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Evaluation of Adenovirus-Delivered Human CD59 as a Potential Therapy for AMD in a Model of Human Membrane Attack Complex Formation on Murine RPE

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Abstract

Purpose—Complement-mediated damage to the retinal pigment epithelium (RPE), Bruch membrane, and choroid has been associated with pathogenesis in age-related macular degeneration (AMD). The terminal step of complement activation involves lysis of cells by the insertion of the membrane attack complex (MAC) in the plasma membrane. The hypothesis that local overexpression of human CD59 (hCD59) delivered by an adenovirus (Ad) vector to primary murine RPE cells in vitro, RPE in vivo, or cornea ex vivo protects those cells from human MAC deposition and lysis was tested.

Methods—A humanized model of MAC deposition on murine cells and murine ocular tissues including RPE and cornea was developed to permit testing of human complement regulators in mice. A recombinant adenovirus-expressing hCD59 was generated, and this virus was injected into the subretinal space of adult mice. Subsequently, eyecups from these mice were exposed to human serum, and the levels of MAC deposition on the RPE were quantified. hCD59 was also expressed on murine cornea ex vivo and in murine hepatocytes, and primary RPE cells in vitro and levels of human MAC deposition and cell lysis were measured.

Results—Adenovirus-mediated delivery of hCD59 to the RPE, cornea, or cells in culture protects those cells from human MAC deposition and MAC-mediated damage and vesiculation.

Conclusions—The humanized model of MAC deposition on murine ocular tissues allows testing of human complement regulators that may have potential in the treatment of AMD or other diseases associated with complement activation.

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in industrialized nations.^{1,2} Although the molecular basis for AMD is not well understood, a growing body of evidence has recently implicated inflammatory processes, specifically the complement system, in the pathogenesis of this disease.^{3–9} Immunohistochemical studies have localized activated complement components, including the membrane attack complex (MAC) in retinal pigment epithelium (RPE) and drusen in the eyes of patients with AMD.^{3,5,6} Epidemiologic and genetic studies have identified

polymorphisms in complement regulatory protein, factor H,^{10–13} as well as complement components factor B, C2,^{14,15} and C3¹⁶ as risk or protective factors for developing AMD. Hence, it appears that an imbalance between the activators and the inhibitors of complement may be responsible for the pathogenesis observed in AMD.

The complement system is an important arm of innate immunity (for reviews, see Walport¹⁷ and Walport¹⁸), mediating numerous inflammatory processes and normally functioning to fight pathogens and to clear potentially dangerous debris. Complement activation results in the generation of several effector molecules, such as the opsonin C3b, the anaphylatoxins C3a and C5a, and ultimately the terminal MAC, which can directly kill cells through the formation of lytic pores in the cell membrane. Complement does not discriminate between self and non-self, and it is constantly activated at low levels by spontaneous hydrolysis of C3.¹⁹ Several regulatory molecules, such as factor H, decay accelerating factor (CD55), membrane cofactor protein (CD46), and CD59 (also called membrane inhibitor of reactive lysis [MIRL] or *protectin*), provide active control at several steps along the complement cascade, keeping complement activation in check and protecting autologous cells from complement attack.^{20,21} This control can, however, be overwhelmed in certain cases because of the presence of specific complement activators or insufficient function or production of the complement regulatory proteins. Complement-activating factors have been implicated in the pathogenesis of AMD. For example, photooxidation products of A2E, which is a bis-retinoid lipofuscin pigment that accumulates in RPE cells, have been reported to activate the complement system,²² and variants of complement regulatory protein factor H confer increased risk for AMD.^{10–13}

We wanted to test the hypothesis that local overexpression of complement regulatory proteins may be used to dampen local complement attack specifically in ocular tissues such as the RPE, a tissue intimately involved in the pathogenesis of AMD. However, testing this hypothesis initially in humans would not be possible, and cross-species differences in some complement proteins limit testing the efficacy of human complement regulatory proteins in nonhuman systems.^{23–26} To allow for the testing of human complement regulators in mice, we developed a humanized murine model of measuring human MAC deposition on murine ocular tissues.

The complement regulatory protein CD59 protects autologous cell killing by preventing the formation of lytic MAC on the cell membrane.^{27,28} Although here we tested human CD59 (hCD59), our model would be useful for testing the efficacy of the other membrane-bound complement regulatory proteins and the synergistic effect of their combined overexpression.

Adenovirus vectors have been shown to allow transgene expression for years in nonhuman primates,²⁹ and “lifetime” persistence and transgene expression in rodents has been observed in vivo.³⁰ In ocular tissues, adenovirus has been shown to persist in the RPE for at least 1 year in vivo, the latest period examined,³¹ and adenovirus has been used in two human ocular gene therapy trials.^{32,33} In these trials, no serious adverse events were recorded.³³ Therefore, inhibiting MAC deposition on RPE by local overexpression of hCD59 with the use of adenovirus vectors may be envisaged in patients with AMD.

Materials and Methods

Adenovirus Constructs

A replication-deficient recombinant human serotype 5 adenovirus vector containing a hCD59 cDNA expressed from a hybrid promoter consisting of a CMV enhancer/chicken β -actin promoter and terminated by a rabbit globin polyadenylation sequence was constructed. As negative control, the same construct devoid of the CD59 cDNA (AdEMPTY) or expressing GFP (AdCAGGFP) was constructed. Cloning details for each vector are in the Supplementary Methods, online at <http://www.iovs.org/cgi/content/full/49/9/4126/DC1>.

