A Novel Choroidal Endothelial Cell Line Has a Decreased Affinity for the Age-Related Macular Degeneration–Associated Complement Factor H Variant 402H

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PURPOSE. Choroidal endothelial cells play a central role in the pathogenesis of age-related macular degeneration (AMD). Protocols for isolating primary choroidal endothelial cells have been described but require access to human donor eyes, which is a limiting factor. Therefore, a conditionally immortalized choroidal endothelial cell (ciChEnC) line has been established.

METHODS. Choroidal endothelial cells were selected by magnetic-activated cell sorting and conditionally immortalized using temperature-sensitive simian virus 40 large T antigen and human telomerase. The cell line obtained was characterized based on expression of endothelial marker proteins and endothelial cell–specific responses to various stimuli. Binding of AMD-associated and non-AMD variants of complement factor H in the context of a recombinant CCP6-8 (complement control protein domains 6-8) construct was determined using ELISA.

RESULTS. ciChEnCs maintained morphology and von Willebrand factor and vascular endothelial cadherin expression for up to 27 passages. The cells internalized acetylated low-density lipoprotein, formed tubes on Matrigel, and increased intercellular adhesion molecule-1 expression in response to tumor necrosis factor-α. Cells grew into dense monolayers with barrier function and showed characteristics of choriocapillary cells, such as expression of plasmalemma vesicle-associated protein, human leukocyte antigen ABC, carbonic anhydrase IV, and membrane indentations reflecting fenestrations. ciChEnCs synthesized glycosaminoglycans chondroitin sulfate and the complement factor H ligand heparan sulfate. Interestingly, binding of the AMD-associated 402H variant of factor H to ciChEnC was significantly decreased compared to the 402Y variant.

CONCLUSIONS. A novel ciChEnC cell line with choriocapillarity characteristics has been established and should greatly facilitate investigation of the pathogenesis of AMD in the context of the choriocapillary microenvironment.

Keywords: age-related macular degeneration, choriocapillaris, cell line, glyocalyx, complement system

Age-related macular degeneration (AMD) is the major cause of blindness in the elderly, with an estimated 300 million people worldwide expected to suffer from the disease by 2040.1 In the early stages of AMD, electron-dense deposits called drusen accumulate in the central retina (the macula), where these form within the Bruch’s membrane, the basal extracellular matrix separating the retinal pigment epithelium (RPE), and choriocapillary vessels. In the advanced stage of the disease progresses into two clinical phenotypes: The “dry” form of AMD is characterized by geographic atrophy of photoreceptor and RPE cells, which is often preceded by pathogenic changes in the underlying choroidal vasculature.2–6 In the “wet” form of AMD, aberrant neovascularization of the choriocapillaris across the Bruch’s membrane and RPE leads to retinal fluid leakage and severely impaired central vision.7 Clinically proven effective treatments for “dry” AMD have not yet been described, whereas monoclonal antibodies inhibiting vascular endothelial cell growth factor (VEGF) signaling have shown promise in the treatment of “wet” AMD.8 Unregulated activation of the complement system, particularly the alternative pathway, has been strongly implicated in the etiology of AMD. Drusen contain complement components,9,10 while mutations and AMD-associated polymorphisms have been identified in several proteins of the complement system, including the alternative pathway inhibitor complement factor H.11–14 In particular, a common factor H
polymorphism (Y402H) confers an increased risk for developing AMD15-17 and has been shown to impair the protein’s interaction with Bruch’s membrane and choroidal vessels.18

Therefore, choroidal endothelial cells (chEnCs) likely play a central role in both the “dry” and “wet” form of AMD. Investigation of their response to angiogenic stimuli as well as complement regulation in the choriocapillaris microenvironment could reveal novel treatment targets for AMD. While protocols for the isolation and culture of primary human chEnCs have been described19,20 they require a steady source of donor eyes, as cell viability in culture is limited. Conditional immortalization of (human) endothelial cells, which proliferate when cultured at 33°C and redifferentiate to primary-like cells during culture at 37°C, has proven successful for the culture of endothelial cells, which were previously difficult to obtain and maintain in culture.21-23

We describe here the generation and characterization of a novel, conditionally immortalized choroidal endothelial cell line (ciChEnC). The ciChEnC cell line has multiple phenotypic and functional features of chEnCs, which will facilitate mechanistic research aimed at AMD, including complement regulation in the microenvironment of the choriocapillaris.

