an *in vitro* model that mimics more faithfully the phenotype of the choriocapillaris and the trilayer architecture *in vitro*. A trilayer culture model was generated with retinal pigment epithelium (ARPE-19) cell cultures on the epithelial surface of amniotic membrane and with human umbilical vein-derived endothelial cells on the other surface. A control model for the effect of retinal pigment epithelium on endothelial changes was generated with corneal epithelial cells replacing the ARPE-19. Both human umbilical vein-derived endothelial and ARPE-19 cells formed confluent monolayers on respective surfaces of the amnion. The human umbilical vein-derived endothelial cells in the trilayer became fenestrated when co-cultured with the ARPE-19 cells, but not with corneal epithelial cells, or when grown as monolayers on the amnion, showing a loss of fidelity of origin in the presence of ARPE-19 cells. These cells also revealed VE-cadherin and ZO-1 at cell–cell contacts from 24 h in the trilayer. The tight junctional molecules, occludin and ZO-1, were localized to cell–cell contact regions in the retinal pigment epithelium, both in the monolayer and in the trilayer system. Permeability of the trilayer was tested by using fluorescein and fluorescein-conjugated tracers under flow. At 72 h the trilayer severely restricted transfer of sodium fluorescein (NaF) (ten-fold reduction) whilst transfer of a 4 kDa FITC-conjugated dextran was virtually occluded, confirming a restrictive barrier. Ultrastructural studies showed the retinal pigment epithelium monolayer was polarized with microvilli present on the apical surface. Paracellular clefts showed numerous tight junctional-like appositions, similar to that seen on amnion alone. This study demonstrates that ARPE-19 and human umbilical vein-derived endothelial cells can be co-cultured on the amniotic membrane and that the resultant cross-talk leads to formation of a fenestrated endothelium, whilst maintaining a polarized restrictive epithelial layer. The fenestrated endothelial phenotype achieved in this human *in vitro* trilayer model is a first and offers an outer-retinal barrier which approaches the *in vivo* state and has potential for studies into induced junctional disruption, endothelial proliferation and migration: features of ARMD.
Key words 3D culture; fenestrated endothelium; human; *in vitro* permeability; outer retinal barrier; retinal pigment epithelium.

Introduction

Reductionist approaches into processes underlying age-related macular degeneration (ARMD) have been hampered by the lack of optimal *in vitro* human models. ARMD is the commonest cause of severe visual loss in people over the age of 65 years (Evans, 1995) in the developed world. It has two major forms, the neovascular (or wet) and the atrophic (or dry) form. It is a disease of the retinal pigment epithelium (RPE) and choriocapillaris (Campochiaro et al. 1999) with involvement of the neuro-retina occurring secondarily. In active neovascular ARMD, breakdown of the outer retinal barrier and endothelial cell proliferation are key process that lead to the neuro-retinal damage and involves, among other factors, vascular endothelial growth factor (VEGF; Fischer et al. 1999; Gragoudas et al. 2004). Therefore, the motivation behind this work was to generate an *in vitro* culture model of the outer blood–retinal barrier and the choriocapillaris to mimic the *in vivo* phenotype as closely as possible as the essential first step.

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The functional site of the outer blood–retinal barrier is at the level of the RPE tight junctions with the underlying choriocapillaris being fenestrated and leaky. ARPE-19 is an RPE-derived spontaneously immortalized cell line that has been shown to have structural and functional properties characteristic of RPE cells *in vivo* (Dunn et al. 1996, 1998).

The RPE and the choriocapillaris are separated by Bruch's membrane *in vivo*. The amniotic membrane can be compared with Bruch's membrane, as it consists of an inner layer of epithelial cells on a basement membrane (BM) which is composed of mainly type IV collagen, laminin and heparan sulphate. This BM interfaces with the vascular stoma which consists mainly of collagen I, III and IV, fibronectin and laminin, ideal extracellular matrix for cultivation of endothelial cells (Malak et al. 1993). This membrane has also been shown to contain elastin (Hieber et al. 1997). RPE grown on the denuded epithelial surface of the amnion has been shown to retain epithelial morphology and cell–cell contacts (Capeans et al. 2003; Ohno-Matsui et al. 2005; Singhal & Vemuganti, 2005; Stanzel et al. 2005).

In polarized epithelial monolayers, tight junctions are the key entities that seal the paracellular pathway between adjacent cells. This prevents diffusion of solutes and differentiation of the apical and basolateral membrane domains to allow active transport across the monolayer. Occludin was the first integral membrane protein to be identified in tight junctions (Furuse et al. 1993). It plays a major role in transepithelial resistance and paracellular permeability. Tight junctions also contain structural proteins such as the claudins and junctional adhesion molecule (JAM) which dictate the complexity of tight junctional strands and paracellular permeability (Furuse et al. 1998; Martin-Padura et al. 1998). Regulation of barrier property and cell signalling is afforded by the cytoplasmic linking molecules with ZO-1 being the predominant molecule of epithelial tight junctions (Anderson & Van Itallie, 1995; Balda et al. 2000). Continued expression of these molecules in any *in vitro* model is therefore desirable.

