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Up-regulation of complement factor B in retinal pigment epithelial cells is accompanied by complement activation in the aged retina

Mei Chen, Elizabeth Muckersie, Marie Robertson, John V. Forrester, Heping Xu*

Department of Ophthalmology, University of Aberdeen, Institute of Medical Sciences, Aberdeen, UK

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ABSTRACT

Complement activation is involved in the pathogenesis of age-related macular degeneration. How complement is activated in the retina is not known. Previously we have shown that complement factor H (CFH) is constitutively expressed by retinal pigment epithelial (RPE) cells and the production of CFH is negatively regulated by inflammatory cytokines and oxidative insults. Here we investigated the production and regulation of complement factor B (CFB) in RPE cells. Immunohistochemistry showed that CFB is expressed at low levels on the apical portion of the RPE cells in normal physiological conditions. With age, CFB expression increases and extends to the basal part of RPE cells. Confocal microscopy and real-time PCR of RPE cultures indicated that the production of CFB by RPE cells is positively regulated by TNF-α, IFN-γ and long-term (30 days) photoreceptor outer segments treatments. Increased CFB expression in RPE cells in vivo is accompanied by the accumulation of complement C3 and C3a deposition at the Bruch's membrane and the basal layer of RPE cells. Our results suggest that RPE cells play important roles in regulating complement activation in the retina. Increased complement activation in the aged retina may be important for retinal homeostasis in the context of accumulating photoreceptor waste products.

1. Introduction

Age-related macular degeneration (AMD) is the largest cause of untreatable blindness in the elderly in developed world (Bressler, 2004; Friedman et al., 2004). In the early stages of AMD, known as age-related maculopathy (ARM) (Bird et al., 1995), the disease is characterized by the deposition of drusen beneath the retinal pigment epithelial (RPE) cells. In the late stages, atrophy of photoreceptors and RPE cells or choroidal neovascularization (CNV) may ensue causing substantial visual loss (Sunness et al., 1999a,b). The pathophysiological mechanisms underlying this common and devastating disease are complex and incompletely understood. It is believed, however, that local inflammation, in particular complement activation, plays an important role (Donoso et al., 2006; Kijlstra et al., 2005).

Drusen, an established risk factor for progression of early AMD to end stage disease, forms in association with local inflammatory processes (Anderson et al., 2002). Immunohistochemical studies performed on AMD eyes implicated the activation of the complement cascade in the process of drusen formation (Anderson et al., 2002; Hageman et al., 2001) but a direct cause and effect link was not identified. Proteomics of drusen studies confirmed that complement components are prominent constituents of drusen in patients with AMD (Crabb et al., 2002; Hollyfield et al., 2003). Recent genetic studies established associations between AMD and single nucleotide polymorphisms in complement factor H (CFH) (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005), C2 and factor B (CFB) (Gold et al., 2006; Spencer et al., 2007), thus linking abnormal complement regulation with the pathogenesis of AMD.

Both CFH and CFB are involved exclusively in the alternative pathway (AP) of complement activation. CFH is a potent fluid state negative regulator and CFB is a positive regulator of the AP. Previously, we have shown that CFH is constitutively expressed by RPE cells and the production of CFH is negatively regulated by cytokine TNF-α, IL-6 and oxidized photoreceptor outer segment (ox-POS) tips (Chen et al., 2007). We proposed that a local complement regulation system exists at the retinal/choroidal interface and dysfunction of this regulatory system may contribute to the pathogenesis of AMD. The aim of this study is therefore to further investigate the retinal complement regulatory system and to understand whether the CFB is produced locally in the eye. Our results show that CFB is produced almost exclusively by RPE cell at the retina/choroidal interface and the production of CFB by RPE cells increases with age. The production of CFB by RPE cells is

* Corresponding author. Department of Ophthalmology, University of Aberdeen, Institute of Medical Science, Foresterhill, Aberdeen AB25 2ZD, UK. Tel.: +44 (0) 1224 555 822; fax: +44 (0) 1224 555 955.
E-mail address: h.xu@abdn.ac.uk (H. Xu).

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regulated positively by inflammatory cytokine TNF-α and IFN-γ, and increased CFB production by RPE cells in the aged retina is accompanied by increased complement activation in the retina.

2. Materials and methods

2.1. Animals

C57BL/6 mice were supplied by the Medical Research Facility of the University of Aberdeen. Twelve to 15 mice with the age of 3 months old (young) and 22–24 months old (old) were used in this study. All mice were housed and bred in a normal experimental room and exposed to a 12-h dark-12-h light cycle. All procedures concerning animals in this study were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and under the regulations of the United Kingdom Animal License Act 1986 (UK).

2.2. Immunostaining

Cryosections of mouse eye were fixed with 2% paraformaldehyde (Agar Scientific Ltd., Cambridge, UK) for 15 min at room temperature. The coverslips with RPE cultures were fixed with 100% ice-cold methanol (Fisher Scientific, Loughborough, UK) and then were permeablized with 0.3% triton X-100 in TBS for 10 min. After blocking with 5% BSA all samples were incubated with goat anti human CFB polyclonal antibody (1:100, Santa Cruz Biotechnology Inc., CA), or biotinylated anti mouse complement C3 (1:100, clone: RMC11H9, Cedarlane Lab., NC), or rat anti mouse C3b/iC3b/C3c (1:100, clone: 3/26, Hycult Biotechnology, Uden, The Netherlands), or biotinylated anti mouse complement fragment C3a (1:100; clone: I87–419, BD Biosciences, Oxford, UK), or anti mouse complement C3a receptor (C3aR, T-2301, Bachem (UK) Ltd., Merseyside, UK) for 1 h, followed by fluorescein isothiocyanate (FITC) conjugated anti goat IgG or FITC conjugated streptavidine, or PE-anti rat IgG, (1:200, all from BD Biosciences), or FITC-anti chicken IgY (1:200, Thermo Scientific, Waltham, MA) for a further hour. Samples were washed and mounted on glass slides with Vectashield Mounting Medium with propidium iodide (PI) (Vector Laboratories Ltd., Peterborough, UK). Immunostained samples were examined with a confocal microscope (LSM510 META; Carl Zeiss Meditec, Gottingen, Germany).

