Decay Accelerating Factor (CD55)–Mediated Attenuation of Complement: Therapeutic Implications for Age-Related Macular Degeneration

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Purpose. Sequence variations in complement proteins are associated with age-related macular degeneration (AMD). The terminal pathway of complement results in the formation of the membrane attack complex (MAC) on the cell surface, resulting in their lysis. MAC has been documented on the retinal pigment epithelium (RPE), choroidal blood vessels, and drusen of AMD eyes. Here the investigators test the hypothesis that increasing the expression of decay accelerating factor (CD55) on RPE cells may result in reduced MAC-mediated damage.

Methods. The investigators constructed a recombinant adenovirus expressing human CD55 (AdCAGCD55). Mouse hepatocytes were infected with AdCAGCD55 or negative controls and subsequently incubated with normal human serum (NHS). Cell lysis and MAC formation were measured by FACS and immunocytochemistry, respectively. Adult mice were injected in the subretinal space with either AdCAGCD55 or controls; after 1 week of CD55 transgene expression, the eyecups were excised, challenged with NHS, and quantified for human MAC formation.

Results. Control-infected or uninfected mouse hepatocytes lyse at a rate of 93% and 94%, respectively. AdCAGCD55-infected mouse hepatocytes lyse at a rate of 29%. Lysis was confirmed to occur in the presence of MAC, which was reduced by 67% when cells were infected by AdCAGCD55. Mice injected in the subretinal space with AdCAGCD55 exhibited a 55.7% reduction in MAC formation on the RPE relative to controls.

Conclusions. Adenovirus-mediated delivery of hCD55 to murine RPE confers protection against human complement. The investigators propose that the expression of hCD55 on RPE cells warrants investigation as a potential therapy for AMD. (*Invest Ophthalmol Vis Sci.* 2010;51:6776–6783) DOI: 10.1167/iovs.10-5887

Age-related macular degeneration (AMD) is the leading cause of vision loss among the elderly in the developed world.[1,2] AMD typically presents itself as an accumulation of lipoproteinaceous deposits known as drusen between the retinal pigment epithelium (RPE) and Bruch's membrane.[3] These early stages of the disease are generally referred to as dry AMD, which may progress to geographic atrophy, characterized by the loss of RPE cells and subsequent degeneration of the retina. In approximately 10% of cases, AMD can further progress to the wet form, involving choroidal neovascularization (CNV) and macular edema. Elevated levels of vascular endothelial growth factor (VEGF) have been associated with the formation of neovascular membranes in AMD patients, and hence the wet form of AMD is amenable to treatment with US Food and Drug Administration–approved VEGF antibody fragment or aptamer, ranibizumab,[4] and pegaptanib,[5] respectively. Off-label use of bevacizumab, a VEGF antibody, has also been applied in the treatment of wet AMD.[6] Anti-VEGF treatments have been found to be highly efficacious and are now the standard of care for wet AMD. However, as yet, there are no treatment options available for the 90% of AMD patients with the early or dry form of the disease.

Although AMD is a complex disorder involving various risk factors, several lines of evidence point toward a central role for complement in the early pathogenesis of AMD.[7–13] Immunohistochemical studies have found a variety of complement proteins or complement activation products in the ocular tissues and drusen of AMD patients.[7,13,14] Recent genetic studies point toward a possible imbalance between complement activation and complement inhibition in AMD patients. Specifically, polymorphisms in a negative regulator of the alternative arm of complement, Factor H, have been implicated in AMD.[8,10,12,15,16] In contrast, polymorphisms in complement component 3 (C3), which constantly activates the alternative pathway of complement, have also been implicated in AMD.[17] Additional complement proteins, such as Factor B, are also known to be involved.[18,19] Recent studies investigating polymorphisms in C2 are suggestive of a role for the classical pathway of complement in AMD.[20,21]

Activation of complement terminates in the formation of the membrane attack complex (MAC) on the surfaces of cells, leading to their lysis.[22] Loss of RPE cells is a hallmark of geographic atrophy, and RPE cells "compromised" with MAC have been documented in AMD.[11] Elevated levels of MAC have also been documented on the choroidal blood vessels of AMD patients.[23] Sublytic levels of MAC are known to increase growth factor release from endothelial cells[24] and to increase the mitogenesis of 3T3 cells,[25] suggestive of a possible link or a transition from dry to wet AMD. Bioactive fragments generated through the activation of complement include the anaphylatoxins C3a and C5a, which are also known to elevate VEGF expression in vitro and to promote CNV in models of wet AMD in vivo.[23]

Complement activity is nonspecific and requires the assemblage of regulatory molecules to dampen and fine-tune the cascade of enzymatic cleavage events to protect host

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tissue damage. Complement regulators expressed specifically on the surfaces of human cells include membrane cofactor protein (CD46),[26] decay accelerating factor (CD55),[27] and protectin (CD59).[28] Each regulator attenuates complement at different stages of the complement cascade. Whereas CD46 prevents the formation of the C3 convertase, CD55 accelerates the decay of C3 convertase in both classical and alternative pathways.[26,29] In contrast, CD59 attenuates the formation of the MAC.[30] Theoretically, increased expression of complement regulator(s) on the surfaces of host cells that are typically damaged by complement attack may provide some protection against complement-mediated damage in AMD.