Primary human umbilical vein cells are extensively used for vascular research. They are experimentally pliable and show phenotypic plasticity ranging from formation of quiescent continuous monolayers with VE-cadherin-rich adherens junctions to angiogenesis/tubulogenesis in three-dimensional culture (Dejana, 1996; Wright et al. 2002). Moreover, isolated human umbilical vein-derived endothelial cells (HUVECs) continue to express signalling molecules which respond to inflammatory mediators (Esser et al. 1998a), hypoxia and angiogenic growth factors such as VEGF (Carmeliet et al. 1999; Wright et al. 2002), features which may play a predominant role in ARMD.

The aim of this study was therefore to establish and characterize an *in vitro* model of the outer retinal barrier using ARPE-19 cells to replace retinal pigment epithelial cells, human amnion to replace the Bruch's membrane and HUVECs to mimic the choriocapillaris endothelium.

Materials and methods

Materials

Local ethical approval and fully informed patient consent was obtained for all materials of human origin (amniotic membrane and HUVECs). The work adheres to the tenets of the Declaration of Helsinki.

Human umbilical vein endothelial cells

HUVECs were isolated from umbilical cords ($n = 6$), as modified according to Jaffe et al. (1973). HUVECs were routinely cultured in full medium: M199 medium (Gibco BRL, Paisley, UK), 20% fetal bovine serum (FBS; Perbio, Chester, UK), 2 mM L-glutamine (Gibco BRL), 2 μg mL^{-1} fungizone, 100 U mL^{-1} penicillin, 200 μg mL^{-1} streptomycin and 50 μg mL^{-1} endothelial cell growth supplement (First Link, UK) on 1% gelatin-coated tissue culture flasks at 37 °C, 5% CO_2. For amniotic membrane assays cells were used from passages 2–3 and grown in standard endothelial medium with reduced FBS (5%).

Retinal pigment epithelium cells

RPE cells (ARPE-19, ATCC CRL-2302) were routinely cultured in Dulbecco's modified Eagle's medium Nutrient Mixture F-12 HAM (Sigma) with sodium bicarbonate at a final concentration of 56 mM, 2 μg mL^{-1} fungizone, 100 U mL^{-1} penicillin, 200 mg mL^{-1} streptomycin (Sigma) and 10% FBS. RPE cells from passages 15–25 were used in all experiments performed.

Human corneal epithelial cells

Human corneal epithelial cells (HCE-T) were a generous gift from Dr R. Clothier, University of Nottingham. They were routinely cultured in EpiLife Medium (Cascade Biologics, Portland, OR, USA), with added calcium chloride (120 μM) and fetal calf serum (FCS; 10%).

Preparation of amnion

Amniotic membrane was obtained from placentas after caesarean section from normal pregnancies. Under sterile conditions, the amnion was processed as previously described by Tseng et al. (1997). Once separated from the chorionic membrane, the amniotic membrane was clamped into tissue carriers (13 mm) or minusheets (Minucells and Minutissue, Germany), which consists of a base ring and a tension ring. Excess membrane was trimmed (so there was no overhang) and the whole placed in thermolysin (125 μg mL^{-1}; Sigma) for 16 h at 4 °C in order to remove the native amniotic epithelium. Membranes were washed gently, placed into the RPE medium (as above) and stored for up to 2 months prior to use.

Methods

Trilayer culture

The amniotic membrane in the minusheet carrier was placed into a 24-well plate and RPE cells carefully seeded

at 1×10^5 mL^{-1} (with 2 mL per well). After 24 h the membranes were washed to remove excess unattached RPE cells and the minusheet (with RPE) turned over. The cells remained bathed in epithelial medium, up to the level of the minusheet. The HUVECs were seeded at 1×10^5 mL^{-1} onto the flipped (interstitial) surface and endothelial medium was placed on to the minusheet. The membrane was washed again 24 h later to remove unattached HUVECs. Once confluence was reached (48 h) the trilayer was placed into a single tissue gradient carrier in the dual perfusion chamber system (Minucells and Minutissue), endothelial side up, with endothelial medium (in the upper chamber) nourishing the HUVECs, and RPE medium (in the lower chamber) nourishing the RPE cells. The chambers were connected to separate reservoirs and a closed circuit containing 25 mL medium was obtained for both epithelial and endothelial circuits. Medium was replaced on a daily basis. This system is amenable to both static and flow culture. The flow system was used to measure permeability of the trilayer to known tracers (see below). The flow rates for the upper and lower chamber were 0.1 mL min^{-1}; this produces a shear stress of less than 0.25 dyne cm^{-2}, i.e. negligible shear stress for cells (Dewey et al. 1981).

For cell-specificity studies RPE cells were replaced by human corneal cells (HCE-T, 1×10^5 mL^{-1}), grown in its culture medium for 24 h.

Permeability studies

After 24 h in the dual perfusion chamber system (i.e. 72 h post HUVEC seeding) under flow, the permeability of the HUVEC/amnion/RPE trilayer was measured by tracer leakage studies. Sodium fluorescein (0.02 mg mL^{-1}) and fluorescein-conjugated dextrans (4, 20 and 40 kDa) at 0.05 mg mL^{-1} were dissolved in the endothelial medium flowing through the upper chamber. The lower chamber was continually bathed in the epithelial medium (as above) without tracer. The motivation for flow being present was to ensure absence of any stagnant boundary layers which would artefactually decrease permeability measurements.

Samples of 200 μL were taken at 15-min intervals from the lower chamber and the fluorescence of the sample examined using a Hitachi F-2000 fluorescence spectrophotometer. The concentrations of dextran in test samples were calculated by comparison against a standard concentration curve. The permeability was tested for amniotic membrane alone, amniotic membrane with RPE cells or HUVECs, and the trilayer (n = 3 in each case). Transfer of Na fluorecein over 2 h was also measured for HCE/amnion/HUVEC trilayers.

