The role of ADGRE5/CD97 in human retinal pigment epithelial cell growth and survival

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Cell surface molecules of retinal pigment epithelial (RPE) cells participate in the pathogenesis of retinal diseases. In an attempt to identify cell surface proteins that play a role in RPE cell–cell interactions, we have considered studying the expression, regulation, and signaling of ADGRE5/CD97, an adhesion G protein–coupled receptor family member, based on its known adhesive function in other cell types such as leukocytes. We showed that RPE cells express three isoforms of CD97 and identified inflammation-related cytokines as important mediators regulating CD97 expression. Whereas TNF-α and IFN-γ upregulated CD97, TGF-β decreased CD97 expression. Due to interaction with CD55, ARPE-19 cells firmly adhered to monocytes and T lymphocytes when overexpressing CD97, suggesting a role for CD97 in controlling leukocyte infiltration across the RPE-based blood–retinal barrier. CD97-mediated signaling acted synergistically with PDGF-BB and IFN-γ to regulate cell growth and survival, ensuring a cellular balance under inflammatory conditions. These findings suggest that CD97 on RPE cells serves to control leukocyte activation and trafficking in uveoretinal inflammation while at the same time regulating second messenger-mediated gene expression, cell growth, and survival.

Keywords: adhesion GPCR; retinal pigment epithelial cell; growth; survival; inflammation; CD97

Introduction

The retinal pigment epithelium (RPE) is formed by a monolayer of terminally differentiated cells between the choroid and neural retina and plays a pivotal role in maintaining vision. Being in close functional relationship with photoreceptors, the RPE is responsible for the degradation and renewal of photoreceptor outer segments. Furthermore, retinal pigment epithelial cells (RPE cells) transport biomolecules between the choriocapillaris and the outer retina and secrete several soluble mediators that ensure the viability of the choriocapillaris and photoreceptors. In addition, the RPE, acting as a protective barrier against a possible immune attack, is essential for the integrity and proper function of the retina. Disruption of the RPE leads to a compromised retinal function and contributes to pathological conditions in inflammatory or degenerative retinal diseases.1

Under pathological conditions, such as choroidal neovascularization (CNV) or retinochoroidal inflammation or injury, the outer blood–retina barrier (BRB) is disrupted and RPE cells may encounter soluble mediators that are normally excluded from the retina, or may interact with invading cells. In CNV conditions or under other situations where the BRB is compromised (e.g., proliferative vitreoretinopathy (PVR)), RPE cells dedifferentiate and undergo aberrant proliferation and migration, with concomitant formation of pre- and/or subretinal fibrocellular membranes. It is important to note that, in these pathological conditions, RPE cells communicate with cells that usually do not occur

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in the chorioretinal compartment, such as proliferating endothelial cells or invading leukocytes, and that such cell–cell communication may interfere with the progression of retinal disease.2,3

It is well established that RPE cells are able to secrete and contribute to altered levels of inflammation- and angiogenesis-regulating cytokines and display functionally related proteins on their cell surface. RPE cells express a multitude of cytokines or growth factors and chemokines, either from their apical or basolateral surface.4–8 While the polarized and balanced release of these soluble factors may protect the integrity of choroid and retina, changes in their expression are involved in modulating retinal inflammation or choroidal endothelial cell angiogenesis.9–11 On the other hand, proteins expressed on the cell surface of RPE cells may be involved in inflammation and/or interactions with leukocytes in several pathologies of the posterior segment of the eye. RPE cells express several adhesion molecules, such as integrins (CD49e–CD29,12 CD54/ICAM-1,2,13,14 and CD106/VCAM-114,15), complement regulatory proteins (CD46, CD55/DAF (decay accelerating factor), and CD5916), and CD4017. Furthermore, RPE cells can be induced by cytokines to upregulate MHC class II molecules, suggesting that they have a role as antigen presenting cells in inflammatory conditions.18

Here, we describe the expression and regulation of ADGRE5/CD97 (hereafter referred to as CD97), an adhesion G protein–coupled receptor (aGPCR), in RPE cells. Combining our results and previous data showing the upregulation of CD97 under oxidative stress and mitochondrial dysfunction in ARPE-19 cells,19 we propose that this receptor may perform an important function in conditions in which the outer BRB is compromised. CD97 belongs to the subfamily E of aGPCRs, also known as the epidermal growth factor seven-span transmembrane (EGF-TM7) family.20–22 It displays the characteristic extracellular architecture of aGPCRs, like the conserved GPCR autoproteolysis-inducing (GAIN) domain23 with a cysteine-rich GPCR proteolysis site that is accessible to autoproteolytic cleavage.24 Proteolytic processing liberates most of the extracellular region, referred to as the N-terminal fragment (NTF), which thereafter can reassociate noncovalently with the C-terminal fragment (CTF), encompassing the residual extracellular fragment at the N-terminus, the 7TM portion, and the cytoplasmic portion. The NTF of CD97 harbors three to five EGF-like repeats, depending on alternative RNA splicing.25,26 Several aGPCRs have been shown to harbor a tethered agonist (summarized in Ref. 27), and there is evidence to suggest that such an agonist sequence is also involved in the signaling of CD97, as the removal of its NTF potentiates the basal Go12/13-mediated signaling through the small GTP-binding protein RhoA.28,29 CD97 expression in cancer cells has further been shown to positively regulate extracellular signal-regulated kinase (ERK)-1/-2 MAP kinase activation.29

The objective of this study was to obtain evidence for a possible role of CD97 in RPE cell interactions with inflammation-related cells as well as for its integration with intracellular signaling. Our findings support the notion that CD97 expression by RPE cells plays a role in regulating adhesive cell interactions in the choroid–retina region, thereby controlling the rate of leukocyte infiltration across the outer BRB. Furthermore, we consider it likely that in posterior uveitis or a situation where RPE cells are induced to proliferate (i.e., after BRB disruption), CD97 is among the modulating molecules controlling RPE cell growth and survival.

