All-trans-Retinal Sensitizes Human RPE Cells to Alternative Complement Pathway–Induced Cell Death

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PURPOSE. Retinal pigment epithelial (RPE) cell death occurs early in the pathogenesis of age-related macular degeneration (AMD) and Stargardt's disease. Emerging evidence suggests that all-trans-retinal (atRal) and alternative complement pathway (AP) activation contribute to RPE cell death in both of these retinal disorders. The aim of this study was to investigate the combined effect of atRal and AP activation on RPE cell viability.

METHODS. RPE cells were treated with atRal and then incubated with a complement-fixing antibody followed by stimulation with C1q-depleted serum to activate AP. Cell viability was assessed by tetrazolium salt and lactate dehydrogenase release assays. Changes in cell surface CD46 and CD59 expression were assessed by flow cytometry. Cells were pretreated with the antioxidant resveratrol, and C1q-depleted serum was incubated with an anti-C5 antibody prior to initiating AP attack to determine the protective effects of antioxidant therapy and complement inhibition, respectively.

RESULTS. Both atRal and AP activation independently caused RPE cell death. When AP attack was initiated following atRal treatment, a synergistic increase in cell death was observed. Following 24-hour atRal treatment, CD46 and CD59 expression decreased, corresponding temporally to increased susceptibility to AP attack. Resveratrol and the anti-C5 antibody both protected against AP-induced cell death following atRal exposure and were most effective when used in combination.

CONCLUSIONS. atRal sensitizes RPE cells to AP attack, which may be mediated in part by atRal-induced downregulation of CD46 and CD59. Despite increased susceptibility to AP attack following exposure to atRal, resveratrol and anti-C5 antibody effectively prevent AP-mediated cell death.

Keywords: age-related macular degeneration, retinal pigment epithelium, oxidative damage, alternative complement pathway, all-trans-retinal

Age-related macular degeneration (AMD) and Stargardt's disease share many clinical features, although similarities in the pathogenesis of these two diseases are less clearly defined. The sequence of events leading to geographic atrophy, the advanced form of nonneovascular AMD, is still being explored; however, histopathologic studies indicate that retinal pigment epithelial (RPE) cell loss precedes photoreceptor cell death and vision loss.^{1–4} The etiology of AMD is complex, but evidence supports a “two-hit” hypothesis in which oxidative stress injures RPE cells, impairing their ability to regulate surface complement deposition, which leads to alternative complement pathway (AP) activation and RPE cell death.^{5–8} The aim of this study was to characterize the combined effects of AP activation and all-trans-retinal (atRal), the pro-oxidant chromophore that accumulates in patients with Stargardt's disease and is proposed to play a causative role in the observed retinal pathology.

Stargardt's disease is a hereditary juvenile macular dystrophy with clinical features similar to AMD. This disease occurs in patients harboring homozygous mutations in *ABCA4*, which encodes a photoreceptor protein involved in processing atRal.⁹ When this enzyme is dysfunctional, free atRal can accumulate.^{10–14} In a mouse model of Stargardt's disease, animals with

double knockouts of *ABCA4* and *RDH8*, another enzyme involved in atRal processing, manifest retinal abnormalities; further, the investigators experimentally demonstrated that in this model, the retinal pathology appears to be caused by free atRal and not A2E or other atRal condensation products.^{13,15} Genetic studies linking AMD and Stargardt's disease provide rationale to explore atRal's role in AMD. Given the phenotypic similarities between these conditions, investigators screened AMD patients for alterations in *ABCA4*. Of the patients screened, 16% had either an amino acid substitution or deletion in this gene. Thirteen independent alterations were observed, three of which have also been detected in patients with Stargardt's disease.¹⁶ Further, a subgroup of AMD patients with unique features on fundus autofluorescence imaging has been identified that is significantly associated with monoallelic *ABCA4* sequence variants, providing support for a complex role of *ABCA4* in the etiology of at least a minor proportion of patients with AMD. These findings suggest the possibility that atRal accumulation may be a contributing factor to macular degeneration, with recessive homozygous mutations in *ABCA4* causing the juvenile onset observed in Stargardt's disease, while the heterozygous state enhances susceptibility to AMD later in life.

