Central serous chorioretinopathy (CSC), similar to other ocular diseases, finds its origin in the choroid. The neuroretinal detachment that characterizes CSC patients presumably occurs secondary to choroidal congestion, thickening, and hyperpermeability. This leads to dysfunction of the retinal pigment epithelium (RPE), which can subsequently cause leakage of serous fluid to under the neuroretina. The exact cause of CSC is unknown, but many risk factors have been addressed. Corticosteroid use is the most well-established exogenous risk factor in CSC, with reported odds ratios of up to 37. Remarkably, the established correlation between exogenous steroid use and the occurrence of CSC is
independent of duration and route of administration and prescribed dosage.19 Because corticosteroids bind to both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), it has been hypothesized that both are involved in the pathogenesis of CSC.5,9-12

Because of the plausible role of the choroidal endothelium in the pathophysiology of CSC, culturing of human choroidal endothelial cells (CECs) is of particular interest.13-16 Endothelial cells (ECs) are heterogeneous cells that show substantial site-specific and biochemical variation due to their interaction with their surroundings.17 This cell type is involved in maintaining the integrity of the blood vessel wall and controlling the transfer of molecules.18 ECs also interact with smooth muscles, platelets, and white blood cells through either paracrine or endocrine actions.19 Ocular ECs have been found to show significant variation in their function and response to certain treatments.20 Moreover, gene expression and cell signaling have been shown to differ significantly between ECs from different origins.21 Thus, the effect of corticosteroids on gene expression and signaling pathways in ECs should be studied preferably on a site- and species-specific basis. The successful isolation and culture of ECs in general, and from the choroid in particular, has been reported before by using various techniques.13-16

The primary goal of this study was to develop a culturing protocol for human primary donor-derived CECs and to assess the responsiveness of such CECs to corticosteroids, as a basis to gain knowledge on diseases associated with CEC dysfunction, such as CSC.

Materials and Methods

Human Donors

Eyes from human cadaveric donors (n = 8) were obtained from the Netherlands Brain Bank (Amsterdam, the Netherlands) (n = 2) or the Department of Anatomy and Embryology of Leiden University Medical Centre (Leiden, The Netherlands) (n = 6) and dissected within 24 hours after death. From every donor, both eyes were used for all experiments, which were processed and cultured separately. The number of donors used for each experiment and their age is stated in the caption of the figures. Donors had no known history of either ocular or vascular disease. The study was approved by the local medical ethical committee and followed the Tenets of the Declaration of Helsinki. Prior written informed consent for organ and tissue use in research was given by each donor.

Primary CEC Isolation and Culture

A flowchart for the isolation of human primary CECs is depicted in Figure 1. To isolate primary CECs from human eyes, an optimized protocol specific for this cell layer was designed based on the outcome of other studies.14-22 In brief, eyes were washed in 70% ethanol for 20 seconds and then placed on Hanks' balanced salt solution (Gibco Life Technologies, Paisley, UK) in a petri dish. Extraocular tissue and the anterior segment structures were removed. The eye was cut into three equal parts. After removing vitreous body remnants and the neuroretina, the peripheral part of the RPE/choroid complex was removed by cutting along the transition zone because CSC most often occurs foveally. The RPE/choroid complex was detached from the sclera, incubated for 1 hour at 37°C in 0.05% trypsin-EDTA (TE), and transferred to cold PBS (B. Braun Medical B.V., Oss, The Netherlands). The loosened RPE was gently removed as much as possible with a camel hair brush by using a dissection microscope (MZ6, Leica Camera, Wetzlar, Germany). The underlying choroid was washed in PBS at 300g for 3 minutes and digested in 2 ml of 0.2% collagenase type II (C2674; Sigma-Aldrich, Steinheim, Germany) on a 37°C shaking water bath. Digestion was stopped by adding 10 ml of Dulbecco's modified Eagle's medium (DMEM) without pyruvate (Gibco Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich). The suspension was filtered through a 30-μm cell strainer (MACS SmartStrainer; Miltenyi Biotec B.V., Leiden, The Netherlands), centrifuged at 300g for 5 minutes, and resuspended in EGM-2MV medium (BulletKit CC-3202 EGM-2MV [including 5% FCS, but without adding hydrocortisone]; Lonza, Walkersville, MD, USA). Cells were seeded in 3 wells of a 6-well plate, 1.5 ml of medium per well, which were coated with 1 ml of 100× diluted fibronectin (catalog no. F1114; Sigma-Aldrich) for 30 minutes at room temperature (RT). Cells were incubated at 37°C with 5% CO2. The medium was changed three times per week, and cell morphology was regularly checked with a microscope (Axiovert 25 CFL; Carl Zeiss AG, Oberkochen, Germany).