MATERIALS AND METHODS
Isolation and Conditional Immortalization of Primary Choroidal Endothelial Cells
Primary chEnCs were isolated based on published protocols.19,20 A ~1-cm² tissue section around the macula was excised from the enucleated eye bulb of a 65-year-old female donor with ocular melanoma that did not affect the macula. There were no signs of macular degeneration in the unaffected or enucleated eye. Informed consent was obtained according to the tenets of the Declaration of Helsinki. Following removal of the neuroretina, the RPE in the macula area was carefully brushed from Bruch’s membrane using a sterile cotton swab. Bruch’s membrane and the underlying choroid were peeled from the sclera and digested using 200 U/mL collagenase II (Life Technologies, Breda, The Netherlands) in sterile Hank’s balanced solution (HBSS) for 4 hours at 37°C. The released cells were then filtered through 70-µm nylon filters (WVR International, Radnor, PA, USA), seeded in bovine fibronectin (1 µg/cm²; Bio-Connect, Huissen, The Netherlands)-coated culture flasks (Corning, Inc., Corning, NY, USA) containing microvascular endothelial cell growth medium (EGM-2 MV; Lonza, Verviers, Belgium), and grown to confluence. Monolayers were detached using 0.0125% (wt/vol) trypsin (Life Technologies)–coated culture flasks (Corning, Inc., Corning, NY, USA) and 0.5% (vol/vol) bovine serum albumin (BSA, Sigma-Aldrich Chemie) in PBS (pH 7.4) for 3 minutes. Subsequently, the cell monolayers were detached in enzyme-free cell dissociation buffer (Sigma-Aldrich) in EGM-2 MV for 18 hours before measuring tube formation assays, 3- and 200 total magnification respectively, using a Coolpix 990 camera (Nikon, Tokyo, Japan). The RPE cell line ARPE-19 was cultured in DMEM/F12 (Life Technologies) as described previously.25

Immunocytochemistry
Cells were cultured in slide flasks, washed twice in phosphate-buffered saline (PBS), and fixed for 10 minutes using 90% ice-cold acetone, or 2% paraformaldehyde for plasmalemma vesicle-associated protein (PLVAP), human leukocyte antigen (HLA)-ABC, and carbonic anhydrase IV staining. Cell monolayers were blocked for 1 hour at room temperature (RT) with 1% (wt/vol) bovine serum albumin (BSA, Sigma-Aldrich Chemie) in PBS. Cells were stained using goat anti-vascular endothelial cadherin antibody (Santa Cruz Biotechnology), rabbit anti-von Willebrand factor antibody (DAKO, Glostrup, Denmark), rabbit anti-PLVAP (Atlas Antibodies, Bromma, Sweden), mouse anti-HLA-ABC (ITK Diagnostics, Uithoorn, The Netherlands), or rabbit anti-carbonic anhydrase IV (Life Technologies) in 1% BSA in PBS for 1 hour at RT. Antibodies were detected using fluorescently labeled donkey anti-goat IgG:Alexa 594 or goat anti-rabbit IgG:Alexa 488 (Life Technologies) in 1% BSA in PBS for 1 hour at RT. For internalization of acetylated low-density lipoprotein (LDL), unfixed ciChEnCs or ARPE-19 cells were incubated with Alexa 488-labeled acetylated LDL (Life Technologies) in EGM-2 MV for 4 hours at 37°C. Stained cells were postfixed using 1% paraformaldehyde in PBS for 15 minutes at RT and embedded in Vectashield H-1000 mounting medium (Brunschwig Chemie, Amsterdam, The Netherlands) containing 4,6-diamidino-2-phenylindole. Pictures were taken at >200 total magnification, or ×100 total magnification for PLVAP, HLA-ABC, and carbonic anhydrase IV using an Axio Imager M1 microscope (Zeiss, Jena, Germany).

Analysis of ciChEnC Activation by Flow Cytometry
ciChEnCs were cultured at 37°C and activated using 10 ng/mL recombinant tumor necrosis factor-α (eBioscience, Vienna, Austria) in EGM-2 MV for 18 hours before measuring intercellular adhesion molecule-1 (ICAM-1) expression. Cells were detached in enzyme-free cell dissociation buffer (Sigma-Aldrich Chemie) for 30 minutes at RT, centrifuged at 5700g for 5 minutes, and resuspended in ice-cold 0.5% (wt/vol) BSA in PBS. ICAM-1 expression was detected using a mouse monoclonal anti-human ICAM-1 antibody (Clone HA58, eBioscience), followed by goat anti-mouse IgG:Alexa 488 conjugate (Life Technologies). Fluorescence was quantified using a FC500 flow cytometer with CXP2.2 software (Beckman Coulter, Miami, FL, USA), and mean fluorescence intensities were corrected for background staining of murine IgG1 isotype control antibody (Sigma-Aldrich Chemie).