While the etiology of Stargardt's disease can be traced to homozygous mutations in a single gene, the etiology of AMD is more complex, with multiple factors contributing to disease occurrence. Two clear underlying factors associated with AMD are oxidative stress and alterations in complement activation. The retina is especially susceptible to oxidative stress because it has high oxygen consumption and is continually exposed to light.⁷ Further, it contains chromophores such as atRal, which generates superoxide anion and singlet oxygen when exposed to visible and UV light, respectively.^{17,18} The demonstrated protective effects of antioxidant supplements for high-risk patients support the notion that oxidative stress is a causative factor in AMD.¹⁹ The AP was implicated in AMD pathogenesis due to a common variant in the complement factor H (CFH) gene (Y402H), which is strongly associated with increased risk for AMD.⁵ CFH, a circulating inhibitor of the AP, is expressed by RPE cells and provides localized cell surface protection against complement attack.²⁰ Similarly, RPE cells express membrane complement regulatory proteins (mCRPs) including CD46 and CD59. Deficiency in either CFH or mCRPs increases susceptibility of mammalian cells to complement-mediated cell stress and death. Notably, *ABCA4* knockout mice that develop Stargardt's-like macular degeneration have reduced expression of the CD46 and CD59 mouse homologues, leading to increased RPE C3 deposition.²¹ This finding suggests a relationship between atRal processing dysregulation and decreased RPE cell mCRP with associated cell surface complement deposition.

In the present study, we hypothesized that atRal serves as a source of oxidative stress, sensitizing RPE cells to AP-mediated cell death. Our lab has previously reported that CD46 and CD59 are robustly expressed on the surface of cultured primary human RPE cells.²² In the current study, we assessed whether atRal downregulates these mCRPs, thus increasing RPE cell susceptibility to AP attack. Despite increasing understanding of the etiology of AMD and Stargardt's disease, effective treatments are limited. We thus determined whether we could prevent oxidative stress and complement activation as a possible treatment approach to prevent atRal- and AP-mediated cell death. To accomplish this, we used resveratrol, an antioxidant shown to attenuate reactive oxygen species (ROS) production in RPE cells, as well as an anti-C5 antibody to inhibit complement activity.²³ Resveratrol was selected as the antioxidant of choice for these experiments due to a growing body of literature pointing to its protective effects in various diseases and a recent report that pretreatment with resveratrol induces a significant, dose-dependent increase of superoxide dismutase, glutathione peroxidase, and catalase activities in RPE cells.²³

MATERIALS AND METHODS

Antibodies and Reagents

Rabbit anti-ZO-1 antibody, goat antirabbit Alexa-488, goat antimouse Alexa-568, and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) were purchased from Invitrogen (Carlsbad, CA). Resveratrol, atRal, and mouse anticytokeratin-18 antibody were purchased from Sigma (St. Louis, MO). Allergan, Inc. (Irvine, CA) generously provided the sheep anti-RPE antibody (S-58). The Cytotoxicity Detection Kit to detect LDH release and WST-1 reagent to detect cell viability were purchased from Roche Applied Science (Penzberg, Germany). Monoclonal mouse anti-C5 antibody (A217) and C1q-depleted serum were purchased from Quidel Corporation (San Diego, CA). Mouse antihuman CD46 and mouse antihuman CD59 antibodies were purchased from AbD Serotec (Kindlington, UK). Antimouse IgG antibody conjugated with horseradish

peroxidase for immunoblotting and fluorescein-conjugated rabbit antimouse IgG antibody for flow cytometry were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Chemicon (Billerica, MA).