Materials and methods

Cells and additives to cell cultures

The use of human RPE cells and leukocytes was approved by the institutional ethics committee of Leipzig University, Faculty of Medicine. Human RPE cells were prepared by enzymatic digestion as described earlier.30 Briefly, eyes without a history of disease were obtained from donors (15 males aged 38–71 years old and 12 females aged 25–89 years old) and the RPE cells were isolated within 60 h of the death of the donors. After the removal of vitreous and retina, the RPE was mechanically dissected from the choroid, digested with 0.05% trypsin/0.02% EDTA (1 h, 37 °C), and rinsed twice with phosphate-buffered saline (PBS). RPE cells were cultured in culture flasks at 37 °C and 5% CO2 in Ham’s F10 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (each from Invitrogen), and were used at passage 4 or less. The identity of RPE cells was verified using immunocytochemical staining with monoclonal antibodies (mAbs) against acidic
type I and basic type II cytokeratins (AE1 and AE3; Merck Millipore, Darmstadt, Germany). The identity was further confirmed for cells from several donors by immunofluorescence staining using mAbs against cellular retinaldehyde-binding protein (CRALBP; clone B2, Acris Antibodies, Herford, Germany) and occludin (Santa Cruz, Heidelberg, Germany). PCR analysis demonstrated that RPE cells expressed RPE-specific protein 65 (RPE65), CRALBP, zonula occludens protein-1 (ZO-1), and bestrophin-1 (data not shown). Freshly isolated RPE cells were obtained after mechanically peeling the RPE layer from the choroid of human donor eyes with subsequent trypsinization (15 min, 37 °C). Where appropriate, RPE cells were exposed to cytokines or to the synthetic inhibitors of the PI3K/Akt signaling pathway, LY294002 (5 μM), wortmannin (5 nM; both from Merck Millipore), and added to RPE cell cultures. Reombinant human interferon γ (IFN-γ), transforming growth factor β2 (TGF-β2), platelet-derived growth factor BB (PDGF-BB), interleukin (IL)-1β, and IL-6 were purchased from R&D Systems (Wiesbaden, Germany), human tumor necrosis factor-α (TNF-α) was obtained from Biosource (Karlsruhe, Germany), and IL-17A from ImmunoTools (Friesoythe, Germany). The human RPE cell line, ARPE-19, as well as Jurkat, U937, K562, and HL-60 cells were obtained from American Type Culture Collection (Rockville, MD). Peripheral blood monocytes and T lymphocytes were isolated from the blood of healthy adult donors and separated by counterflow elutriation using a JE-6B elutriation system (Beckman Instruments, Palo Alto, CA). The purity of these cells was usually >90%, as determined by analyzing CD14 or CD3 expression using immunofluorescence staining and flow cytometry. Monocytes and T lymphocytes were washed twice with PBS at 4 °C and resuspended in serum-free DMEM at 1 × 10⁷ cells/mL before their use in cell–cell interaction assays.

Monoclonal antibodies. The anti-CD97 mAbs used in this study were MEM-180, BL-Ac/F2 (both murine IgG1). MEM-180 was a generous gift from V. Hořejší (Academy of Sciences of the Czech Republic, Prague, Czech Republic). BL-Ac/F2 recognizes EGF domain I of CD97 and ADGRE2/EMR2 (CD312), while MEM-180 is directed against an epitope in the GAIN region of CD97. The anti-CD55 mAb MAB2009 (clone 278803, IgG2b) was obtained from R&D Systems.

Immunofluorescence. CD97 cell surface expression on RPE cells was routinely analyzed by flow cytometry. Cells were detached by the treatment with HEPES (20 mM)-buffered Hank’s balanced salt solution (HBSS), pH 7.4, 2.5 mM EDTA, for 5 min at 37 °C and resuspended in FACS buffer (10 mM HEPES, pH 7.6, HBSS/1% bovine serum albumin (BSA), 1 mM CaCl₂, 1 mM MgCl₂, 0.05% NaN₃) supplemented with 5% normal goat serum. Cells were then incubated at 4 °C with primary mAbs or isotype control IgG for 60 min, washed, and exposed to R-PE- or FITC-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany). After washing, the samples were analyzed on a flow cytometer (FACScan™, BD Biosciences, Mountain View, CA) using CELLQuest software. Gates were set to exclude nonviable cells, and histograms were recorded. Histograms plot the cell number (y-axis) versus fluorescence intensity on a logarithmic scale (x-axis).

For imaging experiments, RPE cells were plated on glass coverslips, cultured, and fixed/permeabilized with ice-cold methanol. Cells were briefly washed in PBS and blocked with FACS buffer supplemented with 1% ovalbumin/5% normal goat serum for 1 h at 37 °C. Subsequently, the specimens were incubated with primary antibodies diluted in PBS/2% BSA overnight at 4 °C. After washing three times with PBS/2% BSA, cells were incubated for 2 h with cyanine (Cy3)-labeled polyclonal anti-mouse IgG (Dianova). Cells were washed, counterstained with Hoechst nuclear dye, and mounted.

Reverse transcription polymerase chain reaction. Total cellular RNA was purified with a commercially available kit (RNaseasy®, Qiagen, Hilden, Germany) and treated with 1 U DNase I (Life Technologies). RNA (1 μg) was reverse-transcribed using 200 U of Superscript II reverse transcriptase (Life Technologies) in a 20-μL reaction volume containing 500 μM of dNTPs, 5 mM DTT, and 0.5 μg oligo (dT)₁₅. Aliquots of cDNA were amplified using Crimson Taq polymerase (New England Biolabs, Frankfurt, Germany). Primers (200 nM) used for polymerase chain reaction (PCR) are listed in Table S1 (online only). Amplified DNA was visualized using agarose gel electrophoresis and ethidium bromide staining.
Quantitation of PCR products. Total cellular RNA was extracted and reverse transcribed as indicated above. CD97 mRNA expression was analyzed in competitive reverse transcription PCR (RT-PCR) experiments using cDNA samples adjusted to equal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA inputs. To determine relative GAPDH levels, cDNA was amplified in the presence of constant amounts of a competitor DNA fragment, with amplified competitor DNA (851 bp) that was readily distinguishable from amplicons derived from cellular GAPDH cDNA (566 bp). cDNA was serially diluted and amplified at PCR cycle numbers that allowed exponential PCR product accumulation, and amplified DNA was visualized using agarose gel electrophoresis. In real-time PCR assays, aliquots of cDNA (2 μL) were added to Maxima SYBR™ Green master mix (Thermo Fisher Scientific, Braunschweig, Germany) containing 200 nM of primers. Samples were denatured at 95 °C for 6 min, followed by 45 cycles of melting at 95 °C for 10 s, annealing at 58.5 °C for 25 s, and extension at 72 °C for 25 seconds. Data were checked for homogeneity by dissociation curve analysis. Fluorescence changes were monitored after each cycle, with assessment of mean \( \Delta C_q \) (quantification cycle) values for amplification of CD97, GAPDH, and \( \beta \)-actin mRNA. Relative CD97 expression levels were obtained by calculating \((1+E)^{\Delta\Delta C_q}\) (fold expression), with \( E \) representing amplification efficiency, \( \Delta C_q \) corresponding to CD97 expression normalized to \( \beta \)-actin expression \( (C_q,CD97 - C_q,\beta\text{-actin}) \), and where \( \Delta\Delta C_q \) was calculated by subtracting experimental and control \( \Delta C_q \) values.

