Article

Encapsulated Cell Technology-Based Delivery of a Complement Inhibitor Reduces Choroidal Neovascularization in a Mouse Model

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Purpose: Age-related macular degeneration (AMD) is a slowly progressing disease, and risk appears to be tied to an overactive complement system. We have previously demonstrated that mouse choroidal neovascularization (CNV) and smoke-induced ocular pathology can be reduced with an alternative pathway (AP) inhibitor fusion protein consisting of a complement receptor-2 fragment linked to the inhibitory domain of factor H (CR2-fH) when delivered systemically. Here we developed an experimental approach with genetically engineered encapsulated ARPE-19 cells to produce CR2-fH intravitreally.

Methods: ARPE-19 cells were generated to stably express CR2 or CR2-fH, microencapsulated using sodium alginate, and injected intravitreally into 2-month-old C57BL/6J mice. CNV was induced using argon laser photocoagulation 4 weeks postinjection. Presence of capsules and progression of CNV was analyzed using optical coherence tomography. Bioavailability of CR2-fH was evaluated in retina sections by immunohistochemistry, and efficacy as an AP inhibitor by C3a ELISA.

Results: Secretion of CR2-fH or CR2 from encapsulated ARPE-19 cells was confirmed. An efficacious concentration of CR2-fH capsules to reduce CNV was identified. Bioavailability studies showed that CR2-fH was present in capsules and retinas of injected mice, and reduced CNV-associated ocular C3a production.

Conclusions: These findings indicate that the AP inhibitor CR2-fH, when generated intravitreally, can reduce CNV in mouse.

Translational Relevance: Encapsulated ARPE-19 cells secreting CR2-fH or perhaps other antiangiogenic or prosurvival factors might be useful as a potential therapeutic tool to treat age-related macular degeneration.

Introduction

Age-related macular degeneration (AMD) is a slowly progressing neurodegenerative disease. AMD can be divided into two categories, wet and dry AMD, depending on the pathology involved. Wet AMD is characterized by the invasion of choroidal blood vessels through the damaged blood retina barrier (BRB) made up of retinal pigment epithelium (RPE) and Bruch's membrane (BrM), which causes fluid accumulation in the subretinal space. If left untreated, the fluid-induced retinal detachment leads to photoreceptor degeneration. Dry AMD, through mechanisms less clearly understood, involves the loss of photoreceptor cells in the macula and concomitant degeneration of the corresponding RPE and choriocapillaris.

AMD is a complex disease, involving both environmental and genetic risk factors.[1] In support of the treatment using anti-vascular endothelial

growth factor (VEGF) therapies in wet AMD, single nucleotide polymorphisms (SNPs) in VEGFa,[2,3] the VEGFR2 receptor,[4] and genes in the proliferation subpathway[5] have been found to be associated with the occurrence of AMD; although surprisingly, there does not seem to be a pharmacogenetic association between a patient's response to anti-VEGF therapy and his/her VEGF$_A$ or VEGFR2 SNPs.[6,7] Multiple SNPs in genes of the complement pathway have been linked with an increased risk of developing AMD. Those include SNPs in the complement inhibitor complement factor H (CFH), with the Y402H variant representing the most abundant polymorphism identified and thus presumably posing the greatest single genetic risk for AMD (reviewed in Ref. 8). Other variants include SNPs in the complement inhibitors complement factor I (CFI)[9,10] and vitronectin (VTN),[8] complement component 2 (C2),[11] which is central to the activation of both the classical and the lectin pathways of complement, complement factor B (CFB),[11] an essential activator of the alternative pathway (AP) of complement, as well as complement component 3 (C3)[12] and complement component 9 (C9),[13] two essential components of the common terminal pathway. Limited pharmacogenetic associations are available for the complement axis in AMD, and clinical trials testing complement inhibitors have mostly been unsuccessful.[14]

Based on the strong correlation between Y402H and AMD, we have focused on examining mechanisms of complement activation in models of AMD pathology and the development of complement therapeutics for the treatment of those pathologies. The complement system is an evolutionarily ancient part of the innate and adaptive immune system, and is triggered in response to the generation of stress and/or injury-exposed antigens. Upon activation of the complement cascade, multiple different biological effector molecules are generated that are involved in recruitment of phagocytes (anaphylatoxins C3a and C5a), opsonization of damaged cell or debris (opsonins C3d and C3dg), and lysis of cells (membrane attack complex [MAC]).[15] In addition to the arms of the complement system that trigger activation, there exists a complement amplification arm driven by the complement AP which, in the absence of proper inhibition by CFH and CHI, can result in pathologically high levels of complement activation resulting in the generation of a proinflammatory micro-environment. We have developed a complement inhibitor molecule (CR2-fH) that was efficacious in both in vitro[16,17] and in vivo models of AMD.[18,19] CR2-fH consists of the N-terminus of mouse CFH (short consensus repeats [SCRs] 1–5), which contains the AP-inhibitory domain, linked to a complement receptor 2 (CR2) targeting fragment that binds complement activation products (see Ref. 20 for comprehensive review). Hence, the CR2 domain targets the inhibitor fH to sites of complement activation, where it is needed to inhibit AP activation,[18] and importantly, the CR2-fH protein does not rely on the endogenous ligand-binding domains present in CFH (SCR 6–8 and SCR 19/20). In vitro, CR2-fH effectively blocks complement activation at the level of the RPE and prevents pathological changes.[16,17,21] In vivo, we have shown that when injected intravenously, it can reduce choroidal neovascularization (CNV) development by reducing complement activation and VEGF production[18,22] or promote the regression of deposits in BrM generated in response to long-term smoke inhalation in mouse.[19] Importantly, CR2-fH has been found to be an effective inhibitor in many different AP-dependent disease models, including acute lung and ischemia reperfusion injury, collagen-induced arthritis, and more.[23–26] Finally, we have confirmed safety and efficacy of subretinal administrations of an AAV vector encoding the CR2-fH inhibitor in the mouse CNV model.[27]