Testing of human complement regulatory proteins in animal tissues in vivo is complicated by the lack of interspecies conservation in some complement regulatory proteins.[31] For example, whereas humans are known to express only one form of membrane-associated CD55, mice express two—CD55a and CD55b—in a distribution different from that found in humans.[32] Hence, testing human complement regulatory proteins in vivo in preclinical studies is technically challenging. To this end, we recently developed an in vivo/ex vivo model of human MAC deposition on murine RPE.[33] This model permits the expression of any human complement regulatory protein on murine RPE in vivo through the use of a recombinant adenovirus vector. In this study we used this recently described model to test the potential of human CD55 in blocking human MAC-mediated damage to murine ocular tissues, specifically the RPE, a tissue intimately involved in the pathogenesis of AMD.

Materials and Methods

Cell Lines

Human embryonic retinoblast (HER) cells[34] were maintained in DMEM/10% fetal bovine serum (FBS), and the mouse hepatoma cell line (hepa1c1c7; ATCC, Manassas, VA) was maintained in α-MEM/10% FBS. Cell culture media, FBS, and reagents were obtained from Invitrogen (Carlsbad, CA).

Adenovirus Constructs

Recombinant adenovirus serotype 5 (Ad5) expressing human CD55 (hCD55) was generated by cloning a *Sal*I/*Not*I fragment from a plasmid containing the human CD55 cDNA (ATCC 5830488) into pCAGEN (a kind gift from C. Cepko) generating pCAGCD55. An *Spe*I/*Bam*HI fragment containing the entire CD55 expression cassette was inserted into pShuttle.[35] pShuttle was cotransformed with pAdEasy1 into *BJ5183* cells, as described previously,[35] to rescue the plasmid pAdCAGCD55. The hCD55-expressing virus was rescued by the transfection of 911 cells with *Pac*I-linearized pAdCAGCD55, and virus was purified with a purification kit (Adenopure; Puresyn, Inc., Malvern, NY). Viral titer was determined using a spectrophotometer set at 260λ. Recombinant Ad5 expressing either GFP (AdCAGGFP) or Ad5 devoid of a transgene (AdCAGpA) have been described by us previously.[33]

Western Blot Analyses

Human embryonic retinoblasts were infected with either AdCAGCD55 or AdCAGpA at a multiplicity of infection (MOI) of 1000 for 24 hours. The cell lysate and media were collected and electrophoresed through a 12.5% Tris-HCl precast gel (Bio-Rad, Hercules, CA) under reducing conditions. After transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) and blocking in 5% skim milk (Becton Dickinson, Sparks, MD), the membrane was probed for hCD55 with a 1:1000 goat anti–human CD55 antibody (R&D Systems, Minneapolis, MN) and a 1:5000 secondary horseradish peroxidase-conjugated bovine anti–goat antibody (Jackson ImmunoResearch Laboratories, Inc., Bar Harbor, ME). The signal was detected using a Pierce (Rockford, IL) chemiluminescent kit. After stripping and blocking as described, the same membrane was probed for β-actin with a 1:5000 mouse anti–β-actin monoclonal antibody (Clone AC-15; Sigma-Aldrich, St. Louis, MO). Secondary detection was performed as described.

Human Serum Cell Lysis Assay

hepa1c1c7 cells were infected with AdCAGCD55 and AdCAGpA at an MOI of 1000 for 65 hours in α-MEM/2% FBS. Cells were washed with 1× PBS and dissociated with reagent (TrypLE Express; Gibco, Grand Island, NY). Cells were collected by centrifugation at 1200 rpm/4°C and resuspended in ice-cold gelatin veronal buffer with Ca^{2+} and Mg^{2+} (GVB^{2+}; Complement Technology, Tyler, TX). Normal human serum (NHS, 1%; Sigma) or 1% heat-inactivated NHS (HI-NHS; 56°C for 1 hour) was added to 5×10^5 cells for 1 hour at 37°C to activate complement pathways. Lysed cells were labeled by propidium iodide (PI), counted by FACS (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ), and analyzed for PI uptake (CellQuest Pro software; Becton Dickinson).

