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Complement Component C3 is a Critical Point in Aged Choroidal Vessels in the Complement Factor H knock Out Model of Retinal Degeneration.

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ABSTRACT

Key words: Choroid, Complement component C3, Immunostaining, Western blot

Deposition of extra cellular material is a feature of retinal ageing resulting in reduced outer retinal perfusion that can be associated with disease. This has been documented for Bruch's membrane (BM) and is elevated in mice that are complement factor H knock outs (*Cfh*$^{-/-}$) and proposed as a model for macular degeneration. Here we ask if aged deposition occurs to the outer retinal blood supply before transmission across BM in 12 month old C57Bl and *Cfh*$^{-/-}$ mice and if differences exist at this level between these mice. We show arterial deposition of age related proteins complement component C3 with immunostaining and Western blot. Results showed deposition of C3 in all internal lamina of choroid blood vessels and three fragments were revealed from western blot which decrease significantly in *Cfh*$^{-/-}$ mice choroid.

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1. INTRODUCTION:

The choroid in the eye constitutes a major point of interaction between systemic circulation and the neural environment (Stolp et al., 2013). Many diseases of ageing are characterized in part, by the buildup of extracellular deposits that contribute to the pathogenesis, as well as the progression of the disease (Anderson et al., 2004) and altration in the function of the choroid have been associated with diseases of the outer retina, including age related macular degeneration (AMD), a disease in which both vasculature and immune system-related mechanisms are implicated (Ambati et al., 2013, Miller et al., 2013).

The central complement component C3 is the point of convergence of the three complement pathways (Fearon, 1984) and, thus, plays a critical role in biological processes mediated by complement activation. Its activation results in the cleavage of C3 by C3 convertase to smaller proinflammatory molecules, C3a and C3b. C3a aids in the recruitment and activation of innate immune effector cells and has antimicrobial and antifungal activity (Nordahl et al., 2004). C3b is an opsonin and will, through a feedback loop, amplify and trigger activation of the complement terminal pathway (Farries et sl., 1990).

The production of the proinflammatory anaphylotoxins, opsonisation and Membrane attack complex (MAC) mediated cellular injury all ultimately lead to cytokine-mediated recruitment and activation of immune cells to the site of complement activation (Murphy and Walport, 2007). The stability of C3 convertase is controlled by positive and negative regulatory proteins. Negative regulatory proteins protect the normal host cells from complement activation. Factor H, the major inhibitor of the alternative pathway in plasma, binds to C3b on the host cells and competes with factor B to displace Bb part from the convertase hence inhibits complement activation (Murphy and Walport, 2007). Factor H accelerates the decay of this convertase and acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b into iC3b and C3dg (Hoh Kam et al., 2010).

The consequences of age-related deposition of C3 in the neural retina are well documented (Coffey et al., 2007, Hoh Kam et al., 2010&2013), but less is known about these consequences in the choroidal blood vessels. To understand the effect of normal aging on deposition of and C3 in retinal choroid, we compared between aged *Cfh*$^{-/-}$ and C57 mouse using immunohistochemistry and western blot.

2. Materials and methods

2.1. Animals:

All animals were used with University College ethics committee approval and under a UK Home Office Project License (PPL 70/8379). All procedures were conformed to United Kingdom

Animal License Act. 12 months C57 BL/6 (N=10) and CFH deficient mice (*Cfh*$^{-/-}$) on a C57 BL/6 background (N=10) were used. They were maintained under standard laboratory conditions and sacrificed by cervical dislocation. A series of experiments were undertaken. In each, one eye from each mouse was used for immunostaining and the other for Western blotting. Analysis was undertaken for C3.