CD97 constructs and short interfering RNA transfection. Plasmids were generated by ligating the expression plasmid pcDps by with full-length human CD97 (EGF 1,2,5) cDNA or cDNA encoding for a NTF-lacking (ΔSer23–Leu437) fragment of the CD97 (EGF 1,2,5) preproprotein (accession #NP_001775.2). These constructs, HF-hCD97 and HF-hCD97-CTF, respectively, have been described previously. For experiments using CD97 knockdown, RPE and ARPE-19 cells were transfected with short interfering RNA (siRNA) (Invitrogen) according to standard procedures. Briefly, siRNAs targeting CD97 ("stealth" siRNA1, sense: 5′-CCU GCC UCU UUG GCC UGU UCA UCU U-3′, antisense: 5′-AAG AUG AAC AGG CCA AAG ACC CAG G-3′; siRNA2, sense: 5′-CCG AGG UCA CCA UCC AGA AU GUC AU[dT][dT] -3′, antisense: 5′-AUG ACA UUC UGG AUG GUG ACC UCG G[dT][dT] -3′) were complexed with Lipofectamine® RNAiMAX (Thermo Fisher Scientific) and added to the cells in antibiotic-free OptiMEM (Life Technologies). Parallel cultures treated with control siRNA (Santa Cruz, Cat# sc-37007, or scrambled "stealth" siRNA1, sense: 5′-CCU UGU CUU GGC CGU UUA CUG GCU U-3′, antisense: 5′-AAG CCA GUA AAC GGC CAA GAC CAA GAC AAG G-3′) were included. Downregulation of the CD97 expression level was routinely verified using quantitative PCR.

Proliferation and cell viability assays. DNA synthesis in RPE cells was assayed in sextuplicate by determining the incorporation of 5-bromo-2′-deoxyuridine (BrdU; Sigma). Briefly, 4 × 10³ cells/well were plated in 96-well tissue culture plates, cultured to ~50% confluence, and serum-starved for 12 h prior to the experiments. Cells were incubated for 20 h at 37 °C in Dulbecco’s modified Eagles medium (DMEM) supplemented with 5 mg/mL BSA, in the presence of TGF-β2, IFN-γ, or PDGF-BB. RPE cells were pulsed for an additional 4 h with BrdU (10 μM), fixed to the wells with an ethanol-based fixative, and stained with a peroxidase-conjugated mouse anti-BrdU antibody as specified in the protocol of a commercially available cell proliferation colorimetric kit (Roche Applied Science, Mannheim, Germany). A colorimetric reaction was then generated using 3,3′,5,5′-tetramethylbenzidine as a chromogen. The absorbance of samples was measured at 450 nm in a spectrophotometer. Results are indicated as proliferation index (BrdU incorporation rate in the presence of cytokines, compared with basal cellular proliferation in DMEM). Transfections were performed with RPE cells grown in 6-well plates using 60 pmol of control or CD97 siRNA (Invitrogen). Cells were plated in 96-well microplates 48 h after transfection, serum-starved overnight, and proliferation assays were carried out as indicated above.

The production of ATP as a proxy indicator for cell growth was measured using the ATPlite kit according to manufacturer’s instructions (PerkinElmer LAS, Rodgau, Germany). Briefly, ARPE-19 cells were split into 96-well plates (3 × 10³...
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cells/well for siRNA-mediated knockdown or \(1 \times 10^3\) cells/well for overexpression experiments) and transfected with 6 pmol of siRNA or 100 ng of the respective CD97-encoding plasmid. The empty plasmid pcDps served as a vector control. The next day, cells were starved overnight and incubated for 48 h at 37 °C in Ham’s F10 medium in the presence of 10% FCS, 5 ng/mL TGF-β, or 100 ng/mL IFN-γ. Assays were stopped by the addition of cell lysis solution and subsequent application of substrate solution. Luminescence was measured with the EnVision Multilabel plate reader (PerkinElmer LAS).

**Cell–cell interaction assay.** An ARPE-19 cell line stably overexpressing CD97 was created for this assay using a plasmid containing the full-length human CD97 (EGF 1,2,5) cDNA ligated into the expression plasmid pcDNA3.1 (Invitrogen) through the HindIII–KpnI restriction endonuclease cutting sites. The plasmid construct was transfected into ARPE-19 cells using Lipofectamine 2000 (Invitrogen), and expression of CD97 in stably transfected cell clones obtained after neomycin resistance selection was screened by FACS analysis. One of several clones overexpressing CD97 (EGF 1,2,5) was selected, propagated in DMEM containing G418 (500 μg/mL), and used in cell–cell interaction assays. ARPE-19 cells stably overexpressing CD97 were plated into 24-well tissue culture plates and assayed in duplicate for each condition. ARPE-19 monolayers were overlaid with \(1 \times 10^6\) peripheral blood T lymphocytes or monocytes and were incubated in serum-free DMEM for 1 h at 37 °C with repeated agitation, either in the presence of anti-CD55 and anti-CD97 mAbs or isotype control mouse IgG. Plates were washed five times with DMEM and adherent cells were visualized using a phase contrast microscope. Adherent leukocytes were counted in two randomly selected fields per well, thereby determining the average number of leukocytes bound per ARPE-19 cell.

