Effects of Cytokine Activation and Oxidative Stress on the Function of the Human Embryonic Stem Cell–Derived Retinal Pigment Epithelial Cells

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PURPOSE. In several retinal complications, such as age-dependent macular degeneration (AMD), oxidative stress is increased and cytokine level is elevated. These are shown to alter the activation and expression of matrix metalloproteinase (MMP) both in human primary and immortalized retinal pigment epithelial (RPE) cells. However, the effects on human embryonic stem cell (hESC)–derived RPE cells remain to be elucidated.

METHODS. The mature hESC-RPE cells were exposed to inflammatory cytokines (IFN-γ or TNF-α) for 24 hours or oxidative stress (H2O2) for 1 hour. Effects on barrier properties were analyzed with transepithelial electrical resistance (TEER), the expression of MMP-1, MMP-2, MMP-3, MMP-9, collagen I, and collagen IV genes with quantitative RT-PCR, and the expression of MMP-1 and MMP-3 proteins with Western blot or ELISA, respectively. Also, activation and secretion of MMP-2 and -9 proteins were analyzed with zymography.

RESULTS. In normal state, mature hESC-RPE cells expressed MMP-1, -2, -3, and -9 genes in low levels, respectively. Tumor necrosis factor-α increased MMP-1 and -2 gene expression, and H2O2 increased MMP-3 and -9 gene expression. Zymography revealed IFN-γ- and TNF-α–induced secretion of MMP-2 and high-molecular-weight species of MMP (HMW MMP), but H2O2 decreased their secretion. Furthermore, TNF-α and H2O2 significantly decreased barrier properties.

CONCLUSIONS. Here, cytokines induced the MMP-1 and -2 gene and protein expression. Also, H2O2 induced MMP-3 and -9 gene expression, but not their protein secretion. These data suggest that oxidative stress and cytokine stimuli, mature hESC-RPE cells resemble their native counterpart in the human eye in regard to MMP secretion and expression and could be used to model retinal disorders involving alterations in MMP activity such as AMD, diabetic retinopathy, or proliferative vitreoretinopathy in vitro.

Keywords: stem cells, matrix metalloproteinase, MMP, retinal pigment epithelium

The retinal pigment epithelium (RPE) is an epithelial monolayer, situated on the Bruch’s membrane (BrM) between photoreceptors and choroid. It has a wide variety of functions necessary to uphold vision, such as the phagocytosis of the photoreceptor outer segments, recycling of the visual pigment and the maintenance of the photoreceptors.1 RPE cells also modulate the extracellular matrix (ECM) of the BrM, which is important for the protection of the BrM as a filter barrier.2 The RPE is probably the most pivotal cell in the development of age-related macular degeneration (AMD), the main cause for blindness in the Western world, and the understanding of RPE biology and pathology is of high scientific and medical interest.1,2

To enhance the knowledge on RPE cells, to investigate new treatment option, or to even be used for RPE replacement therapy, human pluripotent stem cell (hPSC)-derived RPE cells have been generated.3–6 These hPSC-RPE cells are shown to have closer resemblance to the native RPE according to their gene expression,7 barrier properties,8 morphology, and drug transport functionality9 than widely used immortalized RPE cell lines, such as ARPE-19.

Matrix metalloproteinases (MMPs), a group of metzincin proteases, comprise more than 20 zinc endopeptidases that regulate cell matrix composition by organizing and cleaving basal lamina and ECM proteins.10 Most of the MMPs are secreted to the extracellular space as inactive proproteins that get activated when cleaved by ECM proteinases.10 The RPE is known to produce several MMPs, for example, interstitial collagenase MMP-1 and stromelysin-1, MMP-3,11,12 and the gelatinases MMP-2 and MMP-9.11,13 In several retinal diseases, MMP levels are altered: MMP-1 is increased in patients with proliferative vitreoretinopathy (PVR)14,15 but not in AMD.16 MMP-2 levels are elevated in PVR, AMD, and diabetic retinopathy (DR).14,15,17 (For review, see Ref. 18.) Matrix metalloproteinase-3 in turn is increased in PVR,13 and MMP-9 levels are increased in patients with PVR14 and DR.16,19 Otherwise, a reduction of the function of MMP-2 and MMP-9
has been implicated in the thickening of BrM and the development of atrophic AMD.\(^{20}\)