2.2. Immunohistochemistry

Eyes were enucleated and fixed in 4% paraformaldehyde in phosphate buffer saline (PBS), pH 7.4 for 1h. Anterior tissues were removed and the eye cups were cryoprotected in 30% sucrose in PBS and embedded in optimum cutting temperature (OCT) compound (Agar Scientific Ltd). Retinal sections were cut at 10µm and thawed-mounted onto charged slide. Sections were then incubated for 1h with 5 % Normal Donkey serum (NDS) in 0.3% Triton X-100 in PBS, pH 7.4, followed by overnight incubation with primary antibody; goat polyclonal antibody for C3 (1:500, Cappel, UK). The primary antibody was diluted in 1% NDS in 0.3% Triton X-100 in PBS. After primary antibody incubation, sections were then treated with 0.3% H_2O_2 in PBS for 30 mins to quench endogenous peroxidase activity. After several washed, the sections were incubated with biotin-SP conjugated secondary antibodies against goat (1:1000, Jackson ImmunoResearch Laboratories, UK) which was prepared in 2% NDS in 0.3% Triton X-100 in PBS for 1h at room temperature. After the secondary antibody incubation, sections were washed several times and then incubated in a ready to use horseradish peroxidase streptavidin solution (Vector Laboratories, Peterborough, UK) for 30 mins. Chromogenic visualization was achieved with 3, 3-diaminobenzidine (DAB) as peroxidase substrate by incubating for 1min (DakoCytomation, USA). Sections were then washed, cover slipped with glycerol and sealed with nail varnish. Sections were viewed and 24- bit colour images were captured using an Epi-fluorescence bright-field microscope (Olympus BX50F4, Japan).

2.3. Western blot

Eyes were dissected on ice and RPE-choroidal tissues separated and frozen in liquid nitrogen. Samples were homogenized in 2% sodium dodecyl sulfate with protease inhibitor cocktail (Roche Diagnostics, West Sussex, UK) and centrifuged. Supernatants were transferred to new microcentrifuge tubes and protein concentrations were measured with an absorbance of 595nm. Bovine serum albumin was used as a standard protein concentration. Samples (10µg/well) were loaded using 4-20% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes. The nitrocellulose membranes was pre-treated with 5% non-fat dried milk in 1M PBS (pH 7.4) for several hours and incubated overnight at 4^{o}C with Goat C3 (1:1000;Chapel) followed by washes in 0.05% Tween-20 in 1M PBST. Membranes were incubated with the respective peroxidase conjugated secondary antibody; rabbit anti goat HRP (1:10,000; Thermo Fisher Scientific, UK) for 1h. Protein Immunoreactivities were visualized exposing X-ray film to blots incubated with ECL reagent (SuperSignal West Dura, Thermo Scientific). Total protein profile was determined by staining blots with 2% Ponceau S solution. Protein bands were photographed and scanned. Absolute band intensity was measured using Adobe Photoshop CS4 extended (Adobe Systems Software Ireland, Ltd, Dublin, Ireland).

2.4. Data Analysis

The percentage of the area of deposition/expression in blood vessels were measured at magnification of X1000 in 2 separate regions from central retina of each section were analysed for C3 protein using the lasso tool in Adobe Photoshop CS4 Extended. Five central sections of each mouse were used to measure the percentage of blood vessel walls that were coated with deposits. Statistical analysis was undertaken using Mann-Whitney U test to compare the two groups. Data were analyzed using GraphPad Prism, version 5.0 (GraphPad, San Diego, CA).

3. RESULT

The pattern of C3 deposition was found in the internal laminae of all kinds of choroidal blood vessels, arteries, veins and capillaries but were more obvious and heavily immunolabelled in arteries than did any other types of vessels and specially in large ones. There were some differences between both strains as well. In C57 mice C3 deposition was present on70% of the luminal wall of choroidal vessels. Significantly less C3 was found in *Cfh-/-* mice, but it was still present on 50% of the luminal wall where its deposition appeared more nodular and clumped than C57 mice (Fig. 1 D)

In C57, there was marked increase in the deposition and it was as continuous band with symmetrical shape and distribution (Fig. 1 A). However, it was irregular in *Cfh-/-* mice choroid (Fig. 1 B). It was

surprisingly that in *Cfh*-/- mice, the complement C3 protein was detectable in some vessels showing high level of deposition with segmental shape While, others showed no deposition at all.