**Cell surface biotinylation.** The surface of RPE cells was labeled according to a previously described method. Briefly, RPE cells were grown in bottles until confluency was achieved, detached with PBS/5 mM EDTA, washed three times with PBS, and biotinylated using d-biotin-N-hydroxysuccinimide ester (Roche Applied Science; 30 min, room temperature). Cells were then washed with ice-cold PBS, lysed (1 \( \times 10^8\) cells/mL) in 50 mM Tris-HCl, 0.15 M NaCl, pH 8, 2% NP-40, 1 mM PMSE, 1 mM EDTA, and precleared twice using goat anti-mouse IgG–coated protein A Sepharose and mouse IgG-coupled Sepharose beads, respectively. Cell extracts were then immunospecifically depleted of CD97. Eluted CD97 was subjected to analysis by SDS-PAGE and transferred to nitrocellulose. Blots were blocked in 5% powdered nonfat dried milk (2 h at 37 °C) and subsequently probed with alkaline phosphatase–conjugated streptavidin (Roche Applied Science), followed by incubation in 0.5 mg/mL nitro blue tetrazolium/0.25 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (Sigma) dissolved in 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5, 5 mM CaCl₂, and 5 mM MgCl₂.

**Serum response element luciferase assays.** ARPE-19 cells were plated in 96-well microplates and transfected with vector control (empty plasmid pcDps) or plasmids encoding for full-length CD97 and CD97-CTF using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were cotransfected with 100 ng of the respective plasmid and 50 ng of a luciferase reporter plasmid (Agilent Technologies, Waldbronn, Germany), which contained five tandem copies of serum response element (SRE). Starved (DMEM/0.5% FCS, overnight) cells were left unstimulated or were stimulated with 1% human AB serum for 5 h and assayed in quintuplicate. Cell extracts were prepared by lysis of cells in steadylite plus™ (PerkinElmer LAS) 30 h post-transfection and the activity of firefly luciferase was assayed using the EnVision™ Multilabel plate reader (PerkinElmer LAS). Proper surface expression of CD97 constructs on ARPE-19 cells was measured using a cell sandwich enzyme-linked immunosorbent assay (ELISA) as described previously.

**Statistical analysis.** Data are presented as means ± SEM. Data were analyzed using the statistical tests specified in the figure legends for each data set. Significance was accepted at \(P < 0.05\).

**Results**

**CD97 expression in RPE cells**
To investigate the expression pattern of CD97 in the RPE, we analyzed mRNA transcripts in primary RPE cells by RT-PCR or stained RPE cells with anti-CD97 mAbs. Flow cytometry experiments
demonstrated CD97 on the surface of freshly isolated RPE cells (Fig. 1A), and immunofluorescence microscopy revealed that CD97 is expressed in cultured RPE cells (Fig. 1B). Furthermore, RT-PCR analyses demonstrated CD97 mRNA transcripts in RPE cells freshly prepared from donor eyes as well as in cultured RPE cells and, interestingly, also revealed mRNA expression of the closely related molecule ADGRE2/EMR2 (CD312; Fig. 1C). However, we were not able to detect another member of the ADGRE subfamily, ADGRE1/EMR1, in RPE cells (Fig. S1, online only).

As known from cell types of hematopoietic origin, CD97 is expressed as three distinct isoforms, which contain three, four, or five EGF domains in their extracellular portion. The three CD97 isoforms are commonly coexpressed.\textsuperscript{25,26} Comparative RT-PCR coamplification of fragments of isoform-encoding splice variants from RPE cells and other cell types (for primers, see Table S1, online only) revealed identical fragment lengths (Fig. 1D) suggesting an isoform expression pattern in RPE cells that is identical to that in hematopoietic cells, which was determined previously.\textsuperscript{26} Moreover, the immunoprecipitation of CD97 from the surface of RPE cells revealed a protein pattern similar to that of myelomonocytic HL-60 and Jurkat T cells, that is, a zone migrating at $\sim$85 kDa (Fig. 1E). This

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\caption{Freshly isolated (A) or cultured (B) human RPE cells express CD97. Cells were stained with mAb MEM-180 and immunofluorescence was detected using flow cytometry (A) or microscopic imaging (B, left panel, red fluorescence: CD97; right panel: negative control using nonimmune mouse IgG1; blue fluorescence: cell nuclei labeled by Hoechst 33258; scale bar: 20 μm). (C) Freshly isolated (a) or cultured (c) RPE cells express mRNA transcripts for CD97 and ADGRE2/EMR2. Total RNA was extracted from cells of two different donors and mRNA expression was monitored by RT-PCR. Primers were designed to span segments of 7TM portions in both molecules (NC: negative PCR control). (D) RT-PCR analysis of CD97 expression revealed that three isoforms are expressed in RPE cells (lane 1), similar to dermal fibroblasts (lane 2) and cells of hematopoietic origin, such as U937 (lane 3), Jurkat (lane 4), and K562 (lane 5) cells. The primers used spanned CD97 exons 2 and 9. (E) CD97 was immunoprecipitated with mAb BL-Ac/F2 from Jurkat T lymphocytes (lane 1), myeloid HL-60 cells (lane 2), or cultured RPE cells from two different donors (lanes 3 and 4).}
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migration behavior of CD97 in SDS-PAGE is consistent with the predicted molecular weights of the three processed CD97 proproteins, taking into account differences due to glycosylation.

Regulation of CD97 expression in RPE cells

RPE cell viability plays an important role in maintaining normal function of the retina, in particular, by protecting it from degenerative neuronal cell loss, as seen in age-related macular degeneration. Since the phosphoinositide 3 kinase (PI3K)–Akt/protein kinase B (PKB) pathway mediates cell survival and loss of Akt activity increased death of RPE cells in vitro, we were interested to explore whether CD97 expression is altered under conditions of attenuated PI3K-Akt activity. As shown in Figure 2A and B, CD97 expression in cultured RPE cells is sensitive to inhibitors of PI3K–Akt signaling, suggesting that prosurvival factors largely contribute to maintaining the CD97 level in RPE cells.

