Novel Role for a Complement Regulatory Protein (CD46) in Retinal Pigment Epithelial Adhesion

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PURPOSE. There is increasing evidence that the complement system may play a significant role in one of the leading diseases causing blindness in the elderly population, age-related macular degeneration. In this study, a novel role in the retina for a regulatory protein in the complement system, CD46, is proposed.

METHODS. The retinal pigment epithelium (RPE) was obtained from human donor eyes as well as human immortalized RPE cell lines (ARPE19). Immunohistochemistry and confocal microscopy were used to immunolocalize CD46 and β1 integrin. Immunoprecipitation experiments with antibodies to either CD46 or β1 integrin were performed on RPE cell lysates. A cell adhesion assay was used to determine the proportion of RPE cells that adhere to Bruch’s membrane explants from donor eyes.

RESULTS. Immunohistochemistry and confocal microscopy demonstrated that CD46 was polarized to the basal surface of the RPE along with β1 integrin, shown previously to be involved in RPE adhesion. Immunoprecipitation experiments demonstrated that CD46 and β1 integrin coprecipitated from RPE cell lysates when either protein was used as the precipitating antibody. The adhesion assay showed that antibodies to either CD46 or β1 integrin reduced RPE adhesion to the surface of Bruch’s membrane compared with the control.

CONCLUSIONS. These findings suggest that this complement regulatory protein, which protects host cells from autologous complement attack, may have a functional interaction with β1 integrin in the eye that is related to RPE adhesion to its basement membrane and Bruch’s membrane. (Invest Ophthalmol Vis Sci. 2003;44:3669–3674) DOI:10.1167/iovs.02-0815

Evidence is growing that the complement system may play a significant but as yet undefined role in age-related macular degeneration (AMD), the leading cause of blindness in the elderly population. One of the hallmarks of this disease is the formation of extracellular deposits or drusen between the retinal pigment epithelium (RPE), its basement membrane, and the remaining Bruch’s membrane. The disease progression leads to RPE dysfunction, detachment, and, eventually, degeneration that adversely affects the sensory photoreceptors and results in visual loss. Some investigators have suggested on the basis of immunolocalization of terminal complement complexes in drusen that their formation involves complement activation and that a dysfunctional condition in the RPE is an initiating event in AMD. Although some components of the complement system are present in the retina and RPE, very little is known about their role, other than that they serve a protective function in innate immunity. The purpose of the present study was to explore the role of one complement regulatory protein, CD46, in RPE cell adhesion.

CD46 (membrane cofactor protein [MCP]) acts as a serum protease cofactor that degrades C3b and prevents activation of the complement cascade that serves to protect the host cell against autologous attack. It is a transmembrane glycoprotein that is present on most nucleated cells, and it serves as a receptor for measles virus, as well as C3b, C4b, and two other human pathogens. In addition, it is polarized on the basolateral membrane of epithelial cells from nonocular tissue and is highly expressed at the blood–brain barrier. This study, CD46 was also preferentially localized to the basolateral membrane surface of the RPE in situ, along with β1 integrin. In addition, CD46 coprecipitated with β1 integrin, which has been shown to mediate RPE attachment to the basement membrane. Function-blocking experiments with antibodies demonstrated that RPE adhesion to Bruch’s membrane extracts can be reduced by anti-CD46 antibodies. Although it has been shown that CD46 associates directly with multiple β1 integrins in nonocular tissue, this is the first report in ocular tissue of a functional interaction between a complement regulatory protein and a β1 integrin, which may be significant not only in adhesive mechanisms in the retina but in a constellation of functional interactions associated with integrin signaling pathways. Furthermore, by studying the role of this protein in normal RPE, we may gain new insights into the relationship of the complement system and complement regulatory proteins to the dysfunctional RPE observed in AMD.

MATERIALS AND METHODS

Immunohistochemistry

All research on tissue obtained from human subjects adhered to the tenets of the Declaration of Helsinki. Posterior globes of donor eyes (ages, 54–74 years) obtained from the Kentucky Lions Eye Bank were prepared for immunohistochemistry by fixing in 4% paraformaldehyde overnight, dehydrating, and embedding in paraffin. Consecutive 5-μm sections were hydrated, incubated for 2 hours with 0.3% H2O2 in phosphate-buffered saline to quench endogenous peroxidase followed by incubation with 5% goat normal serum. The sections were then incubated with 1:100 mouse anti-human CD46 antibody (BD Pharmingen, San Diego, CA) and a stain (NovaRed Substrate Kit for Peroxidase; Vector Laboratories, Burlingame, CA) that produces a red color in contrast to the gold-brown of the melanin granules in the RPE. Control sections were incubated either with isotype-matched nonimmune serum or without the primary antibody.