VEGF analysis

Using the trilayer in the closed circuit dual chamber system (n = 3), samples of supernatants (2 mL) were taken from reservoirs supplying the upper and lower chambers at 24, 48 and 72 h in culture, snap-frozen and stored at −20 °C until further analysis. Fresh medium replaced the sample at each time point. Cumulative levels of VEGF at 72 h were also sampled, by not replenishing the culture medium. Levels of VEGF production were assayed by an enzyme-linked immunosorbent assay (R&D systems, Abingdon, UK), according to the manufacturer's recommendations. Each sample of medium was tested in triplicate.

Immunocytochemistry

For both static and flow experiments, surface expression (and localization pattern) of adherens and tight junctional proteins in the trilayer were assessed by indirect immunofluorescence every 24 h for over 1 week to ensure the was no change in localization patterns beyond 72 h. The trilayer was not tested beyond 9 days. Each experiment was performed in triplicate; moreover, the experiments were repeated using HUVECs isolated from three different cords. Briefly, the culture medium was aspirated and cells were washed and fixed with 1% paraformaldehyde at room temperature for 10 min. Cells were then washed, permeabilized with 0.5% Triton, blocked with 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with the following antibodies: mouse monoclonal antibodies against VE-cadherin (Clone 55-7H1, Pharmingen, 5 μg mL^{-1}), rabbit polyclonal antibodies against ZO-1 (Clone 61-7300, Zymed laboratories 2.5 μg mL^{-1}), mouse monoclonal antibodies to occludin (Zymed, 4 μg mL^{-1}), rabbit polyclonal antibodies against occludin (Zymed, 10 μg mL^{-1}) and anticellular retinaldehyde-binding protein (ascitic fluid 1 : 250, Affinity Bioreagents, MA3-813). The secondary antibodies used were goat anti-mouse IgG-TRITC and goat anti-rabbit IgG-FITC. Cells were overlaid with PBS/glycerol mixed at a ratio of 1 : 1 and analysed by confocal scanning microscopy (Leica TCS-4d, Leica SP2) and images were analysed with LSM 5-image analysis software. Optical images were compiled and tilted on the x- and z-axes to obtain information on the extent of paracellular clefts occupied by junctional adhesion molecules and to ensure cross-contamination of cells to the incorrect amniotic surface in initial seeding or via migration had not occurred.

Electron microscopy (TEM)

Trilayers and monolayers of RPE cells and HUVECs on amnion, as well as HCE cells and HUVECs on amnion, were fixed in modified Karnovsky's (with 2.5% paraformaldehye, 2% glutaraldehdye in 0.1 M sodium cacodylate buffer, pH 7.4) embedded in resin and processed for TEM. Thin (70 nm) sections were viewed using a JOEL 1010 TEM microscope.

Statistics

One-way analysis of variance (ANOVA) and Kruskal–Wallis post-test was used to analyse differences in trilayer

permeability at set time points. A commercial software, Prism, was used.

Results

Characteristics of RPE and HUVEC monolayers on amnion: junctional organization

As mono-cultures, RPE cells became confluent 24 h after seeding onto the epithelial surface of the amniotic membrane only (Fig. 1). They showed a typical columnar appearance, with no multiple layering, with cell–cell contact regions showing immunoreactivity to the tight junctional markers, occludin and ZO-1, by 24 h (Fig. 1A,B), but were negative for endothelial junctional marker, VE-cadherin (Fig. 1C).

HUVECs reached confluence 48 h after seeding onto the interstitial surface of the amniotic membrane only. It had the typical cobblestone appearance and showed full expression of the adherens junctional molecule VE-cadherin at cell–cell contacts (Fig. 1F). The tight junctional marker ZO-1, but not occludin, was also localized to paracellular clefts (Fig. 1D,E).

Characteristics of RPE–HUVEC co-culture trilayer on amnion (junctional organization and duration of co-culture)

RPE cells and HUVECs were observed to form continuous monolayers on their preferred sides of the amnion (Fig. 2). HUVECs grew to confluence within 24 h of co-culture with RPE cells and amnion. The co-culture conditions did not disturb the molecular phenotype of the endothelial adherens junctions; VE-cadherin was still found at cell–cell contacts throughout this period (Fig. 3A). At 24 h, HUVECs retained the junctional localization of the tight junctional marker, ZO-1 (Fig. 3B), with the surface expression of occludin remaining cytoplasmic. However, increased paracellular localization of occludin was observed by 48 h, and after 72 h co-culture with RPE cells most of the occludin was localized to cell–cell contacts in HUVECs (Fig. 3D).