Cell Lines and Adenovirus Infections

The human embryonic retinoblast cell line 911³⁴ was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), and the mouse hepatoma cell line hepa-1c1c7 (ATCC, Manassas, VA) was maintained in α -MEM supplemented with 10% FBS. For Western blot analyses or the human serum cell lysis assay and for hCD59 immunocytochemistry or the human serum MAC deposition assay, respectively, 1.2×10^6 hepa-1c1c7 cells and 2.5×10^4 hepa-1c1c7 cells were infected with AdCAGGFP or AdCAGCD59 or were left uninfected. All adenovirus infections were performed in media with 2% FBS. Three days after infection, cells were treated as described.

Primary mouse RPE cells were harvested from 6- to 10-week-old C57Bl/6J mice. Eyecups devoid of retina were incubated in 200 μ L of 0.25% trypsin-EDTA for 50 to 60 minutes at 37°C. Eyecups were transferred to α -MEM supplemented with 10% FBS. The RPE was dislodged using fine forceps, and aspirated. 3×10^4 cells were seeded in one chamber of a poly-D-lysine-coated chamber slide (Becton Dickinson, Franklin Lakes, NJ). After 1 week, cells were used as described. Infections were done in media with 2% FBS. Western blot analysis was performed as described in the Supplementary Methods.

Human Serum Cell Lysis Assay

Lyophilized human serum (Sigma, St. Louis, MO) was reconstituted with 1 mL cold sterile deionized water to obtain a volume of serum equal to that of the human plasma from which the powder was obtained. The resultant human serum was separated into aliquots and stored at -80°C . Two lots of serum were used in this study. The first lot, with a hemolytic titer of 43 CH₅₀ U/mL, was used in experiments with hepa-1c1c7 cells. The second lot, with a hemolytic titer of 74 CH₅₀ U/mL, was used in all the other experiments. Hemolytic titers were determined by the manufacturer.

For the human serum cell lysis assay, single-cell suspensions of uninfected or adenovirus-infected hepa-1c1c7 cells in a total volume of 500 μ L were used. Cells were washed twice with $1\times$ PBS and, after trypsinization (0.25% trypsin-EDTA, 4–6 minutes), were suspended in PBS containing 0.5% FBS. Cells were collected by centrifugation at 4°C and were resuspended in ice-cold gelatin veronal buffer with Ca²⁺ and Mg²⁺ (GVB²⁺) (Complement Technology, Tyler, TX). Cells (5×10^5) were exposed to normal human serum (NHS) or heat-inactivated (56°C for 1 hour) normal human serum (HI-NHS) and were incubated at 37°C for 1 hour with gentle rotatory shaking in a 1.5-mL tube. Cell lysis was determined by

the propidium iodide (PI) exclusion method, followed by FACS analysis. Events (2.5×10^4) per sample were counted (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) and analyzed (CellQuest Pro software; Becton Dickinson). Percentage cell lysis was calculated as % Cell Lysis = $[1 - (\% \text{ Live Cells in NHS} / \% \text{ Live Cells in HI-NHS})] \times 100$.

MAC Deposition Assay in Cell Culture

Three days after infection, AdCAGGFP-infected, AdCAGCD59-infected, or uninfected hepa-1c1c7 cells in poly-D-lysine-coated chamber slides (Becton Dickinson) were washed twice with $1 \times$ PBS and incubated with 10% (vol/vol) NHS or HI-NHS in GVB²⁺ (Complement Technology, Tyler, TX) at 37°C for 1, 3, 5, 7, or 10 minutes. Primary mouse RPE cells were incubated with or without 25 $\mu\text{g/mL}$ goat anti-mouse ex-tracellular matrix metalloproteinase inducer (emmprin; R&D Systems, Minneapolis, MN) antibody in GVB++ (Complement Technology) for 1 hour and were washed and fixed for emmprin immunocytochemistry or, alternatively, NHS/HI-NHS (final concentration, 50%) was added for 7 minutes for MAC deposition assay. Immediately thereafter, cells were washed three times with cold $1 \times$ PBS and were fixed with 3.7% formaldehyde (MP Biomedicals, Solon, OH) in $1 \times$ PBS for 15 minutes. Cells were washed three times with $1 \times$ PBS and were stored in this solution at 4°C until immunocytochemical analysis.

Subretinal Injections

The use of animals in this study was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Subretinal injections were performed as described in the Supplementary Methods.