Oxidative stress and the appearance of reactive oxygen species (ROS) are considered to have an essential role in the pathogenesis of several retinal diseases such as AMD\(^{21-23}\) and DR.\(^{24}\) Due to high oxygen tension, constant light exposure, high mitochondrial activity, and lipid peroxidation, the retina is constantly exposed to oxidative stress. In healthy retina, RPE is highly adapted to the high oxygenated environment and reacts to oxidative stress, protecting itself and its surrounding tissue.\(^{24}\) This ability, however, decreases with age and may contribute to the development of AMD.\(^{25}\) Vice versa, oxidative stress may induce premature aging in RPE cells.\(^{26}\) Oxidative stress also alters MMP expression and secretion in RPE, inducing MMP-1 and MMP-3 activity while reducing the activity of MMP-2.\(^{27-28}\)

In addition, proinflammatory cytokines are considered to be of importance in the development of retinal disease such as AMD. Tumor necrosis factor-\(\alpha\) is a potent pleiotropic proinflammatory cytokine with a central role in several retinal diseases\(^{29}\) such as PVR and AMD.\(^{30}\) Anti-TNF-\(\alpha\) treatment for wet-age-related macular degeneration has indeed shown promising results in small-sized patient series.\(^{31}\) The TNF-\(\alpha\) induces migration and proliferation in adult primary and immortalized RPE cells\(^{32,33}\) and causes breakdown in barrier function in porcine primary RPE.\(^{34}\) Moreover, TNF-\(\alpha\) induces several MMPs, such as MMP-1, MMP-2, MMP-3, and MMP-9, both in primary and immortalized human RPE cells.\(^{35,35}\)

Interferon \(\gamma\), a proinflammatory factor found in inflammatory intraocular disorders,\(^{36}\) is proposed to also have an immune modulatory role in AMD.\(^{37}\) Little is known about its effect in RPE cells, but it has been described to slightly reduce MMP-2 production in human primary RPE cells, indicating a possible inhibitory role in these cells.\(^{35}\)

To use human embryonic stem cell (hESC)-RPE cells as a model to study the behavior of native RPE cells, these cells need to be biologically functional and to resemble their behavior under pathologic stimuli conditions. Therefore, in this study, we investigate the influence of oxidative stress and cytokine stimuli, as present in several retinal diseases, on the expression and function of MMPs in hESC-RPE cells.

**MATERIALS AND METHODS**

**Cell Lines**

In this study, we used three pluripotent hESC lines, Regea08/017, Regea08/023, and Regea11/013, that have been derived and characterized in our laboratory\(^{38}\) and cultured and differentiatated as previously described.\(^{39,40}\) Briefly, the hESC lines were cultured on top of mitomycin (10 \(\mu\)g/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) treated human foreskin fibroblast feeder cells (CRL-2249TM; ATCC, Manassas, VA, USA). The hESCs were cultured at 37°C and 5% \(\text{CO}_2\) with hESC culture medium consisting of knockout Dulbecco’s modified Eagle’s medium supplemented with 20% KnockOut Serum Replacement (KO-SR), 2 mM Glutamax-I, 0.1 mM 2-mercaptoethanol (all from Life Technologies, Carlsbad, CA, USA), 1% nonessential amino acids, 50 U/m penicillin/streptomycin (both from Lonza Group Ltd., Basel, Switzerland), and 8 ng/mL human BFGF (Peprotech, London, UK). The culture medium was changed five times a week, and undifferentiated colonies were enzymatically selected onto new feeder cells every 10 days. The undifferentiated hESCs were manually selected and induced to differentiate into RPE cells in floating cell aggregates by reducing the KO-SR concentration to 15% and removing the BFGF, as previously described in Reference 40. Thereafter, floating aggregates were cultured for 70 to 155 days (Table 1). The culture medium (RPEbasic\(^{25}\)) was replenished three times per week. Thereafter, pigmented areas of floating aggregates were dissociated, dissociated with 1× Trypsin-EDTA, and replated on human placental collagen IV (5 \(\mu\)g/cm\(^2\); Sigma-Aldrich Corp.) for enrichment of cell number as described in Reference 39.

