Chapter 20

Isolation and Properties of an In Vitro Human Outer Blood-Retinal Barrier Model

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Abstract

The outer blood–retinal barrier is composed of a monolayer of retinal pigment epithelium (RPE), Bruch's membrane, and the choriocapillaris, which is fenestrated. An in vitro model that includes all these layers within a 3-D architecture confers a clear advantage over traditional monolayer cultures. Cells here, whether endothelial or epithelial, reside in conditions resembling that in vivo and can participate in cell–cell and cell–matrix cross talk. This chapter describes how a human trilayer culture model was generated with RPE (ARPE-19) cells cultured on the epithelial surface of amniotic membrane and with human umbilical vein derived endothelial cells (HUVEC) on the interstitial surface. This model resembles the outer retinal barrier both in restricting transport of small molecules (<4 kDa), possession of occludin-rich tight junctions in the RPE and fenestrated endothelial cells. Techniques used to test the generated trilayer properties are also described and these include imaging of structure and molecular occupancy of tight and adherens junctions, estimation of the barrier efficiency of trilayer by measurement of fluorescein and fluorescein-conjugated tracers under flow, measurement of secreted vascular endothelial growth factor-A and ultrastructural studies, which allow analyses of the fine structure of the tight junctions in the RPE, and the endothelial fenestra.

Key words: 3D culture, Fenestrated endothelium, HUVEC, In vitro permeability, Outer retinal barrier, Retinal pigment epithelium, VEGF-A

1. Introduction

Reductionist approaches into mechanisms underlying diseases of the outer retinal barrier, such as age related macular degeneration (AMD), have been hampered by the lack of optimal in vitro models utilising human cells to provide the 3-D architecture and allow expression of the in vivo phenotype for both the retinal pigment epithelial cells and the endothelium. There is good evidence from in vivo studies that the choriocapillaris is dependant upon the

Sukriti Nag (ed.), *The Blood-Brain and Other Neural Barriers: Reviews and Protocols*, Methods in Molecular Biology, vol. 686,
DOI 10.1007/978-1-60761-938-3_20,

overlying retinal pigment epithelium (RPE) as it atrophies when the RPE is experimentally removed (1). The morphological phenotype of the choriocapillaris appears to be dictated by trophic factors secreted across the basement membrane by the RPE (2, 3). In active neo-vascular AMD, breakdown of the outer retinal barrier and endothelial cell (EC) proliferation are key processes that lead to retinal damage and involve factors such as vascular endothelial growth factor –A (VEGF-A) (3–5).

In this chapter, we describe techniques employed to generate a viable human in vitro model for the outer blood–retinal barrier consisting of the RPE, amnion, and human umbilical vein endothelial cells (HUVEC) (2). This model possesses some of the key properties of the outer barrier and can be experimentally manipulated for mechanistic studies while allowing cross talk between neighbouring tissue layers.

The RPE and the choriocapillaris are separated by Bruch's membrane in vivo. The amniotic membrane can be compared to Bruch's membrane, as it consists of an inner layer of epithelial cells resting on a basement membrane (BM), which is composed of mainly type IV collagen, laminin, elastin, and heparan sulphate (6). This BM interfaces with the vascular stroma, which consists mainly of collagen I, III, IV, fibronectin and laminin, forming an ideal extracellular matrix for cultivation of ECs. We and others have shown that the human amniotic membrane is suitable for the growth of epithelial and endothelial monolayers (2, 7, 8). This chapter describes how placing RPE cells first on the epithelial side of the amnion and then seeding the opposite surface with ECs (see below) results in formation of a polarised RPE layer and a fenestrated endothelial monolayer. Co-cultures of RPE and bovine or human choroidal EC on transwell inserts have been attempted before (5, 9), but the phenotypic endothelial modifications we have reported were not seen on the synthetic membranes.

Primary HUVEC is used in the trilayer since these cells have been 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 3-D culture (10, 11). Moreover, isolated HUVEC continue to express signalling molecules, which respond to inflammatory mediators (12), hypoxia, and angiogenic growth factors such as VEGF-A (11). In our generated trilayer, HUVEC show loss of fidelity of origin by becoming fenestrated (2). Moreover, they still possess paracellular clefts with defined junctional regions, which include presence of VE-cadherin, zonula occludens (ZO)-1, and occludin (see Fig. 1).

In polarised epithelial monolayers, tight junctions are the key entities that seal the paracellular pathway between adjacent cells.