Magnetic-Activated Cell Sorting

After 7 to 10 days, when the primary mixed culture showed cell islands with EC-like morphology, magnetic-activated cell sorting (MACS) was used to isolate CECs. For this purpose, the anti-human CD31 MicroBead Kit and LS columns on a MidiMACS separator (all from Miltenyi Biotec B.V.) were used according to the manufacturer's instructions. A TE concentration of 0.01% was used, and cells were maintained in DMEM + 10% FCS. CECs were resuspended and seeded on fibronectin-coated 6-well plates in EGM-2MV medium (3 to 5 wells per eye, 1 ml fibronectin dilution per well, 1.5 ml EGM-2MV per well). Four to 7 days later, MACS was repeated to obtain sufficient pure CEC cultures, after which cells were seeded on 12-well plates (12 wells per eye, 0.5 ml fibronectin dilution per well, 1 ml EGM-2MV per well). All treatment experiments were performed during the second passage (i.e., after two MACS procedures) at near confluency and TriPure (Roche, Basel, Switzerland) was added directly afterward for RNA isolation.

Immunocytochemistry

Cells were cultured on chamber slides (Nunc Lab-Tek Chamber Slide System, C7182; Sigma-Aldrich) fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes and washed in PBS. Blocking was performed in 1% bovine serum albumin (A7906; Sigma-Aldrich) fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes, and resuspended in EGM-2MV medium (BulletKit CC-3202 EGM-2MV [including 5% FCS, but without adding hydrocortisone]; Lonza, Walkersville, MD, USA) for 30 minutes. The anti-VE-cadherin antibody (ab35168; Abcam, Cambridge, UK) was diluted in blocking buffer at 1:350 and left at 4°C for overnight incubation. Samples were washed with PBST for 5 minutes and incubated with Alexa Fluor 647 (A-21244; Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:250 in blocking solution for 90 minutes at RT. Finally, samples were washed with PBST and mounted with ProLong Gold Antifade with 4',6-diamidino-2-phenylindole (P36931; Thermo Fisher Scientific).

CEC Tube Formation Assay

Capillary-like tube formation is a functional characteristic of ECs when cultured on a 3D matrix.23 To further assess the functionality of the isolated CECs, growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was placed on prechilled μ-slide angiogenesis (Ibidi GmbH, Planegg/Martinsried, Germany), with a final thickness of 0.8 mm. On the same slide, other wells were coated with fibronectin and the slide was incubated for 50 minutes at 37°C to allow the coating material to solidify. An equal amount of CECs suspended in
EGM-2MV growth medium was seeded on either the Matrigel or fibronectin-coated wells and incubated at 37°C. Every 2 hours, CECs on both Matrigel and fibronectin were observed for cell growth and tube formation with a microscope.

Assessing the Optimal Treatment Regimen of Cortisol

To determine the most effective regimen of cortisol (H0888; Sigma-Aldrich) treatment, different treatment schedules were assessed (either short-time [4 hours], intermittent, or continuous treatment) (Table 1). The EGM-2MV medium contained 5 nM cortisol (originating from the fetal calf serum), resembling baseline cortisol levels. CECs were treated with either 10−7 M cortisol dissolved in ethanol or with vehicle (0.1% ethanol dissolved in EGM-2MV medium). Medium was changed for all samples at the four different time points. Every treatment group of all experiments consisted of a technical triplicate (3 wells of a 12-well plate).