Tissue Processing and MAC Deposition on Mouse RPE and Cornea

Six days after injection, mice were killed by CO₂ inhalation, and eyes were harvested and placed in $1 \times$ PBS containing penicillin (100 U/mL) and streptomycin (100 $\mu\text{g/mL}$). Eyecups devoid of retina were fixed immediately in 4% paraformaldehyde in phosphate buffer (pH 7.4) overnight (for CD59 immunohistochemistry) or were incubated with 25 $\mu\text{g/mL}$ goat anti-mouse emmprin antibody (R&D Systems) in cold GVB++ (Complement Technology) at 4°C for 1 hour. Eyecups were then washed three times with cold $1 \times$ PBS and fixed (for emmprin immunohistochemistry), or, for the MAC deposition assay, an equal volume of NHS or HI-NHS (final concentration, 50%) was added and incubated at 37°C for 7.5 or 15 minutes and then washed three times with cold $1 \times$ PBS and fixed as described. Corneas were harvested from uninjected mice and cultured in 300 μL DMEM with 2% FBS. Corneas were infected with 1.5×10^9 virus particles (vp) of the AdCAGGFP or the AdCAGCD59 virus. Three days after harvesting/infection, corneas were treated with the anti-mouse emmprin antibody, as with eyecups, and were washed and fixed for emmprin immunohistochemistry or treated with 50% NHS or HI-NHS for 20 minutes for the MAC deposition assay and washed and fixed as described. Before immunohistochemistry, tissues were washed three times for 10 minutes each with $1 \times$ PBS to remove any remaining fixative. Immunocytochemistry/immunohistochemistry and image analysis were performed as described in the Supplementary Methods.

Results

Adenovirus Constructs and Human CD59 Expression in Adenovirus-Infected Mouse Hepa-1c1c7 cells

To deliver hCD59 to murine RPE in vivo, we generated a recombinant adenovirus (AdCAGCD59) containing the hCD59 cDNA under the control of the chicken β -actin promoter (Fig. 1A). Two control vectors were constructed, one devoid of cDNA (AdEMPTY) and one expressing GFP (AdCAGGFP) (Fig 1A).

Human CD59 is an 18- to 21-kDa glycosylphosphatidyl-inositol (GPI)-anchored membrane protein. To confirm the expression of hCD59, we infected mouse hepa-1c1c7 cells with 1×10^3 vp/cell of AdCAGCD59 or AdCAGGFP. Cell lysates were analyzed by Western blot, which confirmed the presence of hCD59 in cell lysates from AdCAGCD59-infected cells and low levels of hCD59 in the media from those same infections (Fig 1B). We also detected endogenous hCD59 in nontransduced human embryonic retinoblast (911) cell lysates; however, this signal was much weaker than that from the AdCAGCD59-infected mouse cells (Fig. 1B). The small shift in electrophoretic mobility between the endogenous hCD59 in human 911 cells and the recombinant hCD59 produced by AdCAGCD59-infected murine hepa-1c1c7 cells may be attributed to differences in protein modification, such as variation in the protein's glycosylation pattern in the two cell lines.

Immunostaining of nonpermeabilized AdCAGCD59-infected mouse hepa-1c1c7 cells confirmed expression and localization of hCD59 on the cell membrane (Fig. 1C). No hCD59 staining was observed on cells infected with AdCAGGFP (Fig. 1D).

Protection of Mouse Cells from Human Complement-Mediated Lysis by Adenovirus-Delivered hCD59

To test the functional activity of hCD59 expressed from AdCAGCD59, we performed human serum cell lysis assays on mouse hepa-1c1c7 cells. Cell suspensions were incubated with NHS or heat-inactivated NHS (HI-NHS, as a control for non-complement-specific lysis), and the resultant percentage of cell lysis was determined by uptake of PI and quantified by FACS. First, we investigated the kinetics of serum concentration on the lysis of uninfected cells. Mouse hepa-1c1c7 cells effectively activated human complement, and a serum concentration as low as 0.5% (1/200 dilution) lysed more than 50% of cells (Fig. 2A). Lysis of cells was serum concentration dependent. The lowest serum concentration tested that resulted in maximum ($96.06\% \pm 0.87\%$) cell lysis was 1% (1/100 dilution), and this serum concentration was used in subsequent cell lysis experiments.

Hepa-1c1c7 cells were infected with 1×10^3 vp/cell of AdCAGCD59 or AdCAGGFP. Sixty-five hours after infection, they were harvested and subjected to the human serum cell lysis assay. Preinfection of mouse cells with AdCAGCD59 significantly protected those cells from lysis, reducing complement-mediated cell lysis from $96.06\% \pm 0.87\%$ to $12.29\% \pm 0.18\%$ (Figs. 2B, 2D, 2E). However, preinfection with AdCAGGFP instead resulted in $95.27\% \pm 0.01\%$ cell lysis (Figs. 2C, 2E), similar to that of uninfected cells (Figs. 2B, 2E), indicating that protection resulted from the expression of hCD59 and not from the adenovirus infection. The level of protection from cell lysis was dependent on the amount of

AdCAGCD59 used because 250 vp/cell resulted in an approximately 60% inhibition of cell lysis, whereas there was no change in cell lysis at any concentration of AdCAG-GFP tested (Fig. 2F). In conclusion, the expression of recombinant hCD59 significantly protected mouse cells from human complement-mediated cell lysis.