MAC Deposition In Vitro

hepa1c1c7 cells were infected with AdCAGCD55 or AdCAGpA at an MOI of 1000 for 72 hours in α-MEM/2%FBS in poly-D-lysine–coated chamber slides (Becton Dickinson). Cells were washed with 1× PBS and incubated with 10% NHS or HI-NHS in GVB^{2+} for 5 minutes. Cells were subsequently washed three times with cold 1× PBS and incubated with 1:100 mouse anti–human C5b-9 (Clone AE11, Abcam) in 6% normal goat serum (NGS; Jackson ImmunoResearch Laboratories, Inc.) for 2.5 hours after fixation with 3.7% formaldehyde (MP Biomedicals, Solon, OH). Cells were washed and probed with 1:200 secondary Cy3-conjugated donkey anti–goat IgG (Jackson ImmunoResearch Laboratories, Inc.) in 3% NGS for 1.5 hours.

Images were captured using a microscope (IX51; Olympus, Tokyo, Japan) equipped with a camera (Retiga EXi; QImaging, Surrey, BC, Canada), as described previously.[33] Grayscale images were analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) for degree of MAC immunofluorescence in arbitrary units. Representative areas were selected with the polygon selection tool, and mean fluorescence intensity/pixel was measured. Background fluorescence in the hepatocyte images was subtracted from the mean fluorescence measurements.

Subretinal Injections

C57Bl/6J mice (Jackson Laboratories) were maintained in accordance with federal, state, and local regulations. Six- to 10-week-old mice were anesthetized by intraperitoneal injection of xylazine (0.01 g/Kg)/ketamine (0.1 g/Kg). Subretinal injections were performed using the transscleral-transchoroidal approach with a 32-gauge needle attached to a 5-μL glass syringe (Hamilton, Reno, NV). To identify the region of injection, AdCAGpA or AdCAGCD55 preparations were "spiked" with 1:10 AdCAGGFP. A total of 1 μL of each mixture containing 1×10^8 viral particles was injected into each mouse eye.

MAC Deposition on Mouse RPE

Six days after subretinal injection, mice were asphyxiated with CO_2, and eyes were enucleated. The lens and cornea were removed, and the eyecup was incubated in GVB^{2+} containing 140 μg/mL goat anti–mouse emmprin (R&D Systems) for 1 hour at 4°C. Then 50% NHS or HI-NHS was added to the GVB^{2+}/emmprin solution and incubated for 15 minutes at 37°C. Samples were washed in cold 1× PBS and fixed overnight in 3.7% formaldehyde.

To detect successful infection of RPE by AdCAGCD55 and expression of hCD55, eyecups with retina removed were immunostained with 1:100 goat anti–human CD55 and a 1:200 secondary

Cy3-conjugated donkey anti–goat (Jackson ImmunoResearch Laboratories, Inc.) before flat mounting. For cross-sections, immunostained eyecups were embedded (Tissue-Tek; Sakura Finetek, Torrance, CA) before 14 μm frozen sections were collected on glass slides.

To visualize MAC deposition on mouse RPE, fixed eyecups were immunostained with 1:100 mouse anti–human C5b-9, flat mounted, and coverslipped. ImageJ was used to quantify MAC deposition on mouse RPE.

Statistical Analysis

Experiments were performed in duplicate at least three times. Error bars represent SE from the mean. Significance was calculated using ANOVA or paired *t*-test, where appropriate.

Results

Expression of Human CD55 from Adenovirus-Infected Cells

An adenovirus vector expressing human CD55 (hCD55) regulated by a chicken β-actin promoter was generated (Fig. 1a). Expression and processing of hCD55 was confirmed in AdCAGCD55-infected human embryonic retinoblasts (HERs). Examination of cell lysates by immunoblot revealed both the unglycosylated 40-kDa hCD55 precursor and the mature 70-kDa protein (Fig. 1b). Low levels of the unglycosylated, glycosylated, and one species of higher molecular weight hCD55 were also detected in the media of AdCAGCD55-infected cells. Localization of hCD55 to the cell membrane was confirmed by immunostaining of nonpermeabilized mouse hepa1c1c7 cells infected with AdCAGCD55 (Fig. 1c).

Protection of Mouse hepa1c1c7 Cells from Complement-Mediated Lysis by Adenovirus-Delivered hCD55

AdCAGCD55-mediated protection of murine cells from complement-mediated damage was evaluated with a human serum-mediated cell lysis assay on mouse hepatocytes infected with either AdCAGCD55 or AdCAGpA. The AdCAGCD55-infected, AdCAGpA-infected, or uninfected cell suspensions were incubated with either 1% NHS or HI-NHS. FACS analysis of PI uptake was used to quantify cell lysis. AdCAGCD55 infection significantly protected mouse hepa1c1c7 cells from lysis (Fig. 2a). Although complement-mediated cell lysis in the AdCAGpA-infected or uninfected cells was 93.22% $\pm$ 0.65% and 94.00% $\pm$ 0.47%, respectively, lysis of AdCAGCD55-infected cells was 29.29% $\pm$ 1.98% ($P < 0.0001$). Cell lysis after incubation of cells with HI-NHS did not result in any significant differences between viral infections. Specifically, complement-mediated cell lysis was 18.99% $\pm$ 2.03% and 19.58% $\pm$ 2.11%, respectively, in the AdCAGpA-infected and uninfected cells and 24.53% $\pm$ 2.26% in the AdCAGCD55-infected cells ($P = 0.1514$). The percentage of cell lysis observed after incubation in HI-NHS suggests a baseline level of cell damage from cell manipulation during the assay and may account for most of the cell damage observed in AdCAGCD55-infected cells incubated with NHS. A representative FACS from each experiment is presented in Figure 2b. These results suggest that hCD55 expressed from an adenovirus in mouse hepa1c1c7 cells confers significant protection against human complement-mediated cell lysis.