RPE Cell Culture

Human donor eyes were obtained from the North Carolina Organ Donor and Eye Bank in accordance with provisions of the Declaration of Helsinki for research involving human tissue. RPE cells from the eyes of a 62-year-old male donor were harvested as previously described.²⁴ Cells were grown in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37°C in a humidified environment containing 5% CO₂. ARPE-19 cells were grown in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 with 10% FBS and 1% P/S. For all experiments, cells were plated at 0.1×10^6 cells/mL and grown in medium with 10% FBS for 6 days, at which time they were confluent and had a cuboidal morphology (Fig. 1A). Cell cytoplasm and membranes were stained positively for cytokeratin and ZO-1, respectively, confirming the epithelial nature of the cells (Fig. 1B). All experiments were performed in phenol-free medium. All experiments were performed in dim ambient light unless otherwise indicated.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature (RT), blocked for 1 hour in 5% normal goat serum in PBTA (PBS + 1% Triton + 7.5% BSA) at RT, then incubated overnight at 4°C with the following antibodies in PBTA: rabbit anti-ZO-1 (1:1000), mouse anticytokeratin-18 (1:400). Cells were incubated for 1 hour at RT with the following antibodies in PBTA: goat antirabbit Alexa-488 (1:500), goat antimouse Alexa-568 (1:500). Cells were incubated for 5 minutes at RT with 4',6-diamidino-2-phenylindole (DAPI) and mounted on a slide using Fluoromount (SouthernBiotech, Birmingham, AL).

Determination of Cell Viability

Cells were treated with atRal in medium with 1% FBS for 90 minutes or 24 hours. Subsequent steps were performed in serum-free medium. All conditions were tested in triplicate. To activate AP, cells were incubated with 24% sheep anti-RPE antibody for 30 minutes and then treated with 6% C1q-depleted human serum (C1qD). After 90 minutes at 37°C, the supernatant was collected in a 96-well plate and replaced with fresh medium. LDH release was measured in the supernatant using a Cytotoxicity Detection Kit (Roche Applied Science). Cell viability was determined by adding WST-1 reagent directly to cells. When resveratrol was used, it was added to cells in medium with 10% FBS for 24 hours prior to atRal treatment. When the anti-C5 antibody was used to attenuate AP activation, it was mixed with C1qD at a concentration of 25 µg/mL on ice for 10 minutes to neutralize the C5 component of the complement cascade. The mixture of C1qD + anti-C5 antibody was then added to cells, and the effect of the antibody on attenuating AP-mediated cell death was compared to cells stimulated with C1qD alone.

Flow Cytometry

Cells were treated with atRal, hydrogen peroxide, or hydroquinone in medium with 1% FBS for 90 minutes or 24 hours.

FIGURE 1. atRal and AP cause dose-dependent cell death in primary human RPE cells. (A) The experiments in this study were carried out in primary human RPE cells. *Scale bar:* 25 μ m. (B) Experiments were performed 6 days following plating of RPE cells. At this time, cells stain positively for ZO-1 (*green*), a marker of tight junctions, and cytokeratin-18 (*red*), a marker of cells of epithelial origin. *Scale bar:* 10 μ m. In a WST-1 assay, RPE cells undergo dose-dependent decrease in cell viability following incubation with atRal for 90 minutes (C) or following AP activation (D).

mCRP cell surface expression was measured as previously described.²² The percentage of viable cells in each sample was also measured. CD46 and CD59 antibodies were used at a dilution of 1:20. Fluorescein-conjugated rabbit antimouse antibody was used at a dilution of 1:50. All conditions were tested in triplicate.

Determination of ROS Generation

Cells were incubated in serum-free medium with 20 μ M H₂DCFDA fluorescent probe (5-[and-6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester [CM-H₂DCFDA]; Molecular Probes #C6827, Invitrogen, Eugene, OR), treated with atRal in medium with 1% FBS for 90 minutes, then switched to serum-free medium. After addition of the fluorescent probe, exposure of cells to light was limited. Fluorescence was detected using a plate reader (SpectraMax M5 plate reader; Molecular Devices, Sunnyvale, CA) at excitation wavelength 490 and emission wavelength 522. To evaluate the effects of antioxidant-mediated attenuation of ROS generation, cells were treated with 25 μ M resveratrol in medium with 10% FBS for 24 hours, washed with serum- and phenol-free medium, then incubated with

the fluorescent probe as described above. The dose of resveratrol was selected following a dose-response experiment, which showed 25 μ M to be the lowest concentration that reliably caused significant attenuation of ROS production. All conditions were tested in triplicate.