Protection of Mouse Cells from Human MAC Deposition by Adenovirus-Delivered hCD59

MAC is a multimolecular complex composed of C5b, C6, C7, C8, and C9 (C5b-9). On formation of a nascent C5b-8 complex on the cell membrane, multiple copies of C9 can be recruited and polymerized to form a pore in the plasma membrane, leading to osmotic imbalance and lysis of cells. CD59, when present on the cell surface, can bind to C8, C9, or both in the complex, preventing the polymerization of C9 and the formation of MAC.³⁵

To examine whether AdCAGCD59-infected mouse cells were protected from lysis specifically by being able to inhibit the formation of a C5b-9 complex, we used an in vitro MAC deposition assay. Mouse cells were incubated with 10% NHS or HI-NHS at 37°C for 1 to 10 minutes and subsequently were washed and fixed. Incubation of these cells with NHS for 5 minutes caused significant changes in cell morphology (Fig. 3A). Cells lost their extensive cytoplasmic processes and became round and granular. These effects were not seen in cells incubated with HI-NHS (Fig. 3B). Immunocytochemical analysis using a monoclonal antibody directed to a neopeptide on the C5b-9 complex revealed extensive membrane staining at the borders of cells treated with NHS, confirming deposition of the MAC (Fig. 3A). Almost no MAC was observed on cells treated with HI-NHS (Fig. 3B). Under these serum treatment conditions, lysis of almost all NHS-treated cells was confirmed by trypan blue staining (Fig. 3C), whereas almost no lysis was observed on HI-NHS-treated cells (Fig. 3C).

Preinfection of mouse hepa-1c1c7 cells with 1×10^3 vp/cell of AdCAGCD59 significantly protected these cells from human MAC deposition (Fig. 4B). On exposure to NHS for 5 minutes, cells preinfected with AdCAGCD59 maintained their normal morphologic characteristics (Figs. 4B, 4C). Immunocytochemistry revealed almost complete absence of MAC staining (Fig. 4B), and cell lysis was essentially inhibited as indicated by the absence of trypan blue staining (Fig. 4C). In contrast, cells preinfected with AdCAGGFP were not protected against MAC deposition with 5 minutes of NHS treatment, and morphologic changes, MAC immunostaining, and cell lysis of these cells were similar to that of uninfected cells (Figs. 4A, 4C). MAC staining started to appear on some of the AdCAGCD59-infected cells with 7 minutes of NHS treatment (Fig. 4B) and increased after 10 minutes of serum treatment (data not shown). However, after 7 minutes of NHS treatment, MAC staining on AdCAGGFP-infected cells was significantly greater than on Ad-CAGCD59-infected cells, and after 10 minutes of serum treatment almost all AdCAGGFP-infected cells were detached from the cell culture slide, presumably because of complete cell lysis (data not shown). The pattern of MAC immunofluorescence appeared to indicate the extent of cell membrane damage, with strong punctate staining delineating the cell borders, correlating with greater damage, whereas diffuse staining extending throughout the cell membrane correlated with cells that appeared intact. MAC deposition on uninfected and Ad-CAGGFP-infected cells was rapid, and the diffuse staining, indicating lower levels

of MAC deposition, could only be seen on AdCAGCD59-infected cells. The various patterns of MAC immunostaining could be more readily identified when cells were infected with smaller amounts of the AdCAGCD59 virus, as can be seen for cells preinfected with 100 or 500 vp/cell with 5 minutes of NHS treatment (Supplementary Fig. S1; all Supplementary Figures are online at <http://www.iovs.org/cgi/content/full/49/9/4126/DC1>). More important, preinfection with even these smaller amounts of AdCAGCD59 significantly protected mouse hepa-1c1c7 cells from MAC deposition.

Model of Human MAC Deposition on RPE Cells

Next, we extended our MAC deposition assay to primary murine RPE cells and murine eyecups. RPE cells were harvested and identified by the presence of pigmentation, characteristic polygonal morphology, and immunostaining for the RPE cell marker RPE65 (Supplementary Fig. S2). In contrast to hepa-1c1c7 cells, only weak and inconsistent MAC immunostaining was observed on primary (passage 0) mouse RPE cells on treatment with 50% NHS (data not shown). Similarly, eyecups were harvested from C57Bl/6J mice and treated with various concentrations of NHS. Immunohistochemical analysis with the anti-human C5b-9 antibody revealed no fluorescent signal on the RPE, even when eyecups were treated with an NHS concentration as high as 50%. Treatment with 100% NHS for 1 hour resulted in occasional scattered but weak staining (Fig. 5C). This inconsistent and weak signal on primary RPE cells and eyecups proved unreliable as an assay for MAC deposition. To enhance complement activation on murine RPE to the extent reliable as an assay, we used an antibody against the extracellular domain of mouse emmprin, which is an abundantly expressed membrane protein on RPE cells.³⁶ Mouse eyecups treated with anti-mouse emmprin antibody, followed by the addition of NHS (final concentration 50% for 15 minutes), now resulted in extensive MAC immunostaining on the RPE (Fig. 5A). The RPE monolayer often appeared convoluted, and various patterns of staining were observed, presumably because of different amounts of MAC deposition causing various degrees of cell damage (Fig. 5A, 1–3). Under similar conditions, HI-NHS failed to deposit MAC on the RPE of murine eyecups (Fig. 5B). Similarly, pretreatment of primary mouse RPE cells with the anti-emmprin antibody, followed by 50% NHS for 7 minutes, resulted in extensive MAC immunostaining (Fig. 5D). Significantly less MAC was observed on HI-NHS-treated cells (Fig. 5E).