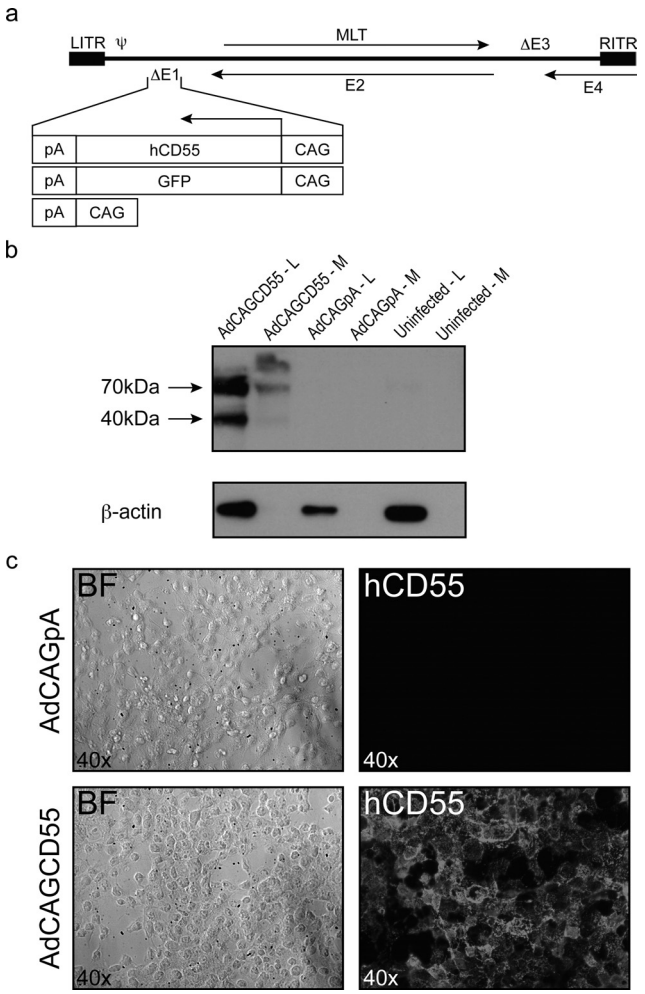

Figure 1. Human CD55 (hCD55) expressed in vitro from an adenovirus is processed correctly and localizes to the cell membrane. (**a**) Expression cassettes were cloned into the deleted E1 region of serotype 5 adenovirus in an antisense orientation with respect to the E1 enhancer. hCD55 and GFP were expressed from a CAG promoter. (**b**) Western blot analysis of cell lysates (L) or media (M) indicated hCD55 expression in AdCAGCD55-infected HERs and not in AdCAGpA-infected HERs. (**c**) Immunocytochemistry of AdCAGCD55-infected mouse hepa1c1c7 cells indicated localization of hCD55 to the cell membrane. LITR, left inverted terminal repeat; ψ, adenovirus packaging signal; ΔE1, ΔE3, deleted E1, E3 regions; MLT, major late transcript; E2, E4, early regions 2, 4; RITR, right inverted terminal repeat; CAG, chicken β-actin promoter; pA, bovine growth hormone polyadenylation signal; BF, bright-field.

Protection of Mouse Cells from Human MAC Deposition by Adenovirus-Delivered hCD55

Complement-mediated cell lysis is caused by the formation of the MAC on the plasma membrane. hCD55 regulates all three complement pathways by binding and accelerating the decay of C3 convertase in the classical and alternative pathways, theoretically preventing downstream MAC deposition on biological surfaces. To test this hypothesis, hepa1c1c7 cells infected with AdCAGCD55, AdCAGpA, or uninfected cells were incubated with 10% NHS or HI-NHS for 5 minutes. Cells were immunostained with an antibody directed against a neoepitope of the C5b-9 complex (MAC). AdCAGCD55-infected cells indicated significantly less staining for MAC than for AdCAGpA-infected (Fig. 3a) or uninfected cells (data not shown), suggesting that inhibition of C3 convertase successfully attenuates