Scanning Electron Microscopy (SEM)
ciChEnCs were grown at 37°C for 5 days on glass coverslips coated with 1 µg/cm² bovine fibronectin. After washing with serum-free EGM-2 MV at RT, the cell monolayer was prefixed with a 1:1 mixture of ice-cold serum-free EGM-2 MV and 2% glutaraldehyde (Sigma-Aldrich Chemie) in 0.1 M sodium cacodylate buffer (pH 7.5) for 5 minutes. Subsequently, the mixture was exchanged against ice-cold 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and the cells were fixated overnight at 4°C. Fixed cells were washed 3× with 0.1 M
sodium cacodylate buffer for 10 minutes, followed by sequential incubation with 1% (wt/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer, 1% (wt/vol) thio-carbohydrate in deionized water (MQ), and 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 15 minutes at RT. The cells were then washed in MQ and dehydrated by sequential incubation with 30%, 50%, 70%, and 90% ethanol in MQ for 5 minutes, followed by 100% ethanol and zeolite-dehydrated 100% ethanol for 15 minutes. Afterward, the cells were dried using hexamethyldisilazane (Sigma-Aldrich Chemie) and gold-coated using a 208HR sputter coater (Cressington, Redding, CA, USA). Pictures were taken using a SEM6340F electron microscope (Jeol, Tokyo, Japan).

**Glycosaminoglycan Isolation and Gel Electrophoresis**

Glycosaminoglycan (GAG) isolation from ciChEnC monolayers and analysis by gel electrophoresis were performed as described previously.28 GAGs from ~8-cm² monolayer were loaded on agarose gels, and electrophoretic mobilities of the observed GAG spots were compared to 0.5 µg/each of commercial standards for heparan, dermatan, and chondroitin sulfate (Sigma-Aldrich Chemie). The identity of ciChEnC GAGs was confirmed by digestion with 1.0 U/mL chondroitinase ABC (Sigma-Aldrich Chemie) in 50 mM Tris-acetate pH 8.0, 60 mM sodium acetate, or 0.1 U/mL heparinas I, II, and III (IBEX Technologies, Montreal, Canada) in 20 mM Tris-HCl pH 7.0, 100 mM sodium chloride, and 1.5 mM calcium chloride for 1 hour at 37°C.

**Transendothelial Electrical Resistance (TEER) Measurement**

TEER measurements were performed essentially as described previously.27 Briefly, ciChEnCs were seeded in polyester transwell inserts (0.4-µm pore size; Corning, Inc.) and cultured for 5 days at 37°C. Control wells were incubated with EGM-2 MV and refreshed simultaneously with the ciChEnC cultures. TEER was determined using a Millicell-ERS Volt-Ohm meter (EMD Millipore, Billerica, MA, USA). After measurement, ciChEnC monolayers were fixed using 1% paraformaldehyde and stained with 0.1% (wt/vol) crystal violet in 10% ethanol in MQ for 15 minutes to evaluate the presence of an intact monolayer. Pictures were taken at ×40 total magnification.

**Recombinant Factor H CCP6-8 Binding in ELISA**

ciChEnCs were seeded in 96-well tissue culture plates (Corning, Inc.) and cultured for 5 days at 37°C. Recombinant 402H and 402Y variants of factor H complement control protein domains 6 to 8 (CCP6-8) were expressed and purified as described previously.28 Cells were washed with HBBS, followed by incubation with 2-fold dilution series of 20 µg/mL CCP6-8 402Y and 402H in 2% BSA in HBSS for 20 minutes at RT. Protein binding was detected using polyclonal goat anti-human factor H antibody (Quidel, San Diego, CA, USA) followed by incubation with a 2-fold dilution series of 20 µg/mL recombinant CCP6-8 proteins in microtiter plates (NUNC, Roskilde, Denmark) followed by incubation with a 2-fold dilution series of anti-factor H antibody (Supplementary Fig. S1).

**Statistics**

Experiments were performed at least in triplicate and values are given as mean ± standard error of the mean. Groups were compared with Student’s t-test or ANOVA (>2 groups) using GraphPad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA).

