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Regulated secretion of complement factor H by RPE and its role in RPE migration

Yeong Hoon Kim^{a,b}, Shikun He^{a,b,c,*}, Satoru Kase^a, Mizuki Kitamura^a, Stephen J Ryan^{a,b}, and David R Hinton^{a,b,c}

^aDoheny Eye Institute, Keck School of Medicine of the University of Southern California, Los Angeles, CA

^bDepartment of Ophthalmology, Keck School of Medicine of the University of Southern California, Los Angeles, CA

^cDepartment of Pathology, Keck School of Medicine of the University of Southern California, Los Angeles, CA

Abstract

Background—Variants in the gene for complement factor H (*CFH*) have been implicated as a major risk factor for the development of age-related macular degeneration (AMD). Little is known, however, about the factors regulating local expression and secretion of CFH by retinal pigment epithelial cells (RPE).

Methods—Cultured human early passage RPE cells, highly differentiated, polarized human RPE cultures, and bovine RPE explants were incubated in the presence or absence of recombinant human or bovine interferon- γ (IFN- γ ; 25 ng/ml). CFH expression in cell lysates, and secretion into culture supernatants were examined by Western blot. CFH expression and localization was analyzed by confocal microscopy. Migration assay was performed in a modified Boyden chamber with early passage human RPE cells after stimulation with recombinant CFH protein (1–100 ng/ml).

Results—CFH was expressed in the cell lysates of RPE cells, and this expression was significantly upregulated by IFN- γ . Immunoreactivity for CFH was detected in RPE cells of bovine explants and highly differentiated human RPE monolayers, and the level of immunoreactivity increased after IFN- γ stimulation. Confocal microscopy revealed that CFH was predominantly localized in the apical cytoplasm of polarized human RPE. Western blot confirmed that IFN- γ increased CFH secretion into RPE supernatants. Dose dependent RPE cell chemotactic migration was induced by CFH.

Conclusion—IFN- γ promotes CFH expression in the apical compartment of RPE cells and increases secretion of CFH into RPE culture supernatants. Furthermore, CFH promotes chemotactic migration of RPE. This study suggests that interactions between CFH and IFN- γ have the potential to play a role in the pathogenesis of AMD.

Keywords

AMD; complement factor H; migration; retinal pigment epithelium; interferon- γ

Corresponding author: David R. Hinton, MD, FRCPC, Department of Pathology, University of Southern California, 1355 San Pablo St. DVRC213, Los Angeles, CA. Tel: (323)442-6617; Fax: (323)442-6688; e-mail address: dhinton@hsc.usc.edu.

*Contributed equally to the work and therefore should be considered equivalent first author

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the elderly in developed countries [1–5]. AMD is a complex disorder stemming from the interaction of aging with multiple genetic and environmental risk factors [6,7,8]. AMD, along with several other chronic disorders including Alzheimer disease, atherosclerosis and glomerulonephritis, is characterized by the presence of extracellular deposits and inflammation, which contribute to the pathogenesis and progression of the disease [3,9–14]. In AMD, these deposits appear as drusen, a hallmark of the early form of AMD [1]. Drusen contain various inflammatory components, suggesting that the innate immune response and the complement cascade may contribute to drusen formation [1,15–20]. Complement factor H (CFH) is the major inhibitor of the alternative complement pathway and is found within drusen [3,21]. Variants in the gene for *CFH* have been implicated as a major risk factor for the development of AMD [2,3,4,5,22,23,24]. Thus, a genetic variation in a regulator of the alternative complement pathway, when combined with a triggering event such as infection, oxidative stress or smoking, may underlie a major proportion of AMD in the human population [3,25].

Retinal pigment epithelial (RPE) cells represent a primary site of pathology in AMD and their function and phenotype may be modified by cytokines and inflammatory mediators. The cytokine interferon gamma (IFN- γ) has been shown to induce expression of CFH [26] in the human ARPE-19 cell line, and upregulate expression of major histocompatibility complex (MHC)-II in primary human RPE cells [27]. In contrast, a recent study by Chen et al. showed that CFH production in a cultured murine RPE cell line was not affected by IFN- γ [28]. Thus, the effects of IFN- γ on the expression of CFH in RPE cells may vary according to species, cell line or culture conditions. Therefore, in order to gain greater relevance to human disease, we determined the effects of IFN- γ on CFH expression in cultured human RPE, on highly differentiated and polarized human RPE cultures [29], and in primary bovine RPE explant cultures. We also hypothesized that CHF secretion into the extracellular microenvironment of the RPE may affect other RPE functions. Since cytokines have been shown to modulate RPE migration [30], and CFH has been reported to be chemotactic to monocytes [31], we evaluated the effects of CFH on RPE chemotactic migration.