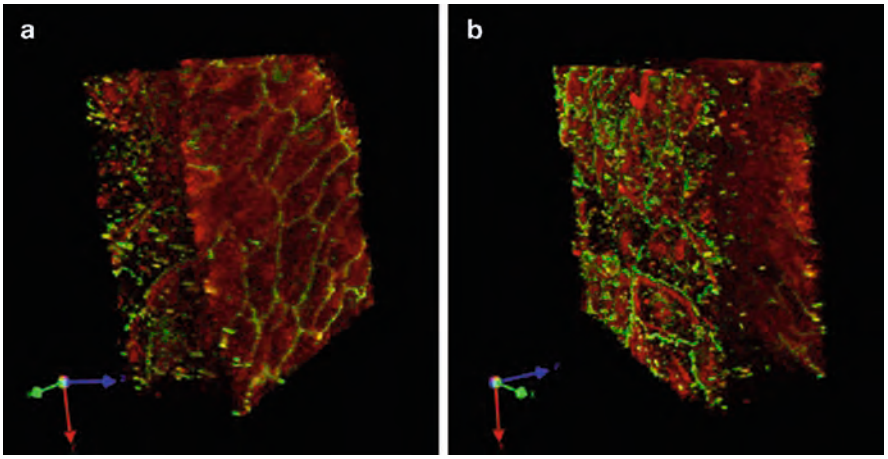

Fig. 1. Laser scanning confocal images of optical sections of the generated outer retinal barrier tilted around its axis showing the RPE cell surface (**a**) and endothelial cell surface (**b**). Monolayers of both surfaces have been immunostained with ZO-1 (*green*) and occludin (*red*).

This prevents diffusion of solutes and differentiation of the apical and basolateral membrane domains to allow active transport across the monolayer (13). The presence of tight junctions must therefore be established in any barrier model, alongside measurements of transepithelial flux of solutes. This chapter describes some of the standard protocols used for localisation of the key structural proteins such as occludin and the cytoplasmic linking molecule ZO-1. Localization can be performed with immunocytochemistry; however, confocal laser scanning microscopes should be used for imaging the precise locations of these molecules (Fig. 1). Tight junction (TJ) proteins such as the claudins, occludin, and junctional adhesion molecule (JAM) dictate the complexity of tight junctional strands and paracellular permeability and their expression should be ascertained (14–16).

The tracer leakage studies are performed using a commercially available MINUCELL system, which allows the upper and lower chambers to be independently perfused with different media, under chosen flow rates. This compartmentalisation also allows frequent sampling of different compartments without disturbance of the monolayers and eliminates the unstirred layer, which can complicate tracer flux studies in static culture. Within this system, the generated model, after full confluence of both layers, excluded 95% of the transfer of 4 kDa dextran and 90% of the smaller sodium fluorescein from the upper (HUVEC chamber) to the lower chamber (RPE chamber) over a 2-h period of sampling at flow rates that do not cause shear stress (17). The system also allows collection of culture medium from the two different compartments over the duration of culture, for later measurement of secreted growth factors by ELISA. In this in vitro

3-D model of the outer retina, VEGF-A was found to be secreted with highest measured levels being reached by 72 h in culture.

Electron microscopy studies are used to detail the TJs present between RPE in the generated model. Despite the high restrictivity to or negligent transfer of small molecules measured, ultrastructural analyses show that the TJs in the RPE do not occupy and occlude the entire length of the paracellular clefts, but are present as discrete points of apposition, more reminiscent of non-CNS endothelial tight junctions seen in vivo (18). This may be an in vitro artefact or perhaps the addition of neural factors may lead to the formation of the archetypical tight junctional strands. Ultrastructural studies also revealed the fenestrated nature of the endothelial layer in our 3-D model. The resolution of the light microscope would not have allowed this observation, leading one to advocate, whenever possible, the addition of an ultrastructural analytical step.

In conclusion, this is a reproducible and near physiological model that mimics, with some limitations, the different layers of the back of the eye both in structure and barrier function and may be used to study the interactions between the RPE and vascular endothelium and for mechanistic investigations into development, functioning, and pathology of the outer retinal barrier.