Co-culture conditions did not affect the morphology of RPE cells or localization of occludin or ZO-1 (Fig. 3E). Initially, immunoreactivity to occludin was both cytoplasmic and junctional at 24 and 48 h co-culture, but at 72 h, the occludin immunoreactivity was predominantly at cell–cell

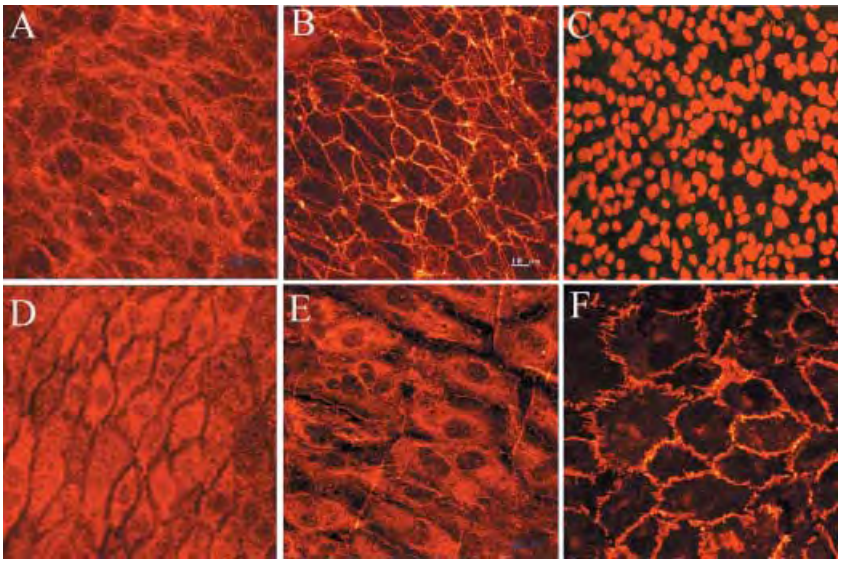

Fig. 1 Confocal micrographs of monolayers of RPE cells on amnion alone (A–C) and HUVECs on amnion alone (D–F). In the RPE, cell–cell contacts were immunostained for occludin (A) and ZO-1 (B) but were immunonegative for VE-cadherin (C). C also shows propidium iodide staining of nuclei. D shows cytoplasmic location of occludin in HUVECs. Cell–cell contacts did possess ZO-1 (E), and the AJ molecule, VE-cadherin (F). Magnification was the same for all images.

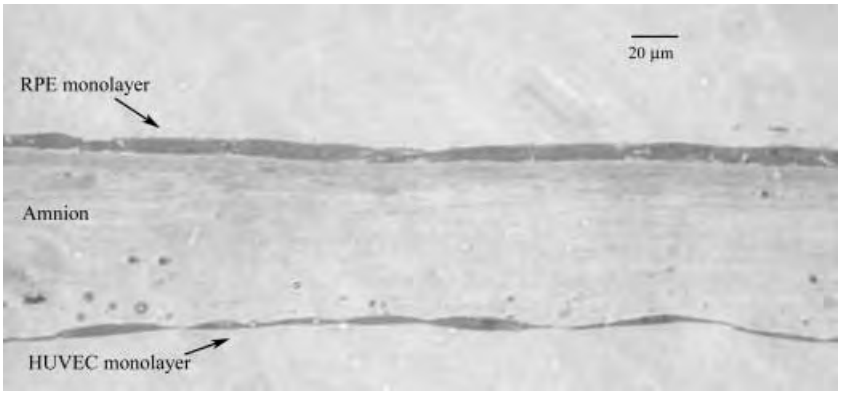

Fig. 2 Toluidine blue-stained trilayer showing monolayers of the retinal pigment epithelium (RPE) (above) and the human umbilical vein endothelial cells (HUVECs) (below). Note the clear separation of the two monolayers.

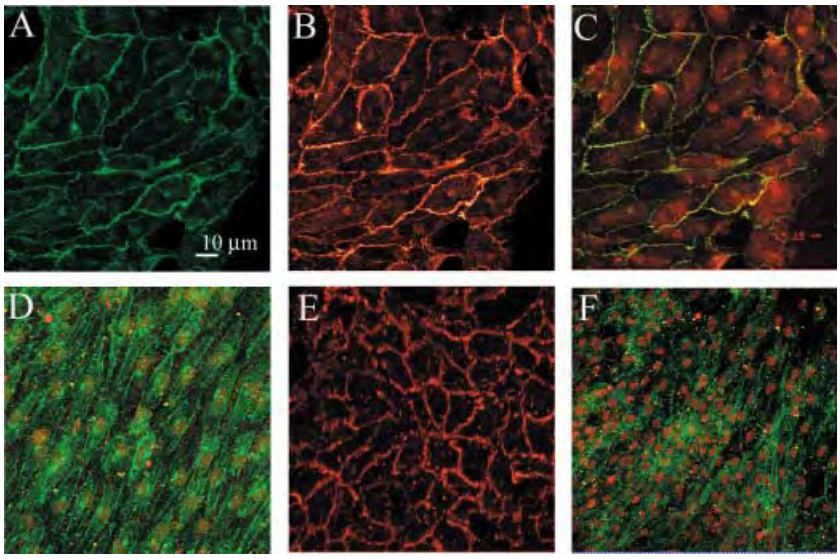

Fig. 3 Confocal micrographs of trilayers of RPE cells and HUVECs at 72 h co-culture. HUVECs still retained VE-cadherin (A) and ZO-1 (B) at cell–cell contacts. (C) Dual labelling of A and B. (D) Localization of occludin in HUVECs is now predominantly at cell–cell contacts as well as cytoplasm (nuclei stained with PI). (E) Double-immunolabelling of RPE, which is immunonegative for VE-cadherin (green) but positive for ZO-1 (red). Occludin is present at cell–cell contacts (F).