We next asked whether RPE cells can be induced to express altered CD97 levels in response to proinflammatory cytokines. These mediators have been shown to account for altered expression of various soluble chemotactic factors and cell surface adhesion proteins by RPE cells. We also tested the impact of TGF-β2, a pleiotropic cytokine associated with an immunosuppressive and/or antiangiogenic cytokine status in the retina. We found that among the proinflammatory cytokines investigated, IL-1β, IL-6, and IL-17 did not induce substantial changes in CD97 cell surface expression on RPE cells (Fig. S2, online only), while increased CD97 cell surface levels were found on TNF-α-stimulated RPE cells after 48 h, as compared with control (unstimulated) cells. However, the strongest CD97 upregulation in RPE cells (approximately threefold) was found in response to stimulation with IFN-γ (Fig. 2C and D). Activation of cells by IFN-γ resulted in increased CD97 cell surface expression when followed over a cell culture period of 3 days, whereas CD97 expression was downregulated by TGF-β2 (Fig. 2E and F). Both cytokines regulated the expression of CD97 in a dose-dependent manner, with changes that were detectable at >100 pg/mL IFN-γ or >10 pg/mL TGF-β2 (Fig. S3, online only). Furthermore, we found that IFN-γ-induced upregulation of CD97 was strongly inhibited by TGF-β2 (Fig. S4, online only). In keeping with these findings, IFN-γ increased RPE cell expression of CD97 mRNA as early as 6 h of stimulation (Fig. 3A), with upregulated transcript levels that progressively increased until 24 h after addition of IFN-γ. During the same time course, TGF-β2 attenuated CD97 mRNA expression (Fig. 3B). These results suggest that IFN-γ and TGF-β2 cross-regulate CD97 expression in RPE cells. Taken together, expression of this particular aGPCR is potentially maintained by cell survival signals and increased in a proinflammatory environment. It remains to be determined how regulated expression of CD97 translates into specific cellular functions.

CD97 mediates interaction with leukocytes through its ligand CD55

Cell–cell interactions mediated by RPE cell surface molecules may be involved in controlling leukocyte transmigration across the outer BRB. Binding to CD55/DAF is a feature of CD97 that is well established for cells of hematopoietic origin. Interaction with CD55 is particularly striking for the most prevalent CD97 variant 2 (isoform CD97 (EGF 1,2,5)), which possesses three consecutive EGF-like domains and binds to CD55 with the highest affinity as compared with molecular configurations of the other isoforms. It was proposed that the first two EGF-like repeats of CD97 interact with the N-terminal short consensus repeats of CD55. Stably transfected ARPE-19 cells with a full-length human CD97 (EGF 1,2,5) expression plasmid and tested whether CD97 overexpression leads to adhesion of CD55+ peripheral blood monocytes and T lymphocytes. CD97 (EGF 1,2,5) cell surface levels were elevated approximately 30-fold in the transfected cells, compared with wild-type ARPE-19 cells (Fig. 4A). As shown in Figure 4B, CD97-overexpressing ARPE-19 cells form adhesive interactions with peripheral blood monocytes or T lymphocytes. These firm cell–cell interactions were blocked by an anti-CD55 mAb (MAB2009) developed to neutralize adhesion of CD55 to CD97 (Fig. 4B) as well as by an anti-EGF domain CD97 antibody (BL-Ac/F2), but were not observed with an mAb directed against the GAIN region of CD97 (MEM-180; Fig. S5, online only). These findings suggest that, in a milieu that supports CD97 upregulation, CD97–CD55-based cell–cell interactions allow the RPE to interact with...
Figure 2. Immunofluorescence flow cytometric analysis of CD97 expression on the cell surface of RPE cells. (A and B) Inhibitors of the PI3K/Akt signaling pathway significantly reduced the number of CD97+ RPE cells when cultured for 24 h in the presence of wortmannin or LY294003, compared with control vehicle–exposed cells. RPE cells were stained with mAb MEM-180; numbers of CD97+ cells are given in %. Histograms from a single experiment (A) and a graph summarizing data from seven independent flow cytometry experiments with cells from different donors (B) are shown (means ± SEM; *P < 0.05; **P < 0.01; one-way ANOVA). The gray shaded area shows CD97 labeling; the white area indicates IgG1 isotype control staining. (C) Staining of RPE cells with mAbs directed against the GAIN domain (MEM-180, upper panel) or EGF-like repeats–containing region (BL-Ac/F2, lower panel) revealed that CD97 upregulation is induced by TNF-α (10 ng/mL) or IFN-γ (50 ng/mL) after 48 h of culture. (E) A progressive change in mean fluorescence intensity (MFI) indicates altered CD97 expression when monitored over 3 days. Black lines represent staining with isotype-matched mAb, the black shaded area shows unstimulated cells, and the gray shaded area represents cells exposed to cytokines. CD97 is upregulated by IFN-γ (upper panel), but downregulated by TGF-β2 (5 ng/mL; lower panel). Cells were stained with mAb MEM-180. Histograms of representative experiments (C, E) and graphs summarized flow cytometry data from independent experiments with cells from different donors (D, F) are shown. Bars represent relative CD97 cell surface levels on RPE cells after treatment with (D) TNF-α and IFN-γ for 48 h (n = 3) or (F) IFN-γ and TGF-β2 for 24 (n = 6), 48 (n = 6), and 72 (n = 4) hours. Data represent means ± SEM of the fold change of CD97 expression relative to CD97 levels on medium control cells and are given as ratios of median fluorescence intensities. One sample t-test was performed for each condition versus medium control (**P < 0.01; ***P < 0.001).
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Figure 3. CD97 mRNA expression in RPE cells is regulated by TGF-β2 and IFN-γ. Cells were treated with or without 5 ng/mL TGF-β2 and 10 ng/mL IFN-γ. (A) At 6 h posttreatment, total RNA was prepared from RPE cells and semiquantitative competitive PCR was performed. The upper two panels show CD97 amplification (middle panel: twofold diluted cDNA), while the lower panel demonstrates that sample cDNA amounts were adjusted to equal GAPDH levels. (B) RNA was isolated at the times indicated and CD97 mRNA expression was analyzed in cells from two different donors by real-time PCR. Results are expressed as relative values (fold change in CD97 mRNA expression) compared with cells of unstimulated control culture at time point 0. Values were normalized to the CD97 level determined for cells cultured in medium only.