2. Materials

2.1. Cell Culture

2.1.1. Chemicals and Media

1. Phosphate Buffered saline (PBS), calcium and magnesium free (Oxoid Ltd, Basingstoke, UK).
2. Phosphate-buffered saline (PBS): 2.69 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4 8.1 mM, Na_2HPO_4, pH 7.6 (Oxoid Ltd).
3. Dulbeccos' modified eagle medium (DMEM), HAM's F12 medium, Hanks Balanced Salt Solution (HBSS); amphotericin B; l-glutamine; and trypsin (0.05%) with ethylene diamine tetra-acetic acid (EDTA) disodium salt (0.02%) in PBS (trypsin/EDTA) (Life Technologies, Paisley,UK) (see Note 1).
4. For HUVEC, supplement Medium 199 with l-glutamine (2 mM), penicillin (100 U/mL), Streptomycin (100 µg/mL) and amphotericin B (0.2 µg/mL). Name this medium M0. Supplement this with 20% (v/v) Hyclone FCS, ECGS (30 µg/mL) and Heparin (90 µg/mL) before use for cell culture and name medium M20.
5. Medium for corneal epithelial cells: Epilife medium (Cascade Biologics, Portland, USA) with calcium chloride (120 µM) and FCS (10%).
6. Foetal calf serum (FCS) (Autogen Bio Clear UK Ltd, Wiltshire, UK).

7. Tracers: FITC-labelled 70 kDa (FD70), 40 kDa (FD40), 4 kDa (FD40), Fluorescein Sodium salt (Sigma-Aldrich).
8. Collagenase type II (Sigma-Aldrich).
9. Chemicals: Dimethylsulphoxide (DMSO), Triton-X-100, paraformaldehyde, phorbol 12-myristate 13-acetate (PMA), thermolysin, sodium acetate, calcium acetate, (Sigma-Aldrich).
10. Bovine serum albumin (BSA) (Sigma-Aldrich).
11. Endothelial cell growth supplement (ECGS) (First Link, Briery Hill, UK).
12. Sodium chloride (Baxter Healthcare Ltd, Thetford, UK).
13. Trigene™ (Scientific Laboratory Supplies Ltd, Nottingham, UK).
14. 1% (w/v) gelatin in sterile water, autoclaved and stored at 4°C.

2.1.2. ARPE-19 Cells

1. RPE Cells (ARPE-19, ATCC CRL-2302) at a passage of 10 (American Type Culture Collection (ATCC), Manassas, Virginia, USA).
2. Medium for RPE cells: Supplement a 1:1 mixture of HAM's F12 medium and Dulbecco's modified eagle's medium with l-glutamine (2.5 mM), and 1.5 g/L sodium bicarbonate with penicillin (100 Units/mL), Streptomycin (100 µg/mL), and amphotericin B (0.2 µg/mL). Call Medium D0. Supplement this was with 10% (v/v) Hyclone FCS prior to use with cell culture and name medium D10.

2.1.3. Tissue Culture Plastics and Accessories

1. NUNC™ tissue culture flasks 25 cm^2 and 75 cm^2, maxisorp wells, plates, chamber slides, and 0.2 mM and 0.4 mM inserts and filters (Life Technologies).
2. Cryopreservation tubes (1 mL) (Sarstedt, Leicester, UK).
3. 3-way stopcocks (Vygon UK Ltd., Gloucester, UK).
4. Suture (Davies and Geck, Gosport, UK).
5. Container to incubate umbilical cord and steel trays (Jencons-PLS, Leighton Buzzard, UK).
6. Cover slips (13 mm, Life Technologies).

2.1.4. Antibodies for Immunocytochemistry

1. Antibodies used, their source and dilution are given in Table 1.
2. Confocal laser scanning microscopy: Leica TCS-4d or Leica SP2.
3. Software: LSM 5-image analysis software.

Table 1
The source of the antibodies used and their dilution are shown

Antibody	Catalogue number	Source	Dilution
Cellular retinoic acid binding protein 1 (CRALBP-1)	(Ascitic fluid 1:250, MA3-813)	Affinity Bioreagents via Cambridge Bioscience	1 µg/mL
Anti-Occludin	ab31721	Affinity Bioreagents	4 µg/mL
Anti-ZO-1	ab59720	Affinity Bioreagents	1 µg/mL or dilute1 in 50–100
Mouse monoclonal anti-human VE-cadherin	Clone 55-7H1, Pharmingen,	Cambio, Cambridge, UK	5 µg/mL
Mouse monoclonal anti E-cadherin	FAB7481P	R&D systems	20 µg/mL
TRITC conjugated goat anti-mouse	T 7782	Sigma-Aldrich	Dilute 1 in 50–100
FITC conjugated goat anti-rabbit	F 0382	Sigma-Aldrich	Dilute 1 in 50–100
Rabbit polyclonal anti-occludin	Clone ZMD.467	Zymed Laboratories, USA	Dispense into 10 µL aliquots 50 µg/mL and stored at −80°CFinal dilution 10 µg/mL
Rabbit polyclonal anti-ZO-1	Clone 61-7300	Zymed Laboratories, USA	Dispense into 10 µL aliquots 20 µg/mL and stored at −80°C.Final dilution 2.5 µg/mL