From the enriched cultures, the pigmented areas were detached and seeded at a density of 100,000 cells/cm\(^2\) either on polyethylene terephthalate (PET) cell culture inserts (Millipore Corporate, Billerica, MA, USA) or 24-well plates (BD Biosciences, San Jose, CA, USA), both coated with collagen IV. The independent experiments are presented in Table 1.

**Treatments**

Mature hESC-RPE cells were subjected to cytokine treatment with 10 (i.e., 200 U/mL) or 50 ng/mL (i.e., 1000 U/mL) TNF-\(\alpha\) (Peprotech, London, UK) according to the protocol presented in Reference 34 or with 100 (i.e., 0.005 ng/mL) or 500 ng/mL (i.e., 0.025 ng/mL) IFN-\(\gamma\) (Peprotech) according to Reference 41. Both cytokines were diluted in RPEbasic medium, and the treatment was carried out for 24 hours in 37°C. Oxidative stress was induced for 1 hour and chased for 24 hours according to the protocol in Reference 28 with 300 or 600 \(\mu\)M H\(_2\)O\(_2\) (Sigma-Aldrich Corp.) in 37°C. Cells were then washed with PBS and cultured for another 24 hours in RPEbasic medium.

**Immunofluorescence Labeling**

Cells were labeled as described in Reference 39. Briefly, the cells were washed 3×5 minutes with 1× PBS (Lonza Group, Ltd.), fixed 10 minutes with 4% paraformaldehyde (pH 7.4; Sigma-Aldrich Corp.), and washed with PBS. Cells were permeabilized with 0.1% Triton X-100/PBS (Sigma-Aldrich Corp.) for 10 minutes and then washed three times with PBS. Nonspecific binding sites were blocked with 3% BSA (Sigma-Aldrich Corp.) in PBS at room temperature for 1 hour. Primary antibody staining was done for 1 hour in 0.5% BSA-PBS with primary antibody mouse anti-Na\(^+\)/K\(^+\) ATPase (1:50) and rabbit anti-bestiophin (1:500), both from Abcam (Cambridge, UK). Cells were then washed three times with PBS. The secondary antibody incubations were done in 0.5% BSA-PBS with secondary antibody donkey anti-mouse IgG (Alexa Fluor 488) or goat anti-rabbit IgG (Alexa Fluor 568; all from Molecular Probes, Life Technologies, Paisley, UK) at a dilution of 1:1500. Nuclei were stained with 4′,6-diamidino-2-phenylindole included in the mounting media (Vector Laboratories, Inc., Burlingame, CA, USA). The labeling procedure was performed at room temperature. Confocal images were obtained with a LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) using a 63× oil immersion objective. Image processing was done in ZEN software (Carl Zeiss). The brightfield images were obtained using a Zeiss AxiosScope A1 (Carl Zeiss) with a 40× objective.

**Transepithelial Electric Resistance**

Effects on barrier function were evaluated by measuring the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system voltohm meter (Merck Millipore, Darmstadt, Germany) for all samples immediately before commencing the experiment. The medium was changed for all samples at the same time. The cytokine treatment was carried out for 24 hours in 37°C, and oxidative stress was induced for 1 hour and chased for 24 hours. The TEER was measured immediately after the experiments were finished.
The control medium was measured 24 hours after change of the medium. The tests were done in duplicate for three independent replicates with three different cell lines. The TEER (Ω cm²) was calculated by subtracting the value of the empty PET filter insert from the ones containing cells and multiplying the value with the cell culture area. The TEER values (Ω cm²) were normalized by subtracting all values with the averages of controls. The results are presented as the ratio against the control sample.

RNA Extraction and cDNA Synthesis

Total RNA was extracted with a NucleoSpin XS-kit (Macherey-Nagel, GmbH & Co, Düren, Germany) or RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturers’ instructions. Total RNA for PCR was extracted from three independent replicates with three different mature hESC-RPE cell lines, and for quantitative real-time PCR (qRT-PCR) was extracted from two independent Regea08/017, Regea08/023, and Regea11/013 hESC-RPE cultures.