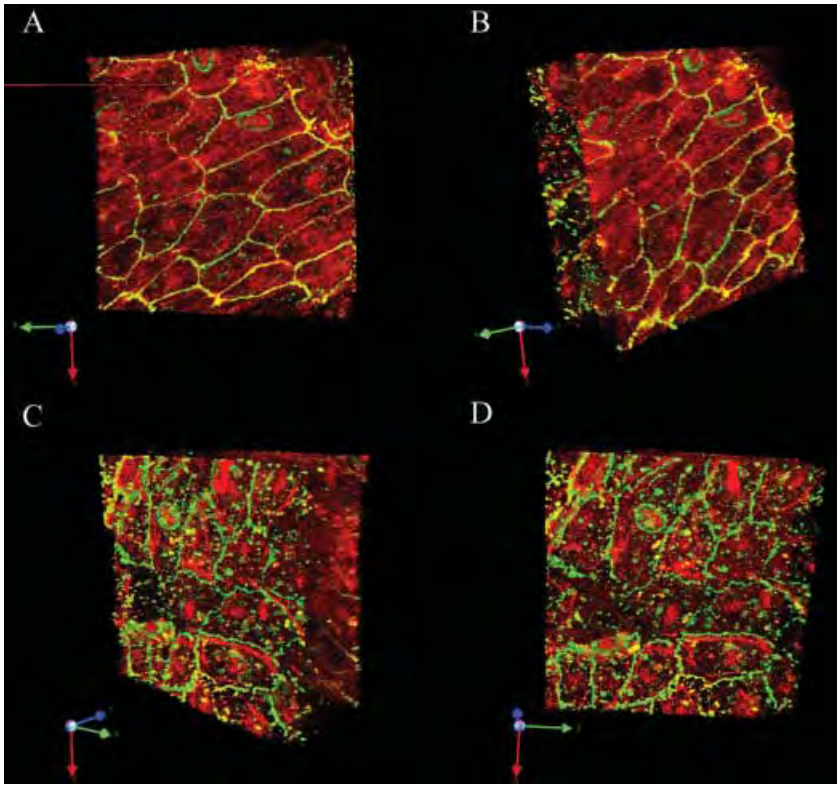

Fig. 4 Confocal micrographs of optical sections of the trilayer tilted around its axis. The images have been tilted at varying angles [starting with the RPE surface (A) to the HUVEC surface (D)] using Velocity software. The trilayer shows immunostaining for ZO-1 (green) and occludin (red) with the yellow showing the areas of dual labelling. Note both HUVECs and RPE cells are present as a continuous monolayer.

contact regions (Fig. 3F). Confocal microscopy and image analysis (using Velocity software) of double-labelled cells showed co-localization of occludin and ZO-1 at paracellular clefts in the RPE monolayer, whilst in HUVECs, the two bands of immunostaining were closely associated at the cell periphery but did not merge (Fig. 4). Confocal imaging and tilting also confirmed that the epithelial and endothelial layers remained as two separate monolayers on either surface of the amnion even after 72 h of co-culture. No migration of cells into the amnion was seen.

Ultrastructural organization of trilayer

TEM confirmed that, at 72 h, in the trilayer RPE cells had formed a continuous monolayer on the amniotic membrane (Fig. 5). Cells showed a degree of overlapping, but

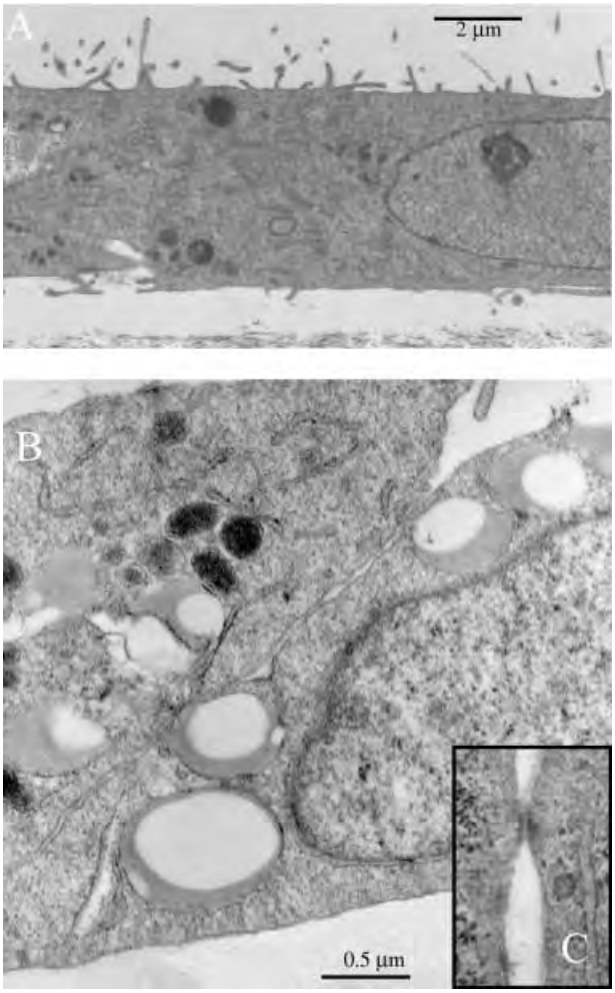

Fig. 5 Electron micrographs of the trilayer. The RPE layer shows a polarized phenotype in possessing microvilli (A) and paracellular clefts between adjacent cells containing junctional regions (B,C). (C) Higher magnification showing that total fusion of membrane does not occur at tight junctional apposition.

did not form multilayers. Apical/basal polarity was established with RPE cells possessing microvilli on the apical surface (Fig. 5A). Paracellular clefts between adjoining cells possessed numerous close appositions of adjoining cell plasma membranes reminiscent of tight junctions, but complete fusion of plasma membranes was not achieved (Fig. 5B,C).