2.2. MINUCELLS Cells and MINUTISSUE System

The 13 mm MINUSHEETs (MINUCELLS and MINUTISSUE, Bad Abbach, Germany) consist of a black base ring, in which the selected support material, i.e. amnion is placed. A white tension ring is used to hold the support in place on the base ring (Fig. 2). The MINUSHEETS optimum sterilisation method is autoclaving at 105°C and 0.3 bar for 20 min or at a maximum of 135°C and 2 bar for 10 min.

2.3. ELISA for VEGF-A

1. Anti-human VEGF-A antibody (Cat no: af-293-na, R&D Systems, UK). To 100 µg add 1 mL of sterile PBS to produce a concentration of 0.1 mg/mL. Store in 50 µL aliquots (1:250 = 0.4 µg/mL).
2. Recombinant VEGF-A (Cat no: 293-ve, R&D systems). Ten micrograms in vial, add 1 mL of 0.1% PBS/BSA to produce a concentration of 10 µg/mL. Store in 10 µL aliquots in the

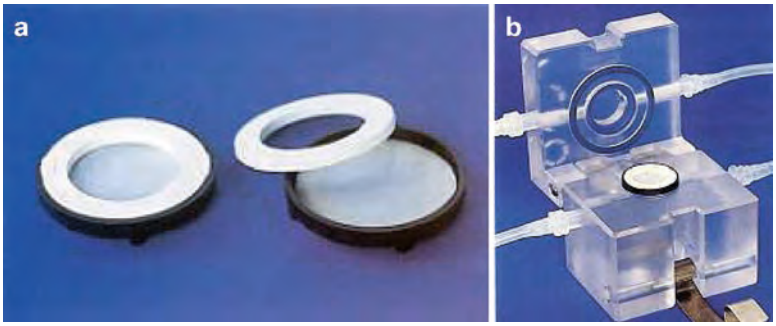

Fig. 2. The MINUCELL SYSTEM (**a**) The amniotic membrane is placed within the black base ring and the white tension ring, prior to seeding with cells. (**b**) The culture system allows independent perfusions of HUVEC (*upper chamber*) and RPE (*lower chamber*) and efficient sampling of tracers from either chamber.

freezer –4°C. Dilute at 1:100 = 100 ng/mL, dilute again for 2,000 pg/mL (first step of standard curve).

3. Biotinylated anti-human VEGF-A Antibody (Cat no: baf293, R&D systems). Fifty micrograms in vial, add 1 mL of tris buffered saline + 0.1% BSA to produce a concentration of 50 µg/mL. Aliquot in 50 µL and freeze. 44 µL in 11 mL is 1:250 dilution to leave 200 ng/mL.
4. Biotyinylated-streptavidin-HRP kit (DY998, R&D systems).
5. TMB kit 50-76-00 www.kpl.com (add substrates at 1:1 less than 15 min before use).
6. Tris buffered saline: To 10 mL of 0.1% BSA, add 24.2 mg of Tris and 67.6 mg of NaCl.
7. 6% H_2SO_4: 6 mL of 100% H_2SO_4 and 94 mL of dH_2O (see Note 2).

3. Methods

3.1. Coating Tissue Culture Plastic and Cover Slips with Extracellular Matrix Protein

1. Coat the culture flasks with sterile gelatin 1% (w/v) in water.
2. Use 2 mL for the 25 cm^2 culture flasks, 5 mL for the 75 cm^2 culture flasks, and 1 mL for each cover slip in an 8-well plate.
3. Incubate at 37°C with 5% (v/v) CO_2 for 30 min.
4. Remove gelatin and air-dry in a laminar flow hood under sterile conditions.

3.2. Preparation of Tissues and Cells

3.2.1. Preparation of Amnion

1. Immediately after clinical inspection of the placenta and umbilical cord by the midwife, transfer the amniotic membrane with chorion still attached, but removed from the placenta, to a 500-mL sterile bottle containing 200 IU/mL penicillin and 200 µg/mL streptomycin (SAL/AB). Store the bottle with the amnion at 4°C for up to 24 h.