Inflammatory cells, such as monocytes and T lymphocytes.

Proliferation and survival of RPE and ARPE-19 cells and CD97-mediated signal transduction

While under physiological conditions RPE cells exist in a resting state, they are induced to enter the cell cycle in several retinal pathologies, such as CNV and PVR. To explore whether CD97 is involved in RPE cell proliferation and viability, we have measured DNA synthesis as well as intracellular ATP levels under control and CD97 knockdown conditions. Results were obtained using different siRNAs, which induced a 95–97% decrease of CD97 transcripts (Fig. S6, online only). Figure 5A shows that RPE cells, when depleted of CD97, demonstrated a significantly (P < 0.001) reduced basal proliferation rate compared with control cells. Proliferation was also attenuated during growth stimulation with PDGF-BB, a potent growth factor for RPE cells. Similarly, the proliferative signals induced by serum (10% FCS) treatment of cells, which increased ATP formation in control siRNA-transfected cells, significantly decreased (P < 0.05) after CD97 depletion (Fig. 5B).

We further determined DNA synthesis and intracellular ATP levels in response to mediators that cause altered CD97 expression. We primarily examined the influence of IFN-γ and TGF-β2, which induced altered CD97 levels in opposite directions (see above). Interestingly, exposure to both cytokines decreased DNA synthesis of RPE cells in a concentration-dependent manner (Fig. S7A, online only), thus indicating that altered CD97 expression levels induced by these cytokines (see above) did not interfere with the resulting IFN-γ- or TGF-β2-induced growth arrest. We therefore tested the effect of CD97 knockdown on ATP production in ARPE-19 cells stimulated with IFN-γ and TGF-β2. Under control conditions and similar to untransfected cells (Fig. S7B, online only), ARPE-19 cells produced significantly less ATP when incubated with IFN-γ compared with nonstimulated cells (P < 0.05), but TGF-β2 apparently had no negative effect in this assay. Knock-down of CD97 with siRNA showed less ATP formation under nonstimulating conditions but reversed the IFN-γ-induced reduction seen in control cells (Fig. 5C).

To elucidate the role of CD97-mediated intracellular signal transduction in cell viability, we overexpressed CD97 (EGF 1,2,5) in ARPE-19 cells. Specifically, we transfected the cells with full-length CD97 and NTF-deficient deletion constructs (CD97-CTF) (Fig. 5D). Overexpression of CD97 and its constitutively active CTF variant led to an overall reduction in ATP formation most robustly under IFN-γ stimulation. Basal activity of the full-length receptor was sufficient to lower ATP levels, while the constitutively active receptor resulted in a significant (P < 0.01) reduction (Fig. 5E). Overexpression of both CD97 variants in ARPE-19
Figure 4. (A) Enhanced CD97 expression was achieved in ARPE-19 cells overexpressing CD97 (EGF 1,2,5) as compared with ARPE-19 wild-type cells. (B) Adhesion of monocytes and T lymphocytes to CD97 (EGF 1,2,5)-overexpressing ARPE-19 cells. A CD55 mAb blocking the binding to CD97 (clone 278803; 10 μg/mL) inhibits adhesion of mononuclear cells, while the presence of a mouse nonimmune IgG2b (control) had no effect. Bar graphs show quantification of the number of leukocytes in the presence or absence of the antibody; representing the mean of two independent experiments with cells from different donors. Mock-transfected cells did not interact with leukocytes (not shown). Scale bar, 50 μm.

cells induced significantly elevated activity in an SRE-luciferase reporter assay (Fig. 5F, left panel), even though CD97-CTF was expressed at significantly lower levels compared with the full length receptor (Fig. 5F, right panel). When ARPE-19 cells were stimulated with human serum, SRE-driven transcriptional activation increased significantly ($P < 0.05$), by about fivefold (Fig. 5G). However, this serum-inducible effect was enhanced when ARPE-19 cells overexpressed CD97 (Fig. 5G). Taken together, these findings strongly suggest that CD97 is on the one hand a mediator of proliferation inducing and survival signals, while on the other hand it is involved in IFN-γ-mediated cell arrest.