The HUVECs were seen to form a continuous monolayer, which at 72 h showed the presence of fenestra (Fig. 6A), as well as a number of caveolar-like structures, which may be showing stages of fenestra formation (Fig. 6B,C). Well-defined paracellular clefts were also found between adjoining cells (Fig. 6D,E). In mono-cultures of HUVEC on amnion no fenestra were seen.

When HUVECs were grown as a trilayer with corneal epithelial cells replacing RPE cells (Fig. 7A), no fenestra could be seen (Fig. 7B). Corneal epithelial cells were seen to form monolayers with apical microvilli and contained paracellular clefts with defined apical regions of close apposition. Again, complete fusion of plasma membranes of adjoining cells was not seen.

Permeability studies

The amniotic membrane alone was freely permeable to sodium fluorescein (NaF) and 4-kDa FITC-conjugated dextran (Fig. 8). Permeability of confluent monolayers of HUVECs to 4-kDa tracers was seen to reflect that or was slightly reduced to that measured for amnion alone. A similar permeability curve but with further reduction in the rate of tracer leakage of 4-kDa tracers was seen for RPE monocultures on amnion. These leakage values of monolayers grown on the amnion were not statistically significant when compared with the amnion alone.

Under flow, 72 h after establishment of the trilayer, permeability to 4-kDa tracer was abolished with less than 5% of 4-kDa dextran being transferred (Fig. 8). Moreover, transfer of NaF was seen to be severely restricted in the trilayer, whereby only 10% of the added fluorescein was transferred from apical to basal wells over a 2-h period in comparison with amnion alone (Fig. 8). Replacement of RPE cells with corneal epithelial cells showed that transfer of NaF in this trilayer was much more rapid, with 11% of NaF being transferred within 15 min.

VEGF levels

Secreted VEGF levels in the trilayer were seen to increase throughout the duration of co-culture and reached 7 ng mL^{-1} (±0.849 SD) by 72 h. Sampling at 24, 48 and 72 h (Fig. 9) revealed a sharp increase (doubling) in VEGF production between 48 and 72 h.

Discussion

In this study we produced a viable human *in vitro* model for the outer blood–retina barrier consisting of the RPE/amnion/HUVEC. The model resembles the *in vivo* phenotype in presenting continuous monolayers of RPE and endothelium separated by a barrier of biological extracellular matrix. The RPE cells show polarity in possessing apical microvilli and containing occludin-rich tight junctions, whilst, remarkably, the endothelium shows fenestration. The *in vitro* model also approaches the *in vivo* model in barrier property, occluding transport of 4-kDa tracers and severely restricting (ten-fold) transfer of sodium fluorescein, which are more readily permeable through monocultures of RPE cells and HUVECs alone. These junctional, structural and barrier changes in the trilayer demonstrate that cross-talk between the two different cell types, epithelial to endothelial and vice versa, is influencing the phenotype of each cell layer, which differs markedly from monoculture alone.

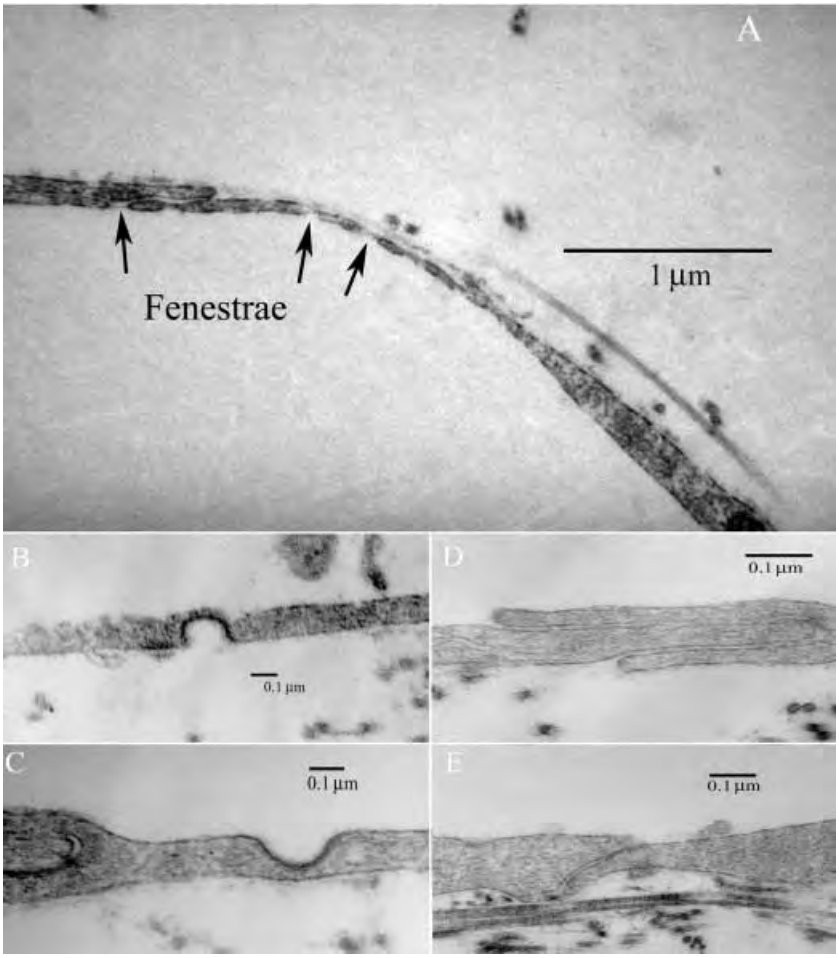

Fig. 6 The endothelial layer of the trilayer shows presence of both paracellular clefts and fenestrae (A); numerous caveoli-like vesicles can be seen, suggestive of fenestra-formation (B,C); paracellular clefts are clearly defined (D,E).