Statistical Analysis

Data are expressed as the mean $\pm$ SD. A Student's *t*-test was used to determine whether there were statistically significant differences between treatment and control groups. To calculate the expected effect of combining atRal and AP, the following formula was used: fractional response to atRal + fractional response to AP $\times$ (1 – fractional response to atRal).

RESULTS

atRal and AP Activation Synergistically Cause RPE Cell Death

To determine the effect of atRal on AP-mediated cell death, we assessed the effect of each treatment alone, then in combina-

tion. atRal alone caused dose-dependent cell death (Fig. 1C). Within the range of 10 to 20 μM , atRal had a small yet reproducible cytotoxic effect on RPE cells; thus doses within this range were used in this study. We have previously shown that AP is activated by priming RPE cells with an anti-RPE antibody followed by exposure to C1qD (Berchuck JE, et al. *IOVS* 2012;233:ARVO E-Abstract 1649). Accordingly, a range of anti-RPE priming antibody concentrations was tested to initiate AP attack; AP activation caused RPE cell death in a dose-dependent manner (Fig. 1D). Twenty-four percent antibody was selected for subsequent experiments as it reproducibly caused significant cell death, yet sufficient cells remained to analyze potential synergy between oxidative stress and complement-mediated cell death.

To examine the combined effects of atRal and AP activation, RPE cells were incubated in atRal for 90 minutes followed by initiation of AP attack. When AP was activated following pretreatment of cells with atRal, the combined effect on cell viability was significantly greater than would be expected from the additive effects of the two independent treatments. Following incubation with 15 μM atRal, cell viability was reduced to 89.6% while AP attack reduced cell viability to 64.2% relative to that in untreated controls. Based on their independent cytotoxic effects, the expected viability following treatment with atRal and AP is 57.5%. Sequential treatment with atRal and AP, however, reduced cell viability to 24.1%, significantly less than the expected viability of 57.5% if the combined treatment had an additive effect (Fig. 2A). To ensure that this phenomenon was not unique to donor cells, this experiment was repeated in ARPE-19 cells, an immortalized RPE cell line, and the same effect was observed (Fig. 2B). To further confirm these findings, an LDH release assay was used to measure the cytotoxic effects of atRal and AP. In both primary human RPE (Fig. 3A) and ARPE-19 (Fig. 3B) cells, LDH release following sequential treatment with atRal and AP was significantly greater than expected given the two independent treatments.

Effect of atRal on mCRP Expression

RPE cells express the mCRPs CD46 and CD59 on their surface to prevent bystander injury when complement is activated. We sought to determine whether atRal mediates sensitivity to AP attack through downregulation of these mCRPs. RPE cells were

treated with atRal 15 μM for either 90 minutes or 24 hours, and cell surface expression was measured using flow cytometry. Ninety-minute treatment with atRal did not alter cell surface expression of CD46 and CD59; however, 24-hour atRal treatment decreased their expression by 58% and 50%, respectively (Fig. 4A). To understand whether this effect was specific to atRal, we treated cells for 24 hours with equivalently cytotoxic doses of other known oxidants (Fig. 4B). Hydrogen peroxide treatment had no effect on CD46 or CD59 levels, and hydroquinone treatment conversely caused a significant increase in levels of both mCRPs (Fig. 4C). Thus, significant downregulation of RPE cell surface CD46 and CD59 was unique to atRal.