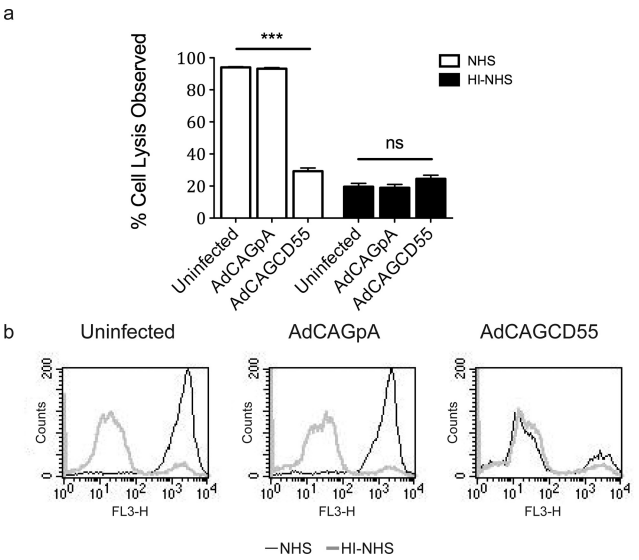

FIGURE 2. Adenovirus expressing hCD55 protects mouse hepa1c1c7 cells from complement-mediated cell lysis. (**a**) Cell lysis after incubation of uninfected, AdCAGpA-infected, or AdCAGCD55-infected mouse hepa-1c1c7 cells (MOI, 1000) in 1% NHS or HI-NHS. Histograms indicate percentage of complement-mediated cell lysis. Data were collected from four independent experiments ($n = 10$). (**b**) Representative FACS histograms of uninfected, AdCAGGFP-infected, or AdCAGCD55-infected cells (MOI, 1000) incubated with 1% NHS or HI-NHS. $^{***}P < 0.0001$ (ANOVA). ns, not significant; FL-3, channel for PI fluorescence detection.

downstream formation and deposition of MAC on cell membranes. Quantification of fluorescence intensity using ImageJ revealed a 67.1% decrease in MAC on mouse cells expressing hCD55 compared with those not expressing hCD55 (Fig. 3b). Mean fluorescence intensity in AdCAGpA-infected and AdCAGCD55-infected cells was 449.9 ± 38.1 and 148.0 ± 13.1, respectively ($P < 0.0001$). In conclusion, human CD55 expressed from an adenovirus in mouse hepatocytes confers significant protection against human MAC deposition.

Protection of Murine Ocular Tissues from MAC Deposition by Adenovirus-Delivered hCD55

Because MAC deposition has been observed on RPE cells in AMD patients,[11] one goal of this study was to determine the efficacy of hCD55-mediated protection of RPE cells against MAC deposition. Adult C57Bl/6J mice were injected into the subretinal space with either AdCAGCD55 or AdCAGpA (each spiked with AdCAGGFP). hCD55 localizes mostly to the nerve fiber layer in healthy human retinas[36] and is not observed on the RPE. We examined the expression and localization of hCD55 on murine RPE cells 6 days after injection. Immunohistochemistry of sclera/choroid/RPE flat mounts of AdCAGCD55+AdCAGGFP-injected eyecups revealed intense hCD55 staining on the membranes of RPE cells coincident with GFP. No significant hCD55 staining was detected on eyecups of mice injected with AdCAGpA+AdCAGGFP (Fig. 4a). Cross-sections through the injection site revealed that hCD55 was expressed on the apical, basal, and lateral membranes of RPE cells (Fig. 4b).

To examine whether this efficiency of hCD55 expression on mouse RPE cells can protect them from complement-mediated MAC deposition, we injected either AdCAGCD55 or AdCAGpA into the subretinal space of adult mice. Again, to each of these viruses, we added AdCAGGFP to demarcate the region of injection. Six days after injection, we harvested the eyes, removed the cornea, lens, and retina, and treated the eyecup with an anti–mouse emmprin antibody (previously shown to be necessary for the activation of complement on RPE cells)[33] and 50% NHS for 15 minutes. Eyecups were subsequently stained for human MAC with the antibody directed against C5b-9. AdCAGCD55+AdCAGGFP-injected eyecups indicated significantly less MAC deposition on the RPE within the region of injection (Fig. 5a) compared with the uninjected region of the same eyecup. AdCAGpA+AdCAGGFP-injected eyecups, however, showed no difference in the intensity of MAC deposition within the injected area compared with the uninjected regions of the eyecup. Unlike AdCAGpA-infected RPE cells, which appeared to have lost some of their hexagonal morphology, AdCAGCD55-infected RPE cells appeared to have relatively healthy, normal morphology to the extent discernible at these magnifications (Fig. 5a). Quantification of the intensity of MAC staining revealed a significant reduction in MAC formation (55.74% ± 5.99%) on the RPE cells of AdCAGCD55-injected eyecups compared with those injected with AdCAGpA ($P < 0.0001$; Fig. 5b).