Complement-Mediated Vesiculation of RPE Cell Membranes

To further investigate the effects of MAC deposition and protection, we preinfected primary (passage 0) mouse RPE cells with either AdCAGCD59+ AdCAGGFP (800 + 200 vp/cell, respectively) or a control mixture of AdEMPTY+ AdCAGGFP (800 + 200 vp/cell, respectively) and, 3 days after infection, subjected these cells to the MAC deposition assay. Examination of cells immediately after 7 minutes of NHS treatment, washing, and fixation revealed the presence of numerous GFP-positive vesicles emanating from, or internalized into, cells (Fig. 6, arrows). The numbers and sizes of these vesicles were substantially higher on AdEMPTY+ AdCAGGFP-infected cells (Fig. 6A) than on AdCAGCD59+ AdCAGGFP-infected cells (Fig. 6B), indicating that the vesiculation was MAC deposition dependent. Furthermore, in AdEMPTY+ AdCAGGFP-infected cells, we noted a reduction in GFP fluorescence relative to AdCAGCD59+ AdCAGGFP-infected cells after treatment with

NHS (Fig. 6). The reduced GFP fluorescence in cells was associated with a concomitant increase in diffuse green fluorescence outside the cells, indicating that GFP had potentially leaked or diffused across the plasma membrane.

Protection of Murine Ocular Tissues and Primary RPE Cells from MAC Deposition by Adenovirus-Delivered hCD59

To assess the efficacy of hCD59 to protect murine RPE from human MAC deposition, we performed *in vivo* subretinal injections of adenovirus. Six days after injection, the expression of hCD59 on murine RPE after subretinal injection of Ad-CAGCD59 was confirmed by immunohistochemistry (Fig. 7A). No staining for hCD59 was observed on AdCAGGFP-injected eyecups (Fig. 7B).

For MAC deposition assay on the RPE, subretinal injections were performed in two groups of mice. Eyes of mice from one group were injected with a mixture of AdCAGCD59+ AdCAG-GFP in a 9:1 ratio (total, 3×10^8 vp/eye; AdCAGGFP was coinjected to allow easy identification of the injection site and area of transgene expression). The eyes of mice from the second group were injected with a control mixture containing AdEMPTY+ AdCAGGFP, also in a 9:1 ratio (total, 3×10^8 vp/eye). Six days after injection, eyes were harvested and eyecups were treated with the anti-mouse emmprin antibody and 50% NHS for 15 minutes.

When eyecups were treated with NHS for 15 minutes, immunohistochemistry for human MAC of AdCAGCD59+ Ad-CAGGFP-injected eyecups ($n = 10$) revealed significantly less staining on the RPE at the area of GFP expression (which should correlate with hCD59 expression) compared with the rest of the eyecup (Fig. 8B). RPE cells in this area appeared undamaged and had defined cell boundaries and normal hexagonal morphology. In contrast, MAC immunostaining at the GFP expressing-area of AdEMPTY+ AdCAGGFP-injected eye-cups ($n = 10$) was similar to the rest of the eyecup (Fig. 8A) and was significantly more extensive and stronger than the MAC immunostaining observed at the area of GFP expression of AdCAGCD59+ AdCAGGFP-injected eyecups. Furthermore, RPE cells at the GFP-expressing area of AdEMPTY+ AdCAG-GFP-injected eyecups appeared extensively damaged, as indicated by their rounded shape, loss of normal hexagonal morphology, and loss of defined cell boundaries (Fig. 8A). This extensive damage resulted in reduced GFP fluorescence on these eyecups (Fig. 8 and Supplementary Fig. S3C, bottom graphs). To discount the possibility that the reduced levels of GFP are a reflection of reduced adenovirus transduction in the AdEMPTY+ AdCAGGFP group, we repeated this experiment, but this time the eyecups were treated with NHS for only 7.5 minutes. Quantification of the levels of GFP between the two groups ($n = 10$ per group) now indicated no significant difference between GFP levels (Supplementary Figs. S3A–C, top graphs). The RPE cell morphology in the AdEMPTY+ AdCAGGFP eyecups was also now more similar to that of the AdCAGCD59+ AdCAGGFP eyecups (Supplementary Figs. S3A, S3B). However, the levels of MAC staining in the AdEMPTY+ AdCAGGFP eyecups was still significantly higher than in the AdCAGCD59+ AdCAGGFP-injected eyecups (Supplementary Figs. S3A, S3B). Quantification of the reduction in MAC immunofluorescence at the area of GFP expression revealed an average reduction of approximately 68% ($P = 0.0018$) at 7.5 minutes of NHS treatment and 56% ($P = 0.0007$) at 15 minutes of NHS treatment in mean fluorescence

intensity on the AdCAGCD59+ AdCAGGFP-injected eyecups compared with AdEMPTY+ AdCAGGFP-injected eyecups (Fig. 8C). Moreover, there was an inverse relationship (more obvious in the 15-minute NHS treatment) between GFP and MAC fluorescence intensities on the AdCAGCD59+ AdCAGGFP-injected eyecups (Supplementary Fig. S3C) that was not apparent in AdEMPTY+ AdCAGGFP eyecups (Supplementary Fig. S3C), providing further evidence that protection from MAC was dependent on the level of hCD59 expression.

When we extended these observations to primary murine RPE cells, immunohistochemistry revealed a significant reduction in MAC on AdCAGCD59+ AdCAGGFP-infected cells compared with AdEMPTY+ AdCAGGFP-infected cells (Figs. 8D, 8E). At this stage, however, the shed vesicles noted earlier had been removed because of the wash steps during MAC staining and could not be readily observed.