The western blot analysis showed that significantly increase in general (185 kDa) and active form C3b (75 kDa) of C3 in C57 mice (Fig. 2 A&B). However, the inactive form C3dg (40 kDa) revealed nearly the same amount between both strains (Fig. 2 C)

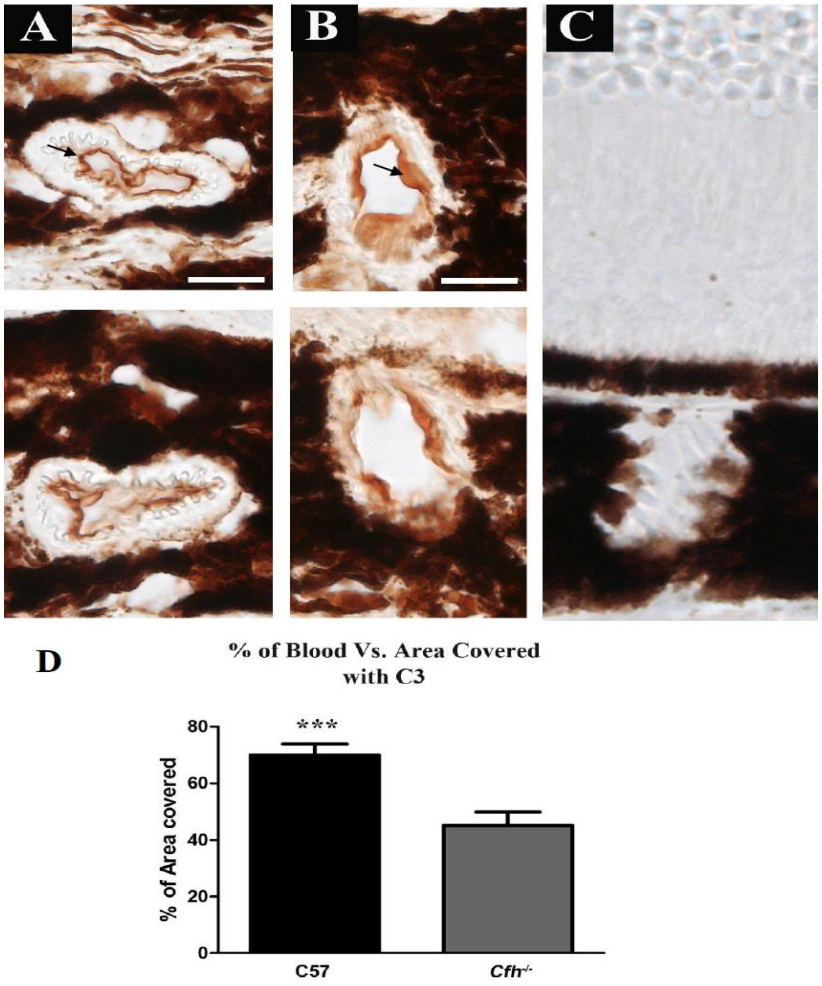

Figure 1: DAB immunostaining of C3 in mice choroid. **A)** C57 choroid showed large artery with stained internal lamina (arrow), **B)** *Cfh*$^{-/-}$ choroid showed large artery with uneven clumped stained internal lamina (arrow), **C)** negative control, **D)** Percentage of blood vessels area covered with C3 showed strongly significant increase in *Cfh*$^{-/-}$ mice. Scale bar =50µm. Statistical significance, *** P>0.00001.