Dose Response Curve

CECs were treated for 4 hours with a serial diluted cortisol dose starting with the highest dose or with vehicle. The maximum efficacy (E_max) between genes and half maximal effective concentration for gene expression response (EC50) were calculated using GraphPad Prism (version 7; GraphPad Software Inc., San Diego, CA, USA).

Treatment of CECs With GR and MR Agonists and Antagonists

The regulation of corticosteroid target genes directly mediated by the GR was assessed by comparing CECs treated with...
cortisol to those treated with different doses of the GR agonist dexamethasone (D4902; Sigma-Aldrich). Moreover, the effect on target gene expression mediated by MR was determined by comparing treatment with cortisol to treatment with different doses of the MR agonist aldosterone (A9477; Sigma-Aldrich). In addition, we evaluated the effect of the GR antagonist mifepristone (M8046; Sigma-Aldrich) and the MR antagonist spironolactone (S3378; Sigma-Aldrich) when administered together with cortisol. An overview of the conducted experiments is shown in Table 2.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

RNA isolation was performed using TriPure (Roche) according to manufacturer’s instructions. For reverse transcription, M-MLV reverse transcriptase was used, and quantitative PCR (qPCR) was performed with SYBR Green master mix, according to the manufacturer’s instructions (all Promega Benelux B.V., Leiden, The Netherlands). Gene expression was normalized to LRPI0 and calculated using the 2<sup>-ΔΔCt</sup> method. Corticosteroid responsive genes FKBP5, PER1, GILZ, and SGK1 were selected based on available literature. Markers for ECs, including vWF, CDH5, CD31, CD34, and PLVAP, the latter as a marker for fenestrated capillaries, were used. The expression of VIM was measured as a marker for fibroblasts to quantify possible contamination of the EC culture. Sequences of primers are shown in Supplementary Table S1.

Table 1. Overview of the Assessed Treatment Schedules

<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>Treatment</th>
<th>Time Points</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>5 nM cortisol</td>
<td>start of treatment 4 hrs 24 hrs 28 hrs</td>
</tr>
<tr>
<td>Continuous Treatment</td>
<td>5 nM cortisol</td>
<td>start of treatment 4 hrs 24 hrs 28 hrs</td>
</tr>
<tr>
<td>4 Hours Treatment</td>
<td>5 nM cortisol</td>
<td>start of treatment 4 hrs 24 hrs 28 hrs</td>
</tr>
<tr>
<td>Intermittent Treatment</td>
<td>5 nM cortisol</td>
<td>start of treatment 4 hrs 24 hrs 28 hrs</td>
</tr>
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Statistical Analysis

SPSS Statistics (version 20.0; IBM Corp., Armonk, NY, USA) was used to analyze the difference in relative gene expression between 10<sup>-7</sup> M cortisol and vehicle treatment. The expressions of eight genes were examined for distribution and were log-transformed to correct for skewness of data. Analysis was performed using a linear mixed effects model (P = 0.00625, corrected for multiple testing) on the log-transformed relative gene expression values per gene. To take into account that multiple samples and qPCR measurements were collected from the same donor (i.e., that data from the same donor are correlated), a random intercept term has been assumed. Only the treatment effect was investigated. The P values are reported with the 95% confidence interval of the log-transformed relative gene expression for both treated and control samples (Supplementary Table S2).

GraphPad Prism was used for statistical analysis for the experiments with dexamethasone, aldosterone, and antagonists, as well as for the dose response curve. To investigate...
differences between treatments, the mean difference between corticosteroid-treated and control samples was estimated using a 1-way ANOVA with Dunnett correction. For the statistical analysis of the dose-response curve, a Friedman ANOVA with Dunn's multiple comparison test was performed. A Student's paired t-test was used to analyze the differences in gene expression between the cultured CECs and the effluent after two MACS procedures. Differences were considered significant when a P value was <0.05. All data (arbitrary units) are normalized to their respective vehicle.