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The RPE-choroid from one donor eye was prepared as a flatmount for confocal microscopy and in situ staining with CD46 antibody (as above) and visualized by incubation with a Cy3-conjugated goat anti-mouse antibody (Sigma-Aldrich). RPE cell nuclei were counterstained with 4’,6’-diamino-2-phenylindole (DAPI, Vector Laboratories).

RPE cells, harvested from donor eyes and maintained as primary cultures in DMEM/F12 with 10% fetal bovine serum (FBS), were also prepared for immunohistochemistry and confocal microscopy by fixing in 4% paraformaldehyde for 20 minutes. Cultured RPE were immunostained for CD46 as just described and, in addition, for the β1 integrin antibody (Chemicon International, Temecula, CA) and then visualized by a Cy3-labeled antibody.

In addition, ARPE19 cells (ATCC, Manassas, VA) derived from human RPE were cultured on chamber slides (Nalge Nunc International, Naperville, IL) in DMEM-F12 containing 10% FBS with 100U/mL penicillin and 100 µg/mL streptomycin and maintained in culture conditions for 4 weeks before preparing for CD46 and β1 integrin immunohistochemistry and confocal microscopy as described for cultured cells.

Reverse Transcription–Polymerase Chain Reaction
ARPE19 monolayers maintained in culture for 4 weeks or confluent RPE cell cultures obtained from human eyes (ages, 35–65 years) and established after two to six passages were used to extract total RNA (RNAeasy Mini Kit; Qiagen, Valencia, CA) according to the manufacturer’s specification. The yield and purity of RNA were estimated by optical density at 260/280 nm. After DNase treatment, cDNAs were synthesized from RNAs with reverse transcriptase (Superscript II; Invitrogen/Gibco, Gaithersburg, MD) with oligo dT as the primer, according to the manufacturer’s specifications. Polymerase chain reactions were performed in an automatic sequencer (Gene-Amp PCR System 2400; Applied Biosystems Inc., Foster City, CA) with advantage cDNA polymerase mix (Clontech, Palo Alto, CA). The following primer for CD46 was used: forward, 5’-CCG CAA AAT GGC ACT TAG G-3’; reverse, 5’-AAA AAC CCT TAT CGC ATT CAA AC-3’. PCR products were sequenced by DNA autosequencing (CEQ 2000; Beckman Instruments, Fullerton, CA) and the sequence identity verified by using a BLAST search of the Genome Systems Data Bank, available at http://www.ncbi.nlm.nih.gov/blast/.

Immunoblot Analysis
To detect the expression of the complement regulatory protein CD46 in RPE, Western blot analysis was performed with RPE cells from donor eyes (ages, 60–70 years) and ARPE19 cell lines. Posterior globes were frozen within 12 hours after death and were stored at −80°C, thawed, and dissected. RPE cells were collected by adding Hanks’ medium into the eyecups and gentle pipetting. Total protein extract of thawed, and dissected. RPE cells were collected by adding Hanks’ balanced salt solution containing 5% nonfat dry milk and incubated with mouse anti-human RPE were cultured on chamber slides (Nalge Nunc International, Naperville, IL). Fifty micrograms of each protein sample was separated under reducing conditions for 4 weeks before preparing for CD46 and β1 integrin immunohistochemistry and confocal microscopy as described for cultured cells. Five percent of each protein sample was separated by SDS-PAGE on 10% gels under nonreducing conditions. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked overnight at 4°C in TBS-T solution containing 5% nonfat dry milk and incubated with mouse anti-human antibodies against CD46 (BD PharMingen International, San Diego, CA) in TBS-T solution. The antibody binding was detected with horseradish peroxidase-conjugated anti-mouse (Dako, Glostrup, Denmark) secondary antibody and enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham Life Science, Buckinghamshire, UK).