Discussion

An important physiological function of the RPE is to maintain the outer BRB, which highlights the vital role that RPE cells play for retinal integrity. Under several pathological conditions, however, the outer BRB is compromised, which poses a threat to retinal tissue and vision. Inflammatory cells may enter the retinal tissue and, as a consequence, RPE cells are exposed to cytokines that otherwise do not occur in the retina in significant amounts. TGF-β2, a pleiotropic cytokine with immunoinhibitory effects in the eye, can upregulate the production of matrix metalloproteinases by RPE cells to facilitate leukocytes or choroidal endothelial cells.
Figure 5. CD97 is involved in basal and PDGF-BB–stimulated RPE cell proliferation. (A) CD97 was depleted from RPE cells from different donors using two different siRNAs. BrdU incorporation in cells cultured for 24 h was determined in the absence (*P < 0.05; **P < 0.001) or presence (*P < 0.05) of 10 ng/mL PDGF-BB, comparing the proliferation of CD97 siRNA– versus control siRNA–treated cells (means ± SEM, n = 6; one-way ANOVA). Untransfected cells without treatment tended to display higher proliferation rates (1.6-fold) compared with control siRNA–treated cells, but this difference was not statistically significant (P = 0.14, unpaired two-tailed t-test). (B, C) Levels of intracellular ATP used as a live cell marker were determined at 48 h after culture to assess the viability of ARPE-19 cells under siRNA-mediated CD97 knockdown and control conditions upon addition of (B) FCS (10%) and (C) TGF-β2 and IFN-γ. ATP production was expressed as fold change relative to control siRNA–treated cells in the absence or presence of additives (means ± SEM; n = 3). (B) An unpaired two-tailed t-test was performed to compare the effect of CD97 knockdown on basal (*P < 0.05) and FCS-induced (*P < 0.05) ATP production. (C) Significance comparing the effect of TGF-β2 and IFN-γ on control (*P < 0.05) or CD97 knocked-down (*P < 0.05) cells was determined using one-way ANOVA with Dunnett’s multiple comparisons test. (D) Schematic diagram of CD97 constructs based on the human isoform, CD97 (EGF1,2,5). For detection purposes, the molecules were N- and C-terminally tagged with a hemagglutinin (HA) and a FLAG epitope, respectively. Left panel: full-length CD97. Right panel: the NTF-deficient deletion variant, CD97-CTF. NTF, N-terminal fragment; CTF, C-terminal fragment; GAIN, GPCR autoproteolysis-inducing; GPS, GPCR proteolytic site; the tethered agonist is shown in red. (E–G) ARPE-19 cells were transiently transfected with indicated plasmids. (E) Intracellular ATP production was determined at 48 h after culture under the influence of the cytokines indicated. ATP production was expressed as fold change relative to vector control–transfected cells (mean ± SEM, n = 3). The effect of transfected construct under specific stimulation was evaluated using two-way ANOVA and Tukey’s multiple comparisons test (***P < 0.01). (F) Left panel: CD97-overexpressing ARPE-19 cells were cotransfected with a plasmid encoding for SRE-luciferase and assayed for SRE activation (mean ± SEM, n = 15; versus vector control (pcDps): *P < 0.005 and **P < 0.001; effect of NTF deficiency: *P < 0.05; two-way ANOVA). Right panel: Cell surface levels were determined using a cell surface sandwich ELISA and expressed as relative optical densities. Bars represent percentages of human P2Y12 expression on cells transfected with an HA-/FLAG-tagged P2Y12 construct, which served as a positive control (*P < 0.05; unpaired two-tailed t-test; OD₄₉₂ readings: P2Y₁₂: 0.30 ± 0.18; negative control/pcDps: 0.02 ± 0.004). (G) CD97-overexpressing ARPE-19 cells were assayed for serum-induced activation of SRE. Data represent SRE reporter gene activation relative to the signal obtained with mock-transfected cells. Significant differences (mean ± SEM) from values corresponding to cells transfected with vector control, each in the absence (*P < 0.05) or presence (*P < 0.05) of human serum, are indicated; n = 4; two-way ANOVA.
to transmigrate basement membranes toward the retina. Infiltrating leukocytes may secrete proinflammatory mediators, like IFN-γ, TNF-α, and IL-17,3,49 or vasostimulatory cytokines, such as VEGF,50,51 to regulate the expression of cell surface adhesive proteins on RPE cells.13,15,17,52 These cell surface molecules can contribute to adhesive interactions of RPE cells, thereby playing an important role in the context of chorioretinal pathologies. In this study, we report that RPE cells express members of the aGPCR family (Fig. 1C) and that expression of CD97 by RPE cells is regulated by IFN-γ, TNF-α, and TGF-β (Figs. 2 and 3).

Our data are consistent with the idea that, under physiological conditions, CD97 is expressed at low levels in the RPE but upregulated in inflammatory settings, for example, by IFN-γ, which may be released from dendritic cells, macrophages, and a subset of effector T lymphocytes (Th1 cells). Conversely, elevated CD97 levels are counterregulated by TGF-β2 (Fig. S4, online only), which is produced by many retinal cells and regulatory T cells. It is likely that the balance between inflammatory cells adjacent to the RPE, that is, activated T effector cells as well as mono- and polymorphonuclear leukocytes, and TGF-β-producing cells is involved in the control of CD97 expression. Results from a previous transcriptional analysis suggest that CD97 levels in the aging RPE increase in response to a state of chronic inflammatory response, due to increased cellular oxidative stress and mitochondrial dysfunction.19

CD97 possesses structural elements that potentially interact with multiple ligands expressed on leukocytes, mainly through binding to CD55, which has been previously identified as a cellular ligand for CD97.35,44 Our results suggest that both lymphocytes and monocytes can interact with RPE cells in a CD55–CD97-dependent manner (Fig. 4). However, the precise role of this CD55–CD97-mediated interaction for RPE cells remains to be determined. Previous experiments addressing the function of CD97 suggested that it facilitates cell–cell interactions of leukocytes.35,44 On the other hand, the interaction of CD97 with activated CD4+ effector T lymphocytes can generate a costimulatory signal in the T cells via CD55, resulting in proliferation and cytokine production.53 This could conceivably play a role in conditions where RPE cells stimulate T effector cell responses, through a mechanism similar to that suggested for a nonclassical pathway of T cell activation involving CD2 and its RPE cell ligands, CD48 and CD59.54 Increased CD97 expression may also promote the contact of leukocytes with the RPE to regulate the rate of leukocyte infiltration across the RPE-based barrier. On the other hand, CD97 could assist in inhibiting the release of adherent leukocytes from the RPE cell surface, thus playing a role in counteracting unlimited leukocyte trafficking or in limiting inflammation.

In various pathological conditions that potentially involve a breakdown of the outer BRB, RPE cells release and respond to growth factors, such as PDGF-BB, which causes them to proliferate, as is known to occur in PVR.48 It has been shown previously that the overexpression of CD97 enables tumor cells to evade apoptosis.55 This cell growth-promoting activity of CD97 may be pathophysiological important in conditions of retinal injury, which are accompanied by exudation of blood plasma from the vasculature, and where RPE cells are exposed to a variety of serum components.56 From our data, it can be inferred that CD97 contextually regulates cell growth and survival. Using siRNA-mediated knockdown of CD97 in RPE cells, we show that CD97 is essential to promote growth factor–mediated effects on RPE cell proliferation (Fig. 5A) and survival (Fig. 5B). On the other hand, CD97 acts synergistically with IFN-γ, which inhibits RPE cell proliferation (Fig. S7A, online only; see Ref. 57), leading to decreased cell viability (Fig. 5C, E).