2. Handle the amnion and umbilical cord in a class two laminar flow hood vented to the outside, and decontaminate all surfaces that have come into contact with the tissue, cells or blood with Trigene™ as a precaution against the potential infectious elements in human tissue.
3. Place the amnion in a bowl for manipulation. Under sterile conditions, process the amnion as previously described by Tseng and co-workers (19) and described below.
4. The chorion interstitial side is easily distinguishable from the epithelial surface because it is more irregular and messy. Peel the chorion carefully off the amnion with blunt dissection, making sure to remove all the interstitial material with it and leave the amnion alone behind.
5. During this process, clip one of the minusheets onto the amnion with the black side to the epithelial surface to maintain the amnion polarity. Completely remove and dispose of the chorion (see Note 3).
6. Wash the amnion repeatedly in further SAL/AB to remove all blood and protein remnants, and clip the rest of the MINUSHEETs onto the amnion.
7. Once all the clips are attached, perform careful dissection of the clips with curved scissors to produce a clean edge.
8. Place each of the clips into a sterile 12 well plate and suspend in 2 mL of SAL/AB and store overnight at 4°C.
9. After 24 h, remove any further chorionic material that is visible by light microscopy in the laminar flow hood under sterile conditions.
10. The clips can remain in the sterile 12 well plate suspended in 2 mL of SAL/AB and stored at 4°C for up to 1 month (see Note 4).

3.2.2. Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)

1. Obtain umbilical cord and amnion (see Subheading 3.2.1).
2. Isolate HUVEC based on the method by Jaffe (20).
3. Inspect the cord for any damage that could affect the isolation process or the endothelium of the umbilical vein; e.g. needle stick holes or clamp marks.
4. Blot the ends of the cord on sterile absorbable paper to dry and remove blood residues. Tease open vein at either end with forceps.
5. Cannulate the vein at either end using the 3-way stopcocks, and tie securely to prevent any leak.
6. Flush the vein three times with SAL/AB to remove blood cells and proteins. Fill vein completely to full turgor to ensure no leak or puncture marks are present and then empty.

7. Fill with a 0.5-mg/mL solution of collagenase type II in Medium M0 until the vein is under pressure and close the second stopcock.
8. Incubate the cord in a water bath for 10 min at 37°C. Using two syringes attached at the stopcocks at either end of the vein, agitate the collagenase solution back and forth through the vein to ensure that all the endothelial cells have been removed from the vessel wall.
9. Transfer the cell suspension from the syringe into a 25-mL sterile tube and centrifuge at 10 × *g* for 5 min.
10. Siphon off the supernatant and re-suspend the pellet in 5 mL of full medium M20 with 50 µg/mL ECGS. Seed this onto a gelatin coated flask. Incubate at 37°C with 5% (v/v) CO_2 in air, and re-feed every 2–3 days with fresh medium.
11. Cells can be subcultured when they reached 90% confluence.

3.2.3. HUVEC Subculture

1. Aspirate medium from 25cm² culture flasks or 75cm² culture flasks and wash the cells with calcium and magnesium free PBS (pre-warmed to 37°C).
2. Incubate these cultures at 37°C, 5% (v/v) CO_2 with 2 mL trypsin/EDTA (0.05/0.02%) for 5 min. Observe cells rounding up and assist detachment by tapping the flask gently.
3. Once the cells have all detached, add medium M20 to inhibit the action of the trypsin/EDTA, and transfer the suspension to a sterile 25 mL tube.
4. Centrifuge at 10 × *g* for 5 min and re-suspend pellet in 5 mL of the M20 medium.
5. Seed cells on to 75 cm² culture flasks at a split ratio of approximately 1:4 or 1:5. Add medium to flasks to give a final volume of 15 mL and add 50 µg/mL ECGS.
6. Maintain cultures at 37°C with 5% (v/v) CO_2 in the air, and re-feed with fresh medium every 48–72 h (see Note 5).

3.2.4. Culture of Retinal Pigment Epithelium (RPE)

1. Culture RPE cells routinely in the D10 medium.
2. Maintain cultures at 37°C with 5% (v/v) CO_2 in air, and re-feed with fresh medium every 48–72 h.
3. Antibodies to cellular retinoic acid binding protein 1 (ascitic fluid 1:250) should be used periodically to prove the cells retain their phenotype.