Effect of atRal-Mediated mCRP Downregulation on Susceptibility to AP Attack

Following the discovery that atRal induces time-dependent downregulation of mCRPs, we sought to determine whether duration of atRal exposure affects sensitivity to AP attack. To assess whether less complement activation is required to cause cell death following atRal-mediated downregulation of mCRPs, we initiated AP attack with varying concentrations of the anti-RPE priming antibody following 90-minute or 24-hour atRal treatment. Cells treated for 24 hours were significantly more susceptible to AP-induced cell death at lower concentrations of antibody compared with cells treated for 90 minutes (Fig. 5A). In multiple independent experiments, cell viability data were fit with a linear regression; and the EC_{50} , the concentration of antibody needed to kill 50% of cells following atRal treatment, was calculated. Following 24-hour treatment, the EC_{50} , 20.7%, was significantly lower than the EC_{50} following 90-minute treatment, 27.4%.

atRal-Mediated ROS Production and Its Effect on Susceptibility to AP Attack

All-*trans*-retinal is known to generate ROS. We therefore asked whether atRal-mediated RPE cell death and sensitization to AP attack is caused by ROS generation. ROS production was measured using a H_2DCFDA fluorescent probe (5-[and-6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester [CM-H₂DCFDA]; Molecular Probes #C6827, Invitrogen,

FIGURE 2. atRal sensitizes RPE cells to complement attack. Primary human RPE cells (**A**) and ARPE-19 cells (**B**) were incubated in atRal for 90 minutes prior to initiation of AP attack. In a WST-1 assay, a significantly greater decrease in cell viability was observed when AP attack was initiated following atRal pretreatment than would be expected given the combined effect of the two independent treatments alone (* $P < 0.005$, # $P < 0.0005$). The dotted line represents the expected additive effect of atRal and AP attack as independent treatments.

FIGURE 3. In the LDH release assay, complement attack increased RPE cell membrane permeability. Ninety-minute atRal treatment alone caused minimal cell permeability; however, it sensitized primary human RPE cells (A) and ARPE-19 cells (B) to complement attack leading to greater cell permeability than would be expected given the combined effect of the two treatments (* $P < 0.01$, # $P < 0.0001$). The dotted line represents the expected additive effect of atRal and AP as independent treatments.

FIGURE 4. atRal alters mCRP cell surface expression. CD46 and CD59 levels are expressed as the mean fluorescence intensity of the population of viable cells measured for that condition normalized to untreated cells. (A) Treating RPE cells with atRal 15 μM for 90 minutes did not affect CD46 and CD59 cell surface expression; 24-hour treatment with the same dose of atRal, however, caused a significant decrease in both CD46 and CD59 surface levels (* $P < 0.00005$). (B) Cells were treated for 24 hours with doses of atRal (15 μM), hydrogen peroxide (400 μM), and hydroquinone (100 μM) that caused similar levels of cytotoxicity. (C) Following 24-hour treatment with these oxidants, only atRal significantly decreased CD46 and CD59 levels (* $P < 0.001$); hydrogen peroxide treatment had no effect on CD46 or CD59 levels, and hydroquinone significantly increased cell surface expression of both mCRPs (# $P < 0.01$).

FIGURE 5. Duration of atRal treatment affects susceptibility to complement attack. Cells were treated with atRal for 90 minutes or 24 hours followed by initiation of AP attack with varying concentrations of the anti-RPE priming antibody. Cell viability data were then fit with a linear regression; and the EC_{50} , the concentration of antibody needed to kill 50% of cells following atRal treatment, was calculated. This experiment was repeated multiple times, and the EC_{50} was calculated for each experiment; the average was 20.7% for 24-hour treatment and 27.4% for the 90-minute treatment. Using the values generated from these independent experiments, cells treated for 24 hours required significantly less antibody to kill 50% of the cells than did cells treated for 90 minutes ($P < 0.0005$).

Eugene, OR). Following 90-minute treatment, atRal generated ROS in a dose-dependent manner (Fig. 6A). Cells were then pretreated with the antioxidant resveratrol for 24 hours prior to atRal treatment, which significantly attenuated atRal-mediated ROS production (Fig. 6B). An LDH release assay was used to determine whether attenuation of ROS production prevents atRal-mediated sensitization to AP attack. Significantly more LDH was released when AP attack was initiated on atRal-treated cells compared to AP attack on untreated cells. Pretreatment with resveratrol, however, significantly reduced AP-induced LDH release following atRal treatment to a level that was no different from AP-induced LDH release on untreated cells (Fig. 6C). Following this result, we hypothesized that resveratrol protects against sensitization to AP attack by preventing mCRP downregulation. Using flow cytometry, we found that pretreating cells with resveratrol prior to 24-hour atRal treatment significantly attenuated atRal-mediated downregulation of CD46 and CD59 (Fig. 6D).

