Complement and UV-Irradiated Photoreceptor Outer Segments Increase the Cytokine Secretion by Retinal Pigment Epithelial Cells

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PURPOSE. Age-related macular degeneration (AMD) is accompanied by increased complement activation, and by lipofuscin accumulation in retinal pigment epithelial (RPE) cells due to incomplete degradation of photoreceptor outer segments (POS). The influence of POS, ultraviolet (UV)-irradiated POS and human complement sera (HCS) on cytokine secretion from RPE cells was therefore examined.

METHODS. RPE cells were incubated with POS or UV-POS every other day for 1 week. The autofluorescence (AF) was measured photometrically and by flow cytometry. Senescence-associated genes were analyzed by RT-PCR. Internalization and degradation of POS were determined using phagocytosis and degradation assays, and lysosomal function by neutral red uptake. RPE cells in polycarbonate cell culture inserts were incubated apically with POS or UV-POS and afterward basally with HCS. C7-deficient HCS was used as control. The integrity of the cell monolayer was assessed by measuring the transepithelial electrical resistance (TER) and the permeability. Interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1, and vascular endothelial growth factor were quantified by ELISA.

RESULTS. POS treatment led to an increased AF and senescence marker expression, which were further elevated in response to UV-POS. UV-POS were preferentially accumulated over POS and the lysosomal function was impaired due to UV-POS. HCS intensified the cytokine production compared with controls. POS had no effect, though UV-POS combined with HCS induced a significant increase in all cytokines.

CONCLUSIONS. RPE cultivation with UV-POS might serve as a model to investigate the accumulation of lipofuscin-like structures. The enhanced cytokine secretion due to UV-POS with HCS may account for an increased susceptibility for lipofuscin accumulations in AMD.

Drusen are extracellular deposits located between the basal lamina of the RPE and the innermost layer of BrM. The presence of drusen is a significant indicator for early AMD and for the development of neovascular membranes. The characterization of drusen revealed proteins involved in inflammatory processes including several complement components. The role of complement in AMD was further elucidated when a single nucleotide polymorphism (SNP) in the complement factor H (CFH) gene was found in approximately half of AMD patients. This SNP is associated with an increased activation of complement. The complement system as an important part of innate immunity constitutes a first, nonspecific defense against potential pathogens. It provides a cascade of proteins, whose activation leads to the assembly of C5b-9 on surfaces of target cells to induce cell lysis. However, sublytic amounts of C5b-9 are capable of activating several cell populations and exerting chemotactic activity.
AMD is regarded as a chronic subclinical inflammatory disease. \(^{27,28}\) Local inflammatory responses in the RPE may be conducted through the secretion of interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1). The expression of IL-6 was observed to be elevated in laser-induced murine CNV, \(^{29}\) and IL-8 concentration in aqueous humor has been associated with the CNV severity. \(^{30}\) The aqueous MCP-1 concentration in patients with exudative AMD has been correlated with the occurrence of macula edema. \(^{31}\) Vascular endothelial growth factor (VEGF) participates in angiogenic processes and intensifies vascular permeability. \(^{32}\) VEGF is produced by the RPE and contributes essentially to the development and maintenance of the choriocapillaris. \(^{33}\) Increasing amounts, however, were associated with abnormal vessel growth within exudative AMD. \(^{34}\)

In this study, RPE cells were incubated with UV-irradiated (UV)-POS to induce the accumulation of structurally altered POS, similar to lipofuscin-like structures in RPE cells in vivo. We analyzed autofluorescence (AF) and expression of senescence-associated genes apolipoprotein J (Apo J), fibronectin, osteonectin, and transglamin (SM22), which are considered markers for cellular age. \(^{35,36}\) We also investigated the effect of human complement sera (HCS) on the polarized secretion of IL-6, IL-8, MCP-1, and VEGF by untreated, POS-, or UV-POS-treated RPE cells.

**METHODS**

**Isolation of POS**

POS were isolated from porcine eyes as described previously \(^{37}\) and stored at \(-80^\circ\)C until further use.

**UV-Irradiation of POS**

UV-irradiation was performed as previously described, \(^{38}\) accompanied by a few modifications. POS (1 \(\times\) 10\(^7\)) were diluted in 1 mL PBS in a 24-well plate and exposed to a UV-light source with a wavelength of 254 nm and 2 \(\times\) 15 W for 3 hours at a distance of 20 cm. After the irradiation period, POS were thoroughly removed from the well, centrifuged at 6000 \(g\) for 10 minutes and directly used in experiments.