MATERIALS AND METHODS

Human RPE cell cultures

The Institutional Review Board of the University of Southern California approved the use of cultured human RPE cells. RPE cells isolated from human fetal eyes (20 weeks gestation; Advanced Bioscience Resources, Inc., Alameda, CA) were cultured in Dulbecco's minimal Eagle's medium (DMEM; Fisher Scientific, Pittsburgh, PA) with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA). All experiments were performed using first to fourth passage cells. Cells were confirmed to be RPE by their typical morphology, their immunoreactivity for cytokeratin (>95%) and the lack of immunoreactivity for macrophage or endothelial cell markers.

Western blot

Passage 2–4 human RPE cells (5×10^5) were cultured in Dulbecco's minimal Eagle's medium with 10% heat-inactivated fetal bovine serum (FBS) [30] on 6-well plates until they reached confluence. Twenty four hours prior to IFN- γ stimulation, the confluent cells were gently washed with serum-free medium and then the medium was replaced with Dulbecco's minimal Eagle's medium with no added serum; subsequently, recombinant human IFN- γ (25 ng/ml;

Sigma, St. Louis, MO) was applied and incubated for 24, 48 or 72 hours, after which the cells and supernatants were harvested. Parallel control non-IFN- γ -treated cultures were also evaluated after 24, 48 or 72 hours of incubation. Cell lysates were prepared using cell lysis buffer (Pierce Biotechnology, Rockford IL). The cell lysate (10 μ g protein/lane) and supernatant (40 μ g protein/lane) proteins were resolved on Tris hydrochloride 7.5% polyacrylamide gels (Ready Gel; Bio-Rad Laboratories, Hercules, CA) at 120 V. The proteins were transferred to a polyvinylidene fluoride blotting membrane (Millipore, Billerica, MA). These membranes were then probed with anti-CFH antisera (Quidel Corporation, San Diego, CA) 1:500, followed by horseradish peroxidase-conjugated anti-goat antibody (1:5000; Vector Laboratories) for 30 minutes at room temperature. Images were developed by adding chemiluminescence detection solution (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). As a loading control for cell lysates, membranes were also probed using antibodies against the housekeeping gene GAPDH. Band intensity was quantified using Image Pro Plus software. The integrated optical density, defined as the integrated total intensity of all pixels in each band was determined and corrected for background. The resulting absolute numerical values were expressed relative to GAPDH. Student-T test was then used to compare the integrated optical density between control and IFN stimulated RPE at each time point. The accepted level of significance was $p < 0.05$.

Preparation of polarized human RPE monolayer cultures

Highly differentiated, polarized, human RPE monolayer cultures were prepared as previously described [29]. Briefly, primary cultured, human fetal RPE cells were trypsinized and resuspended in DMEM supplemented with 100 U/mL penicillin-streptomycin, 2 mM L-glutamine, and 1% FBS. Cells (1.7×10^5 cells/cm²) were seeded onto individual fibronectin-coated polyester permeable supports (0.4 μ m pore size, 12 mm diameter; Transwell; Corning Costar, Corning NY) in 0.5 ml of medium. The medium was changed twice a week. Cells were cultured for at least a month to form differentiated monolayers, with the apical domain corresponding to the retinal facing side of the RPE monolayer. Trans-epithelial resistance (TER) reached a plateau at the time of experimentation ranging between 200 and 300 $\Omega \cdot \text{cm}^2$. Recombinant human IFN- γ (0, and 25 ng/ml, Sigma, St. Louis, MO) was added to the medium and incubated for 48 hours.

Bovine RPE-choroid explants

In order to determine CFH expression in situ, we evaluated CFH expression and its regulation by IFN- γ in bovine RPE-choroid explants. Fresh bovine eyes were obtained from a local abattoir and the eyes were cut circumferentially posterior to the limbus, and the vitreous and retina were removed. A 1-cm² section of RPE with choroid was obtained and incubated in 12-well plates with DMEM containing 1% FBS for 24 hours, and then recombinant bovine IFN- γ (0, and 25 ng/ml; Sigma) was added for an additional 48 hours.