Examining the Protective Effects of an Anti-C5 Antibody Against AP Attack

Since the discovery that aberrant AP activation contributes to RPE cell injury, significant efforts have been made to develop complement inhibitors as a novel class of therapies for AMD. Using our model, we sought to determine whether an anti-C5 antibody protects against AP-mediated cell death following exposure to atRal. In an LDH release assay, the anti-C5 antibody prevented 75% of AP-mediated LDH release alone and 85% of LDH release following atRal treatment. Despite significantly greater cytotoxicity when AP attack was initiated following atRal compared to untreated cells, addition of the anti-C5 antibody was equally, if not more, effective at preventing AP-mediated cell permeability (Fig. 7A). Having observed the protective effects of resveratrol and the anti-C5 antibody when used independently, we assessed their efficacy in combination. Using a WST-1 assay, we observed that both resveratrol and anti-C5 antibody independently prevented the atRal- and AP-mediated decrease in cell viability. Further, their protective effect in combination was greater than either treatment alone

and restored cell viability of atRal- and AP-treated cells to 99% compared to untreated cells (Fig. 7B).

DISCUSSION

In this study, we found that both atRal and AP activation independently cause dose-dependent RPE cell death. Further, following 90-minute or 24-hour treatment, atRal sensitizes primary human RPE cells to AP-induced cell death. When exposed to atRal for 24 hours, RPE cells undergo time-dependent downregulation of the mCRPs CD46 and CD59. Decreased cell surface expression of these mCRPs corresponds temporally to enhanced susceptibility to AP-induced RPE cell death. Pretreating RPE cells with the antioxidant resveratrol prevents atRal-mediated mCRP downregulation and attenuates sensitization to AP attack. Treatment with an anti-C5 antibody prevents AP-mediated cell death, and when used in combination with resveratrol, completely reverses the adverse effects of atRal and AP attack on cell viability.

RPE cell death is one of the initial events leading to macular degeneration in both Stargardt's disease and AMD.¹⁻⁴ Understanding the mechanisms by which cellular stresses such as atRal and AP activation contribute to RPE cell injury will help guide novel approaches to treating these diseases. We therefore assessed how sublethal doses of atRal sensitize RPE cells to AP attack. The doses of atRal used in this study (10–20 μ M) are likely achievable under aberrant conditions, as rod outer segments contain 5 mM rhodopsin, and bleaching of even 0.5%, if not properly cleared, would generate atRal concentrations exceeding those used in the current experiment.²⁵

It has previously been shown that ARPE-19 cells exposed to sublethal doses of hydrogen peroxide followed by stimulation with human serum have decreased transepithelial resistance compared to untreated cells. Based on existing models, we performed atRal treatment and AP activation sequentially instead of simultaneously because (1) they were optimized under different treatment conditions and (2) this approach helped to elucidate the mechanisms by which pre-exposure to atRal sensitizes RPE cells to subsequent AP attack, which would be difficult to ascertain if the treatments were administered simultaneously.

One unique aspect of our approach is that the priming antibody, under the conditions used in this series of experiments, is able to function through specific activation of the AP. It is clear that complement activation is involved in AMD, and immunoglobulins are components of drusen, which are associated with complement activation in eyes with AMD. However, the nucleating event associated with complement activation remains to be determined. Clearly, multiple factors are likely to be involved and not just immunoglobulins. We have, however, developed the IgG priming antibody used in this series of studies to achieve a model of complement attack that is tightly controlled. Since the alternative pathway is associated with AMD, we have implemented a more relevant model than previously reported studies in which the mannose or classical pathways are the primary pathways activated. As such, these reagents and experiments become more relevant and open up the opportunity to ask questions on how the alternative pathway and its end products affect RPE cell biology. We have, however, taken care to not extrapolate the priming antibody to induction of complement activation in AMD patients.

