Isolation, culture, and characterisation of human macular inner choroidal microvascular endothelial cells

A C Browning, T Gray, W M Amoaku

MATERIALS AND METHODS
Isolation of macular inner choroidal endothelial cells

Human posterior segments free of any known ocular disease were obtained from UK Transplant. The research had the approval of the local research ethics committee (Nottingham 2 LREC). After the iris had been removed, an 8 mm biopsy punch was positioned over the macula and pressure was applied, sufficient to penetrate the full thickness of the sclera. The macular sample was removed and transferred to a petri dish where the retina was discarded and retinal pigment epithelium (RPE) removed by gentle brushing with a sterile spatula and irrigation with sterile phosphate buffered saline (PBS). The choroid was then gently teased from the attached sclera and turned upside down. With the use of a dissecting microscope, the outer choroidal vessels along with adherent pigmented fibrous tissue were peeled off, leaving the relatively non-pigmented inner choroid and choriocapillaris/Bruch’s membrane complex. The tissue was cut into 1 mm pieces and washed three times in minimum essential medium (MEM) (Invitrogen Ltd, Paisley, UK) containing 30 mM HEPES, 0.25 g/ml amphotericin B, 100 µg/ml streptomycin, 50 µg/ml kanamycin, and 30 µg/ml penicillin (isolation medium). The pieces were incubated in 0.1% collagenase I (Sigma-Aldrich) in MEM for 2 hours at 37 ˚C, (isolation medium). The pieces were incubated in 0.1% collagenase I (Sigma-Aldrich) in MEM for 2 hours at 37 ˚C, and then Trypsin EDTA was used to separate the choroid from the retina. The choroid was then gently teased from the attached sclera and turned upside down. With the use of a dissecting microscope, the outer choroidal vessels along with adherent pigmented fibrous tissue were peeled off, leaving the relatively non-pigmented inner choroid and choriocapillaris/Bruch’s membrane complex. The tissue was cut into 1 mm pieces and washed three times in minimum essential medium (MEM) (Invitrogen Ltd, Paisley, UK) containing 30 mM HEPES, 0.25 g/ml amphotericin B, 100 µg/ml streptomycin, 50 µg/ml kanamycin, and 30 µg/ml penicillin (isolation medium). The pieces were incubated in 0.1% collagenase I (Sigma-Aldrich) in MEM for 2 hours at 37 ˚C, with frequent agitation. The collagenase was neutralised with MEM containing 10% fetal calf serum (Invitrogen Ltd, Paisley, UK), and the mixture filtered through sterile 40 µm and 20 µm filters (Millipore Ltd, Watford, UK). The eluate was centrifuged (75 g) and washed three times in isolation medium, and resuspended in 1 ml of PBS/BSA. The endothelial cells were then isolated as in the manufacturer’s instructions (Dynal Ltd, Wirral, UK). The Dynabeads were resuspended in endothelial growth medium (EGM2-MV with hydrocortisone omitted, Cambrex Biosciences, Wokingham, Berks, UK) and seeded onto fibronectin coated 35 mm culture dishes (Beckton Dickinson, Oxford, UK). The cells were incubated at 37 ˚C in a humidified atmosphere of 5% CO2 and the medium changed every 2 days. Cells were passaged with 0.025% trypsin and 0.01% EDTA in sterile PBS. All reagents were from Sigma-Aldrich, Poole, Dorset, UK unless otherwise specified.

Endothelial cell characterisation

After growing the cells (passage 1) on 1% gelatin coated glass cover slips (VWR Ltd, Poole, UK) and fixing them in ice cold 4% paraformaldehyde, the cells were stained with fluorescein isothiocyanate (FITC), fluorescein isothiocyanate, ICECs, inner choroidal endothelial cells; MEM, minimum essential medium; PBS, phosphate buffered saline; RPE, retinal pigment epithelium; TEM, transmission electron microscopy; VEGF, vascular endothelial growth factor.
methanol at −20°C for 20 minutes, a standard two stage immunofluorescence technique was applied, using primary antibodies detailed in table 1. (E-selectin expression was examined after exposure to TNF-α (100 pg/ml) (R and D Systems, Abingdon, UK) or PBS for 4 hours before fixation.) The secondary antibody used was rabbit anti-mouse F(ab')2 fragment, fluorescein isothiocyanate (FITC) conjugated (1:20 dilution) (Dako, Ely, Cambridgeshire, UK) in all cases except vWF, for which swine anti-rabbit F(ab')2 fragment FITC conjugate was used (1:20 dilution) (Dako). The slides were mounted in glycerol containing 2.5% 1–4 diazabicyclo [2, 2, 2]-octane (DABCO) (Sigma-Aldrich) and observed by confocal fluorescence microscopy (Leica TCS40D, Leica, Milton Keynes, UK). Frozen sections of fresh human placenta were used as a positive control for the vascular endothelial growth factor (VEGF) receptor antibodies, while HUVEC cells were used as a positive control for all other antibodies except for anti-α smooth muscle actin (anti-α-SMA) and anti-human fibroblast, for which human retinal pigment epithelial cells and human Tenon’s capsule fibroblasts were used respectively.

Transmission electron microscopy (TEM)
Isolated cells were grown to confluence on 35 mm fibronectin coated culture dishes (Becton Dickinson, Oxford, UK) The

### Table 1 Primary antibodies used in study

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Figure 1
Histological section of human submacular choroid stained with toludine blue (A) before and (B) after removal of the outer choroidal vessels. (B) Shows the residual choriocapillaris and inner choroidal vessels attached to Bruch’s membrane.

Figure 2
Phase contrast photomicrograph of confluent primary culture of ICECs demonstrating a typical cobblestone appearance (20× original magnification).