2.3. RPE cell culture

Human retinal pigment epithelial cell line ARPE19 was purchased from the American Type Culture Collection (ATCC, LGC Priomochem, Middlesex, UK) and cultured in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) (PAA Laboratories Ltd., Somerset, UK). For immunostaining of CFB, ARPE19 cells were cultured in 8-well chamber slides (Nunc International, NY). In some experiments, serum free epithelial medium Quantin 286 (PAA Laboratories Ltd.) was used to polarize ARPE19 cells.

2.4. Preparation of photoreceptor rod outer segment (POS)

POS were isolated from bovine eyes by sucrose gradient density centrifugation method (Molday and Molday, 1987) and described previously (Chen et al., 2007). Briefly, batches of five bovine retinas were placed in 5 ml homogenizing solution (20% w/v sucrose, 20 mM Tris acetate, pH 7.2, 2 mM $MgCl_2$, 10 mM glucose and 5 mM taurine). The suspension was shaken gently and then filtered through a 100 μm BD Falcon cell strainer (BD Biosciences, Oxford, UK) to remove tissue fragments. The samples were layered on 25–60% w/v continuous sucrose gradients containing 20 mM Tris acetate pH 7.2, 10 mM glucose and 5 mM taurine and centrifuged at 25,000 rpm for 45 min at 4 °C. The pink band containing the POS was collected and washed with storage buffer (10 mM sodium phosphate, pH 7.2, 0.1 M NaCl and 2.5% sucrose). Isolated POS aliquots were stored at −80 °C at a concentration of 10^8 POS/ml.

Oxidized POS (ox-POS) were generated according to a method described previously (Wihlmark et al., 1996). Briefly, normal POS (n-POS) aliquots were placed to 9-cm Petri dishes and were exposed to 302-nm ultraviolet light (Ultraviolet Products, Cambridge, UK) in a laminar air-flow box for 6 h. Samples were then collected and washed with distilled water. The irradiated POS were pelleted by centrifugation at $12{,}000 \times g$ for 20 min and re-suspended in RPE culture medium. Previously we have confirmed that ox-POS preparations have high levels of thiobarbituric acid reactive substances (TBARS) and carbonyl (Chen et al., 2007).

2.5. Treatment of RPE cells with POS and cytokines

2.5.1. RPE phagocytosis

The ARPE19 cells were seeded into 12-well plates (2×10^4/well) in DMEM/F12 medium with 10% FCS. When cells reached confluence, 1×10^7/ml of n-POS or ox-POS were added to the cultures. Twenty-four hours later RPE cells were collected for CFB mRNA analysis. For long-term POS treatment, RPE cultures were incubated with 1×10^7/ml of n-POS or ox-POS for 7 or 30 days. POS tips were replaced twice a week. At the end of the incubation, un-digested POS were removed with PBS washes and RPE cells were collected for total RNA extraction.

2.5.2. Pro-inflammatory cytokine treatment

Previously we have shown that pro-inflammatory cytokines TNF-α (20 ng/ml) and IL-6 (10 ng/ml), but not IFN-γ (100 U/ml) down-regulated the expression of CFH in RPE cells (Chen et al., 2007). We therefore investigated whether CFB production by RPE cells is also affected by these pro-inflammatory cytokines. TNF-α (20 ng/ml), or IL-6 (10 ng/ml) or IFN-γ (100 U/ml) (all from R & D Systems, Abingdon, UK) were added to the confluence RPE cultures. Two, 6, 16 and 24 h later, RPE cells were collected for CFB mRNA analysis. In each experiment all treatments were triplicated. The concentrations of inflammatory cytokines used in the in vitro study were optimized from previous published work (Andoh et al., 1993; Circolo et al., 1990; Timmerman et al., 1995) as well as our own experience in RPE cells (Chen et al., 2007).

2.6. Real-time polymerase chain reaction (RT-PCR)

Total RNA were isolated using RNeasy mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instruction. cDNA was synthesized by a reaction of 5 μg of total RNA with 2 μg of oligo-DT primer (Thermo Electro GmbH, Germany) at 70 °C for 10 min, chilled on ice for 3 min, followed by a further addition of 5 mM of nucleotide mixture (Roche Diagnostics GmbH, Germany), 20 mM DTT and 200 U reverse transcriptase in 5× First-strand buffer (Invitrogen) at 42 °C for 50 min and 95 °C for 10 min.

Real -time PCR was performed in a total of 20-μl mixture solution in 96-well plates using the LightCycler® 480 system (Roche Applied Science, Mannheim, Germany). Each 20 μl of reaction mixture contains 1 μl of Taqman® Gene Expression Assay for CFB (Hs00156060_m1, Applied Biosystems, CA), 1 μl of endogenous control housekeeping gene huGAPDH Assays (Applied Biosystems), 10 μl of LightCycler® 480 Probe Master (Roche Diagnostics GmbH, Mannheim, Germany) and diluted cDNA. The standard cycling started with an initial single cycle at 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s and 60 °C for 30 s. PCR products were quantified by the LightCylcer® 480 software. Expression levels were normalized to the GAPDH mRNA level.