DISCUSSION

Inflammatory processes and specifically activated complement have been implicated in the pathogenesis of a number of diseases, including but not limited to Alzheimer's,[37] atherosclerosis,[38] and glomerular basement membrane kidney disease.[39,40] More recently, a role for complement has been established in the pathogenesis of AMD. Consequently, a

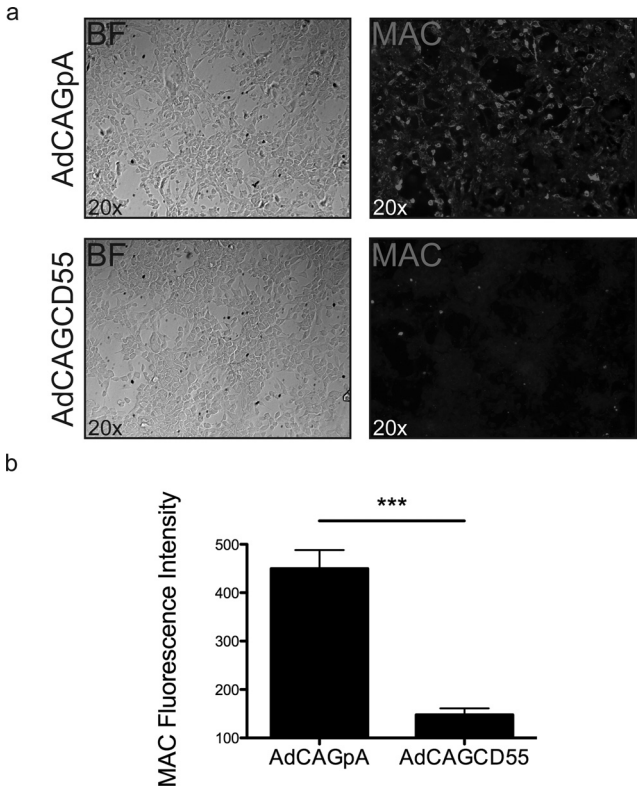

FIGURE 3. AdCAGCD55 protects mouse hepa1c1c7 cells from complement-mediated MAC deposition. (**a**) MAC staining of mouse hepa1c1c7 cells infected with either AdCAGpA or AdCAGCD55 (MOI, 1000) and incubated with 10% NHS. (**b**) Quantification of MAC deposition indicates a 67.1% reduction in staining in AdCAGCD55-infected cells compared with AdCAGpA-infected cells. Results were obtained from four independent experiments ($n = 11$). $^{***}P < 0.0001$ (paired *t*-test). BF, bright-field.

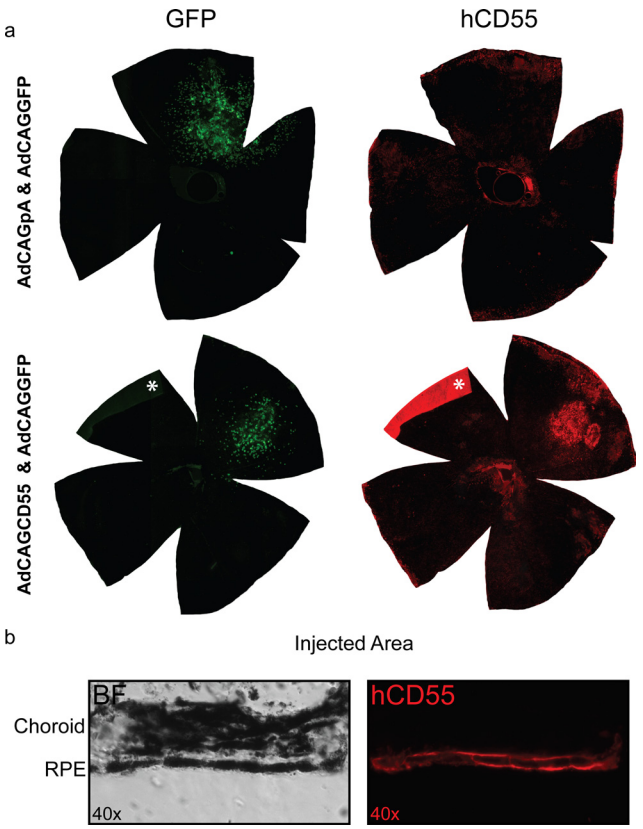

FIGURE 4. hCD55 localizes to the apical, basal, and lateral membranes of mouse RPE cells after subretinal injection of AdCAGCD55. (**a**) Immunohistochemistry of AdCAGCD55-injected eyes reveals a patch of hCD55 expression coincident with the coinjected AdCAGGFP. (**b**) Cross-sections through the injection site indicate that hCD55 expression occurs on the apical, basal, and lateral surfaces of the RPE cells ($n = 3$). *Asterisk*: portion of eyecup that folded onto itself. BF, bright-field.