RESULTS

CEC Culture and Characterization

CECs were characterized by their EC-specific morphology, island formation (Fig. 2A), and cobblestone appearance (Fig. 2B), which was observed after 4 to 7 days. After two MACS procedures, CECs showed a convex polygonal monolayer with less than 5% of other cell populations. CECs showed clear capillary-like tube formation when cultured on Matrigel after 2 to 4 hours, resembling microvascular structures with a clear lumen (Figs. 2C, 2D). CECs stained positive for the intercellular junction marker vascular endothelial (VE)-cadherin (Fig. 2E), whereas MACS-isolated CD31-negative cells stained negative (Fig. 2F). Moreover, qPCR analysis of both the CEC cultures and the MACS effluent, showed high expression of the CEC-specific markers CD31, CD34, CDH5, vWF, and PLVAP in the CD31-positive cell population (Fig. 2G). For one donor, all EC markers were measured and the immunofluorescence staining was performed; for the other donors, CDH5 expression was measured as an indicator for purity of the CEC cultures.

Assessing the Optimal Treatment Regimen of 10⁻⁷ M Cortisol

When treated with cortisol for either 4 hours or according to the intermittent or continuous regimen, CECs showed an overall transcriptional upregulation of the investigated target genes (Fig. 3A). PER1 showed the highest fold change in expression, whereas FKB5 and GILZ showed moderate transcriptional upregulation. SGK1 showed no significant change in any of the conditions. In general, the effect of continuous cortisol on gene expression was less than that of either the 4-hour treatment or intermittent treatment. Intermittent treatment was used for the other experiments, as it mimics the diurnal rhythm of physiologic cortisol exposure.

We next analyzed in detail the effects of cortisol treatment on gene expression of primary corticosteroid responsive genes, the corticosteroid receptor coding genes, and endothelial/fibroblast cell markers in the CECs. GR (NR3C1), MR (NR3C2), four target genes (FKBP5, PER1, GILZ, and SGK1), VE-cadherin (CDH5) (EC marker), and vimentin (VIM) (fibroblast cell marker) were tested in all cortisol and vehicle-treated samples (Fig. 4). Both NR3C1 and NR3C2 were expressed in cultured CECs, with cycle threshold values of 24.6 ± 0.11 and 26.0 ± 0.14, respectively. The treatment of CECs with 10⁻⁷ M cortisol significantly induced the expression of FKB5, PER1, and GILZ (P < 0.0001 for all), whereas NR3C2 was downregulated in cortisol-treated samples compared with controls (P = 0.004). Finally, differences in NR3C1 and SGK1 between treated and control samples were nonsignificant, which was also the case for CDH5 and VIM (Supplementary Table S2). To test for possible transient upregulation of SGK1, a treatment for 45 minutes was also performed (Supplementary Figure S3E).

To assess whether in vitro culture might diminish MR or GR expression, mRNA of directly postmortem isolated CECs and protein of cultured CECs were analyzed. Expression of the MR appeared to be variable and substantially lower than the GR and did not differ from the cultured CECs (Supplementary Figure S3E).

Dose-Response Curve

The three target genes FKB5, PER1, and GILZ were dose-dependently induced by cortisol (Fig. 5B), with different E_max and EC₅₀ values. PER1 had the highest E_max, whereas FKB5 and GILZ had a lower and very similar E_max, respectively. FKB5 induction had a low EC₅₀ (6.3 nM), whereas PER1 and GILZ had higher EC₅₀ values (30 nM and 55 nM, respectively). SGK1 did not respond to any of the cortisol doses.

Effect on Target Gene Expression of Treatment With GR and MR Agonists and Antagonists

The administration of the GR agonist dexamethasone resulted in comparable induction of corticosteroid target genes to that induced by cortisol, whereas the GR agonist aldosterone did not induce changes in gene expression in any of the target genes (Figs. 3C, 5A).