Immunoprecipitation
RPE cells harvested from human donor eyes or ARPE19 cells were washed with ice-cold PBS and lysed for 30 minutes at 4°C in nonionic detergent (Brij 98; Sigma-Aldrich; 20 mM Tris [pH 7.5], 150 mM NaCl, 1% Brij 98, 1 mM CaCl2, 1 mM MgCl2, 10 µg/mL antipain, 10 µg/mL leupeptin, 10 µg/mL pepstatin A, 2 µg/mL aprotinin, and 100 µM PMSF). The insoluble material was removed by centrifugation at 12,000g for 15 minutes at 4°C, and the supernatants were preincubated by incubation with Sepharose protein G (Amersham Pharmacia Biotech) for 1 hour at 4°C. Precleared supernatants were centrifuged and transferred to new tubes and then incubated overnight at 4°C with either anti-CD46 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β1 integrin monoclonal (Chemicon International) precipitating antibody. The purified rabbit IgG or isotype-matched IgG served as the control. The immune complex was precipitated by adding 1:10 volume of Sepharose protein G and incubating at 4°C. After a 1-hour incubation under constant rotation, samples were centrifuged and washed five times with lysis buffer containing 0.5% Brij 98. Bound proteins were eluted with two times lysis buffer and boiling for 10 minutes. Immunoprecipitated proteins were resolved in a 7.5% SDS gel under nonreducing conditions and transferred to PVDF membranes. Coprecipitated β1 integrin or CD46 were probed either by a combination of biotin-labeled mAb (Ansell, Bayport, MN) plus horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech) or a combination of unlabeled mouse mAb (BD Pharmingen International) plus HRP-conjugated rabbit anti-mouse secondary antibody (Chemicon International).

Cell Adhesion Assay
Explants of human Bruch’s membrane were prepared from human donor eyes (60–70-year-old donors), as described previously. After a full-thickness circumferential incision was made posterior to the ora serrata and the vitreous and anterior segment removed, the posterior poles were isolated and discarded if there was any evidence of subretinal blood, drusen, or irregular pigmentation of the macular RPE. The neural retina was removed and 0.02 N ammonium hydroxide was pipetted into the eyecup to remove adherent RPE, followed by washing with PBS three times. A 6.5-mm diameter corneoscleral trephine was used to punch out explants of human Bruch’s membrane from the macula and periphery of the eyecups. Six to eight explants were harvested per eye.

Second-passage human RPE cells were harvested from donor eyes (53–65-year-old donors) by incubating in 0.25% trypsin/0.25% edetic acid in Hanks’ balanced salt solution for 20 minutes. Ten milliliters of MEM/15 was added for quenching and the cell suspension was centrifuged for 5 minutes at 800 revolutions per minute. The cell pellet was incubated on a shaker table at room temperature for 1 hour in one of the following antibodies: 0.1 to 25 µg/mL of mouse anti-human CD46 monoclonal antibody (Accurate Chemical & Scientific Corp., New York, NY), 10 µg/mL of mouse anti-human β1 integrin monoclonal antibody (Chemicon International), or 1:500 mouse non-specific IgG1k MOPC21 monoclonal antibody (Sigma-Aldrich). The cell pellet was washed three times and resuspended in MEM without serum, and aliquots of 5 × 104 viable cells were applied to each Bruch’s membrane explant. Cells were allowed to attach for 24 hours. Unattached cells were removed by picking up the tissue with fine forceps and dipping three times in Hanks’ balanced salt solution before placing the explants in a new well of a 96-well plate. Three explant buttons were used for each experimental condition. Data were analyzed by one-way analysis of variance followed by a multiple comparison test or a two-tailed t-test.

Cell adhesion assays were performed using an established MTT-based cell assay, as described previously. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) is a dye with characteristics that change when it is dehydrogenated by cellular mitochondrial dehydrogenase, and the activity of the latter enzyme is proportional to the number of live cells exposed to the dye. The amount of yellow-reduced tetrazolium was quantified with an enzyme-linked immunosorbent assay reader with a 570-nm filter after the solid tissue was removed from the wells containing explants and 96-well plates read. The number of cells attached to the surface was calculated by comparing the enzyme-linked immunosorbent assay readings ob-
Results

Immunolocalization and Expression of CD46

In histologic sections of the normal human eye, CD46 staining showed a preferential distribution on the basolateral surface of the RPE, but not in Bruch’s membrane (Fig. 1). Confocal microscopy of horizontal sections of RPE harvested directly from human donor eyes and prepared as a flatmount demonstrated a complete absence of CD46 staining on the apical RPE membranes in situ (Fig. 2A), as well as an absence of autofluorescent pigment granules (Fig. 2B). CD46 staining was present in sections of the basolateral RPE surface (Fig. 2C), and some autofluorescence was present, due to pigment granules in the basal RPE cytoplasm (Fig. 2D). Confocal microscopy of cultures immunolabeled for CD46 and β1 integrin also revealed a basolateral membrane localization of both proteins in horizontal and vertical views of the RPE monolayer in primary cultured RPE from donor eyes (Fig. 3) and in RPE cell lines (Fig. 4). When the RPE monolayer was viewed at different confocal planes from apical to basolateral surfaces, immunolabeled CD46 and β1 integrin were present only on the basolateral membrane surfaces. When viewed in vertical sections, antibody staining for CD46 and β1 integrin clearly labeled the basal RPE surface.