number of phase I and II clinical trials aimed at attenuating complement activation in AMD patients have recently been initiated. These trials are targeting either C3 (compstatin/POT-4 peptide), C5 (*eculizumab* antibody, ARC1905 aptamer), or Factor D (TNX-234 antibody).[41,42] One goal of the present study was to develop an approach to attenuate complement activation by accelerating the concomitant decay of the classical and alternative C3 convertases on RPE cells of mice. Theoretically, any perturbation upstream in complement activation through C3 convertase activity should result in significantly altered levels of MAC assembled in the terminal pathway. Because MAC is a major component of complement-mediated cellular damage, we quantified levels of MAC deposition on RPE cell surfaces, a tissue intimately involved in AMD pathogenesis. To our knowledge, this is the first study to demonstrate hCD55-mediated reduction of MAC deposition on ocular tissues.

There are limited data on the localization of the different complement regulators in the human retina. However, current evidence suggests that the complement regulators hCD55 and hCD59 are found primarily in the inner retina, whereas hCD46 is present exclusively in a polarized fashion on the RPE.[36] Recently, it has been shown that the Y402H variant of factor H that is associated with AMD may result in decreased binding of Factor H to the RPE, ultimately leading to reduced protection of these cells from complement-mediated attack.[43] Hence, one may envision increasing RPE resistance to MAC-mediated damage by increasing the cell surface levels of complement regulators such as hCD55, as described here. Phase I clinical trials of adenoviral vector-mediated ocular gene transfer for the treatment of ocular disorders, such as AMD[44] and vitreous tumor seeding from retinoblastoma,[45] have demonstrated that intraocular injections of adenoviral vectors is a safe and well-tolerated approach. Adenovirus vectors can be engineered to express transgenes for extended periods of up to 1 year, the longest time period examined.[46–48] Important questions regarding the consequences of long-term expression of hCD55 on the RPE, a tissue in which it is not normally found at high levels, still remain to be addressed.

Cross-species differences in some complement proteins and key differences between the complement systems of humans and rodents limit testing the efficacy of human complement regulatory proteins in nonhuman systems.[31] For example, whereas humans have one CD55 gene, mice have two (CD55a and CD55b).[49] Similarly, whereas humans have one CD59 gene, mice have two (CD59a and CD59b).[50,51] CD55b and CD59b are exclusively expressed in the mouse testis, whereas CD55a and CD59a are expressed broadly. Based on such functional and structural differences between murine and human complement genes and proteins, it would be prudent to assume that the murine complement system has limited predictive value for the testing of human complement regulators. Moreover, before any clinical trials, it would almost always be preferable to test the actual human complement regulator in the context of human complement (serum) instead of extrapolating data from the murine homolog. To this end, we recently developed an in vivo/ex vivo model of human complement-mediated damage to murine ocular tissues. This model was used previously by us to demonstrate the use of human CD59 (hCD59) in inhibiting human MAC formation on murine RPE cells.[33] The current findings indicate that in the context of MAC formation, hCD55 is as potent as hCD59. Given that hCD59 acts specifically during the assembly of MAC and that hCD55 acts by accelerating the decay of classical and alternative C3 convertases, this result is surprising. Hence, we conclude that in terms of MAC formation, attenuation of the convertases in this model is as efficacious as direct attenuation of the downstream assembly of MAC.

Expression of hCD55 has in some studies been documented on human choroidal endothelial and human RPE cells in vitro.[52] Interestingly, exposure of human RPE cells to inflammatory cytokines and repetitive nonlethal oxidative damage results in the upregulation of hCD55, a phenomenon not observed for hCD59 or hCD46.[53] It is, hence, tempting to suggest that hCD55 may play a selective role not served by either hCD59 or hCD46 in protecting RPE cells during complement-mediated damage.

We found that some fully processed hCD55 was secreted or shed from the cell surfaces of AdCAGCD55-infected cells. Several recent studies[54–56] have described the soluble version of CD55 as biologically active. Further studies will be required to test whether soluble hCD55 also has an effect on complement inhibition.

Although CD55 is a potent regulator of complement at the level of C3 convertase, it would be beneficial to explore whether a synergistic effect can be achieved from overexpression of multiple complement inhibitors such as CD55, CD59, and CD46. Additional studies are necessary to determine which stage or stages of complement inhibition would be most effective as a treatment for AMD. In addition, any anticomplement therapy must be carefully titrated such that natural host defenses against infection are not compromised.