**Cell Culture and Treatment**

Human ARPE-19 cells (ATCC number CRL-2302) \(^{39}\) were cultivated in Dulbecco’s modified Eagle’s medium and Ham’s F12:1:1 (DMEM/F12; Biochrom, Berlin, Germany) at 37°C with 5% carbon dioxide. The media was supplemented with 100 U/mL streptomycin/penicillin (PAA Laboratories GmbH, Pasching, Austria) and 10% fetal calf serum (FCS). When grown to confluence, cells were incubated with 2 \(\times\) 10\(^7\) POS or UV-POS/mL medium and 1% FCS for every other day of a week. The polarized secretion of cytokines was analyzed using RPE cells grown on polycarbonate cell culture inserts (Transwell, \#3401, 0.4 \(\mu\)m pore size; Corning, Amsterdam, the Netherlands).

At 5 weeks postconfluence, when the transepithelial electrical resistance (TER) was stable, a permeability assay was performed to ensure the integrity of the cell monolayer. POS were added to the apical compartment. Subsequent incubation with 2% and 4% HCS was carried out in the basolateral compartment for 24 hours. Medium alone and 4% of the commercially available C7-deficient HCS were used as controls. Unless stated otherwise, all reagents were purchased from Sigma (Steinheim, Germany).

**Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)**

Total RNA was isolated using a kit (RNeasy Plus Mini Kit; Qiagen, Hildesheim, Germany) following the manufacturer’s instructions. Total RNA (0.5 \(\mu\)g) was transcribed into cDNA with a kit (OmniScript RT Kit; Qiagen). The cDNA template was applied to RT-PCR, accomplished with a PCR kit (HotStarTaqPlus Master Mix; Qiagen). The expression of Apo J, SM22, fibronectin, and osteonectin was analyzed with specific primers \(^{39,40}\) under the following conditions (Table 1) using a PCR thermal cycler (Thermocycler Gradient T; Biometra, Göttingen, Germany). The PCR products were separated by electrophoresis on a 1.5% agarose gel at 100 V. Relative fold change in each cytokine expression was calculated by normalizing values to GAPDH (primer designed by Teresa Hsi, Harvard NeuroDiscovery Center) expression. Expression values of treated samples were compared with respective values of untreated samples.

**AF Measurement**

For photometric measurement of the cellular AF, cells were washed, covered with 100 \(\mu\)L PBS, and analyzed using a plate reader at 490 nm wavelength. For flow cytometry analysis, cells were detached with trypsin-EDTA, resuspended in 100 mM PBS-EDTA, and the AF was analyzed with a flow cytometer (BD FACS Calibur; BD Bioscience, Heidelberg, Germany). The emitted fluorescence of 488 nm for 50,000 events was measured and analyzed with computer software (Win MDI 2.9, freeware created by Joseph Trotter, Scripps Research Institute, La Jolla, CA).

**Phagocytosis Assay**

To differentiate between bound and internalized POS, cells were incubated with 0.2% trypsin blue for 10 minutes to quench the fluorescence of externally bound POS. \(^{41}\) PBS control treated cells reflected bound and internalized POS. After excessive washing, the cells were detached and the AF was analyzed via flow cytometry as described above.

**In Situ Distribution of POS**

POS were labeled with AlexaFluor 555 (Invitrogen, Darmstadt, Germany) and fed to RPE cells in polycarbonate cell culture inserts (Transwell, Corning, Amsterdam, the Netherlands).

**Table 1. Primer Sequences and PCR Conditions**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence from 5’ to 3’</th>
<th>bp</th>
<th>Initial Step</th>
<th>Denaturation, Annealing, Elongation</th>
<th>Number of Cycles</th>
<th>Final Elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo J</td>
<td>F gaa atg aag cgg ccg ecg</td>
<td>286</td>
<td>5’ at 95°C</td>
<td>45 s at 94°C, 45 s at 57°C, 1’ at 72°C</td>
<td>24</td>
<td>10’ at 72°C</td>
</tr>
<tr>
<td></td>
<td>B gaa act gaa cgg gcc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM22</td>
<td>F tgg tgg ccc gac acc ca</td>
<td>367</td>
<td>5’ at 94°C</td>
<td>45 s at 94°C, 45 s at 57°C, 1’ at 72°C</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B ctc ctc ctc acc ca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Tgc cca ccc ctc ctc ctc act ca</td>
<td>492</td>
<td>5’ at 94°C</td>
<td>45 s at 94°C, 45 s at 57°C, 1’ at 72°C</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Osteonectin</td>
<td>Tcc gac gag acc gga gga ga</td>
<td>207</td>
<td>5’ at 94°C</td>
<td>45 s at 94°C, 45 s at 57°C, 1’ at 72°C</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Tcc gac gag gaa gga gga ga</td>
<td>177</td>
<td>5’ at 94°C</td>
<td>45 s at 94°C, 45 s at 55°C, 1’ at 72°C</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

Illustrated are forward (F) and backward (B) primer sequences of Apo J, SM22, fibronectin, osteonectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), corresponding base pairs (bp) of the respective products, and PCR conditions.