The effects of cortisol were blocked after cotreatment with the GR antagonist mifepristone but not by the MR antagonist spironolactone (Fig. 5B). Moreover, the continuous administration of spironolactone did not lead to transcriptional upregulation of the genes of interest, arguing against transcriptional activation via MR caused by cortisol present in the used serum (Fig. 5C). An overview of (additional/replication) experiments that concerned treatment with MR agonists and antagonists is shown in Supplementary Figure S3.
Figure 2. Culture and characterization of human primary CECs. (A–D) Phase contrast photomicrographs of primary CEC cultures. (A) Primary choroidal mixed cell culture showing an EC-like island after 2 to 3 days of culture on fibronectin in EGM-2MV medium. (B) Primary culture after MACS showing characteristic EC morphology (cobblestone appearance) with little or no noticed growth of other cell types. (C) Primary CECs seeded on Matrigel show characteristic formation of capillary-like structures after 2 hours of culture. (D) After 4 hours, CECs cultured on Matrigel continue to form tubular networks, indicating the ability of these cells to simulate the capillary forming function of ECs. Scale bars are shown in each panel. (E, F) Immunofluorescence photomicrographs of MACS-isolated CECs (E) and the effluent (F), stained for VE-cadherin (anti-VE-cadherin...
antibody; 1:350 dilution), using Alexa Fluor 647-conjugated goat anti-rabbit IgG secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Staining for VE-cadherin in MACS-isolated CECs (E) is localized at the plasma membrane. 40x original magnification. (G) Expression of vimentin and CEC-specific markers after two MACS procedures for the CECs and the effluent cultured, which were cultured under the same conditions. Gene expression of the effluent was normalized to CECs. Paired t-test, *P < 0.05; **P < 0.01. n = 1 donor (age 94).

**FIGURE 3.** Experiments with glucocorticoid and MR agonists. (A) Expression of corticosteroid target genes (FKBP5, GILZ, PER1, and SGK1) in CECs in response to the different treatment regimens with cortisol. Error bars represent standard deviation (SD). ***P = 0.0002; ****P < 0.0001, n = 3 donors (ages 77, 68, 93). (B) Dose-response curve of cortisol. Expression of corticosteroid target genes in CECs after treatment with increasing concentrations of cortisol. PER1 has the largest maximum efficacy (E_{max}) compared with FKBP5 and GILZ. Error bars represent SD. SGK1, in contrast to the other genes, did not respond to the treatment. n = 1 donor (age 92). (C) Assessment of the most effective glucocorticoid. Expression of corticosteroid target genes in response to intermittent treatment with either cortisol or dexamethasone in two different concentrations (and vehicle as negative control). Comparable results were observed after dexamethasone and after cortisol administration. Error bars represent SD. ***P = 0.0002; ****P < 0.0001. n = 1 donor (age 66). EC_{50}, half maximal effective concentration for gene expression response.
DISCUSSION

In this study, we established a human CEC culture that is responsive to corticosteroid hormones. The administration of cortisol to human CECs in an intermittent rhythm led to the upregulation of primary corticosteroid target genes, and this was mediated via the GR. As exogenous corticosteroid exposure is the single most important risk factor for CSC, we propose that this system may be used for research into this common chorioretinopathy. Conversely, corticosteroids are an important means of treatment for other ocular diseases that appear to originate in the choroid, such as several forms of posterior uveitis. We therefore propose that the described human CEC culture model can also be used for research into these ocular diseases that involve the choroid and choroidal endothelium. In contrast with available literature on isolating and culturing CECs, we separated the RPE and the choroid by using 0.05% TE at 37°C for 1 hour, which was already sufficient for brushing the RPE from the choroid and resulted in sufficient CEC culture. Vimentin has been proposed as a specific fibroblast marker, but its expression levels did not correspond to our gene expression analysis. Therefore, our study lacks a suitable marker to detect fibroblast contamination. This would need attention in future studies because the assessment of the purity of our CEC cultures can only be interpreted by expression of EC-specific markers, such as VE-cadherin. Besides the fact that we performed a comprehensive characterization to confirm the CEC phenotype, we also describe the first detailed protocol to study the effects of corticosteroids on CECs.