**RESULTS**

**Choroidal Endothelial Cells Have Successfully Been Immortalized**

Conditionally immortalized and differentiated choroidal endothelial cells (ciChEnCs) grew into “cobblestone” monolayers comparable to primary chEnCs, with cell morphology being unaffected up to 27 passages (Figs. 1a, 1c, 1d). ciChEnC
expression of vascular endothelial cadherin (VE cadherin) and von Willebrand factor (vWF), both marker proteins for an endothelial phenotype, mirrors the expression by primary chEnCs and remained unchanged during prolonged cell culture as well (Figs. 1e, 1g, 1h). Culture at the proliferative temperature of 33°C resulted in significantly reduced VE cadherin expression and general absence of vWF-positive cells indicating dedifferentiation (Figs. 1b, 1f).

**ciChEnCs Show Endothelial Cell–Characteristic Responses to Various Stimuli**

To further investigate the endothelial phenotype, ciChEnCs were exposed to a number of stimuli that result in endothelial cell–specific responses. Endothelial cells express a scavenging receptor for acetylated LDL. Therefore, the ability of ciChEnCs to internalize fluorescently labeled acetylated LDL was investigated. ciChEnCs ingested acetylated LDL (Fig. 2a), whereas no uptake was observed for ARPE-19 cells, a RPE cell line (Fig. 2b). When cultured on Matrigel, a sarcoma-derived extracellular matrix rich in angiogenesis-promoting factors including VEGF, ciChEnCs formed intricate tubular networks, mimicking the formation of new blood vessels in vivo (Fig. 2c).

In response to inflammatory stimuli, endothelial cells increase the expression of cellular adhesion molecules such as ICAM-1, allowing leukocytes to adhere to the blood vessel wall and extravasate toward the site of inflammation. Accordingly, ciChEnCs increasingly expressed ICAM-1 after exposure to tumor necrosis factor-α (TNF-α) (Fig. 2d).

**ciChEnCs Share Characteristics With Choriocapillary Cells**

The choriocapillaris is a highly polarized, attenuated and fenestrated endothelial tissue. Choriocapillary fenestrations are generated in response to locally produced growth factors, particularly RPE cell–derived VEGF which is also present in the cell culture medium. Scanning electron microscopy of ciChEnCs reveals oval membrane indentations of 50- to 100-nm diameter consistent with fenestrations within the ciChEnC membrane (Figs. 3a, 3b). ciChEnCs stained strongly positive for HLA-ABC (Fig. 3c) and showed granular membrane staining for carbonic anhydrase IV, which are enriched and exclusively expressed, respectively, in the choriocapillaris. Similarly, PLVAP, which is contained in fenestral diaphragms, has been found to be selectively expressed in the choriocapillaris but not larger choroidal vessels. Probing ciChEnCs with antibodies specific for PLVAP (Fig. 3d) resulted in pronounced...
FIGURE 3. Conditionally immortalized choroidal endothelial cells contain membrane indentations consistent with fenestrations and express choriocapillary cell-specific proteins. Scanning electron microscopy of ciChEnCs reveals oval membrane indentations (a, arrowheads; scale bar: 1 μm) that are approximately 100 nm in diameter (b, scale bar: 0.1 μm) and reminiscent of fenestrae. ciChEnCs stain strongly positive for human leukocyte antigen (HLA)-ABC (c). Staining of ciChEnCs for plasmalemma vesicle-associated protein (PLVAP) (d) and carbonic anhydrase IV (e) results in granular membrane staining, whereas staining with isotype-matched rabbit IgG control is negative (f).
granular membrane staining, whereas the isotype-matched rabbit IgG controls revealed no staining (Fig. 3f).

ciChEnCs Express Endothelial Glycocalyx Components Heparan Sulfate and Chondroitin Sulfate

In vivo, endothelial cells are covered by a thick glycan layer called glycocalyx, which contains among others the GAG heparan sulfate (HS). Since HS plays a central role in controlling complement activation in the cellular microenvironment by binding to the alternative pathway inhibitor complement factor H, the expression of GAGs in ciChEnCs was evaluated. After resolving extracted GAGs by agarose gel electrophoresis, two distinct GAG spots were observed, which comigrated with commercial standards for HS and chondroitin sulfate, and which were efficiently degraded by incubation with the GAG-specific glycosidases heparinase I, II, and III, and chondroitinase ABC (Fig. 4).

ciChEnCs Form Dense Endothelial Monolayers With Barrier Function In Vitro

In glomerular and other endothelial cells, the glycocalyx provides a selective barrier against leakage of plasma proteins through endothelial fenestrations. Since the choriocapillaris is fenestrated and ciChEnCs express glycocalyx components in vitro, cells were cultured in transwell insert and the TEER was determined as model for endothelial barrier function. Crystal violet staining revealed dense monolayers (Fig. 5a), which increased TEER over the transwell insert by approximately 20 $\Omega\cdot$cm$^2$ compared to empty control wells (Fig. 5b).