PCR Reaction

Complementary DNA was used as a template in the following PCR reaction according to the protocol that has been previously presented in Reference 40. Complementary DNA was used as a template in the following PCR reaction, which was carried out using 5 U/μL Taq DNA Polymerase (Fermentas; Thermo Fisher Scientific, Inc., Leicestershire, UK) with 5 μM primers specific for particular genes (Biosmers.net GmbH, Söflinger, Germany) (Table 2). The PCR reactions were carried out in PCR MasterCycler ep gradient (Eppendorf AG, Hamburg, Germany) with the following program: 95°C for 5 minutes, 95°C for 30 seconds, annealing for 30 seconds, 72°C for 1 minute, and 72°C for 5 minutes for 38 cycles. The primers annealing temperatures are presented in Table 2. Polymerase chain reaction products were investigated as described in Reference 40.

Quantitative RT-PCR

The qRT-PCR for the quantification of MMP-1, MMP-2, MMP-3, MMP-9, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was performed using the LightCycler 2.0 instrument (Roche, Mannheim, Germany) and Universal Probe Library probes and associated primers (Roche) (Table 3), with initial denaturation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The amount of mRNA was quantitated by using a standard curve. The expression levels of MMPs were normalized against the housekeeping gene GAPDH, and the samples of the different treatments were then compared to the nontreated sample.

Expression of ECM genes COL1A1 (Hs00164004_m1) and COL4A1 (Hs00266237_m1) and a housekeeping gene GAPDH (Hs99999905_m1) was assessed and analyzed similarly as in Reference 39.

Enzyme-Linked Immunosorbent Assay

Potential activation of total MMP-3 secretion was evaluated 24 hours after treatment from the culture media collected from apical compartments of two biological duplicates from three independent replicates of three different cell lines of mature hESC-RPE cells cultured on inserts. The MMP-3 concentration was determined using Quantikine ELISA Human total MMP-3 according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

Zymography

Medium from the apical and basal compartments from two biological duplicates from three independent replicates of the

<p>| Table 1. Mean Culture Period of Cell Samples (Range of Culture Periods Is in Parentheses) |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Adherent Culture (SD)</th>
<th>Entire Culture Period (SD)</th>
<th>Gene Expression (RT-PCR and qRT-PCR)</th>
<th>Protein Expression (WB)</th>
<th>TEER and Zymography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regea08/017</td>
<td>d111 (97–125)</td>
<td>d509 (277–416)</td>
<td>d155 (113–225)</td>
<td>d268 (237–288)</td>
<td>d225</td>
</tr>
<tr>
<td>Regea08/023</td>
<td>d111 (97–125)</td>
<td>d274 (227–298)</td>
<td>d197 (113–211)</td>
<td>d234 (70–302)</td>
<td>d70</td>
</tr>
</tbody>
</table>

<p>| Table 2. Reverse Transcriptase–PCR Primer Sequences and Used Annealing Temperatures (Tm) |
|-----------------|-----------------|-----------------|-----------------|
| Primer Sequences (5’–3’) |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTTGGACATGCTAGCCGCCATC</td>
<td>GGAATTTGCCAATGGGTTGA</td>
<td>55</td>
</tr>
<tr>
<td>OCT 3/4</td>
<td>CGTAGAAGCTGTGAGAGAGACTG</td>
<td>AAAGGCCGCACCTACCATATTC</td>
<td>55</td>
</tr>
<tr>
<td>RAX</td>
<td>CTTAAGACAGGAGCACATT</td>
<td>CTCTGGGATTGCCGAAGTTT</td>
<td>55</td>
</tr>
<tr>
<td>PAX6</td>
<td>AACAGACACAGCCCTCCACAACA</td>
<td>CGGGAATCTGGAATGGAATC</td>
<td>60</td>
</tr>
<tr>
<td>MITF</td>
<td>AAGTTGAGGCCCTGTCCATGT</td>
<td>GGCAGACCTTTGTTCCATA</td>
<td>52</td>
</tr>
<tr>
<td>Bestrophin</td>
<td>GAATTGAGGCTGTCCCTGT</td>
<td>ATCCAGGAGAGCAAGGAT</td>
<td>60</td>
</tr>
<tr>
<td>RPE65</td>
<td>TCCCCAATACAAATGCACAT</td>
<td>CCCACCACTACAGAATCTA</td>
<td>52</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>TGCCACGATCTATCTCTCC</td>
<td>GACACAGCAAGCTCAACAGC</td>
<td>52</td>
</tr>
</tbody>
</table>
three different cell lines was collected 24 hours after treatment. Zymography was conducted as previously described. In brief, medium from the apical and basal compartments from three separate replicates of three different cell lines, Reg08/007 hESC-RPE, Reg08/023, and hESC-Reg011/013 hESC-RPE cells was collected 24 hours after treatments. For zymography, 10 μL conditioned media was mixed with 20 μL nonreducing zymogram sample buffer and loaded onto 0.1% gelatin-containing SDS-PAGE gel (10% Ready Gel; BioRad, München, Germany). After electrophoresis, gels were incubated with re- nuration and development buffer (both BioRad). Afterward, gels were stained with Commassie blue (BioRad) and destained with a destaining solution (BioRad). Digital images of the gels were obtained with ChemiDoc chemiluminescence system (Biostep, Jena, Germany). For quantification, the inverted gelatinase bands were analyzed with one-dimensional gel analysis software (TotalLab TL100; TotalLab Ltd., Newcastle, UK). Molecular weight and band volume (density) was assessed. Band of the untreated control of the respective MMP was set as 1 (arbitrary unit).