Immunoblot analysis of RPE obtained from donor eyes and the ARPE19 cell line demonstrated the presence of a protein doublet at 55 and 65 kDa, which corresponds to the lighter and heavier isoforms of CD46, respectively (Fig. 5). Primary RPE cultures established from donor eyes and cultured ARPE19 cells grown to confluence and maintained for 4 weeks or longer demonstrated the presence of mRNA for CD46 (Fig. 6). A PCR product was obtained in the expected base pair range of 448 bp for CD46, and sequencing confirmed a 99% sequence identity with its appropriate cDNA.

Coimmunoprecipitation of CD46 and β1 Integrin in RPE Cells

To characterize the association of CD46 with β1 integrin, immunoprecipitation experiments were performed on cell lysates of RPE harvested from human donor eyes and the ARPE19 cell line and immunoblotted with antibodies to CD46 and β1 integrin. β1 integrin coprecipitated with CD46 from both human RPE (Fig. 7A) and RPE cell lines. Reciprocally, CD46 coimmunoprecipitated with β1 integrin in both human RPE (Fig. 7B) and ARPE19 cell lysates. Control samples incubated with purified rabbit IgG or isotype-matched IgG failed to precipitate either CD46 or β1 integrin.

Figure 1. Histologic section of a donor eye immunostained with anti-CD46 (A). Staining was present on the basolateral membrane surface of the RPE. Inset: higher magnification of basolateral staining. Control sections (B) showed little or no specific staining. Bar, 20 μm.

Figure 2. Confocal microscopy of horizontal sections through RPE from a human donor eye, flatmounted and immunolabeled for CD46. (A, B) represent sections from the same apical RPE plane showing the absence of staining for CD46 (A), as well as the absence of autofluorescent pigment granules (B). (C, D) represent sections from the same basolateral RPE plane showing membrane staining for CD46 (C) and autofluorescence due to pigment granules in the basal RPE cytoplasm (D). Bar, 20 μm.

Figure 3. Confocal microscopy of horizontal sections through the RPE monolayer from primary cultures showing the absence of labeling for CD46 and β1 integrin on the apical plane of the section and the presence of basolateral membrane staining only in the basal plane of the monolayer. Polarized labeling on the basal RPE surface was clearly visible in vertical sections. Bar, 20 μm.
FIGURE 4. Confocal microscopy of horizontal sections through the ARPE19 cell monolayer showing the same absence of apical labeling and intense basolateral labeling for both CD46 and \( \beta_1 \) integrin in both horizontal and vertical sections. Bar, 20 \( \mu \)m.

RPE Cell Adhesion Assay

We used the cell adhesion assay previously described in which RPE attachment to their native physiological substrate, the basal lamina of human Bruch’s membrane, is determined (Fig. 8). RPE cells that were preincubated with an irrelevant IgG (mouse IgG1 MOPC21 monoclonal antibody) before seeding onto Bruch’s membrane served as the control for all the adhesion studies. Anti-CD46 reduced RPE adhesion to human Bruch’s membrane by approximately 40% to 50% compared with the control in a dose-dependent manner (Fig. 8A), with half-maximum inhibition at a concentration of approximately 1 \( \mu \)g/ml of anti-CD46. We have demonstrated that anti-\( \beta_1 \) integrin antibodies decrease RPE adhesion to human Bruch’s membrane and RPE-derived extracellular matrix. In the present study, 10 \( \mu \)g/ml of anti-\( \beta_1 \) integrin, previously shown to be a saturating dose, produced inhibition comparable to anti-CD46 alone (Fig. 8B). Incubation with both anti-CD46 and anti-\( \beta_1 \) integrin did not produce further inhibition compared with either antibody alone (Fig. 8B).