The current standard of care treatments for wet AMD (anti-VEGF therapy) as well as a potential future treatment for dry AMD (e.g., anticomplement therapy) requires monthly (or as needed) intraocular injections. However, one of the challenges for a slowly progressing disease such as AMD is the requirement for long-term treatment. With each injection, there exists a risk for retinal detachment, infection, and other complications. For protein or antibody delivery directly to the back of the eye, the traditional approaches are limited. An FDA-approved route for short-term treatment is intraocular injections. Other strategies are currently under investigation, which include the use of encapsulated cells for the production of the therapeutic locally within the eye, or the use of devices for the local delivery of exogenous, stable compounds, using microelectro-mechanical system drug-delivery devices, injectable intravitreal implants, microcatheters, and biodegradable microparticles.[28] Most of the systems have been tested for biologics, and some are even refillable. The encapsulated cell technology (ECT) utilizes cells encapsulated in porous material that allows the exchange of nutrients and waste products, but prevents the efflux or influx of cells. The cells are genetically modified to

secrete the product of choice. Neurotech is spearheading this technique, aiming to develop ciliary neurotrophic factor (CNTF; NT-501, a growth factor that has been shown to slow down photoreceptor degeneration in animal models of retinitis pigmentosa [RP]) into a treatment for neurodegeneration in RP, dry AMD or glaucoma, and soluble antivascular endothelial growth factor receptor protein for a treatment of wet AMD.[29] Hence, for a biologic such as CR2-fH with potentially limited long-term stability in a 37°C environment, we consider the ECT using immortalized cells, which guarantees long-term and continuous production of the biologically active compound, as one of the more promising drug delivery systems currently under investigation.

As proof of principle, we investigate the efficacy of CR2-fH delivery via cell-encapsulation of genetically modified ARPE-19 cells in alginate in the mouse CNV model. Alginate capsules were used due to their small size required for injection into a mouse vitreous. We compare efficacy between cells expressing the CR2 portion when compared to CR2-fH, a strategy similar to that employed in our original characterization of the fusion protein.[18]

Methods

Stably Transfected ARPE-19 Cells

The plasmid constructs of CR2 and CR2-fH were previously described.[18] In short, the CR2-fH expression plasmid (PBM vector) contains a CD5 signal peptide sequence required for secretion,[30] the sequence encoding the four N-terminal SCRs of mouse CR2 (residues 1–257 of mature protein, RefSeq accession number M35684), a $(G_4S)_2$ linker, followed by the five N-terminal SCRs of mouse fH (residues 1–303 of mature protein, RefSeq NM009888).

ARPE-19 cells (ATCC® CRL-2302™), a human RPE cell line that displays the differentiated phenotype of RPE cells,[31,32] were used for encapsulation. These cells were grown in DMEM-F12 (Gibco; Thermo Fisher Scientific, Waltham, MA) with 10% fetal bovine serum (FBS) and 1 × penicillin to streptomycin ratio. The plasmid construct was transfected into ARPE-19 cells with FuGene HD transfection reagent, according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN), and stable expression of a mixed population of drug resistant cells was generated using L-methionine sulfoximine selection. Protein secretion into the apical and basal compartment was monitored in polarized RPE grown on transwell plates (Fig. 1E).[16] CR2-fH gave a single band of appropriate molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; data not shown).

Cell Encapsulation

Stably transfected ARPE-19 cells were cultured to 70%–80% confluence, washed with phosphate-buffered saline (PBS), detached with trypsin/ethylenediaminetetraacetic acid (EDTA) counted, pelleted, and suspended in sodium alginate. Cells were microencapsulated as previously described.[33] Briefly, electrospray method of microencapsulation was used to encapsulate the targeted cells at a final cell concentration of 1×10^6 in alginate solution using sodium alginate (high maluronic acid content, low viscosity; Sigma-Aldrich Corp., St. Louis, MO) at a concentration of 2% w/v and purified by filtration at 0.2 mm. Alginate (~300 µL aliquots) was loaded into a 3-cc syringe and attached to a syringe pump, and pumped through a needle (30G blunt tip; Small Parts, Inc., Logansport, IN) into a gelling bath placed in a beaker below the syringe at 7 mm of a needle to bath spraying distance. Constant parameters of 60 mm/h flow rate and 8.0 kV voltage were adjusted to produce microcapsules with a size of ~150 µm. The gelling bath contained a volume of 40 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline containing 100 mM calcium chloride ($CaCl_2$) (Sigma-Aldrich Corp.), and 0.5% w/v poly-L-ornithine (PLO) (Alfa Aesar, Haverhill, MA) that forms a second coating to the microcapsules, using a one-step method for adjusting the porosity. Cell containing microcapsules were washed with washing solution (10 mM HEPES buffered saline containing 1.5 mM $CaCl_2$, pH 7.4) twice, and then incubated with suitable media at 37°C and 5% CO_2.