Evaluation of Emmprin Expression and Anti-Emmprin Antibody Binding Following Adenovirus Infections

It is possible that the difference in MAC deposition between AdCAGCD59 and control injected eyecups or primary RPE cells could be explained by a difference in mouse emmprin expression or a difference in anti- emmprin antibody binding after the expression of hCD59. To assess the levels of bound anti- emmprin antibody, we performed immunohistochemistry for mouse emmprin on AdCAGCD59+ AdCAGGFP or AdEMPTY+ AdCAGGFP-injected eyecups. Anti-mouse emmprin antibody treatment was performed exactly as it was for the MAC deposition assay, and then eyecups were washed, fixed, and incubated with an appropriate Cy3-conjugated antibody. No significant differences in emmprin immunofluorescence on the RPE were observed between the area of transgene expression and the rest of the eyecup (Figs. 9A, 9B) or uninjected eyecups (data not shown). No differences in emmprin immunofluorescence were observed between the areas of transgene expression of AdCAGCD59+ AdCAGGFP or control injected eyecups (Figs. 9A, 9B, and Supplementary Fig. S4). Similarly, no changes in emmprin expression levels attributed to the expression of hCD59 were observed on primary mouse RPE cells (Figs. 9C, 9D). In conclusion, protection from MAC deposition could not have resulted from differences in emmprin expression or anti- emmprin antibody binding because immunocytochemistry for mouse emmprin revealed no differences between control and AdCAGCD59-infected cells. Finally, the collective data described in this study clearly demonstrated the destructive effects of human MAC deposition on the RPE and on primary RPE cells and their significant protection by expression of hCD59.

Protection from MAC Deposition on Murine Corneal Endothelium

We examined the potential of using the murine corneal endothelium as a surface to measure MAC deposition and protection by adenovirus-delivered hCD59. Apart from providing an additional tissue to confirm our results, the value of using the cornea in these experiments was that it is an easily accessible tissue that can be easily cultured and infected *ex vivo*. Although not directly relevant to AMD, the corneal endothelium allows for much more efficient and homogenous transduction of the endothelial cells and may provide a more reliable result when measuring the efficiency of complement regulators that must be highly

expressed. This ex vivo assay may, hence, be more useful for the initial screening of inhibitors of MAC deposition before their testing in the RPE in vivo. Human CD59-mediated protection of corneal endothelium was demonstrated in experiments similar to those described earlier and those data are presented in Supplementary Results (<http://www.iovs.org/cgi/content/full/49/9/4126/DC1>) and Supplementary Fig. S5.

Discussion

AMD is a slowly progressive, chronic disease involving inflammation and complement-mediated damage in the outer retina, more specifically at the RPE, Bruch membrane, and choroid.⁸ Available therapies for AMD, such as *lucentis* and *macugen*,³⁷ inhibit vascular endothelial growth factor (VEGF) activity and, hence, treat only late-stage, or wet, AMD involving choroidal neovascularization (CNV). No therapy is available to slow the progression of early-stage, or dry, AMD, which accounts for approximately 90% of AMD cases. We envision that because dry AMD progresses slowly over many years, its treatment will potentially require regular injections of anticomplement molecules in patients with relatively good visual acuity. Apart from patient discomfort, such frequent injections expose patients to increased risk for endophthalmitis, retinal detachment, and increased or decreased intraocular pressure. Although the risk for these side effects is limited in any one procedure, it becomes cumulative over long periods.³⁷ A gene therapy approach that locally dampens complement activation may provide long-lasting effects and may require infrequent intraocular injections. In this context, adenovirus is known to persist in the RPE for more than 1 year in animals,³¹ and adenovirus has been proven to be safe in human ocular gene therapy.^{32,33} To the best of our knowledge, the approach described herein is the first gene therapy approach aimed at locally dampening complement-mediated cell lysis and MAC deposition in ocular tissues. Furthermore, because complement has important systemic and visual functions, it may be deleterious to inhibit complement activity systemically or in all ocular tissues by intraocular injection. The preferred approach would be to inhibit complement-mediated damage of those tissues particularly susceptible to and involved in the pathogenesis of AMD, such as RPE, Bruch membrane, and choroid.

One model on the origin of drusen formation is that RPE cells shed membrane-enclosed cytoplasm, or parcels, from their basal surfaces.³⁸ In support of this hypothesis, membrane-bound organelles have been found in drusen.³⁸ Furthermore, in eyes of AMD patients, MAC-positive RPE cells and drusen-associated vesicle-like structures immunoreactive for activated complement components have been documented.⁶ One may postulate that such parcels of cytoplasm are generated from complement-mediated attack on the RPE. Interestingly, in cell cultures we did observe vesicle shedding from primary mouse RPE cells after exposure to activated complement. More interesting was that such vesiculation could be significantly reduced by the expression of hCD59 on these cells, indicating that the vesiculation process was MAC dependent. Indeed, vesiculation caused by MAC is a well-documented process that has been observed in numerous nucleated cells and is a protection mechanism that cells use to clear MAC.^{39–41} However, one may envision that such a process on the RPE can have negative consequences if these vesicles accumulate between the RPE and Bruch membrane. Hence, a scenario in which RPE cells are protected from MAC (e.g., by CD59 overexpression) could reduce direct MAC-mediated damage to RPE

cells and could reduce the need for the RPE cells to clear MAC by vesiculation, but this remains a hypothesis yet to be tested.