Immunocytochemistry

Immunocytochemistry was performed on bovine RPE/choroid explants, cryostat frozen sections (6 μ m thick) of bovine RPE/choroid explants, and human polarized RPE monolayers which had been incubated with or without IFN- γ . Samples were fixed in 4% paraformaldehyde for 30 min, and then permeabilized using 0.2% Triton-X 100 at 37° C for 15 min. Blocking was achieved by addition of 1% rabbit serum for 20 min. The samples were incubated with primary anti-CFH antibody (Quidel Corporation; 1:1,000) for 1 hr at room temperature. After washing with PBS, secondary FITC-conjugated rabbit anti-goat antibody (1:400; Chemicon, Temecula, CA) was applied to the slides for 30 min at room temperature. As a negative control, sections were treated with secondary antibody alone. Sections were mounted on glass slides with mounting medium including propidium iodide (Vector Laboratories). Fluorescence was

examined and photographed with a high resolution Zeiss laser-scanning microscope (LSM2, Carl Zeiss, Thornwood, NY) with a 40x oil-immersion lens.

Migration assay

Migration assay was performed in a modified 24 well plate Boyden chamber (Fibronectin coated, 1 μ m thick polycarbonate inserts; 8 μ m pore size; pore density of 1×10^5 pores/cm²; Becton Dickinson Labware, Franklin Lakes NJ) using passage 2–4 human fetal RPE cells (5×10^4 cells/ml in media containing 0.4% FBS). At the time of cell counting, the cells were evaluated microscopically to ensure a single cell suspension. Recombinant CFH (Quidel) was added to the lower chamber alone (chemotactic assay), or both lower and upper chambers (chemokinesis assay) at equal concentrations of 1, 10, 50, 100 ng/ml as the stimulant. After incubating for 5 hours, the inserts were washed 3 times with PBS, fixed with cold (4°C) methanol for 10 minutes, and counterstained with hematoxylin for 30 minutes. The number of migrated cells on the entire insert was counted using a phase-contrast microscope ($\times 200$). Three independent experiments were performed using triplicate inserts. Cross sectional analysis of the mean number of migrated cells in each group was performed to compare groups using the Poisson model for aggregate count data. The accepted level of significance was $p < 0.05$.

RESULTS

CFH protein expression in cell lysates of RPE cells treated with IFN- γ

Western blots of RPE cell lysates revealed the presence of a major CFH immunoreactive band at 155 kDa CFH (Fig. 1a). CFH protein expression in RPE cell lysates was significantly upregulated by stimulation with IFN- γ at 24, 48, and 72 hours (Fig 1a). Semi-quantitative analysis of CFH protein expression by densitometry revealed that expression of CFH was significantly increased at all 3 time points with maximal expression at 48 hours after stimulation with IFN- γ ($P < 0.02$) (Fig1 b).

Immunoreactivity for CFH in bovine RPE-choroid explants treated with bovine IFN- γ

Cross reactivity of anti-CFH antibody with bovine CFH was confirmed by Western blot of bovine RPE cell homogenates; a major CFH immunoreactive band was identified at 155 kD similar to that seen in Figure 1 (results not shown). The expression of CFH in bovine RPE-choroid explants is shown in Fig 2. In untreated explants, scattered RPE cells showed weak to moderate cytoplasmic immunoreactivity for CFH (Fig. 2b). After treatment with IFN- γ (25 ng/ml) expression of CFH appeared to be much stronger and labeled almost every RPE cell in a punctuate pattern (Fig. 2c). No primary antibody controls revealed focal, weak, background staining (Fig. 2a). Cryostat sections of the RPE-choroid explants revealed that there was weak to moderate cytoplasmic staining for CFH in a subset of RPE cells; weak staining of cells within the choroid was also seen (Fig. 2e). Treatment with IFN- γ resulted in a prominent increase in CFH immunoreactivity in essentially all cells of the RPE layer (Fig. 2f). Very weak CFH immunoreactivity was present in the “no primary antibody” control (Fig. 2d)

Immunoreactivity for CFH in polarized human RPE cultures treated with IFN- γ

Evaluation of CFH protein expression in polarized, human RPE cell monolayers, revealed diffuse, cytoplasmic expression in most of the RPE cells in the untreated cultures (Fig. 3; d–f,). After treatment with 25 ng/ml IFN- γ (Fig. 3; a–c), the intensity of expression became more prominent. No primary antibody control sections showed insignificant background staining (Fig. 3; g–i). Higher magnification confocal images of the polarized, human RPE cell monolayers (Fig. 4) confirmed that CFH staining was cytoplasmic and punctate and that the intensity of the immunoreactivity was greater in the IFN- γ –treated cells. Confocal vertical

sections (X–Z) revealed that expression of CFH was predominantly localized in the apical compartment of both untreated and treated cells (Fig. 4; right panel).