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minutes, dehydrated in ascending series of alcohol (70%-100%) for 20 minutes each and embedded in paraffin. Paraffin sections of 7 μm were prepared with a microtome (RM 2135; Leica, Wetzlar, Germany), deparaffinized and the nuclei were stained with Hoechst 33342 (10 μg/mL). Cells were covered with 10% glycerol and analyzed with a fluorescent light microscope (BX40; Olympus, Hamburg, Germany).

**POS Degradation Assay**

POS and UV-POS treated cells were lysed in 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 50 mg/mL deoxycholate, 0.1% SDS, 1% Triton X-100, 10% glycerol, 1 mM Na3Vo4, 1 mM DTT, 1 μM peptatin, 10 μM leupeptin, and 1 mM PMSF.42 The lysate was incubated on ice for 15 minutes and sonicated for 3 × 20 seconds. The samples were separated on an SDS-PAGE at 120 V and transferred to a nitrocellulose membrane at 350 mA for 50 minutes. The degradation of phagocytosed POS was determined using a mouse monoclonal anti-rhodopsin antibody (clone 1D4; Santa Cruz Biotechnology, Heidelberg, Germany). The detected bands were visualized by chemiluminescent reaction (Western Blotting Luminol Reagent Kit, Santa Cruz Biotechnology) and exposed to x-ray film. Densitometric analysis of obtained bands was performed by software developed by Wayne Rasband (ImageJ; National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) and the data were normalized to the effective growth area in cm2.43

**Lysosomal Function**

The lysosomal function was analyzed using a neutral red assay. Cells were washed and incubated with 0.005% neutral red for 2 hours until the dye accumulated in the cells. After efficient washing, the internalized dye was released using 50% ethanol and 1% glacial acetic acid. The optical density (OD) was measured at a wavelength of 550 nm.

**TER Measurement**

The TER was measured weekly with an epithelial volt-ohm meter (Epithelial Voltohmeter; World Precision Instruments, Berlin, Germany). Additional measurements were taken before and after the HCS treatment. The TER was calculated by subtracting the value of an insert without cells from the experimentally measured value. This value was multiplied with the surface area of the insert to obtain the resistance of the effective growth area in Ωcm2.43

**Permeability Assay**

The permeability of the RPE cells grown in polycarbonate cell culture inserts (Transwell; Corning) was determined by measuring the movement of sodium fluorescein from the apical to the basolateral compartment.44 One blank filter without cells was used as a control. The dye (25 μg/mL) was added to the apical side, and 50 μL was collected from the basolateral compartment at each time point (0, 1, 2, 4, and 24 hours). These 50 μL were replaced by equal amounts of medium. The OD of the removed sample was measured with an eight-channel photometer (MRX; Dynatech, Denkendorf, Germany) at a wavelength of 490 nm.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Cell culture supernatants were harvested after 24 hours, stored at −20°C, and analyzed for IL-6, IL-8, MCP-1 (human IL-6/IL-8/MCP-1 OptEIA ELISA Set; BD Bioscience) and VEGF (human VEGF Duoset; R & D Systems, Wiesbaden, Germany) by sandwich-ELISA according to the manufacturer’s protocol. The OD was measured at a wavelength of 450 nm.

**Statistical Analysis**

Data were presented as means with standard deviations. One-way ANOVA and Tukey post hoc were performed for normally distributed data to analyze the differences between more than two groups. P < 0.05 was considered statistically significant, marked with an asterisk (*)

**RESULTS**

**POS and UV-POS Increased the Expression of Senescence-Associated Genes**

Apo J, fibronectin, SM22, and osteonectin were constitutively expressed by RPE cells as detected by RT-PCR. The expression of these senescence-associated genes tended to be elevated in response to POS when compared with untreated cells. The treatment with UV-POS led to a significant rise in the expression of Apo J, fibronectin, osteonectin, and SM22 (Figs. 1A, 1B).