Our data suggest that downregulation of mCRPs by atRal sensitizes RPE cells to AP attack. We have previously shown that primary human RPE cells express the mCRPs CD46 and CD59.²² These cell surface proteins act through unique

FIGURE 6. (A) Ninety-minute treatment with atRal causes dose-dependent ROS production in RPE cells as measured by relative fluorescence units (RFU). (B) Incubating RPE cells in resveratrol for 24 hours prior to treating with atRal significantly attenuated atRal-mediated ROS production ($*P < 0.0001$). (C) In an LDH release assay, pretreatment with resveratrol protected against atRal-mediated sensitization to AP attack ($*P < 0.05$ compared to 24% complement alone, $\#P < 0.05$ compared to atRal + 24% complement with no resveratrol treatment). (D) Pretreating cells with resveratrol 25 μM significantly attenuated downregulation of the mCRPs CD46 and CD59 by atRal 12.5 μM as measured by flow cytometry ($*P < 0.0001$ compared to untreated cells, $\#P < 0.005$ compared to atRal-treated cells). CD46 and CD59 levels are expressed as the mean fluorescent intensity of the population of viable cells measured for that condition normalized to untreated cells.

FIGURE 7. Anti-C5 antibody protects RPE cells from AP-induced cell death. (A) RPE cells were incubated in medium or atRal 20 μM for 90 minutes. The effect of AP activation with and without an anti-C5 antibody was then assessed. Incubation with the anti-C5 antibody significantly protected against AP-induced cell death in a LDH release assay. Significantly more LDH was released when AP attack was initiated following atRal treatment compared to AP attack on untreated cells ($*P < 0.05$); however, incubation with the anti-C5 antibody abrogated the atRal-mediated sensitization to AP-induced cell death. (B) In a WST-1 assay, resveratrol ($*P = 0.052$) and the anti-C5 antibody ($**P = 0.016$) each individually protected against the decrease in cell viability induced by the combination of atRal and AP attack. Using resveratrol and the anti-C5 antibody in combination had an even greater protective effect ($***P = 0.0054$) and completely prevented atRal- and AP-mediated cytotoxicity, rescuing cell viability to 99.1% of that of untreated cells.

mechanisms to prevent bystander injury when complement is activated. In the current study we assessed the effects of atRal on mCRP expression in primary human RPE cells. Following 90-minute atRal treatment, CD46 and CD59 cell surface expression does not change; however, mCRP cell surface levels significantly decrease following 24-hour treatment. Notably, RPE cells are significantly more susceptible to AP attack following 24-hour atRal compared to 90-minute treatment. In a mouse model of Stargardt's disease, atRal accumulation resulted in decreased expression of the CD46 and CD59 mouse homologues, leading to increased C3 deposition in the RPE.²¹ Together with our data to show the temporal relationship between atRal-mediated downregulation of mCRPs and RPE cell sensitivity to complement attack, the studies support the hypothesis that atRal increases susceptibility to AP attack by downregulating mCRPs.

Downregulation of mCRPs appears to be regulated at least in part by oxidative stress, as treatment with resveratrol attenuated atRal-induced ROS production and also prevented atRal-mediated mCRP downregulation. This effect, however, is likely not caused exclusively by oxidative stress, as (1) doses of resveratrol that completely attenuate atRal-induced ROS production did not completely rescue mCRP levels and (2) functionally equivalent doses of hydrogen peroxide and hydroquinone, two known oxidants, did not decrease mCRP expression in the same manner as atRal. Furthermore, we observed increased susceptibility to AP attack following 90-minute atRal treatment when downregulation of mCRPs was not observed, which also supports a role for nonoxidant-mediated mechanisms. Possible insight into this secondary process can be deduced from the finding that when ARPE-19 cells were treated with equivalent doses of a number of retinoids, 9-*cis*-retinal and atRal caused significantly greater cell death than other retinoids, including A2E, and that the effect was identical in the presence and absence of blue light exposure.¹⁵ Notably, the two retinoids causing the most cytotoxicity were both aldehydes. Although retinaldehyde toxicity was not experimentally addressed in the present study, aldehyde cytotoxicity is well established.²⁶ It is possible that in addition to causing oxidative stress, atRal's intrinsic properties as a reactive retinaldehyde play a role in causing irreversible damage to the mitochondria, nucleus, and integrity of cellular proteins in general, resulting in the effects observed in the current study.