CFH secretion into culture supernatants induced by IFN- γ

Since we showed that RPE cells show regulated expression of CFH in their cytoplasmic compartment, we next wanted to determine whether CFH was secreted, consistent with a previous finding that human keratinocytes released CFH into the culture medium [32]. To determine whether CFH was secreted into the serum-free RPE cell culture medium, CFH expression was examined in the cell-free supernatants of RPE cells with or without IFN- γ treatment using Western blot analysis. High molecular weight CFH was detected in the supernatants in control samples and was increased in amount after treatment with IFN- γ for 24, 48 and 72 hours when compared with the time-matched non-IFN- γ treated controls (Fig. 5). In order to determine whether the level of CFH secreted from RPE cultures was relevant to subsequent migration experiments, we established a Western blot standard curve using the pixel density of immunoblotted serum free culture media spiked with recombinant CFH (5, 10, 20, 30, 50, and 100 ng/ml). When 48 hour, IFN- γ -treated cultures (3 independent samples) were run along side the spiked standards, an average concentration of 22 ± 6 ng/ml was found (results not shown).

Modulation of RPE cell migration by recombinant CFH protein

Our results showing that RPE cells secreted CFH protein into the culture supernatant, prompted us to determine whether CFH could affect RPE function. This is relevant, since Lommatzsch et al [33] found that CFH was highly expressed extracellularly in basal laminar deposits from human CNV membranes associated with AMD. The finding that RPE cells migrate into the stroma in response to growth factors found in CNV membranes [34–36] suggested that we evaluate RPE cell migration induced by exposure to CFH protein. Since RPE migration typically occurs in the context of a provisional extracellular matrix, and fibronectin has been localized in human CNV membranes [37] and in diffuse drusen [38], we first evaluated whether a migratory response to fibronectin was present. Baseline migration doubled in the presence of fibronectin and therefore all subsequent migration experiments were performed in the presence of fibronectin (Fig 6). When equal concentrations of CFH were placed in the upper and lower compartments, there was a modest but statistically insignificant increase in migration indicating that RPE lack a significant increase in random migration with CFH. However, a much stronger, dose-dependent chemotactic response was obtained when CFH was added to the lower compartment alone. A maximal chemotactic effect was found at 50 ng/ml ($p < 0.01$; Fig. 6). Interestingly, CFH-induced RPE chemotactic migration was as strong as that stimulated by the known RPE chemotactic factor platelet derived growth factor (PDGF)-BB. In separate experiments, RPE migration was completely blocked by pre-incubating CFH with anti-CFH antibody (data not shown).

Discussion

The importance of CFH in pathogenesis of AMD has been well established and the localization of CFH in drusen suggests local synthesis of CFH by RPE [3,18]. Previous studies in the human ARPE-19 cell line suggested that CFH was not constitutively expressed in RPE [26], while immunostaining and RT-PCR studies in mice showed constitutive expression in RPE [39]. Our results indicate that non-immortalized, passage 2–4 human RPE cell cultures constitutively express CFH as demonstrated by Western blot analysis of cell lysates, and immunofluorescence images of cultured bovine RPE explants and highly differentiated, polarized human cultured RPE monolayers. RPE cells are known to respond to stimulation by pro-inflammatory cytokines such as IFN- γ by up-regulating expression of cytokine receptors and MHC class II molecules [27,40]. These results suggest that IFN- γ may participate in and magnify local

inflammatory responses in the outer retina; however, IFN- γ may also up-regulate expression of genes that dampen inflammatory responses in the delicate environment of the retina. In the current study, we found that IFN- γ stimulation results in up-regulation of CFH expression. This result differs from that published by Chen et al. [28]. They reported that in murine RPE, IFN- γ had no effect on CFH expression while TNF- α and IL-6 downregulated CFH expression. These conflicting results on cytokine regulation of CFH expression may be due to the difference between species, as varying results are obtained even among different cell lines [21,41]. Similar to our results, Wu et al. demonstrated that CFH was upregulated by IFN- γ in RPE, however, their study, performed using the ARPE-19 cell line, did not show constitutive expression of CFH [26]. In summary, our work clearly supports the contention that IFN- γ up-regulates CFH expression in RPE and that in vitro studies evaluating regulated gene expression in immortalized cell lines should be re-evaluated using differentiated, human primary cell cultures, in situ evaluation or explant cultures.

This study is the first to demonstrate polarized expression of CFH in RPE. Using highly differentiated human RPE monolayer cultures we showed that CFH was clearly localized to the apical compartment. We also demonstrate that CFH is actually secreted from RPE cells where it accumulates in the culture supernatants and that IFN- γ promotes this secretion. Importantly, we show that CFH accumulates in RPE culture supernatants at a level that promotes RPE chemotaxis in Boyden chamber assays suggesting that the level is physiologically relevant. Apical secretion of CFH would promote a CFH gradient that is highest in the subretinal space, where it may be acting to protect photoreceptors from effects of inappropriate complement activation. We also demonstrate here, for the first time, that CFH promotes the chemotactic migration of RPE cells consistent with its effects as a chemotactic agent for monocytes [31]. Therefore, apical secretion of CFH from RPE may have the potential to promote migration of RPE into the stroma of choroidal neovascular lesions.