Cell Recovery

The stably transfected cells were released from alginate microcapsules by dissolution in 55 mM sodium citrate in 10 mM HEPES buffered saline (pH 7.4) with gentle shaking for 5 minutes at room temperature (RT). Dissolution occurs when calcium ions replace sodium ions in the microcapsule structure, resulting in a diluted sodium alginate and gentle degradation resulting in the releasing of the cells from the microcapsules. Once the capsules were dissolved, the cells were centrifuged at 1200 rpm for 5 minutes and incubated for 24 to 48 hours with media and
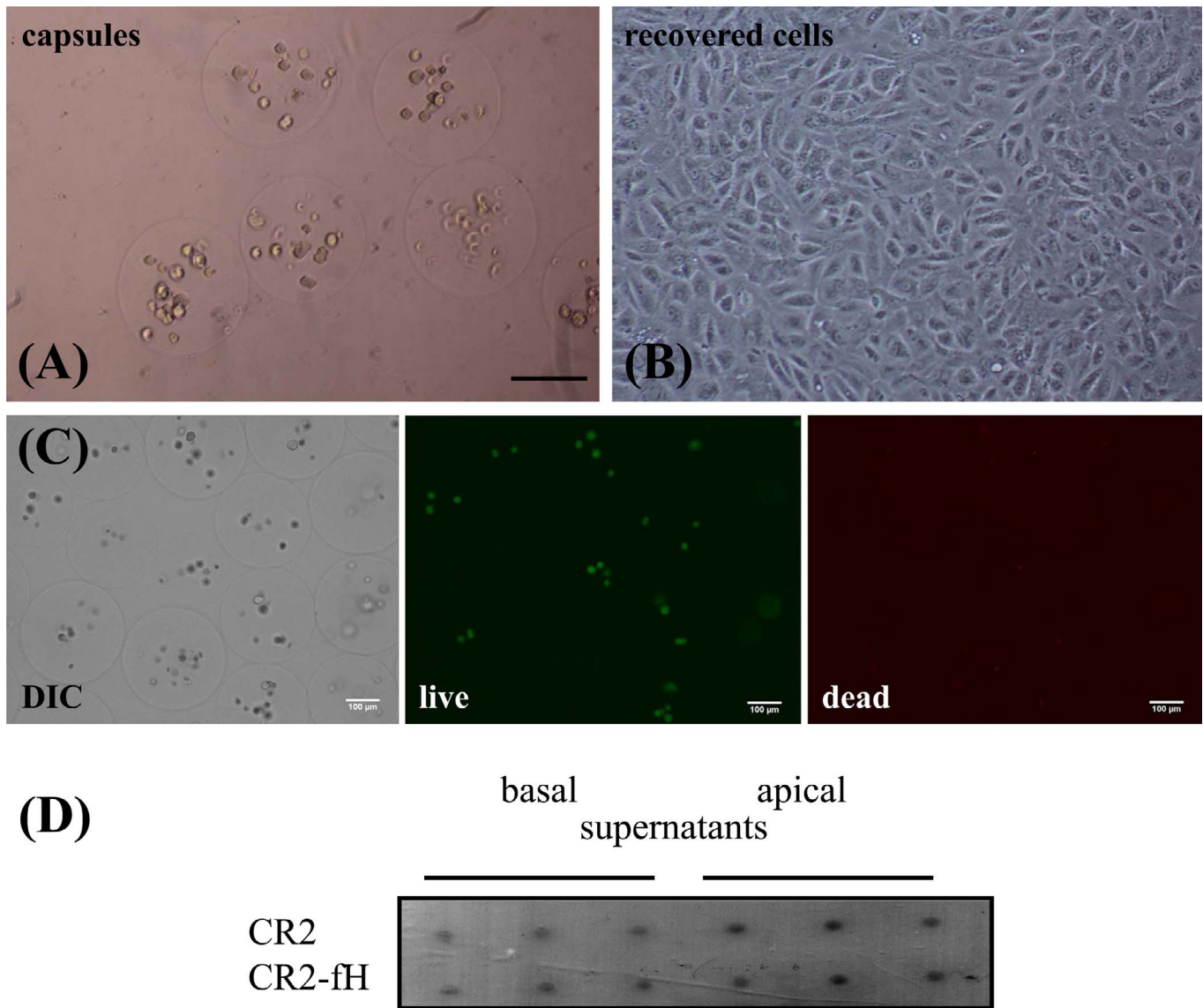
Figure 1. ECT to deliver CR2-fH—tool development. (A) ARPE-19 cells encapsulated in alginate were spotted onto a glass slide for imaging; and (B) plated after dissolving the alginate wall to document viability. Cell survival in the capsules was assessed using Calcein AM (C) indicating viable cells by green fluorescence and Ethidium homodimer-1 indicating dead cells by red fluorescence. (D) Stably transfected ARPE-19 cells secrete CR2 and CR2-fH toward both the apical and basal side when grown as monolayers on transwell plates (supernatants from three different cultures).

replated at 37°C and 5% CO_2. Each batch of cells that was encapsulated had a small portion processed for recovery to ensure that the cells within the capsule were still capable of growth. Replated cells were maintained at 37°C and 5% CO_2 for 3 to 5 days.

Cell Viability (Live/Dead Assay)

To assess the viability of the encapsulated cells, approximately 300 microcapsules containing cells were incubated for 24 hours and then were washed twice in washing solution (10 mM HEPES buffered saline containing 1.5 mM $CaCl_2$) and stained using a live-dead viability assay. The Live/Dead assay kit (Molecular Probes; Thermo Fisher Scientific, Waltham, MA) was used to evaluate the viability of cells after encapsulation according to the manufacturer's instructions. Encapsulated cells were mixed with Calcein AM and Ethidium homodimer-1 at final concentrations of 2 and 4 μM, respectively, in washing solution, and incubated for 30 to 45 minutes in the dark at RT. After staining, microcapsules were washed twice with washing solution and imaged using the Invitrogen EVOS

FL Auto Cell Imaging System (Thermo Fisher Scientific, Waltham, MA). Calcein AM indicates viable cells by green fluorescence (excitation/emission ~495/515 nm), whereas Ethidium homodimer-1 indicates dead cells by red fluorescence (excitation/emission ~495/635 nm). Based on the analysis of 50 capsules, <1% of cells were positive for ethidium homodimer 1.