3.2.5. Subculture of RPE

1. Culture RPE cells in 75 cm² culture flasks and subculture at approximately 90% confluence. Aspirate medium and wash the cells with calcium and magnesium free PBS (pre-warmed to 37°C).

2. Incubate cultures at 37°C, 5% (v/v) CO_2 with 2 mL trypsin/EDTA for 5 min.
3. Observe cells rounding up and assist detachment by tapping the flask gently as previously described for HUVEC. Add Medium D10 to inhibit the action of the trypsin/EDTA, and transfer the suspension to a sterile 25 mL tube.
4. Centrifuge at $125 \times g$ for 10 min and re-suspend the pellet in 5 mL of the D10 medium. Seed cells on to 75 cm^2 culture flasks at a split ratio of approximately 1:4 or 1:5.
5. Add Medium D10 to give a final volume of 15 mL. Cultures are maintained at 37°C with 5% (v/v) CO_2 in air, and re-fed with fresh medium every 48–72 h (see Note 6).

3.3. Culture on Amnion

3.3.1. Culture of RPE on Amnion

1. The native epithelium of the amnion can be neutralised using distilled water or remove using 4% deoxycholate for two h at 20°C (21), or 0.25 M NH_4OH for 1–2 h at RT (22), or by Thermolysin 125 µg/mL buffered with sodium acetate 5 mM and calcium acetate 10 mM for 16 h at 4°C (22–24) (see Note 7).
2. Gently wash the membrane three times with sterile SAL/AB and remove any cells remaining on the amniotic membrane by gently rubbing with a sterile cotton bud.
3. After treatment as above, transfer each clip to a fresh 24 well plate containing 2 mL of Medium D0 per well. Store at 4°C until use.
4. Carefully seed the RPE cells at 1×10^5/mL (with 2 mL per well) onto the epithelial surface of the amnion. Wash carefully after 24 h to remove excess unattached RPE cells.
5. Results: The RPE cells exhibit normal epithelial morphology when grown on the amnionitic membrane. As mono-cultures, RPE cells became confluent 24 h after seeding onto the epithelial surface of the amniotic membrane only. They show a typical columnar appearance, with no multiple layering. Cell–cell contact regions show immunoreactivity to the TJ markers, occludin, and ZO-1 by 24 h but are negative for the endothelial junctional marker, VE-cadherin.

3.3.2. HUVEC Growth on Amnion

1. In a mirrored experiment to the above, the amniotic membrane in the MINUSHEET is placed into a 24-well plate (Fig. 2).
2. Carefully seed HUVEC cells at 1×10^5/mL (with 2 mL per well).
3. Results: HUVEC exhibit the typical cobble-stone appearance and reach confluence 48 h after seeding onto the interstitial surface of the amniotic membrane only. They fail to grow on

the other surface of the membrane. Immunocytochemistry confirms full expression of the adherens junction molecule VE-cadherin at cell–cell contacts. The TJ marker ZO-1, but not occludin, is also localised to the paracellular clefts.

3.3.3. Production of the Trilayer

1. Seed RPE cells onto amnion (as described in Subheading 3.3.1).
2. After 24 h, wash the membranes to remove unattached RPE cells and turn over the minusheet (with RPE attached).
3. The cells remain bathed in epithelial medium, up to the level of the minusheet. Seed the HUVECs at 1×10^5/mL onto the other (interstitial) surface and place endothelial media on to the minusheet.
4. Wash the membrane again 24 h later to remove unattached HUVECs.
5. Once confluence has been reached (~48 h), the trilayer can be placed into a single tissue gradient carrier in the dual perfusion chamber system (Minucells and Minutissue), endothelial side up, with endothelial medium (in upper chamber) nourishing the HUVECs, and RPE medium (in lower chamber) nourishing the RPE.
6. Connect the chambers to separate reservoirs to form a closed circuit containing 25 mL media for both epithelial and endothelial circuits. Replace Medium on a daily basis. This system is amenable to both static and flow culture (see Note 8).
7. Results: RPE and HUVEC form continuous monolayers on their preferred sides of the amnion. HUVEC grow to confluence within 24 h of co-culture with RPE and amnion.

3.4. Immunocytochemistry

1. Transfer the trilayers contained within the MINUSHEETS from the tissue carriers to 12 well plates.
2. Aspirate the culture media, wash cells and fix with 1% paraformaldehyde at room temperature (RT) for 10 min.
3. Wash cells 3 times with normal saline.
4. Permeabilise with 0.5% Triton in PBS.
5. Block with 5% BSA in PBS for 30 min at RT.
6. Incubate with the primary antibody for 1 h at 37°C or for 24°h at 4°C.
7. Wash with PBS.
8. Incubate with the secondary antibodies for 1 h at 37°C or for 24 h at 4°C.
9. Overlay with PBS/glycerol mixed in a ratio of 1:1 (see Note 9).