In conclusion, we demonstrate that CFH is constitutively expressed in human RPE cell cultures and bovine RPE explants and that CFH expression is localized to the apical compartment of polarized RPE cells. The expression and secretion of CFH was significantly increased by the stimulation with IFN- γ . Furthermore, the secretion of CFH by RPE may have functional significance beyond that of modulating complement activation by stimulation of RPE migration. This study suggests that interactions between CFH and inflammatory cytokines may play an important role in the pathogenesis of AMD.

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Fig. 1.

Time course of complement factor H (CFH) upregulation by recombinant human IFN- γ in human RPE cell homogenates as demonstrated by Western blot. High molecular weight CFH fragment (155 kDa) is seen in the Western blot (a). CFH protein expression is significantly upregulated by stimulation with IFN- γ (25ng/ml). Fig 1b shows the relative absolute "Integrated Optical Density" of each CFH band relative to the GAPDH band. All values were corrected for background. Values represent mean and standard deviation of three independent determinations. Asterisk denotes $P < 0.05$; Double Asterisks $P < 0.02$

Fig. 2.

Immunolocalization of CFH in RPE cells of bovine RPE/choroid explants (a–c) and cryostat sections of bovine explants (d–f) after 48 hrs incubation with 0 ng/ml or 25 ng/ml of bovine recombinant IFN- γ . Cytoplasmic immunoreactivity for CFH (green fluorescence) is detected in untreated RPE cells (b,e) and its level increases after treatment with IFN- γ (c,f). Weak staining is also seen in the choroid in both untreated and treated explants. Negative control specimens, in which primary antibody was omitted, show negligible background staining (a,d). Mounting media for cryostat sections (d–f) contains propidium iodide to label cell nuclei (red).

Fig. 3.

Immunolocalization of CFH (b, c, e, f, h, i; green) in human polarized monolayer RPE cultures after 0 ng/ml (e–f), and 25 ng/ml (b–c) of recombinant human interferon (IFN)- γ . Cytoplasmic immunoreactivity for CFH is detected in RPE cells (e–f) and its level increases after treatment with IFN- γ (b,c). Propidium iodide staining provides nuclear counterstain (red). Negative control specimens, in which primary antibody was omitted, show negligible immunoreactivity for CFH (h–i).

Fig. 4.

Immunolocalization of CFH (green) in differentiated human RPE monolayer cultures after 0 ng/ml (lower panel), and 25 ng/ml (upper panel) of recombinant human interferon (IFN)- γ . Left and right panels show confocal horizontal (X-Y), and vertical sections (X-Z), respectively. Immunoreactivity for CFH is weak and punctate in RPE cells with no IFN- γ addition (left lower panel). The CFH immunoreactivity is strongly and diffusely detected in the cytoplasm of RPE cells treated with 25 ng/ml IFN- γ (left upper panel). Vertical section of confocal microscopy (X-Z) reveals immunoreactivity for CFH is located in the apical cytoplasmic compartment of RPE cells (right panel).

Fig. 5.

Secretion of CFH by human RPE cultures measured by Western blot. Accumulation of complement factor H (CFH) in the supernatants of human RPE cultures (serum free medium) treated with 25 ng/ml of IFN- γ was measured after 24, 48 and 72 hours. Western blot for CFH was performed using 40 μ g of total protein in each lane. After treatment with IFN- γ , increased secretion of CFH is found in the 24, 48 and 72 hour supernatants when compared to time-matched non- IFN- γ treated control supernatants.

Fig. 6.

Complement factor H (CFH) induces RPE cell migration in a Boyden chamber assay. Inserts coated with fibronectin (FN) show increased migration in the absence of any factors added to upper or lower compartment ($P < 0.05$). Chemokinesis was evaluated by added equal concentrations of CFH in the upper and lower compartments; no significant increase was seen at 10, 50 or 100 ng/ml CFH. Chemotaxis was evaluated by adding CFH to the lower compartment alone. Dose dependent chemotactic migration was found with a maximal effect at 50 ng/ml. ($p < 0.01$). PDGF-BB (20 ng/ml) is used as a positive control. Values represent mean number of migrated cells/insert and standard deviation from three independent experiments. *; $P < 0.05$; ** $P < 0.01$