DISCUSSION

A complement regulatory protein, CD46, preferentially localized to the basolateral membrane of the RPE from human donor eyes in situ and in primary cultures of human RPE, as well as in ARPE19 cell lines. Immunoprecipitation experiments of RPE lysates from the same sources demonstrated that CD46 coprecipitated with \( \beta_1 \) integrin and, in the reverse immunoprecipitation protocol, \( \beta_1 \) integrin coprecipitated with CD46, indicating a physical relationship between the two proteins. Functional blocking of RPE adhesion with antibodies to CD46 confirms that anti-CD46 reduces RPE cell adhesion, similar to the effect that has been shown with anti-\( \beta_1 \) integrin antibodies. Incubation with antibodies to both CD46 and anti-\( \beta_1 \) integrin inhibited RPE adhesion to the same extent as either antibody alone, thus suggesting that both antibodies affect the same site.

CD46 (membrane cofactor protein [MCP]) is expressed on all nucleated human cells and acts to protect the host cell against autologous complement attack by degrading C3b. It is also present on the basolateral surface of polarized epithelial cells, similar to the localization shown in the current study in the RPE. Further studies of the basolateral targeting mechanism have shown that there is a functional interaction between CD46 and DLG4, a member of the guanylate kinase family and that the polarized expression of CD46 in epithelial cells requires the DLG4-binding domain.

One of the proteins belonging to the DLG4 family may also have a functional interaction with CD46 in RPE cells. DLG4 is one of a family of four human proteins that share a single homologue with a tumor-suppressor called disc large (DLG) from Drosophila. DLG4 is also called postsynaptic density (PSD) or synapse-associated protein (SAP) because of its localization in the postsynaptic density region of neurons. These proteins have multiple protein–protein interaction motifs, including three PDZ domains, which derive their acronym from the three proteins first characterized as having these recognition domains (PSD from neurons, DLG from Drosophila, ZO1 from tight junctions). These proteins are polarized and interact with other PDZ domain-containing proteins, membrane receptors, cell adhesion molecules, and the cytoskeleton to regulate epithelial cell polarization and assemble signaling cascades. In view of this, it is of interest that recent studies of rat RPE have shown that one of these PDZ domain-containing proteins, SAP97, localizes to the basolateral surface of RPE and may have a direct interaction with ezrin, an actin-binding protein associated with the morphogenesis of RPE apical microvilli and basal infoldings. Ezrin belongs to the ERM family of proteins, which collectively defines three highly homologous proteins (ezrin, radixin, moesin) that constitute a group of plasma membrane–cytoskeleton linkers that regulate cell adhesion and morphogenesis of the actin-rich cell cortex. Future work in our laboratory will investigate the relationship of CD46 to ezrin and SAP97 and whether there are any functional associations with RPE adhesion.

Cell adhesion to the extracellular matrix is a crucial regulator of cell behavior and the large protein complexes of signal-
ing proteins and cytoskeleton are assembled into functional units at the sites of integrin–matrix adhesion.36

We have shown that the β1 subunit of integrins partially mediate the adherence of human RPE cells to RPE-derived extracellular matrix and the basal lamina layer of human Bruch’s membrane.18 Recently, it has been shown by immunoprecipitation experiments in carcinoma-derived cell lines that CD46 associates with multiple β1 integrins19 and indirectly with a superfamily of surface molecules,37 tetraspanin, known also to associate with a subset of β1 integrins38–41 and to form a web with common functions related to migration, proliferation, intracellular signaling and adhesion.42–46 CD46 is a newly discovered component of this web.19 The relevance of these protein associations to RPE cells is that they may form the functional units underlying normal adhesion mechanisms, maintaining a healthy RPE phenotype that is not proliferative or migratory. When there is disease, these functional units may become disrupted, the RPE may lose attachment to Bruch’s membrane, and RPE cells may break away from the monolayer and undergo apoptosis.47 Therefore, the loss of RPE cells, which is one of the first signs of AMD,48 may be preceded by the loss of RPE attachment to Bruch’s membrane through a dysfunctional CD46–β1 integrin complex. A recent study, in which flatmount preparations of human cadaveric eyes were stained with the TUNEL technique, provides direct evidence that human RPE undergoes age-related apoptosis in situ, with apoptotic human RPE confined mainly to the macula of older human eyes.49 Another similar report suggests that human RPE die by apoptosis around the edges of geographic atrophy.50 Secondary atrophy of the underlying choriocapillaris and overlying photoreceptors would then follow and signal the clinical recognition of AMD. More studies are needed to determine how specific molecular interactions of integrin with CD46 and other protein partners play a role as a functional unit in maintaining RPE adhesion and phenotype.

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References