10. Add Vectashield with or without Propidium iodide (if desirable to discern cell nuclei) on the slide under the trilayer and over the trilayer under the cover slip.
11. Optical images can be compiled and tilted on the *x* and *z* axes to obtain information on the extent of paracellular clefts occupied by JAMs (2).
12. Results: The co-culture conditions do not disturb the molecular phenotype of the endothelial adherens junctions since VE-cadherin is still found at cell–cell contacts throughout this period. At 24 h, HUVEC retain localisation of the TJ marker, ZO-1, with the surface expression of occludin remaining cytoplasmic. However, increased paracellular localisation of occludin is observed by 48 h, and after 72 h co-culture with RPE most of the occludin is localised to cell–cell contacts in HUVEC.

3.5. Trilayer Culture Under Flow

1. The trilayer can be placed into a flow culture system (Minucells and Minutissue), 48 h after seeding the HUVEC at known concentrations.
2. Flow above and below the membrane can be set to the desired flow rate. Ensure that endothelial medium is nourishing the HUVECs, and epithelial medium is nourishing the RPE.
3. Medium should be replaced on a daily basis.

3.6. VEGF-A Secretion of the Trilayer

1. After creating the trilayer in flow (see Subheading 3.5), 2 mL samples can be taken at the desired time points.
2. Samples should be snap-frozen and stored at −20°C until further analysis.
3. Fresh medium should only replace the sample volume (2 mL) taken at each time point.
4. Coat Maxisorp wells with capture mAb (Anti-human VEGF-A antibody, from the 50 μL aliquots) at a dilution of 1:250 in PBS and leave at 4°C overnight. Use 100 μL per well for a 96-well plate.
5. Wash with PBS.
6. Block with 2% BSA for 1 h at RT.
7. Wash with PBS.
8. Prepare a standard curve of rVEGF-A from aliquots. Dilute in 0.1% PBS/BSA at 1:100 = 100 ng/mL, dilute again 1:50 for 2,000 pg/mL (first step of standard curve). Standard curve will be from 2,000 to 31 pg/mL (maybe further) in 1:2 dilutions. Dilute samples if necessary and leave standards and samples for at least 2 h at RT or preferably at 4°C overnight.
9. Wash with PBS.

10. Dilute with 0.5% PBS/BSA the detecting biotinylated anti-hVEGF to 200 ng/mL (44 μL of aliquoted Ab in 11 mL is 1:250 dilution) and leave for at least 1 h at RT.
11. Wash with PBS.
12. Add Biotinylated-streptavidin-HRP at 1: 2,000 in 1% PBS/BSA and leave for 1 h at RT.
13. Wash with PBS.
14. Add TMB and stop with acid after 10 min or whenever the colour changes.
15. Results: Secreted VEGF levels in the trilayer were seen to increase throughout the duration of co-culture and reached 7 ng/mL (+ 0.849 s.d.) by 72 h. Sampling at 24, 48, and 72 h revealed a sharp increase (doubling) in VEGF production between 48 and 72 h.

3.7. Measurement of Permeability or Tracer Flux

1. Place the trilayer in the MINUSHEET in the single tissue gradient carrier, endothelial side up.
2. Dissolve known concentrations of fluorescein and fluorescein-conjugated dextrans (4, 20 and 70 kDa) singly in the endothelial medium flowing through the upper chamber (see Note 10).
3. The lower chamber is bathed in the epithelial medium (as above) without tracer.
4. The chambers were connected to separate reservoirs and a closed circuit containing 25 mL media is obtained for both epithelial and endothelial circuits.
5. Samples of 200 mL can be taken at known time intervals from the lower chamber.
6. Examine fluorescence of the sample using an appropriate spectrometer, for example the Hitachi F-2000 Fluorescence Spectrophotometer.
7. The concentration of fluorescein and fluorescein-dextran in test samples are calculated by comparison against a standard concentration curve.
8. Results: The Amniotic membrane alone is freely permeable to sodium fluorescein (NaF) and 4 kDa FITC-conjugated dextran. Permeability of confluent monolayers of HUVEC to 4 kDa tracers was similar or slightly reduced to that measured for amnion alone. A 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 amnion show no statistical significance when compared to amnion alone. Under flow and 72 h after establishment of the trilayer, permeability to 4 kDa tracer is abolished with less