### Western Blotting

Cell samples from the three different mature hESC-RPE cell lines were collected 24 hours after treatment, and the Western blotting was done similarly as in Reference 39. The cultures were washed with 1× PBS; thereafter, cells were lysed in Laemmli buffer consisting of 62.5 mM Tris-HCl (pH 6.8 at 25°C) (Trixa base; Sigma-Aldrich Corp.), 17% glycerol (Merek Millipore), 2% w/v SDS (Bio-Rad Laboratories, Hercules, CA, USA), 0.01% w/v bromophenol blue (Sigma-Aldrich Corp.), and 1.43 M β-mercaptoethanol (Sigma-Aldrich Corp.). The protein degradation was inhibited by adding the Complete Mini protease inhibitor (Roche). The samples were run in 7.5% SDS-polyacrylamide gels and transferred to Hybond-P PVDF Transfer membranes (GE Healthcare, Buckinghamshire, UK) in semidyndy conditions. Blocking of unspecific binding sites was done with 5% fat-free dry milk in 0.05% Tween20 (Sigma-Aldrich Corp.) at room temperature for 1 hour. The primary antibody incubations were done in 5% milk solution in Tris-buffered saline (TBS) and 0.05% Tween20 by using rabbit anti-MMP-1 or anti-MMP-2 (both 1:1000; Abcam, Cambridge, UK) primary antibody for 1 hour in room temperature. After primary antibody incubation, the membranes were washed in 0.5% Tween20 in TBS. The secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz, Dallas, TX, USA) was diluted in 5% milk TBS solution with 0.05% Tween20 and incubated at room temperature for 1 hour. Protein-antibody complexes were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). After detection of the label, the membranes were stripped in stripping buffer consisting of 62.5 mM Tris-HCl (pH 6.8 at 25°C), 0.1 M β-mercaptoethanol, and 2% w/v SDS for 30 minutes at +56°C. All membranes were blocked and restained with mouse anti–β-Actin (1:5000; Santa Cruz). The protein expression was densitometrically quantified with band area calculation in ImageJ Image Processing and Analysis Software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). MMP-1 and MMP-3 protein expression was normalized against the β-actin from the same lane of the membrane. The results are presented as relative changes compared with the results of the untreated control samples.

### Statistical Analysis

Statistical significance of the results was analyzed with PASW Statistics, version 18; the gene expression and TEER results with a two-tailed Mann-Whitney U test, and the protein expression and zymography results with Student’s t-test.

### Ethical Issues

We have approval from the National Authority for Medicolegal Affairs Finland research with human embryos (Dnro 1426/32/300/05), and a supportive statement was obtained from the local ethics committee of the Pirkkanmaa hospital district Finland to derive and expand hESC lines from surplus embryos not used in the treatment of infertility by the donating couples and to use these lines for research purposes (R05116). No new cell lines were derived in this study.