Capsule Injections

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University Animal Care and Use Committee. C57BL/6J (Jackson Laboratory, Bar Harbor, ME) were bred in house. Intravitreal injections were performed on mice 8 to 10 weeks of age of both sexes, under visual inspection, using a dissecting microscope. Prior to intravitreal injection, mice were anesthetized by intraperitoneal injection (xylazine and ketamine, 20 and 80 mg/kg, respectively). Phenylephrine HCL (2.5%) and atropine sulfate (1%) were used to dilate the mouse pupils before an amount of 1 μL of DMEM containing capsules with CR2-fH- or CR2-expressing ARPE-19 cells, native ARPE-19 cells, or no content was injected into the vitreous. Injections were performed by puncturing the sclera at the limbus with a 25G needle and injecting the capsules using a 27G needle attached to a Hamilton syringe at a 45-degree angle, avoiding the lens. Following injection, eyes were treated with antibiotic ointment. The effective concentration to reduce CNV progression when comparing CR2- versus CR2-fH expressing cells was determined to be ~10 capsules (data not shown).

Laser CNV

One month following the injection, argon laser photocoagulation (532 nm, 100-μm spot size, 0.1-second duration, 100 mW) was used to generate four laser spots around the optic nerve of each eye.[18] As described previously, bubble formation at the site of the laser burn was used to confirm BrM rupture, and only those lesions were assessed for lesion growth.[34] Five days following laser-induced CNV, mouse eyes were imaged by optical coherence tomography (OCT) before being sacrificed for tissue collection on day 6.

Dot Blot and Western Analysis

To determine whether stably transfected cells produce detectable CR2-fH, lysates were collected following centrifugation (20,000g for 30 minutes at 4°C) and total protein (25 μL) was loaded directly into the wells of a 96-well plate. Using the Bio-Dot® Microfiltration Apparatus (Bio-Rad Laboratories, Inc. Hercules, CA), samples were transferred onto a nitrocellulose membrane. The dotted membrane was then rinsed with Tris-buffered saline, 0.1% Tween 20 (TBST) wash buffer before being blocked for 2 hours at RT with 5% nonfat milk in TBST buffer. CR2-fH was detected using an anti-CR2 primary antibody (10 μg/mL; rat anti-mouse CD21, clone 7G6; purified in house[30]) incubated in 5% nonfat milk/TBST (1:1000) overnight, and visualized with a horseradish peroxidase-conjugated secondary antibody (anti-rat; Santa Cruz Biotechnology, Inc., Dallas, TX) followed by incubation with Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, Inc.).

To determine whether mice generate antibodies against CR2-fH secreted intravitreally by ARPE-19 cells, supernatant from CR2-fH-expressing cells was added to Laemmli sample buffer and boiled. Samples at two different concentrations were separated by electrophoresis on a 4%–20% Criterion™ TGX™ Precast Gels (Bio-Rad Laboratories, Inc.), and proteins transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with primary antibody against CR2, or serum (1:50) from mice treated with intravitreal CR2- or CR2-fH-capsules. Proteins were visualized with horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG and IgM; Santa Cruz Biotechnology) followed by incubation with Clarity™ Western ECL Blotting Substrate and chemiluminescent detection.

Immunohistochemistry

Eyecups were collected as previously described, with the lens and anterior chamber removed, fixed overnight with 4% paraformaldehyde (PFA), washed in PBS,[18] embedded in Neg-50 cutting medium (Richard-Allen Scientific; Thermo Fisher Scientific) and sectioned using a cryostat (14-μm sections). Sections were incubated in blocking solution (10% normal goat serum, 3% bovine serum albumin factor V, and 0.4% Triton-X in PBS) before staining with CR-2 (7G6) antibody (1:200) overnight at 4°C. Sections were washed with blocking solution before adding secondary antibody (Alexa Fluor 488 goat anti-mouse IgG; 1:500; Invitrogen), mounted using Fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL), and examined using a fluorescence microscope (Zeiss, Thornwood, NY) equipped with a digital black-and-white camera (Spot

camera; Diagnostic Instruments, Sterling Heights, MI).

Optical Coherence Tomography

OCT was used to visualize capsules and to analyze CNV lesion size on day 5 post laser treatment as previously described,[35–37] using an SD-OCT Bioptigen® Spectral Domain Ophthalmic Imaging System (Bioptigen, Inc., Durham, NC). For CNV lesion analyses, rectangular volume scan images set at 1.6 × 1.6 mm, consisting of 100 B-scans (1000 A-scans per B scan) were acquired and the cross-sectional area of the lesion measured, using the en-face fundus reconstruction tool. The center of the lesion was determined passing through the RPE-BrM rupture with the axial interval positioned at the level of the RPE/choroid complex,[38] and Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) was used to measure the area around the hyporeflective spot produced on the fundus image, with vertical calipers set at 0.100 mm at the site of each lesion.

C3a ELISA

The C3a ELISA was carried out according to the manufacturer's instructions, using the mouse complement C3a ELISA from LifeSpan Biosciences, Inc., (Seattle, WA). In short, the RPE/choroid tissues were quickly rinsed with ice cold PBS to remove excess blood. Tissues were lysed by ultrasonication using 500 µL ice cold PBS. The final homogenate was centrifuged at 5000g for 5 minutes and then subjected to the assay procedure as directed by the manufacturer, using 100 µL per well. Purified mouse C3a at known concentrations was used as a standard. Measurements were obtained using the microplate reader set at 450 nm.