An alternative hypothesis on the pathogenesis associated with complement activation is that MAC deposition occurs on Bruch membrane and RPE independently and concomitantly. In this context, we did observe hCD59 protein in the media of AdCAGCD59-infected cells. This hCD59 is shed from the cell membrane of AdCAGCD59-infected cells or is a soluble form of hCD59 secreted by those cells. Soluble forms of CD59 have been shown in the urine, serum, and other bodily fluids in humans.^{42,43} This form of CD59 is biologically active.^{42,43} Therefore, when overexpressed on the RPE, hCD59 produced by RPE cells may impact the formation of MAC in the immediate environment, such as on Bruch membrane, in addition to the RPE, but this hypothesis remains to be tested. Incidentally, it has recently been shown that soluble murine CD59a protein injected directly into murine ocular tissues can protect against the formation of CNV in the laser burn-induced CNV model of wet AMD.⁴⁴ Hence, we postulate that de novo synthesis and overexpression of hCD59 in murine ocular tissues may also inhibit CNV in the laser model of AMD.

Although the complement cascade can be regulated at various steps and by various regulatory proteins, CD59 provides specific inhibition of MAC deposition on the cell surface. This approach has the advantage of allowing upstream events of the complement cascade to proceed unhindered and to exert their effector functions against infections. However, targeting the terminal step of complement-mediated cell lysis may be insufficient as a therapy for dry AMD because the upstream effector molecules C3a and C5a generated on complement activation may also be contributing to AMD pathogenesis. At least one study has reported the presence of C3a and C5a in the drusen of a patient with AMD.⁴⁵ This study further reported that C3a and C5a levels were elevated in the laser injury-induced CNV mouse model of wet AMD and that this led to the induction of VEGF expression and contributed to the development of CNV.⁴⁵ However, in this same mouse model, MAC deposition has also been shown to be essential for the development of CNV.⁴⁶ More work is needed to determine the exact stage of the complement cascade at which inhibition would be most beneficial without resulting in adverse effects, and our humanized model should provide a useful tool to further test the efficacy of hCD59 and other complement regulators, which act at various steps of the complement cascade.

In summary, we have demonstrated that murine ocular tissues may provide an ideal platform for testing the ability of human complement regulatory proteins to protect against human complement attack. We also demonstrated that hCD59 delivered by a human adenovirus vector is one ideal approach to prevent MAC-mediated damage on RPE cell membranes. Although more work is needed, one may envision use of the approaches described herein as a potential avenue for developing a therapy for complement-mediated damage to RPE in patients with AMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of recombinant adenovirus constructs. **(A)** Adenovirus constructs used in this study. Expression cassettes were cloned in the deleted E1 region of an E1/E3-deleted adenovirus. **(B)** Western blot analysis of cell lysates and media from infected and uninfected cells using an anti-hCD59 antibody. **(C, D)** Immunocytochemistry using the hCD59 antibody of mouse hepa-1c1c7 cells infected with 1×10^3 virus particles/cell of the indicated adenovirus. Western blot and immunocytochemistry images are representative of three independent experiments. pA, polyadenylation signal; CAG, cytomegalovirus chicken β -actin β -globin promoter; Ψ , adenovirus-packaging signal; ITR, adenovirus-inverted terminal repeat; Δ , deleted; E, early region.

Figure 2.

Adenovirus-delivered hCD59 protects mouse hepa-1c1c7 cells from human complement-mediated cell lysis as measured by FACS analysis of PI uptake. (A) Cell lysis after incubation of uninfected murine hepa-1c1c7 cells with various dilutions of human serum. Data were obtained from two independent experiments ($n = 4$ samples for each serum dilution). (B–D) Representative FACS histograms of uninfected cells (B) or cells preinfected with 1×10^3 vp/cell of the AdCAGGFP (C) or the AdCAGCD59 (D) adenovirus and treated with 1% NHS or HI-NHS as indicated. (E) Quantification of cell lysis from FACS histograms as the ones shown in (B–D) expressed as percentages of complement-mediated cell lysis. Data were obtained from four independent experiments ($n = 10$ samples in each group). (F) Cell lysis analysis of cells preinfected with different amounts of the indicated adenovirus and subjected to serum treatment as in (B–E). Data were obtained from two independent experiments ($n = 4$ samples for each different amount and type of adenovirus). All data are expressed as mean $\pm$ SD. *** $P < 0.0001$; ** $P < 0.001$. N.S., not significant; NHS, normal human serum; HI-NHS, heat-inactivated NHS; FL-3, channel for detection of PI fluorescence.

Figure 3.

Human MAC deposition and trypan blue lysis assays on mouse hepa-1c1c7 cells. (**A**, **B**) Cells were treated with 10% NHS (**A**) or HI-NHS (**B**) for 5 minutes and subjected to immunocytochemistry for MAC (red) using an antibody against a neoepitope on the C5b-9 complex followed by an appropriate Cy3-conjugated secondary antibody. Cell nuclei were labeled with DAPI (blue). (**C**) Cells treated with 10% NHS or HI-NHS, followed by incubation in 0.1% trypan blue solution for 5 minutes. Images are representative of three independent experiments each for the MAC and trypan blue assay. NHS, normal human serum; HI-NHS, heat-inactivated NHS.