In vitro tube formation
A 1:1 mixture of chilled Matrigel (Beckton Dickinson Biosciences, Oxford, UK) and endothelial growth medium (EGM2-MV) was dispensed into pre-chilled wells of a 96 well plate. The Matrigel was allowed to solidify at 37°C for 30 minutes before isolated cells suspended in endothelial growth medium were seeded at a density of 4.8×10⁴ per well. The wells were observed hourly for the formation of tubes. For TEM, the endothelial growth medium was removed and replaced by 2.5% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4) for 16–24 hours and then fixed for 16–24 hours at 37°C. The fixed Matrigel was then gently removed from the well and processed for TEM as described previously.

RESULTS
Human posterior segments of six donors were obtained. The age of the donors (years) and the time from death to endothelial cell isolation (hours) were: 45 (52), 76 (42), 70 (43), 37 (60), 83 (60), and 56 (60).

Light microscopy after dissection showed the sample to comprise Bruch’s membrane along with adherent choriocapillaris and inner choroidal vessels (fig 1A and B). After isolation, the cells from all donors grew with a cobblestone morphology typical of endothelial cells (fig 2). The isolated cells showed a positive expression for vWF (fig 3A), CD31 (fig 3B), CD105 (fig 3C), VEGF receptor 1 (fig 3D), and receptor 2 (fig 3E), E-selectin before and after stimulation with TNFα (100 pg/ml) (fig 3F and G). The cells were negative for mouse anti-rat isotype control (fig 3H), α-SMA (fig 3I) and anti-fibroblast surface protein (fig 3J). Human RPE cells and Tenon’s capsule fibroblasts showed expression of α-SMA (fig 3K) and fibroblast surface protein (fig 3L), respectively.
Fenestrations were found scattered throughout the cells on TEM (fig 4). Cells seeded onto Matrigel formed capillary-like tube structures within 3 hours (fig 5), and TEM showed that these structures possessed a lumen (fig 6) and each lumen was surrounded by up to three cells joined by junctional complexes.

**DISCUSSION**

In view of the known heterogeneity of endothelial cells, it would appear logical to study endothelial cells derived from the macular inner choroidal/choriocapillaris area of humans when studying the disease mechanisms of ARMD. While methods have been described for the isolation of human choroidal endothelial cells that contain a mixture of both inner and outer choroidal endothelial cells, to date it has not been possible to reliably isolate human macular inner choroidal/choriocapillaris endothelial cells.

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**Figure 3** Immunofluorescent photomicrograph of human inner ICECs staining for (A) vWF, (B) CD31, (C) CD 105, (D) VEGF receptor 1, (E) VEGF receptor 2, (F) E-selectin (unstimulated), (G) E-selectin (post-TNF-α stimulation), (H) mouse anti-rat isotype negative control, (I) α-SMA, (J) fibroblast surface protein, (K) human RPE cells stained for α-SMA, (L) human Tenon’s capsule fibroblasts stained for fibroblast surface protein. (All 63 x original magnification.)

**Figure 4** Transmission electron micrograph of human ICECs grown on a collagen IV coated Thermox coverslip showing a typical fenestration (arrow) (bar = 200 nm).
We describe a method to isolate human macular ICECs cells that utilise observations made around 100 years ago. In 1892, Nuel described a natural cleavage plane within the human choroid between the layers of Haller (inner choroid) and Sattler (outer choroid), while in 1912, Salzmann noted that dissection of the choroid was easiest by tearing apart the layers from the outside. With the aid of a dissecting microscope and a microdissection technique, we easily peeled the large outer choroidal vessels from the underlying inner choroid/choriocapillaris complex. The subsequent isolation of endothelial cells was achieved using anti-CD31 coated paramagnetic beads. This purification technique replaces the laborious manual method of cell sweeping or the reliance on preferential EC proliferation by using endothelial cell specific culture medium and produces isolates with a purity in excess of 99%.

When grown in microvascular endothelial culture medium, the isolated cells formed typical cobblestone patterns consistent with their endothelial cell lineage.

The cells were found to stain strongly for CD31, a ubiquitous endothelial cell marker important in cell-cell adhesion, vWF, VEGF receptors 1 and 2, and CD105. The latter is a subunit of the TGF-β receptor found on proliferating vascular endothelial cells and has been suggested as a suitable antigen for antibody directed attack (immunotherapy) on the proliferating endothelial cells within choroidal neovascularisation complexes. The cells also showed expression of E-selectin (CD62E) after stimulation with TNF-α, a feature that is reported to be unique to endothelial cells. There appeared to be no contamination with RPE cells or fibroblasts.

In this study, the isolated cells also formed fenestrations when grown on fibronectin and formed capillary-like tubes when cultured in Matrigel. The lumen of these tubes was surrounded by two or more cells joined by junctional complexes, suggesting that the cells had not simply aggregated together randomly.

In summary, we have developed a method to isolate and culture human ICECs. During early passages, these cells possess the morphological characteristics of vascular endothelial cells, form fenestrations and capillary-like structures, and express a number of surface markers consistent with their endothelial cell origin. These cells may be useful in studying the pathophysiology and cellular mechanisms involved in choroidal neovascularisation.

ACKNOWLEDGEMENTS

We are grateful to the Kelman Foundation, USA, the British Eye Research Foundation (formerly the Iris Fund), and QMC University Hospital Trust for grant funding this project. The authors thank S Anderson and I Ward, School of Biomedical Sciences, University of Nottingham, for their assistance in conducting the confocal microscopy of the cells and I Zambrano, Eye Bank, Manchester Royal Infirmary, for the provision of the donor eye tissue.

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Competing interests: none declared

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Accepted for publication 13 May 2005

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