AMD-Associated 402H Polymorphism Reduces ciChEnC Binding of Factor H CCP6-8

The common Y402H polymorphism in the CCP7 domain of complement factor H, 402H, confers an increased risk for AMD and alters the protein’s specificity for HS compared to the non-disease-associated 402Y variant. Since ciChEnCs were found to express HS, binding of both the non-AMD (402Y) and AMD-associated (402H) variant of factor H in the context of the recombinant CCP6-8 construct to ciChEnCs was determined. While both proteins showed a concentration-dependent increase in signal, binding of the AMD-associated 402H variant
The current study describes the successful conditional immortalization of human chEnCs, as cells could be maintained in culture at the permissive temperature of 33°C for up to 27 passages without signs of senescence. Cell morphology reflected primary chEnCs,19,20 as well as other conditionally immortalized human microvascular endothelial cells.22 ciChEnCs expressed the endothelial marker proteins vWF and VE cadherin at stable levels up to 27 passages at the nonpermissive temperature, whereas the reduced expression of these markers at 33°C suggests dedifferentiation during culture at the permissive temperature. The endothelial phenotype of ciChEnCs is further confirmed by their ability to internalize acetylated LDL, tube formation on Matrigel, and increased ICAM-1 expression in response to TNF-α. Angiogenesis inhibitors targeting VEGF have shown promise in treating the “wet” form of AMD.8 The strong response to angiogenic stimuli in Matrigel suggests that ciChEnCs can be applied in the screening of novel antiangiogenic drugs in a choroidal capillary context.

The choriocapillaris consists of endothelial cells that express fenestrations in response to VEGF produced by RPE cells.53 The EMG-2 MV cell culture medium used in the current study is supplemented with VEGF and, accordingly, ciChEnC membranes were found to contain membrane indentations ~100 nm in diameter that resemble fenestral openings. While the density and symmetric distribution of ciChEnC fenestrations are reduced compared to human choroidal endothelium in vivo,14 the scattered presence of fenestrations in endothelial cell membranes has also been described for primary choroidal microvascular endothelial cells when grown on fibronectin.19 However, the low density of fenestrations, as well as the low probability of capturing one complete, cytosol traversing fenestration in a single cut, makes confirmation of the cells’ choriocapillary origin by electron microscopy almost impossible. Therefore, the microvascular phenotype of the cell line was additionally evaluated through the expression of choriocapillaris-associated protein markers. ciChEnCs stained strongly positive for HLA-ABC, and showed granular membrane staining for PLVAP and carbonic anhydrase IV, which have been shown to be either enriched or selectively expressed in the choriocapillaris, but not larger choroidal vessels in vivo.35,36,38 Together, these results suggest that ciChEnCs have characteristics of choriocapillary cells in vitro.

ciChEnCs expressed the GAGs HS and chondroitin sulfate in vitro. Both components constitute the endothelial glycocalyx and have previously been detected in choroidal vessels using macular tissue sections.45 Since HS has been found to determine complement activation on self-surfaces by binding complement inhibitor factor H,40,42 ciChEnCs could provide a suitable substrate for investigating complement control within the choriocapillary microenvironment. The endothelial glycocalyx furthermore impedes vascular permeability.39 Although the choriocapillaris is generally considered highly permeable to small molecules in order to supply the retina with nutrients and oxygen, early and current studies indicate a selective barrier function of choriocapillary vessels.36–38 Accordingly, ciChEnC monolayers were found to increase TEER across transwell inserts by ~20 Ω·cm² in vitro, matching reported TEER ranges (13.4–26.5 Ω·cm²) for other human, non–brain-derived microvascular endothelial cells.90

Finally, ciChEnCs show a significantly reduced affinity for the AMD-associated 402H variant of recombinant factor H CCP6-8 compared to the non-AMD 402Y variant, reflecting earlier results obtained using human retinal tissue.18 Since the disease-associated polymorphism alters the protein’s specificity for heparin and HS,16,28,51 and the CCP6-8 region of factor H has been reported to be the primary binding site for HS in macular tissue,52 the reduced affinity of factor H for choroidal endothelium could result in unregulated alternative pathway activation in the choriocapillary microenvironment.

In conclusion, a ciChEnC line with choriocapillary characteristics has successfully been established and should contribute to unraveling the pathogenic mechanisms of AMD within the choriocapillary microenvironment.

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