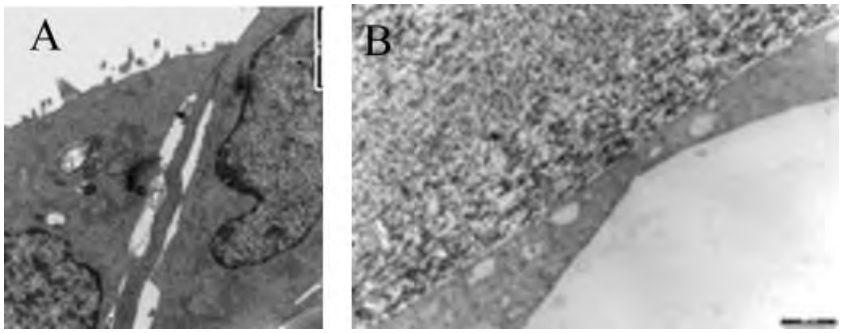

Fig. 7 Electron micrographs of trilayer where corneal epithelial cells are co-cultured with HUVECs. (A) Both apical microvilli and paracellular cleft are present in HCE-T, whilst the HUVECs remain as a continuous monolayer; no fenestrae can be seen (B).

The human amniotic membrane appears to be very suitable for growth of epithelial and endothelial co-culture when placed on the appropriate surfaces. Growth of human RPE cells on amnion, as a monolayer, has shown previously (Capeans et al. 2003), and is not surprising given that the amnion has many of the components common to the Bruch's membrane including elastin (Hieber et al. 1997) and collagens I and IV (Cooper et al. 2005). However, ours is the first study to show that RPE cells can grow on the epithelial side of the amnion with endothelial monolayers lining the interstitial surface of the amnion and that this induces fenestration in the endothelial cells. Co-cultures of RPE cells and bovine or human choroidal endothelial cells on trans-well inserts have been attempted previously (Hartnett et al. 2003; Geisen et al. 2006) but the phenotypic endothelial modifications we report was not seen on the synthetic membranes.

There is good evidence from *in vivo* studies that the choriocapillaris is dependent upon the overlying RPE as it atrophies when the RPE is experimentally removed (Garner, 1994; Del Priore et al. 1996; Pollack et al. 1996; Nasir et al. 1997). Not only is the choriocapillaris dependent

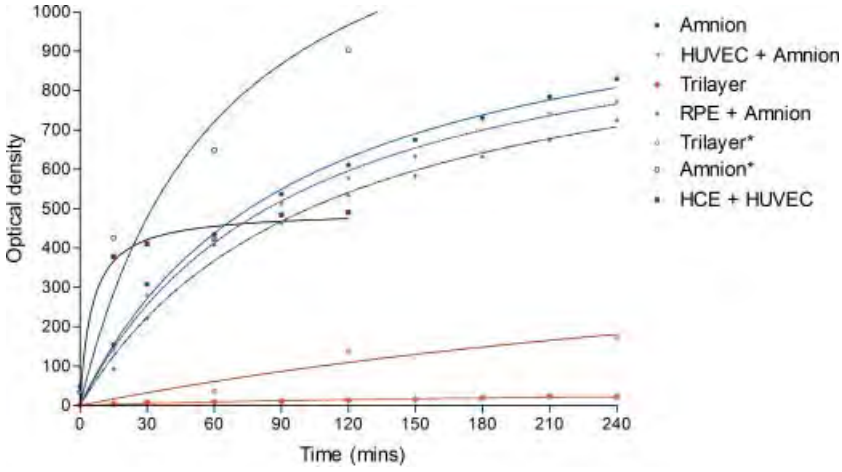

Fig. 8 Graph plotting leakage of 4-kd fluorescein dextan tracer across amnion alone, amnion + HUVEC, amnion + RPE, HCE-T + HUVEC co-culture, and the trilayer (RPE + HUVEC co-culture) (*shows sodium fluorescein as a comparison). Severe restriction of tracers can be seen in the trilayer plots.