As a member of the aGPCR family, CD97 can likely combine its role as an interaction partner for surrounding cells with modulation of second messenger signaling in the cell on which it is expressed.20 Known signaling pathways regulated by CD97 involve engagement of Ga12/13 and signaling through RhoA and serum response factor (SRF) or activation of ERK1/2 and Akt/PKB.29,58 SRF-, ERK1/2-, and Akt/PKB-dependent signaling is also stimulated by serum factors and known to regulate the expression of target genes important for cell growth and survival. We demonstrate that CD97 activates SRE-mediated transcriptional activation in RPE cells (Fig. 5F and G). The degree of SRE-luciferase reporter stimulation was more pronounced when ARPE-19 cells overexpressed the NTF-lacking CD97 variant instead of full-length CD97. This is in line with a previous study showing...
that CD97, in the absence of its extracellular domain, exhibits a higher constitutive signaling activity compared with the full-length molecule. Serum and CD97 promote the induction of SRE-mediated transcription synergistically (Fig. 5G). It is likely that, although both signaling inducers may activate Go12/13-RhoA–regulated SRF signaling, they also stimulate SRE-dependent transcription through an alternative pathway, that is, Ras-Raf–controlled ERK1/2 MAP kinase activation. As a possible mechanism of synergy, CD97 and serum factors could exert a collaborative effect with each other by exploiting both pathways to regulate SRE-mediated transcription. Interestingly, the MAP kinases ERK1/2 have been discussed as essential control elements of IFN-γ–induced cell death in airway epithelial cells, thus possibly representing the missing link in the seemingly contradictory CD97 effects on RPE cell viability. In this context, IFN-γ induces the proapoptotic protein Bcl-2–interacting killer (Bik), which inhibits the translocation of ERK into the nucleus and leads to the subsequent activation of death-associated protein kinase.

A potential scenario for the differential growth-regulating activity of CD97 (for details, see Fig. 6) is that the receptor, in its basal active state, is involved in maintaining RPE cell growth and survival. Yet, under proinflammatory conditions, that is, during effector T lymphocyte response with IFN-γ production, CD97 is upregulated and can guide interaction with cells of the immune system, for example, through CD55, to induce a coordinated response. The interaction between CD97 and CD55 does not lead to ERK1/2 activation, but instead results in reduced CD97 cell surface expression. Thus, the loss of RPE cell viability induced by IFN-γ would no longer be supported by CD97, allowing RPE cells to survive. When the CD97–CD55 interaction is disrupted, not formed at all, and/or leads to a dissociation of the NTF (revealing the constitutively active CTF), elevated CD97 signaling activity induces a drastic increase in downstream effectors that mediate cell death. However, the details of the outlined signaling events require further investigation.

Taken together, we have investigated the expression and regulation of CD97 in RPE cells, as well as its involvement in RPE cell growth and survival. Although not addressed in the present study, it is likely that, similar to malignant tumor cells, CD97-activated signaling in RPE cells is

Figure 6. CD97 is a regulator of cell growth and survival in RPE cells. (A) CD97 is involved in mediating serum- and growth factor–induced cell growth, as exemplified in PDGF-stimulated cells. Known signaling cascades engaged by CD97 include Go12/13-dependent activation of RhoA, with subsequent stimulation of SRF, as well as the ERK1/2 MAP kinase pathway, which is shared with PDGF signal transduction. These pathways promote RPE cell viability and growth. However, when the cell is stimulated by IFN-γ, CD97 is upregulated and expression of the proapoptotic protein Bcl-2–interacting killer (Bik) is induced. Bik inhibits the translocation of phosphorylated ERK1/2 to the nucleus, thus interrupting cell proliferative effects and, at the same time, inducing cell arrest through activation of death-associated protein kinase (DAPK).

(B) CD97 on RPE cells fulfills an important role in the control of uveoretinal inflammation through its interaction with CD55/DAF, by controlling leukocyte adhesion and migration as well as T cell activation. Importantly, CD97–CD55 interaction may lead to CD97 downregulation, thereby preventing pronounced ERK1/2 signaling under inflammatory conditions and subsequent cell death. In an acute Th1-dominated inflammatory response, this would ensure temporary activity of CD97 to modulate the function of leukocytes as long as a functional interaction with these cells is preserved. Once IFN-γ is absent, CD97 can reinforce serum- and growth factor–induced cell growth, as indicated in A.
also involved in regulating cytoskeletal organization and cell adhesion. In this way, CD97 would be able to regulate the invasion of RPE cells under conditions in which the RPE is activated, such as in PVR or CNV development. The present findings open the possibility that modifying CD97 expression, function, or its accessibility is appropriate for future therapeutic approach directed at RPE cell functions involved in posterior uveitis and/or retinal diseases.

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Author contributions

W.E. and I.L. designed the study, planned experimental work, wrote the paper, and analyzed data. W.E., A.L., K.U.S., S.K., J.L., S.B., and I.L. performed experiments. All authors edited and approved the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Oligonucleotides used for PCR.

Figure S1. ADGRE1/EMR1 is not expressed by RPE cells.

Figure S2. IL-6, IL-1ß, and IL-17 do not induce significant changes in CD97 cell surface levels on RPE cells.

Figure S3. IFN-γ and TGF-β2 regulate the expression of CD97 in a dose-dependent manner.

Figure S4. IFN-γ-induced CD97 upregulation on RPE cells is inhibited by TGF-β.

Figure S5. Adhesive interactions of peripheral blood lymphocytes with CD97 (EGF 1,2,5)-overexpressing ARPE-19 cells were blocked by the anti-EGF domain CD97 antibody BL-Ac/F2, but not by an mAb directed against the GAIN region of CD97 (MEM-180).

Figure S6. Efficacy of siRNA-mediated CD97 silencing in RPE cells.

Figure S7. Proliferation of RPE cells derived from different donors. Cells were cultured for 24 h in medium or in the presence of IFN-γ and TGF-β2.

Competing interests

The authors declare no competing interests.

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