Figure 4.

Protection of mouse hepa-1c1c7 cells from human MAC deposition and ensuing lysis by adenovirus-mediated delivery of hCD59. (**A**, **B**) Cells preinfected with 1×10^3 vp/cell of AdCAGGFP (**A**) or AdCAGCD59 (**B**) were treated with 10% NHS for 3, 5, or 7 minutes, followed by immunocytochemistry for human MAC (red) as in Figure 3. Cell nuclei were labeled with DAPI (blue). (**C**) Cells preinfected as in (**A**) and (**B**) with the indicated adenovirus were treated with 10% NHS for 5 minutes and incubated in 0.1% trypan blue

solution for 5 minutes. Images are representative of three independent experiments each for the MAC and trypan blue assay.

Figure 5.

Human MAC deposition on the RPE of murine eyecups and on primary murine RPE cells. Flatmounts of eyecups (A–C) and primary RPE cells (D, E) incubated with (+) or without (–) a complement-activating anti–mouse emmprin antibody, followed by treatment with the indicated NHS or HI-NHS concentration at 37°C for the specified time and followed by immunohistochemistry for human MAC (red). Primary RPE cell nuclei were labeled with DAPI (blue). Images are representative of at least three independent experiments each for eyecups ($n = 4$ eyecups for each condition) and primary RPE cells. NHS, normal human serum; HI-NHS, heat-inactivated NHS.

Figure 6.

Inhibition of MAC-dependent vesiculation on primary mouse RPE cells by adenovirus-mediated delivery of hCD59. (A, B) Three days after infection with the indicated adenovirus mixture (4:1 ratio, total of 1×10^3 vp/cell), primary mouse RPE cells were treated with the anti-mouse emmprin antibody and NHS, as in Figure 5D, and were photographed immediately after washing and fixation. Cells were coinfecting with AdCAGGFP to allow easy visualization of vesicles (*arrows*). Images are representative of three separate experiments.

Figure 7. hCD59 expression on the RPE of in vivo adenovirus-injected mouse eyes. (**A**, **B**) Subretinal injections of the indicated adeno-virus (total, 3×10^8 vp/eye) were performed in C57Bl/6J mice. Six days after injection, eyes were harvested, and eyecups were subjected to immunohistochemistry for hCD59 (red). (**B**, bottom) GFP expression on the RPE of the AdCAGGFP-injected eyecup. Images are representative of at least three eyes injected with each adenovirus.

Figure 8.

Protection from MAC deposition on the RPE of murine eyecups and on primary murine RPE cells by in vivo or in vitro adenovirus-mediated delivery of hCD59. (**A**, **B**) Flatmounts of eyecups from mouse eyes injected subretinally with the indicated adenovirus mixtures and subjected to the MAC deposition assay, as in Figure 5A, 6 days after injection. Immunohistochemistry for human MAC (red) shows reduced staining at the area of GFP expression of the AdCAGCD59+ AdCAGGFP-injected eyecup (**B**) compared with the control injected eyecup (**A**). Images are representative of at least three independent experiments ($n = 10$ eyecups for each group of injections). (**C**) Quantification of MAC immunofluorescence intensity (arbitrary units) at the area of GFP expression of eyecups from eyes injected as indicated or from random areas of uninjected eyecups. Graph includes data from experiments shown in (**A**) and (**B**), Figures 5A and 5B, Supplementary Figures S3A and S3B, and data not shown. Indicated times specify length of serum treatment. Values from the 7.5-minute serum treatment are plotted on the left y-axis, whereas values from the 15-minute serum treatment are plotted on the right y-axis. (**D**, **E**) MAC immunostaining (red) on primary RPE cells subjected to the MAC deposition assay (as in Figs. 5D and 6) 3 days after infection with the indicated adenovirus mixtures. Primary RPE cell nuclei were labeled with DAPI (blue). Images are representative of three separate experiments. Data are

expressed as mean $\pm$ SEM. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. N.S., not significant; NHS, normal human serum; HI-NHS, heat-inactivated NHS.

Figure 9.

Immunohistochemistry for mouse emmprin on adenovirus-infected murine RPE cells. (**A**, **B**) Flatmounts of eyecups from eyes injected as in Figures 8A and 8B with the indicated adenovirus mixture, followed by emmprin immunostaining (*red*) 6 days after injection. Images are representative of three eyecups for each group of injection. (**C**, **D**) Double-labeling experiment showing no hCD59 (**D**, *green*)-dependent differences on anti-mouse emmprin (*red*) antibody-binding compared with GFP (**C**, *green*). Three days after infection with the indicated adenovirus, cells were subjected to immunocytochemistry with the first primary goat anti-mouse emmprin antibody, followed by the first secondary Cy3-conjugated donkey anti-goat IgG antibody. After washings and brief fixation of these antibodies, cells

were incubated with the second primary mouse anti–hCD59 antibody, followed by the second secondary Cy2-conjugated goat anti–mouse IgG antibody. Cell nuclei were labeled with DAPI. Images are representative of three separate experiments.