**Elevated Intracellular AF after UV-POS Treatment**

POS-treated RPE cells revealed an increased AF measured photometrically at a wavelength of 490 nm compared with untreated cells. The incubation with UV-POS led to an additional significant increase in AF (Fig. 2A). Flow cytometry analysis demonstrated a constitutive fluorescence at a wavelength of 488 nm for untreated RPE cells and a geo mean at 4.1 ± 2.3. The POS treatment revealed a shift toward an increased AF compared with untreated cells and an increased geo mean (9.6 ± 1.3). The incubation with UV-POS represented an additional rise in the geo mean (16.8 ± 1.7), accompanied by an additional RPE cell population when analyzed by flow cytometry (Fig. 2B). To distinguish whether this enhanced AF is based on extracellular binding or internalization of POS or UV-POS, the intracellular AF was measured. We recorded a

![FIGURE 1.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933464/)
shift toward an elevated intracellular AF due to POS, verified by a rise in the geo mean from $5.2 \pm 1.1$ to $9.7 \pm 1.1$ ($P < 0.001$). A further increase in the intracellular AF was observed using UV-POS treated RPE cells (geo mean: $12.7 \pm 1.5$; $P < 0.001$) (Fig. 2C). Moreover, the geo mean of the total AF of UV-POS treated cells was higher than the intracellular AF (Figs. 2B, 2C).

**Increased Accumulation of UV-POS in RPE Cells**

Paraffin embedded ARPE-19 cells, incubated apically with AlexaFluor 555-labeled POS, revealed sporadic accumulation of POS in the cells (Fig. 3A). In comparison, incubation with UV-POS revealed an increased accumulation within cells and...
an increased binding to the cell surface (Fig. 3B). Proteins samples analyzed by Western blot revealed distinct bands at 40 kDa corresponding to the POS constituent rhodopsin. POS treated ARPE-19 cells showed a slightly thinner band and a decrease in rhodopsin approximately 44% in comparison with UV-POS treated cells (Figs. 3C, 3D).

**Decreased Neutral Red Uptake in Response to UV-POS**

The RPE cell viability, measured by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, was not altered by POS, UV-POS, or HCS treatment (data not shown). A neutral red assay was performed to determine the lysosomal function. Incubation of RPE cells with POS had no significant impact on the neutral red uptake. Treatment with UV-POS, however, revealed a significant decline in the lysosomal incorporation of the dye (Fig. 4).

**Integrity of a Dense Cell Monolayer**

The time-dependent increase of the TER reflected a saturation curve. After 5 weeks the TER was stable at 65 ± 8.1 Ω cm² (Fig. 5A). Permeability assays revealed intact RPE cell monolayer. Increasing amounts of the apically added sodium fluorescein were detected basally from blank polycarbonate cell culture inserts (Transwell; Corning) without cells. Approximately 6 μg/mL of the dye passed the permeable insert quickly during the first 4 hours. The cell monolayer however retained the fluorescent protein for at least 24 hours, the time period of the HCS treatment (Fig. 5B).

**Decreased TER in Response to UV-POS Combined with HCS**

The TER was not impaired in response to C7-deficient HCS, HCS, or POS incubation. The TER we observed due to UV-POS and combined treatment with UV-POS and C7-deficient HCS tended to be lower compared with that of untreated or POS treated cells. However, a significant reduction in the TER was detected as a result of UV-POS and HCS in combination (Fig. 6).

**Enhanced Basal Secretion of Cytokines due to UV-POS with HCS**

Untreated ARPE-19 cells cultured in polycarbonate cell culture inserts (Transwell; Corning) produced IL-6, IL-8, MCP-1, and VEGF constitutively. The addition of HCS led to a significant increase in the cytokine secretion, independent of any pre-treatment with POS or UV-POS. C7-deficient HCS revealed only minor effects in this respect. POS and UV-POS incubation did not alter the cytokine production compared with untreated cells. However, secretion of IL-6 (P < 0.001), IL-8 (P = 0.015), MCP-1 (P = 0.001), and VEGF (P < 0.001) was strongly elevated into the basal compartment due to UV-POS/4% HCS compared with POS/4% HCS (Figs. 7A–D). The apical secretion of these cytokines was also increased due to HCS. However,
we observed no significant elevation in the secretion in response to UV-POS with HCS (data not shown).

**DISCUSSION**

Cellular aging is a complex and protracted process associated with the progressive accumulation of detrimental changes. This process is characterized by the aggregation of debris, waste products, and lipofuscin. Underlying mechanisms such as light damage, oxidative stress, lipid peroxidation, and modifications in life-supporting organelles like mitochondria and lysosomes are also seen during AMD pathogenesis. Build-up of lipofuscin within RPE cells can cause degeneration leading to photoreceptor degeneration and the development of AMD.