Statistics

Data are presented as mean ± SEM. Single comparisons were analyzed using unpaired t-tests, data consisting of multiple groups were analyzed by ANOVA (Fisher PLSD), with mean value differences considered significant at $P \leq 0.05$ (StatView, Cary, NC).

Results

Generation of Encapsulated ARPE-19 Cells

ARPE-19 cells were transfected using FuGene HD, and stable expression in a mixed population was enforced by antibiotic selection. Apical and basal secretion of CR2-fH and CR2 was confirmed by dot blot analysis of supernatants collected from cells grown on transwell plates as stable monolayers (Fig. 1D). Following confirmation of stable secretion of CR2-fH, subconfluent cells were detached, counted, pelleted, and suspended in 1.2% sodium alginate for encapsulation in alginate capsules.[33] The presence of cells in the alginate capsules was confirmed by bright-field imaging (Fig. 1A), and long-term viability of ARPE-19 cells in the capsules was confirmed by dissolving the capsules in sodium citrate and replating the cells (Fig. 1B) as well as live-dead assays, which indicated greater than 90% viability post encapsulation (Fig. 1C).

Intravitreal Delivery of Encapsulated ARPE-19 Cells

The optimal size of capsules suitable for injection through a 27G needle (inner diameter of 210 µm) to minimize shredding of the capsules was determined empirically in vitro on pools of capsules ranging from 100 to 200 µm (data not shown), with 150 µm being optimal. After intravitreal injections of encapsulated ARPE-19 cells, OCT imaging was performed to confirm the presence of intact capsules in the vitreous chamber of the mouse. Minor amounts of vitreous debris were also identified (Fig. 2A). The presence of CR2-fH in the capsules in the mouse vitreous and retinal tissue was confirmed by immunohistochemistry, staining for the CR2 portion of the fusion protein (Fig. 2B). Finally, transport and retention of CR2-fH in eyes with complement-dependent lesion was confirmed by dot blot analysis of RPE/choroid fractions of capsule-injected mice with and without CNV lesions. Similar to reports in other systems,[39] we found injury-site specific retention of CR2-fH (Fig. 2C).

To confirm that long-term production of secretable CR2-fH in the vitreous chamber, which could potentially gain access to the blood stream, does not induce anti-CR2-fH antibody production, we examined mouse serum 1 month after the injection of the capsules for the presence of anti-CR2-fH antibodies. Comparing serum from experimental animals with CR2-fH capsules when compared to controls (no treatment), neither IgG nor IgM antibodies recognizing CR2-fH could be identified (Fig. 3). These results were similar to those reported previously in mice systemically injected with the same fusion protein.[40]
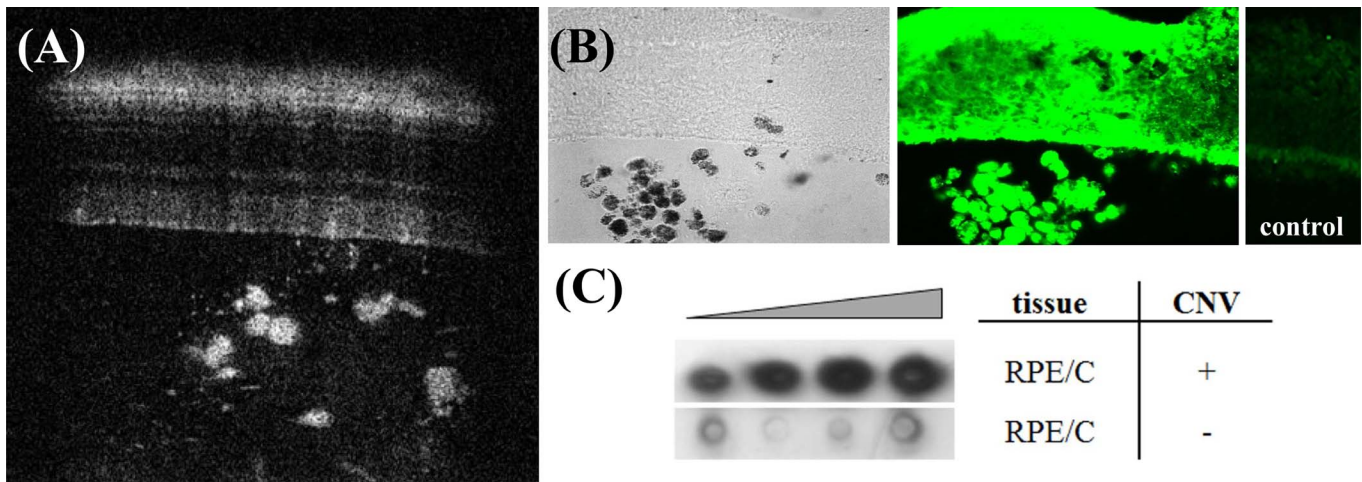
Figure 2. ECT to deliver CR2-fH—documentation in the eye. (A) Capsules can be imaged in the eye using OCT. (B) Production of CR2-fH within the capsules and diffusion of the fusion protein throughout the retina layers was confirmed by immunohistochemistry using an antibody against CR2. The corresponding DIC and fluorescence image is presented. CR2 antibody staining was negative in uninjected control eyes. (C) After intravitreal capsule delivery, CR2-fH was detectable in the RPE/choroid fraction of eyes with CNV lesions (breach of the BRB) but not in those with without. A dot blot of RPE/choroid samples with 2-fold dilution steps is presented. Representative examples from more than three independent experiments are shown.