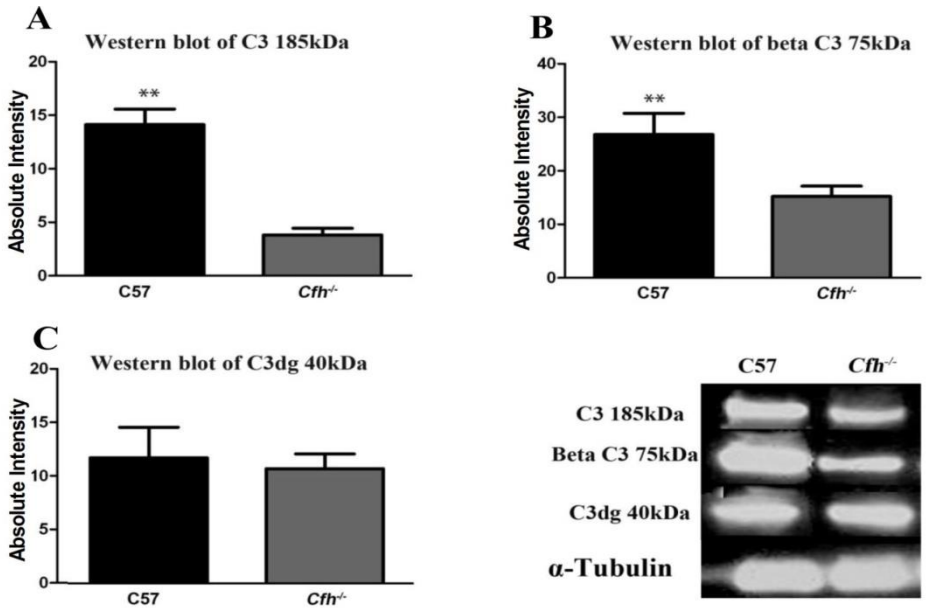

Figure 2: Western blot analysis of Complement component C3 in mice choroid. **A)** General C3 185 kDa with significant reduction in *Cfh*$^{-/-}$ mice**. B)** C3b (beta C3) 75 kDa with significant reduction in *Cfh*$^{-/-}$ mice, **C)** C3dg 40 kDa with nearly the same level in both strains. Statistical significance ** P>0.01.

4. DISCUSSION

CFH is the negative regulator of C3 activation through the alternative complement pathway. Complete deficiency of CFH in humans (Rougier et al., 1998, Levy et al., 1986) pigs (Hogasen et al., 1995) and mice ((Pickering, 2002) results in uncontrolled C3 activation through this pathway and secondary severe plasma C3 deficiency (Pickering, 2002).

Our results revealed that Immunostaining for C3 in aged C57 mice showed a marked increase in C3 deposition with continuous band and symmetrical shape distribution. While, *Cfh*$^{-/-}$ mice of the same age showed lesser deposition with lumpy-shape appearance. Wyss-Coray et al., 2002 showed that complement activation products may be protective against Aβ -induced toxicity and may reduce the accumulation or promote the clearance of Aβ. It is possible that C3 plays a role in suppressing Aβ aggregation.

In brain, CFH deficiency was associated with C3 deposition within the neuroretina that may be derived from systemic sources, due to increased leakage of the posterior blood–retinal barrier, or possibly from local production (Mullins et al., 2000). Unexpectedly, the absence of CFH$^{-/-}$ resulted in a reduction in BM thickness. This may be explained by the dual role of complement in possessing both beneficial and pathologic functions (Walport 2001). Thus, uncontrolled C3 activation may result in neuronal damage on the one hand while stimulating the subretinal debris clearance mechanisms on the other (Walport 2001).

Our results revealed that C3 protein in mice choroid has a selective deposition pattern in some vessels while others showed no deposition at all. A similar result in mice choroid of both strains was reported by Lundh von Leithner et al., (2009). Mason et al., (2002) found that there is a relationship between VEGF and C3 deposition. VEGF is secreted, and its actions are largely confined to endothelial cells. It may promote the up-regulation of the complement regulatory protein decay-accelerating factor (DAF; CD55), which is able to reduce C3 vessel binding (a possible explanation for the selective C3 detection). This is considered to be a cytoprotective effect of VEGF (Hoh Kam et al., 2013). DAF binds to C3, thereby accelerating the decay of the two C3 convertases, C3Bb (alternative pathway) and C4b2a (classical and mannose-binding lectin pathways) (Lublin et al., 1989; Meri et al., 1990) and this leads to decrease C3 deposition.

CFH is a negative regulator of the complement system. Once be absent there is uncontrolled activation of complement component C3, resulting in secondary severe fluid-phase C3 consumption (Pickering, 2002). This result in *Cfh*$^{-/-}$ mice having low levels of C3 in their plasma which is reflected in our western blot results where there was a decrease in the C3 protein in *Cfh*$^{-/-}$ mice.

CFH, membrane cofactor protein (MCP) and complement receptor 1 (CR1) are decay accelerating factor (DAF) proteins whose function is to prevent activated C3 (C3b) from binding to factor B, and hence negatively regulate the complement system. In the absence of CFH, MCP and CR1 are the only DAF proteins protecting cells from lysis (Kindt et al., 2006). MCP and CR1 are membrane bound proteins on photoreceptors. As there are significantly fewer photoreceptors in aged *Cfh*$^{-/-}$ compared with wild type (Hoh Kam et al., 2013), cell loss will reduce hence with their loss there is less available MCP and CR1 necessary to convert activated C3b into inactivated C3dg, which explains our western blot results that show reduced C3dg in *Cfh*$^{-/-}$.

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