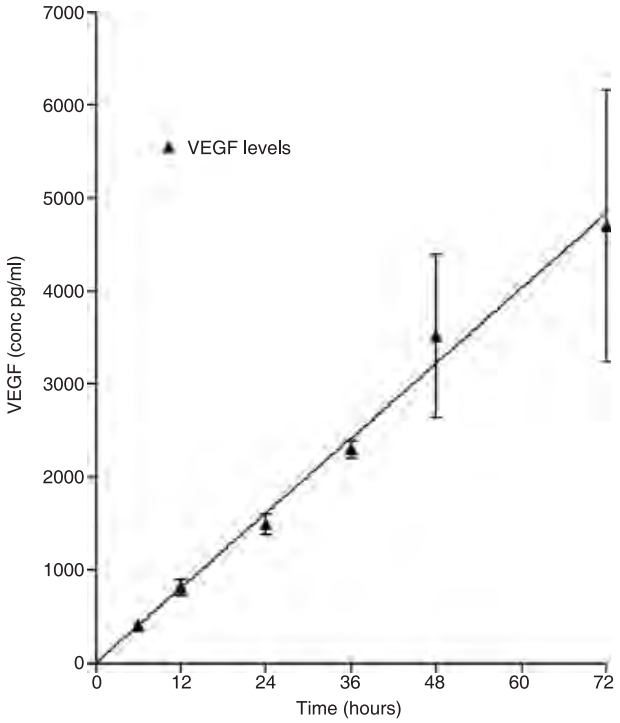

Fig. 9 Graph showing levels (pg mL^{-1}) of VEGF produced by the trilayer over 72 h.

upon the RPE for survival, but its morphological phenotype may be dictated by trophic factors secreted by the RPE. Certainly this type of modification has been shown in culture. Adrenal cortex capillary endothelial cells will develop fenestrations when cultured in the presence of choroidal plexus epithelial cells stably transfected with VEGF (Esser et al. 1998b). In the current study, the appearance of fenestra in HUVECs after 3 days of co-culture with RPE is a novel and exciting finding. *In vivo* and in monocultures of HUVECs show a continuous non-fenestrated phenotype. This fidelity of origin appears to be lost when co-cultured with RPE cells in a trilayer architecture. This is not simply a consequence of co-culture with another epithelial cell type; tri-layering with corneal epithelial cells did not induce such a change in phenotype, suggesting this is an RPE-specific cross-talk. This study strengthens the hypothesis that the endothelial cell phenotype is plastic and that its environment is a more important determinant than the site of origin.

To test the functional viability of this model, we wished to ascertain that the RPE in this co-culture was capable of producing growth factors. To that extent we measured VEGF levels in the first instance. In our model, pan-VEGF levels in the trilayer increased with time, being at its highest (7 ng mL^{-1}) at 72 h. There was no detectable level of VEGF from HUVEC monolayers in culture, which showed a continuous non-fenestrated phenotype. *In vitro*, endothelial fenestrations have been achieved by perfusing exogenous VEGF in capillaries and venules of rat cremaster muscle and skin (Roberts & Palade, 1995). This suggests a possible mechanism for fenestration induction in our model which requires further detailed investigation and is currently underway.

HUVECs in the trilayer also possessed paracellular clefts with defined junctional regions, which included the presence of VE-cadherin, ZO-1 and occludin. The presence of VE-cadherin here is consistent with its presumed function in promoting quiescence by contact inhibition and VEGF-induced endothelial survival (Carmeliet et al. 1999). This raises the possibility that the choriocapillaris phenotype is dependent upon continuous VEGF release from the RPE.

A previous RPE and endothelial co-culture model found that co-culturing the RPE cells with endothelial cells resulted in a marked decrease, rather than increase, in the RPE barrier properties and that this decrease was mediated, in part, by VEGF. This disruption in the barrier properties was greatest when there was direct contact between the RPE cells and the endothelial cells (Hartnett et al. 2003). Separation of the two cell types by an appropriate barrier

has an important effect on their physiology. RPE cells also release other growth factors, including pigment epithelium-derived factor (PEDF) (Steele et al. 1993), which has powerful anti-angiogenic activity (Alberdi et al. 1998). However, it should be noted that human amniotic membrane is a source of PEDF (Shao et al. 2004).

The monoculture of ARPE cells with amnion allowed cell confluence and appearance of occludin at cell–cell contact regions. However, tracer leakage studies showed that whilst this monolayer reduced leakage of 4-kDa tracers, it did not occlude this transfer. Only in the trilayer, i.e. in the presence of amnion and HUVECs, 72 h after co-culture, did the trilayer occlude 95% of the transfer of 4-kDa dextran and 90% of the smaller sodium fluorescein. This restriction was not simply a decrease in the rate of transfer of tracers across an increased diffusion pathway created by having two monolayers on either side of the amnion. Replacing RPE cells with corneal epithelial cells showed that there was a reduction in transfer of NaF from apical to basal wells over 2 h; however, this trilayer was more permissive than the RPE/HUVEC trilayer, allowing similar amounts of tracer leakage at much earlier time points. Surprisingly, molecular occupancy of tight junctions with ZO-1 and occludin appear not to explain this increased restrictivity in our outer blood–retinal barrier model. Immunocytochemically, we could not show any difference in occludin/ZO-1 immunoreactivity in RPE monolayers or trilayers. Other transmembrane molecules such as claudins and JAM may very well show different dynamics in this model. Electron microscopy studies further highlighted that the tight junctions between RPE cells did not occupy the entire paracellular clefts, but were present as discrete points of apposition. Addition of a neural layer, or usage of a different source of RPE cells, may enhance the tight junctional restrictivity of the epithelial layer further and is a valuable future step for this model.

In conclusion, we have succeeded in producing a reproducible and near physiological model that mimics, with some limitations, the different layers of the back of the eye both in structure and in barrier function and may be used to study the interactions between the RPE and vascular endothelium. Our trilayer data suggest that soluble factors from the HUVECs as well as extracellular matrix ligand may be influencing the RPE; a two-way cross-talk exists between the RPE and the endothelium, as evidenced by the altered phenotype in the endothelial layer.

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