Interestingly, the administration of cortisol to human CECs in an intermittent rhythm rather than a continuous administration in this study showed a stable response of corticosteroid target genes in all samples, and this response was mostly higher than in the continuous cortisol administration. The strong response of these genes to intermittent cortisol administration may be explained by the fact that this intermittent stimulation comes closer to the in vivo situation with diurnal rhythm in blood cortisol levels as compared with continuous stimulation. Continuous high cortisol levels may lead to desensitization of the response due to homologous downregulation or other mechanisms.

All four tested corticosteroid responsive genes have been shown to be regulated by both the GR and the MR in several studies. Although the target genes were not responsive to aldosterone treatment in our CEC experiments, studies have demonstrated that these genes are regulated by the MR in other tissues, such as cortical-collecting duct cells. The mRNA of FKBP5, GILZ, and SGK1 showed significant upregulation after cortisol treatment in our study. The lack of any effect on SGK1 may be related to its transient induction upon hormone stimulation. However, after treatment for 45 minutes, no SGK1 upregulation was found, which argues for the possibility that SGK1 is regulated in a tissue-specific manner (Supplementary Fig. S5E). Interestingly, GR-mediated responses to corticosteroids were evident, but MR-mediated responses could not be established in these experiments. Dexamethasone induced a similar effect on the target genes as cortisol, whereas the MR-specific agonist aldosterone was without effect. Furthermore, blocking GR activation during cortisol treatment with the MR antagonist mifepristone demonstrated similar results as the vehicle treatment, suggesting that the GR is essential for the induction of corticosteroid target genes in CECs. On the other hand, the MR antagonist spironolactone, which is the most potent MR antagonist currently used in clinical practice, did not attenuate cortisol-induced target gene expression. Of note, we included 5% of cortisol-containing calf serum in our culture medium, under the assumption that a model for disease development should compare (normal) low to moderate exposure to (pathogenic) high exposure, rather than an all-or-nothing response. The lack of an effect of continuous MR-antagonist treatment argues against the possibility that low basal cortisol levels may already activate the high-affinity MR.

Our study results were obtained from a CEC culture model and cannot be compared directly to in vivo animal and human studies as our results do contrast with these previous studies. After all, these studies emphasized the importance of the MR and the possible role of MR antagonists in CSC. After intravitreal injection of the glucocorticoid corticosterone in rat eyes, choroidal enlargement and hyperpermeability have been observed, resembling abnormalities in CSC. Comparable results were seen after injection of aldosterone, pointing to a role for MR activation in CSC. Furthermore, the finding of CSC-like changes in primary hyperaldosteronism indicate a role for the MR in the pathogenesis of CSC. Zhao et al. have proposed an underlying mechanism of this association: aldosterone could lead to transcriptional upregulation of the endothelial vasodilatory potassium channel KCa2.3, and blockade of this channel prevents aldosterone-induced choroidal thickening (pachychoroid). which is typical for CSC and which has led to the introduction of MR antagonists for CSC in clinical practice. Nonetheless, the variation in the KCa2.3
channel was very small at the protein level, suggesting the involvement of other mechanisms in inducing the observed choroidal changes in the rat model. Moreover, no serous fluid under the retina was observed during these studies, which is a typical finding for CSC. Despite several retrospective reports on a possible reduction and/or resolution of subretinal fluid after treatment with the MR antagonists eplerenone and spironolactone in CSC patients, the efficacy of these medications is under debate, and the spontaneous resolution of subretinal fluid may also occur in CSC. Large
prospective for Central Serous Chorioretinopathy

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