Encapsulated ARPE-19 Cells Expressing CR2-fH Reduce Complement Activation and Attenuates CNV Development

We evaluated the effectiveness of encapsulated ARPE-19 cells expressing CR2-fH on CNV lesion size 5 days following laser-induced photocoagulation. Mice were injected intravitreally with capsules containing either CR2- (control) or CR2-fH-expressing ARPE-19 cells, native ARPE-19 cells, or with empty alginate capsules of the same size. Successful delivery of the capsules was confirmed by OCT (as shown in Fig. 2A), and mice were allowed to recover following the injection. After ∼1 month, mice underwent laser-induced CNV in all four quadrants of the eye. Here we report a significant decrease ($P \leq 0.02$) in lesion size in animals receiving capsules with CR2-fH-expressing cells when compared to those receiving the cells expressing CR2, encapsulated native ARPE-19 cells or alginate capsules alone (Figs. 4A, 4B).
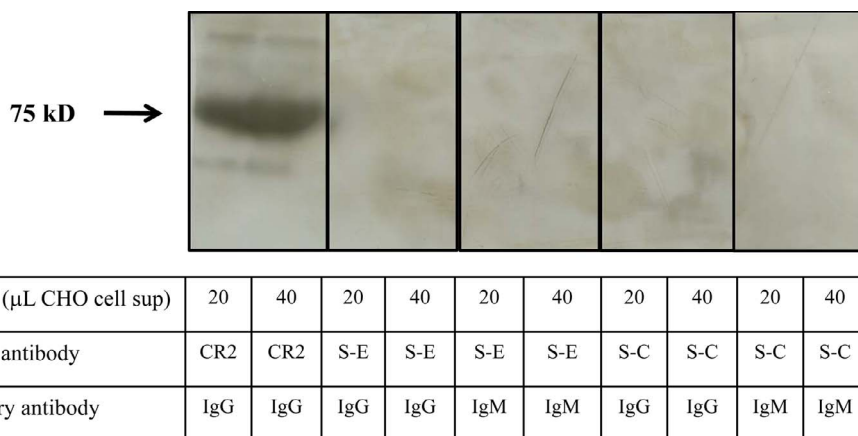

CR2-fH (µL CHO cell sup)	20	40	20	40	20	40	20	40	20	40
primary antibody	CR2	CR2	S-E	S-E	S-E	S-E	S-C	S-C	S-C	S-C
secondary antibody	IgG	IgG	IgG	IgG	IgM	IgM	IgG	IgG	IgM	IgM

Figure 3. CR2-fH produced in the eye does not cause an immune response. Intraocularly produced CR2-fH could potentially gain access to the circulation and lymph nodes. Lack of IgG or IgM antibody production was confirmed 1 month after capsule injection harboring ARPE-19 cells expressing CR2 or CR2-fH. Supernatants from cells expressing CR2-fH were run at two different concentrations and probed for the presence of CR2-fH using the anti-CR2 antibody to identify the size of the protein (positive control). Identical lanes were probed with serum from experimental animals (S-E; injected with CR2-fH capsules) or control animals start with (S-E; age-matched animals without injections S-C) to match the description of the experiment at 1:50, followed by appropriate secondary antibodies.
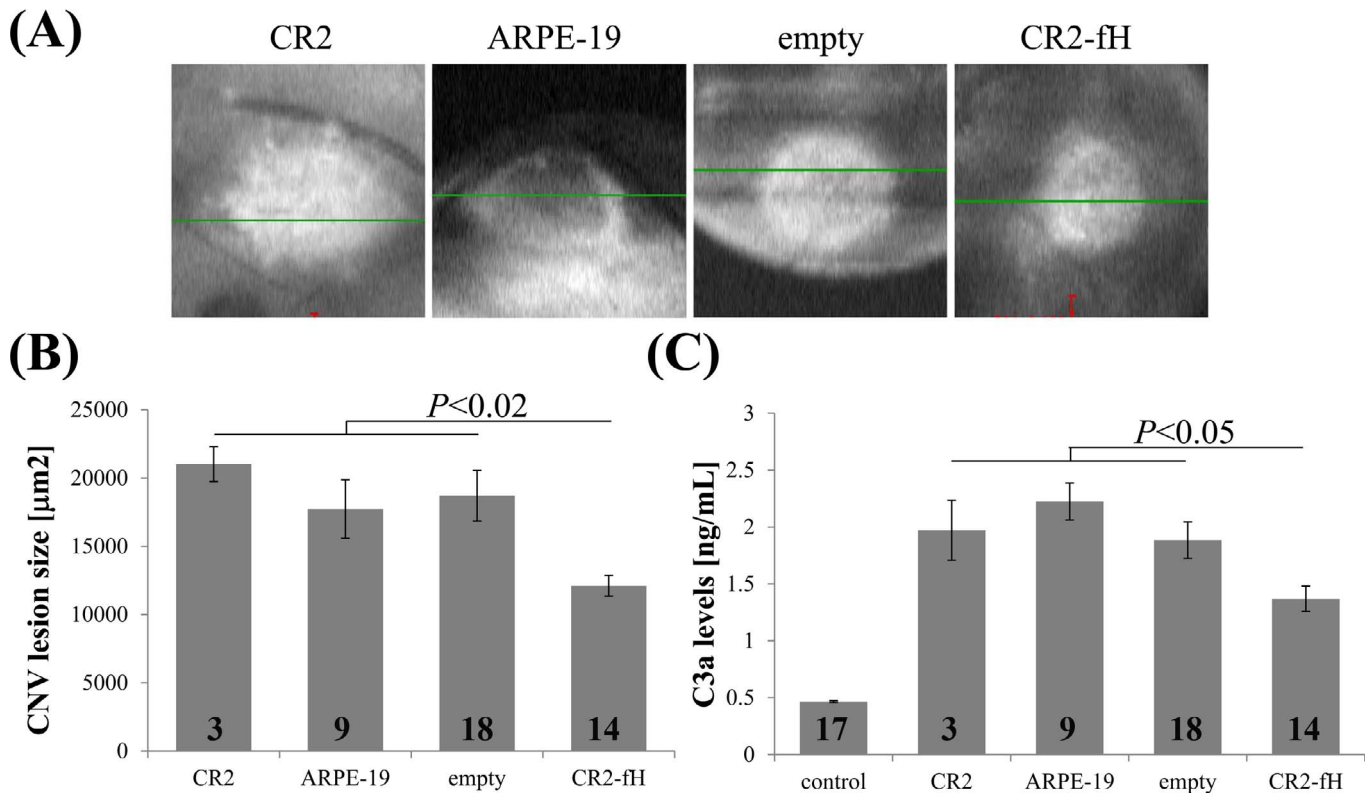
(A)