We observed a significant increase in the expression of Apo J, fibronectin, osteonectin, and SM22 after incubation with UV-POS. Consistent with this, the expression of these senescence-associated genes has been found upregulated by RPE cells or human diploid fibroblasts by hydrogen peroxide or transforming growth factor-β1. The extracellular matrix (ECM) component fibronectin and Apo J have been determined constituents of drusen in AMD patients, and raised amounts of Apo J were observed in a couple of apoptosis models. Osteonectin and SM22 participate in the ECM turnover, cell proliferation, and in senescence-associated changes. Given that overexpression of senescence-associated genes indicates a criterion for stress-induced premature senescence, the increase in Apo J, fibronectin, osteonectin, and SM22 may imply that treatment with UV-irradiated POS induce RPE cell aging.

Our results showed an increase in the total AF in response to POS treatment and a further significant elevation after treatment with UV-POS. The accumulation of autofluorescent material has previously been found in the cytoplasm of postconfluent RPE cell cultures. The bis-retinoid fluorophore A2E, the main component of lipofuscin, was visualized by spectrophotometric fundus AF imaging, and increased lipofuscin fluorescence was observed at the edges of areas with geographic atrophy in AMD eyes. We further detected an increase in the intracellular AF, indicating a preferred accumulation of UV-POS over POS. Moreover, the difference in the geo mean between total and intracellular AF of UV-POS treated cells may give evidence for increased external binding of UV-POS. The elevated AF of RPE cells fed with UV-POS as well as the increased intracellular deposition of UV-POS may reflect the elevated accumulation of structurally altered POS. Up to 33% of the RPE cytoplasmic space was shown to be occupied by lipofuscin from the age of 70 onward. The higher amount of rhodopsin in UV-POS treated cells suggested a decrease in UV-POS degradation, which may support the hypothesis of the accumulation of altered POS as lipofuscin-like structures.

We further found a significant decrease in the neutral red uptake by UV-POS-treated RPE cells, possibly reflecting an impaired lysosomal function. The RPE lysosome is responsible for the terminal degradation of overaged proteins and accumulating waste products. Lysosomes of aged postmitotic cells become enlarged and more lipofuscin-loaded with advancing age, and are predominantly affected by senescence processes. The inability of the lysosomal compartment to completely degrade proteins contributes toward lipofuscin accumulation. Accumulation of the lipofuscin component A2E was investigated to decrease the effectiveness of lysosomal enzymes via increase in the lysosomal pH. A2E also inhibits the degradation of POS and contributes toward blue light-mediated tearing of the lysosomal membranes. The impaired lysosomal function due to UV-POS implicates the accumulation of structurally altered POS in RPE cells as a result of incomplete degradation. Therefore, the ARPE-19 cell culture treatment with UV-POS might represent a model to investigate senescent and lipofuscin-loaded RPE cells.

To mimic the morphologic polarization of RPE cells in the human eye in vivo and to evaluate the directed secretion of mediators, we cultured ARPE-19 cells in polycarbonate cell culture inserts (Transwell; Corning) as previously described. The ability of HCS to influence the TER of ARPE-19 cells was also evaluated by sodium fluorescein retention and a constant TER, before experiments.

We measured a significant decline in the TER due to UV-POS combined with HCS, suggesting an impaired integrity of the cell monolayer. Recent findings demonstrated a decreased TER in RPE cells treated with hydrogen peroxide and complement-sufficient serum. HCS only had an impact on the barrier function, when a second influencing parameter affected the cells. This indicated UV-POS damaged cells to be more suscep-
tible to HCS, arguing for complement-mediated attack exerting a significantly stronger influence on dysfunctional RPE cells. We found no impact of POS on UV-POS treatment on the cytokine release. Contrary to that, other investigators revealed elevated amounts of IL-8 and MCP-1 in response to UV-POS, though the UV-irradiation was performed under a longer time period, a lower radiation force, and the POS concentration fed through complement might only be achieved in addition of a secondary stimulus. These data provide evidence for an enhanced susceptibility for senescent and lipofuscin-loaded RPE cells to complement, which could be relevant during AMD development.

Acknowledgments

The authors thank Martin Busch at Ophtha-Lab, Department of Ophthalmology, Muenster, for his help in processing the statistical data and Jennifer Williams for proofreading and helpful comments.

References

RPE Cytokine Secretion by Complement and UV-POS 1413