### RESULTS

#### Cell Maturation

This study was carried out using mature hESC-RPE cells; thus, the maturation status was ascertained with confocal and brightfield microscopy, RT-PCR, and TEER. All hESC-RPE cells were highly polarized as seen in confocal micrographs with apically localized Na⁺/K⁺ ATPase and basolateral localization of bestrophin (Figs. 1A–C). Reverse transcriptase-PCR demonstrated that the hESC-RPE cells did not express the pluripotency marker gene, Oct3/4, nor the eye-specific lineage marker RAX, and only a trace amount of Pax6. All assessed RPE-specific genes, MITE bestrophin, and RPE65, were expressed by hESC-RPE cell samples (Fig. 1G), verifying the maturity of used cell material.

Transepithelial electrical resistances were measured just before the experiment in Reg08/007 hESC-RPE cells (average, 266 Ωcm²; range, 97–441 Ωcm²), in Reg08/023 hESC-RPE cells (average, 598 Ωcm²; range, 258–1014 Ωcm²), and in Reg08/11013 (average, 386 Ωcm²; range, 176–545 Ωcm²) prior to the stimuli, showing that cell cultures were polarized and formed tight junctions.

#### Barrier Properties

Oxidative stress induced by H₂O₂ with both concentrations (300 and 600 μM) resulted in a statistically significant (P < 0.001) decrease in barrier function measured with TEER in all
three cell lines (Fig. 2). Ten nanograms TNF-α reduced the TEER on average from 1 to 0.41 (SD, 0.18), which was statistically highly significant \((P < 0.001)\). Similar changes were seen in all three cell lines (Supplementary Fig. S1). To our surprise, in all cell lines, 50 ng TNF-α resulted in a smaller but statistically significant \((P < 0.001)\) decrease in TEER than 10 ng. Interferon γ (500 μU) induced only a small statistically insignificant reduction in TEER values.

**Expression of MMP Genes**

Quantitative RT-PCR showed that untreated Regea08/017 hESC-RPE cells, Regea08/023 hESC-RPE cells, and Regea11/013 hESC-RPE cells expressed MMP-1, -2, -3, and -9 genes. The qRT-PCR revealed that MMP-1 gene expression was increased after TNF-α treatment. On average, the higher concentration (50 ng) induced more than eight times higher expression compared with the untreated control (Fig. 3; \(P = 0.001\)). On the other hand, \(H_2O_2\) and IFN-γ treatments induced only a minor decrease in MMP-1 expression, which did not reach statistical significance. The MMP-2 gene expression was increased after 50 ng TNF-α and decreased after \(H_2O_2\) treatment (Fig. 3). Only the changes caused by \(H_2O_2\) were statistically significant \((P = 0.041, 0.001< P < 0.003\) for 300 μM and \(P = 0.003\) for 600 μM \(H_2O_2\)). The MMP-3 gene expression was strongly induced after 600 μM \(H_2O_2\) (\(P = 0.001\) for 600 μM \(H_2O_2\); 300 μM \(H_2O_2\) induced a change, but it was not statistically significant due to the high variation. Treatments did not alter MMP-3 gene expression. Both 100 μU IFN-γ and 300 μM \(H_2O_2\) induced an approximately 5-fold increase in MMP-9 gene expression; however, the variance was too high to reach statistical significance. From Supplementary Figure S2, it can be seen that, although there are differences between the cell lines, TNF-α induces MMP-1 expression, and \(H_2O_2\) reduces the expression of MMP-2 and increases the expression of MMP-3.

**Expression of MMP Proteins**

Densitometric analysis of Western blots from cells revealed that TNF-α treatment increased MMP-1 protein expression 2-fold, but it was statistically insignificant; \(H_2O_2\) induced a minor, statistically insignificant decrease in MMP-1 expression (Fig. 4A). The TNF-α treatment increased MMP-1 protein expression in all cell lines (Supplementary Fig. S5A). The ELISA showed that all three cell lines secreted on average 0.150 ng/mL MMP-3 protein to the media. The treatments with cytokines did not have any effect on MMP-3 secretion (Fig. 4B). With both concentrations, \(H_2O_2\) reduced MMP-3 secretion from 0.19 to 0.14 ng/mL, and changes were statistically significant \((P = 0.005\) for 300 μM \(H_2O_2\) and \(P = 0.002\) for 600 μM \(H_2O_2\)). The \(H_2O_2\) reduced MMP-3 protein expression in all cell lines (Supplementary Fig. S3B).