An important motivation for the present series of experiments was to identify treatment approaches for advanced non-neovascular AMD and Stargardt's disease. Treatments for Stargardt's disease are nonexistent, and treatment for AMD-associated geographic atrophy is limited. There appears to be a subpopulation of patients with AMD that may benefit from therapy directly targeting atRal-mediated RPE cell damage. It was recently discovered that a subgroup of AMD patients with fine granular pattern with peripheral punctate spots seen on fundus autofluorescence imaging is significantly associated with monoallelic *ABCA4* sequence variants.²⁷ This finding has important implications. Previous studies looking at alterations in the *ABCA4* gene alterations found no significant difference between patients with AMD and controls.¹⁰ This new finding, however, suggests that *ABCA4* mutations do play a role in the pathogenesis of a proportion of patients with AMD and that a study of all patients with AMD may simply not be powered to detect real sequence variation in a subpopulation of patients with the disease. This study also shows the ability to use imaging and genetic data to identify those patients who may benefit from targeted therapy preventing atRal accumulation.

Another strategy to prevent RPE injury in AMD is complement inhibition. This approach includes blocking various effector molecules in the complement pathway. C5

inhibition has the advantage of preventing terminal complement activity while maintaining more proximal complement functions such as production of C3a anaphylatoxin and C3b, which are required to prevent bacterial infection and may preserve desired complement-mediated activities.²⁸ In the current study we found an anti-C5 antibody to be a potent inhibitor of AP-mediated cell death. Further, despite AP activation causing significantly greater cell death after cells were exposed to atRal, the anti-C5 antibody abrogates the synergistic cytotoxic effect observed when these stimuli are applied in combination. When the anti-C5 antibody and resveratrol are used together, they have a greater protective effect than either treatment alone and completely prevent atRal- and AP-induced cytotoxicity. The COMPLETE trial is an ongoing study looking at various outcomes in patients with nonneovascular AMD being treated with ecluzimab, an antibody to human complement factor 5. Preliminary results revealed that ecluzimab failed to detectably slow the progression of GA or decrease drusen volume in treated patients relative to controls (Garcia Filho C, et al. *IOVS* 2012;53:ARVO E-Abstract 2045). Many possibilities can explain the failure to obtain a significant finding, including the following: (1) The delivery method and/or dosing was inadequate to stop complement inhibition at the critical site; (2) the trial duration was too short; (3) the trial had too few patients, so was inadequately powered to identify a therapeutic response; and (4) complement inhibitors may need to be used in combination with antioxidants to be most effective. While the efficacy of inhibiting complement activity remains to be proven in clinical trials, the results of the current study provide rationale for testing antioxidants and complement inhibitors in combination to prevent the RPE cell damage observed in AMD and Stargardt's disease.

This study has limitations. First, the conditions in the in vitro model do not accurately represent the complexity of the in vivo environment. Second, because cells were grown in a monolayer, complement attack was primarily initiated on the RPE cell apical surface. In AMD it is generally believed that complement-mediated attack occurs primarily on the basal surface. Preliminary studies in our laboratory performed in transwell permeable supports that allow basal application of complement suggest that similar cytotoxic effects are observed regardless of whether complement attack is initiated on the basal or apical surface.

In conclusion, atRal sensitizes RPE cells to AP-mediated cell death. This process may be mediated in part by atRal-induced downregulation of mCRPs caused by both ROS production and secondary mechanisms independent of oxidative stress. mCRP downregulation is prevented by treating cells with the antioxidant resveratrol. Despite the synergistic cytotoxic effects of atRal and AP activation, antioxidant therapy combined with complement inhibition presents a promising therapeutic approach to prevent the RPE cell death observed in Stargardt's disease and AMD.

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