(B) **(C)**

Figure 4. ECT-mediated delivery of CR2-fH reduces CNV and complement activation. One month following intravitreal injection of alginate capsules, laser-induced CNV was performed. Lesion spot sizes were analyzed 5 days later using OCT, complement activation using ELISA for the anaphylatoxin C3a. (A, B) CNV sizes were reduced in eyes loaded with alginate capsules containing ARPE-19 cells expressing CR2-fH as opposed to those expressing CR2, no extra cargo (native ARPE-19), or empty capsules containing media only. The three control groups did not differ from each other. (C) CNV-induced complement activation as demonstrated by elevated levels of C3a when compared to animals with no lesions (control). C3a levels were elevated in RPE/choroid fractions of animals exposed to CR2, native ARPE-19 cells, or empty capsules, and significantly reduced by CR2-fH. Data shown are average values ($\pm$SEM) ($n = 3$–18 animals per condition as indicated).

Lesion sizes did not differ among the three control groups.

Complement activation in the RPE/choroid fractions were assessed by ELISA for C3a, the cleavage product of the C3-convertase. Since factor H accelerates the decay of the AP C3-convertase and acts as a cofactor for factor I to inactivate C3b to prevent convertase formation, a reduction in C3a in the presence of CR2-fH is expected. ELISA measurements demonstrated that CNV (four lesions per eye) resulted in a ~4-fold increase in C3a in RPE/choroid fractions from eyes with capsules containing CR2-expressing cells, native cells, or alginate capsules alone when compared to naïve age-matched control eyes. In comparison, C3a levels in mice with capsules containing CR2-fH-expressing cells had significantly reduced C3a levels (Fig. 4C).

Discussion

The goal of this study was to obtain proof of principle that ECT-mediated delivery can be used as a therapeutic strategy to deliver CR2-fH to the eye to reduce complement-dependent pathology. The main results of the current study are as follows: (1) ARPE-19 cells encapsulated in alginate are viable (i.e., can be recovered) and secrete CR2-fH; (2) alginate capsules can be delivered intact into the vitreous of the mouse and encapsulated cells secrete CR2-fH that can be visualized by immunohistochemistry; (3) CR2-fH secreted into the vitreous does not lead to anti-CR2-fH antibody production; and (4) CR2-fH expressed and secreted by the ARPE-19 cells reduces the development of CNV and the CNV-induced increase in complement activation. In summary, these results

provide proof of principle that ECT-mediated expression of CR2-fH is an effective means of reducing local complement activation in the eye in the context of CNV. In future experiments, we wish to determine the viability of this method in the model of smoke-induced ocular pathology, which we have shown is also AP-dependent,[19] to further test the feasibility of ECT-mediated delivery of a complement inhibitor as a potential treatment for dry AMD. For those experiments, however, the capsules might need to be deposited in the suprachoroidal space for CR2-fH to gain access to the RPE, BrM and choroid, as we have shown here that CR2-fH generated in the vitreous does not reach those structures in intact eyes (Fig. 2C) unless the outer BRB is severely breached. Our data on the lack of diffusion of CR2-fH past the RPE is similar to that reported on Fab fragments (same approximate molecular weight as CR2-fH), which also do not get transported past the RPE.[41]