**MMP Secretion**

In untreated cells, all three cell lines expressed a band of 58.3 ± 5.3 kDa, corresponding to MMP-2 and a high-molecular-weight band in zymography (HMW), with a molecular weight of 121.7 ± 8.0 kDa.
Expression of MMP genes. Relative expression of MMP-1, -2, -3, and -9 genes were determined after 24-hour incubation with 100 μU IFN-γ, 500 μU IFN-γ, 10 ng TNF-α, or 50 ng TNF-α or 1-hour treatment with 300 or 600 μM H₂O₂ followed by 24-hour chase in RPEbasic medium of all three cell lines. For better visualization, fold changes of MMP-3 and MMP-9 are represented on a logarithmic scale. Data are expressed as mean ± SD of two to six replicates. *P < 0.05, **P < 0.01.

Expression of MMP1 and secretion of MMP3 proteins. Relative expression of MMP-1 protein after treatments (A) quantitated from the Western blot combined from all three cell lines, and the secretion of MMP-3 protein to the culture media of all three cell lines (B) analyzed from ELISA: 24-hour incubation with 100 μU IFN-γ, 500 μU IFN-γ, 10 ng TNF-α, or 50 ng TNF-α or 1-hour treatment with 300 or 600 μM H₂O₂ followed by 24-hour chase in RPEbasic medium. For densitometric evaluation of the Western blot, β-actin was used as a loading control and calibrator. Data are expressed as mean ± SD.
Both 300 and 600 l MH 2O2 reduced the secretion of MMP-2 and HMW MMP in the apical (P < 0.001) and basal sides in all three cell lines (Fig. 5). There were no statistically significant differences between the apical and basal sides. The direction of induced changes was similar in all three cell lines (Supplementary Fig. S4A).

Both the 100 l IFN-γ and 10 ng TNF-α induced a significant secretion of MMP-2 and HMW MMP on the apical side of all three cell lines (Fig. 5). At the basal side, 10 ng TNF-α also induced a statistical significant induction of MMP-2 and HMW MMP (Fig. 5). The direction of induced changes was similar in all three cell lines (Supplementary Fig. S4B).

Expression of Collagen I and IV Genes

Quantitative RT-PCR showed that 500 l IFN-γ resulted in a statistically significant (P = 0.03) 2.1-fold increase in Coll expression. The TNF-α treatment increased Coll gene expression 3.9-fold at 10 ng and 2.8-fold at 50 ng. The results were statistically significant (P = 0.000 and 0.04, respectively); H2O2 treatment decreased Coll expression, but this result was not statistically significant (Fig. 6). The same analysis showed that 50 ng TNF-α resulted in a 25-fold increase in Coll IV expression (P = 0.000), whereas 10 ng TNF-α (P = 0.000), 100 l IFN-γ, and 500 l IFN-γ resulted in an approximately 5-fold increase (P = 0.04 for 100 l IFN-γ and P = 0.01 for 500 l IFN-γ). There was a high variation between the outcomes in three cell lines (Supplementary Fig. S5).

Discussion

The MMPs have a variety of functions in the retina, and their expression has been shown in RPE cells in several studies.11,43
Our hESC-RPE cells expressed the mRNA of MMP-1, MMP-2, MMP-3, and MMP-9, mirroring the results published for human adult primary RPE cells.35,44 Furthermore, zymography showed that the main gelatinase secreted in our hESC-RPE cells was MMP-2, with only occasional traces of MMP-9 found. This corresponds well to the published data, as generally MMP-2 is strongly secreted, and no or small amounts of MMP-9 are found in the supernatant of primary RPE cells.35,44,46

Zymography also exhibited that HMW MMPs (123.7 ± 7.6) were secreted in our mature hESC-RPE cell cultures. In a very early study regarding MMP expression in the primary human RPE cell, a molecular band of 120 was found, corresponding well to our data.11 Moreover, in the BrM, HMW bands of 120 to 130 kDa are generally found.20,47,48 Those are thought to be homo- and heteropolymers of MMP-9 and possibly MMP-2, which would sequester and inactivate MMPs.48