In summary, we have shown that adenovirus-mediated delivery of hCD55 to murine RPE protects those cells against human complement-mediated lysis and MAC formation. The protection conferred by attenuating the C3 convertases through hCD55 is as potent an approach as blocking the

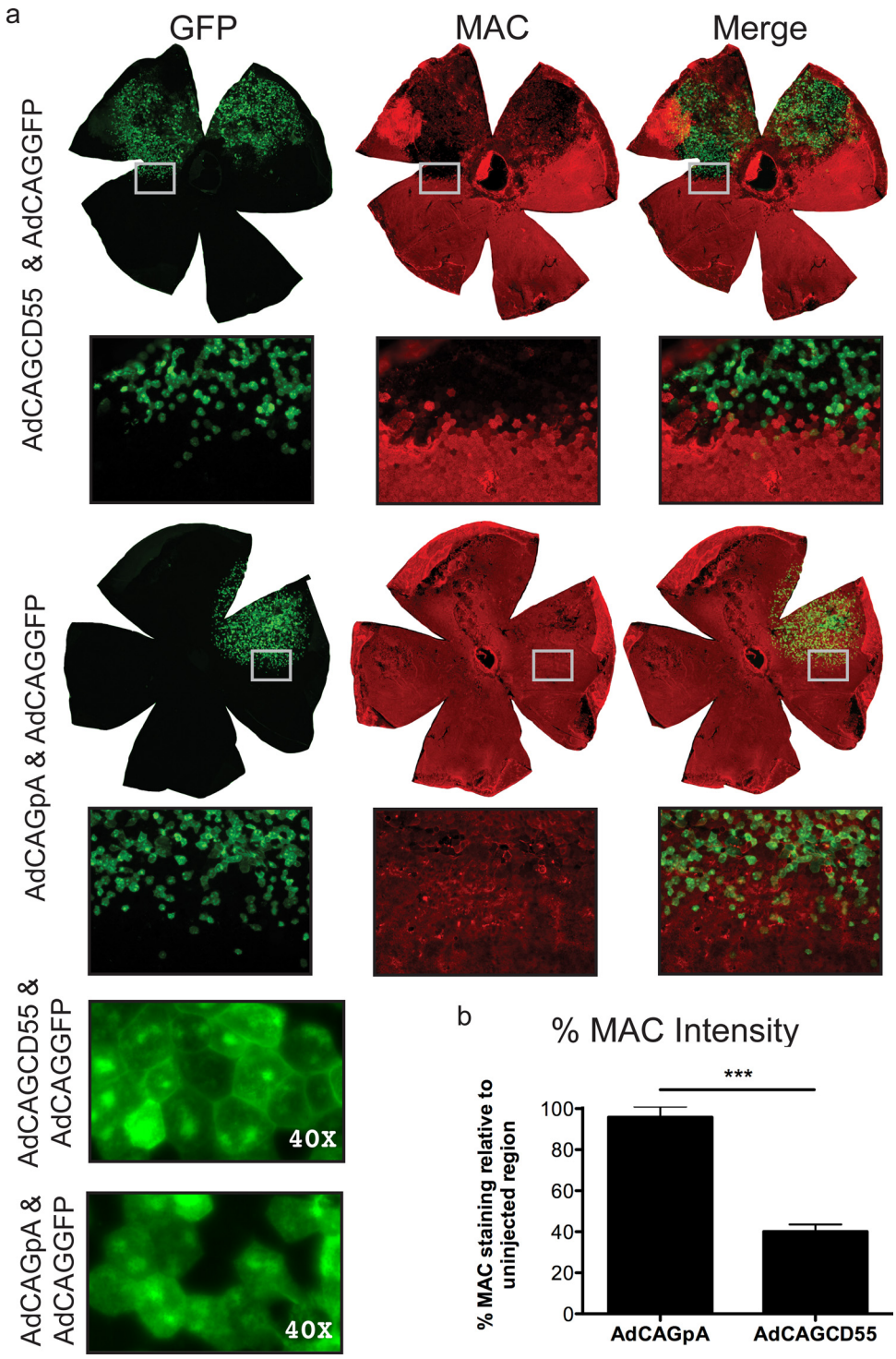

FIGURE 5. Adenovirus expressing hCD55 delivered to mouse RPE cells in vivo protects against human MAC deposition. (**a**) MAC staining of flat mounted eyecups injected with either AdCAGCD55 or AdCAGpA and incubated with NHS indicated reduced MAC deposition in the presence of hCD55. Images are representative of three independent experiments (AdCAGCD55, $n = 11$; AdCAGpA, $n = 8$). *Boxed areas*: regions shown below flat mounts at higher magnification. *Lower left*: higher magnification of individual RPE cells. (**b**) Quantification of MAC staining intensity shows that adenovirus-delivered hCD55 confers a significant (55.7%) reduction in MAC deposition on mouse RPE. $^{***}P < 0.0001$ (paired *t*-test).

assembly of MAC directly on the cell surface through the expression of hCD59. Further studies are necessary to assess the toxicity of long-term expression of hCD55 on the RPE, but our data suggest that further exploration of hCD55 as a potential therapy in AMD may be warranted.

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