Cell-Encapsulation Technology

A very recent manuscript discusses the pros and cons of the ECT technique.[29] This manuscript summarizes that "the pinnacle goal of ECT is to provide sustained delivery of fresh therapeutics secreted by the encapsulated cells at the target sites without causing any immune reactions." We have achieved both of these goals. First, we have confirmed that healthy cells can be recovered from the capsules upon dissolution of the alginate wall, and dot blot analysis of ocular tissues confirmed that CR2-fH is secreted 1 to 2 months post capsule formation and injection. Second, by testing for CR2-fH antibodies (IgG and IgM), we have confirmed that the mouse does not generate antibodies against the active compound (mouse fusion protein) secreted from the ARPE-19 cells. We did not test for antibody formation against ARPE-19 cell epitopes, since in human patients, a number of clinical trials have shown that devices loaded with ARPE-19 cells did not require immunosuppressants.[42–44] As indicated in the introduction, Neurotech has tested ECT devices in the eye using ARPE-19 cells. NT-501 was designed to deliver CNTF and NT-503 a soluble anti-VEGF-R protein. NT-501 had good safety outcomes, but did fail to reach primary clinical endpoints in GA[42] and RP[44] in protecting photoreceptors. This lack of efficacy may be due to the fact that photoreceptor degeneration in mouse or dog versus man has different underlying disease mechanisms and hence the wrong type of therapeutic might have been chosen. Additional trials in glaucoma and Macular Telangiectasia type 2 are currently under way. The phase II trial for NT-503, on the other hand, has been abandoned due to "a larger than anticipated number of patients requiring rescue medication in the treatment arm" (www.neurotechusa.com, 2016). It has been postulated that the ECT-device did not produce high enough concentrations of anti-VEGF to counteract the amount of VEGF produced under CNV conditions, although their studies in rabbit predicted efficacious VEGF inhibition based on NT-503 over the course of 2 years (Landeros C, et al. *IOVS*. 2016;57:ARVO Abstract 4009). However, importantly, and critical for both Neurotech therapeutic targets as well as our approach, ARPE-19 cells consistently secrete proteins other than the therapeutic proteins, which could potentially interfere with the efficacy of the therapeutic, or worse, augment disease. An and coworkers have determined the secretome of APRE-19 cells under resting conditions and have identified ∼200 proteins (146 highly abundant and 43 low abundant proteins),[45] many of which are known to play a role in angiogenesis or the immune response. It is important to note that the composition of the secretome and actual quantities of the specific proteins may differ significantly under ECT conditions and/or in the eyes of a specific disease recipient. We did not attempt to determine the protein profile of the secretome of the ARPE-19 cells under encapsulation conditions, but it seems clear from our own data that ARPE-19 cells present in the mouse eye do not interfere with CNV lesion growth. Rather the injection into the mouse eye alone appears to affect the size of the CNV lesion (a typical CNV lesion in 3-month-old animals using OCT measurements is ∼12,000 μm^2 in size). Nevertheless, against the increased floor of CNV lesions, a protective effect could still be determined when comparing the active compound (CR2-fH) against the inactive compound (CR2), the native cells or the empty capsules (Fig. 4B). Furthermore, based on the complement C3a measurements, the presence of ARPE-19 cells did not affect complement activation, since there was no difference in C3a levels between CNV animals treated with alginate capsules, capsules loaded with native ARPE-19 cells, or ARPE-19 cells expressing CR2 (Fig. 4C). In preliminary experiments to determine the optimal concentration of capsules, we observed that a 3-fold higher load of capsules (∼30) completely reduced complement activation in CR2-fH-treated eyes to baseline (no CNV control versus CNV+CR2-fH capsules: $P = 0.14$); however, the capsule load interfered negatively with CNV development and masked the protective effect (CR2 versus CR2-fH: $P = 0.40$). Finally, it is important to note that the therapeutic dose of CR2-fH delivered via ECT reduced

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complement activation, but did not reduce complement below baseline levels (i.e., below levels present in animals without CNV). These levels will allow for normal homeostatic functions of the complement system as well as low level surveillance of the immune system.[46]

Long-Term Delivery of Anti-Complement Biologics in AMD

Based on the potential complications of the presence of ARPE-19 cells on disease outcome, anti-complement biologics may need to be delivered by alternative means. Although the current standard of care treatments for wet AMD utilize monthly (or as needed) intraocular injections, other strategies should be explored. An attractive means is gene therapy. Ever since the successful application of AAV gene therapy in Leber's Congenital Amaurosis (reviewed in Ref. 47), gene therapy has been utilized and advanced for additional diseases or disease models.[48] Importantly, new advances in gene regulatory systems may make this approach feasible. For an anti-complement gene therapy in a disease such as wet or dry AMD, the expression of the inhibitor should be inducible when needed (e.g., upon the development of a new CNV lesion) and repressed as quickly as possible when the inhibitor is no longer needed to avoid overtreatment or potential toxic effects. In unpublished results, we have noted that constitutive CR2-fH expression driven in mouse RPE under the VMD2 promoter impairs retinal structure and function when infectivity is too high, an effect not noted when expressing a control gene (mCherry) at equal infectivity. Hence an inducible system using an exogenously regulated gene expression system based on antibiotics, rapamycin or RU486 regulation, or an endogenously controlled gene expression system such as the hypoxia response element (HRE)[49] might be more suitable. HRE may be particularly promising since oxygen deprivation and hypoxia are common features of AMD. Likewise, promotors of the complement system like C3 or C1q should be explored since they are consistently upregulated in affected tissues in retinal degeneration.[18,50]

The eye is an attractive organ for drug-delivery devices, since it is easily accessible. However, ocular delivery systems have the same issue as those for other organs, and that is that they require long-term stability of the biologic at 37°C. Based on the amino acid sequence and molecular structure of the therapeutic protein, critical sites within the active domain of the protein could become modified by oxidation, deamidation, hydrolysis, etc., and become inactivated and/or lead to protein aggregation. Hence, protein engineering and identification of appropriate excipients for the stabilization of the protein in the device is essential. Elsaid and coworkers[51] are approaching this question using nanoparticles for ranibizumab (Lucentis) delivery. They tested the biodegradable polymer poly(lactic-co-glycolicacid) (PLGA) to entrap chitosan-based nanoparticles (chitosan-N-acetyl-L-cysteine[CNAC]) containing the antibody. PLGA microparticles containing CNAC showed significantly improved loading with ranibizumab and exhibited slow release of the antibody that was structurally intact and biologically active, with the added benefit that chitosan itself provides anti-angiogenic activity.[51] CR2-fH has recently been humanized (TT30) and efficacy confirmed.[22,52] However, no data on long-term stability in solution or polymers is currently available and no specific data exists on protein modifications either.

In summary, we provided evidence that ECT can be used effectively to deliver the AP-inhibitor CR2-fH to sites of complement activation in the eye, reducing complement activation and slowing down CNV progression. Inhibiting the AP of complement reduces overall complement activation, but allows for the retention of physiological levels of complement required for cellular homeostasis and immune surveillance.[46] In the future, we expect to investigate this mode of delivery for long-term complement inhibition in the smoke-induced ocular pathology model.[19] Finally, given the advances in drug delivery and the demonstration of efficacy using ECT-based delivery of a complement inhibitor, the focus will now be on the development of long-term treatment strategies in AMD and other complement-dependent diseases.

Acknowledgments

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