Inflammation and appearance of inflammatory mediators are predicted to be fundamental in the pathogenesis of several retinal diseases such as AMD,21,22 DR,23 and PVR.20 Cytokines such as TNF-α can in turn induce the activation of MMP-2/9 and MMP-9,30 whereas IFN-γ has been described to reduce MMP production.53 It has been previously shown that TNF-α increases the production of MMP-2 in ARPE-19 cells,57 as well as MMP-1 and MMP-3, and induces MMP-9 secretion in ARPE-19 cells, and also in human fetal primary RPE cells.33,35,45,46 Here hESC-RPE cells exhibited a concentration-dependent increase of MMP-1 expression in our mature hESC-RPEs. Considering MMP-2, we also could confirm an increase of MMP-2 secretion at 10 ng/mL TNF-α, confirming the results of studies using human fetal primary RPE cells.46 Although no reproducible induction of MMP-9 could be found in our cells, we did see an increase in HMW MMP secretion. As this HMW MMP is highly likely to contain MMP-9, this in turn may indicate some induction of MMP-9 in our cells.

For IFN-γ, little has been published on its effect on MMPs in human RPE cells. We found no statistically significant effect of IFN-γ in either concentration used on MMP gene expression or protein secretion, corresponding to the results from human primary RPE cells.53 Taken together, our results show that, in regard to MMPs, our hESC-RPE cells are fully functional to react to cytokine stimulation in a manner similar to what has been published for human primary RPE cells.

Oxidative stress is an important pathologic factor in a variety of ophthalmologic diseases.21,23,24 Oxidative stress, applied for 24 or 1 hour, has been shown to increase MMP-1 expression in ARPE-19 cells28 and in human adult primary RPE cells,12,28 respectively. In our cells, a 1-hour oxidative stress, by both 300 and 600 μM H2O2, induced only a minor decrease in MMP-1 gene and protein expression. Oxidative stress applied for 24 hours on ARPE-19 cells has been shown to increase MMP-2 mRNA expression in ARPE-19 cells,27 and to decrease after 1 hour and 24-hour chase in primary RPE cells,28 but in both studies, oxidative stress decreased MMP-2 secretion and activity. In our study, the 1-hour oxidative stress decreased the expression of the MMP-2 gene and secretion of the MMP-2 protein, corresponding well to the previous studies with ARPE-19 cells.28 In contrast to MMP-1 and MMP-2, we saw a major induction of MMP-3 and high induction of MMP-9 mRNA expression after oxidative stress. This was in concordance with previous results obtained with human adult primary RPE cells.3,12,28 Interestingly, the changes seen in our study concerning the mRNA level with MMP-3 and -9 were not translated to an induction in the protein levels after 24-hour treatments, corresponding to previous results.28

In human fetal RPE cells, 24-hour 5 ng/mL IFN-γ treatment increases the transcellular flux and causes a major reduction in barrier function.53 In our hESC-RPE cell cultures after 1-hour 500 μIU (i.e., 0.025 ng/mL) IFN-γ treatment, we saw only a minor change in the TEER. The TNF-α, which is the major inflammatory cytokine, has been shown to weaken the barrier function in ARPE19 cells53 and porcine primary RPE cells.54 In our study, this was also observed in all hESC-RPE cells with both concentrations of TNF-α, suggesting that the TNF-α was effective in our hESC-RPE cells.

The effects of reactive oxygen species on TEER in RPE cells have been evaluated in two studies.54,55 When 24-hour oxidative stress was induced by tert-butyl hydroperoxide (TBHP), it resulted in a decrease in TEER in ARPE19 cells and in primary human adult RPE cells,55 but when primary RPEs were stimulated with 500 μM H2O2, it did not alter the TEER in human fetal primary RPE cells.54 In our study, the 1-hour treatment, even with a lower concentration of H2O2, induced a major drop in TEER.

To our knowledge, direct effects of cytokines on ECM production have not been studied in RPE cells. In a previous study with fibroblasts, TNF-α induced a small increase (1.32-fold) in CollIV gene expression but had no effect on Coll gene expression.56 In the same study, IFN-γ treatment did not induce any changes on Coll or CollIV gene expression.56 In our mature hESC-RPE cells, we detected a great induction in Coll and CollIV gene expression after TNF-α treatment, and we assume that this is a direct effect of the cytokine on hESC-RPE cells. A


26. Yu AL, Fuchshofer R, Kook D, Kampik A, Bloemendal H, Wege-Lüssen U. Subtoxic oxidative stress induces senescence...
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