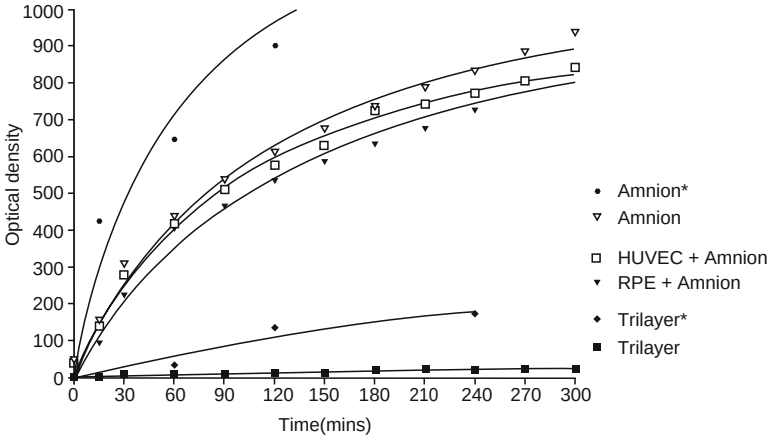

Fig. 3. Graph plotting leakage of 4 kDa fluorescein dextan tracer across amnion alone, amnion + HUVEC, amnion + RPE, amnion + HUVEC co-culture, and the trilayer (RPE + HUVEC co-culture) (*asterisks* shows sodium fluorescein as a comparison). Severe restriction of tracers can be seen in the trilayer plots.

than 5% of 4 kDa dextran being transferred. Moreover, transfer of NaF was seen to be severely restricted in the trilayer (Fig. 3).

3.8. Transmission Electron Microscopy (TEM)

1. To fix the trilayers and monolayers of RPE and HUVEC on amnion, as well as HCE and HUVEC on amnion, use modified Karnovsky's fixative (with 2.5% paraformaldehye, 2% glutaraldehdye in 0.1 M sodium cacodylate buffer, pH 7.4). Embed in resin and process for TEM, using standard protocols.
2. Thin (70 nm) sections should be viewed with an available TEM microscope (JOEL 1010 TEM microscope in our studies).
3. Results: In our study, the appearance of fenestrae in HUVEC after 3 days of co-culture with RPE was a novel exciting finding. In vivo and in monocultures HUVEC show a continuous non-fenestrated phenotype. Thus fidelity of origin appears to be lost when co-cultured with RPE in the trilayer architecture. This was not simply a consequence of co-culture with another epithelial cell type, since tri-layering with corneal epithelial cells did not induce such a change, suggesting this is an RPE-specific cross talk (2). The ultrastructural data highlights the usefulness of high resolution microscopy and strengthens the hypothesis that the EC phenotype (and indeed epithelial phenotype) is plastic and that its immediate environment is a more important determinant than the site of origin.

4. Notes

1. Store all media at 4°C and use within 4 weeks.
2. To make up the acid, goggles must be worn because of the exothermic nature of the reaction.
3. Maintaining the epithelial surface on the side of the black clip prevents confusion in later experiments.
4. Cryopreservation of amnion was not used (23) since freezing the amnion may produce breaks in otherwise healthy tissue that would then affect further studies, particularly the permeability studies.
5. For all RPE assays, it is recommended that the cells be used prior to passage 19 in order to maintain integrity of the phenotype. The cells should be regularly checked for phenotype by using antibodies to CRALBP.
6. For HUVEC assays, cells are used from passages 2 to 3 and are routinely checked for their endothelial nature by using antibodies to vascular specific VE-cadherin.
7. The authors prefer the use of neutralisation of the native epithelium of the amnion rather than using Thermolysin.
8. The flow system can be used to measure permeability of the trilayer to known tracers (see below).The flow rates for upper and lower chamber can be altered as necessary but if used at 0.1 mL/min, a shear stress of less than 0.25 dyne/cm^2 is present. This produces negligible shear stress for cells (17).
9. All steps are 100 µL per well. Wash with PBS containing 0.01% Tween between each step. Use 1:250 dilution (44 µL in 11 mL).
10. The reason for flow being present is also to ensure absence of any unstirred boundary layers, which would lead to an underestimation of solute flux.

Acknowledgements

This study was supported by grants from the Wellcome Trust.

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