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The alternative pathway is required, but not alone sufficient, for retinal pathology in mouse laser-induced choroidal neovascularization

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ABSTRACT

Human genetic studies have demonstrated that polymorphisms in different complement proteins can increase the risk for developing AMD. There are three pathways of complement activation, classical (CP), alternative (AP), and lectin (LP), which all activate a final common pathway. Proteins encoded by the AMD risk genes participate in the AP (CFB), CP/LP (C2), or in the AP and final common pathway (C3). Here we tested which pathway is essential in mouse laser-induced CNV. CNV was analyzed using single complement pathway knockouts (i.e., eliminating one complement pathway at a time), followed by a double knockout in which only the AP is present, and the CP and LP are disabled, using molecular, histological and electrophysiological outcomes. First, single-gene knockouts were analyzed and compared to wild type mice; $C1q^{-/-}$ (no CP), $MBL^{-/-}$ (no LP), and $CFB^{-/-}$ (no AP). Six days after the laser-induced lesion, mice without a functional AP had reduced CNV progression ($P < 0.001$) and preserved ERG amplitudes, whereas those without a functional CP or LP were indistinguishable from the wild type controls ($P > 0.3$). Second, AP-only mice ($C1q^{-/-}$ $MBL^{-/-}$) were as protected from developing CNV as the $CFB^{-/-}$ mice. The degree of pathology in each strain correlated with protein levels of the angiogenic and anti-angiogenic protein VEGF and PEDF, respectively, as well as levels of terminal pathway activation product C5a, and C9. The analysis of complement activation pathways in mouse laser-induced CNV allows for the following conclusions. Comparing the single pathway knockouts with those having only a functional AP showed: (1) that AP activation is necessary, but not alone sufficient for injury; and (2) that initial complement activation proceeds via both the LP and CP. Thus, these data indicate an important role for the AP in the generation of complement-dependent injury in the RPE and choroid via amplification of CP- and LP-initiated complement activation. Improving our understanding of the local regulation of this pathway in the eye is essential for developing improved treatment approaches for AMD.

1. Introduction

Age-related macular degeneration (AMD) is a slowly progressive multifactorial disease involving genetic abnormalities and environmental insults. It is the leading cause of blindness for Americans over the age of sixty (Brown et al., 2005). Dry AMD is characterized by drusen, retinal pigment epithelium (RPE) damage and photoreceptor cell loss (Anderson et al., 2002; Chong et al., 2005; Hageman et al., 2001; Johnson et al., 2001). In some patients, the dry form can transition to wet AMD. Wet AMD is characterized by breakdown of RPE/Bruch's membrane (Hageman et al., 2001), increased release of the pro-angiogenic factor vascular endothelial growth factor (VEGF, e.g., van Wijngaarden and Qureshi, 2008) and development of choroidal neovascularization (CNV, Hageman et al., 2001). While mechanistic studies have shown that inflammation (Hageman et al., 2001) and oxidative stress (Snodderly, 1995) are fundamental components of both forms of AMD, genetic studies have demonstrated that polymorphisms in different complement proteins each independently increase the risk for developing AMD (e.g., Edwards et al., 2008; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005; Lee et al., 2010; McKay et al., 2009). There are three pathways of complement activation,

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classical (CP), alternative (AP), and lectin (LP), which all activate a final common pathway (Sarma and Ward, 2010). The proteins encoded by the AMD risk genes participate in the AP (complement factor B; CFB), CP/LP (complement C2), or in the AP and final common pathway (complement C3). Overall, it has been hypothesized that inadequate control of complement-driven inflammation may be a major factor in disease pathogenesis in AMD. While no effective treatment is available for dry AMD, therapies blocking VEGF ameliorate wet AMD (e.g., van Wijngaarden and Qureshi, 2008).

The complement cascade is activated when ligands for one of three pathways engage their respective pattern recognition molecules (Sarma and Ward, 2010). Classical pathway activation is usually antibody-dependent and is initiated when C1q binds to an immune complex, although other C1q ligands have been identified. The lectin pathway is activated when mannose binding protein (MBL) or ficolins bind to conserved carbohydrate structures, and structures on IgM have also been shown to activate the lectin pathway. The alternative pathway is activated by spontaneous hydrolysis of C3 to C3(H_2O) that binds factor B (fB), following which cleavage of fB to Bb and Ba by factor D (fD) leads to formation of the alternative pathway C3 convertase [C3(H_2O)Bb]. The alternative pathway also provides an amplification loop for the classical and lectin pathways when fB bound to C3b again undergoes cleavage by fD to create the C3bBb amplification C3 convertase which is stabilized by properdin. Thus, all pathways converge at C3 activation with the subsequent cleavage of C5. During this process, the anaphylatoxins C3a and C5a are generated, and C5 cleavage initiates the terminal complement pathway that culminates in the formation of the membrane attack complex (MAC). The MAC can be directly cytolytic and alternatively can stimulate the production of proinflammatory molecules when inserted in cell membranes at sublytic concentrations. Important for our study; since the 3 pathways require unique entry molecules, pathway-specific mutants can be generated ($C1q^{-/-}$: no CP, $MBL^{-/-}$: no LP, $CFB^{-/-}$: no AP and $C1q^{-/-}$ $MBL^{-/-}$: AP-only mice).

The most studied animal model for wet AMD is the CNV model in rodents. In this model, laser-induced injury is generated by argon laser photocoagulation, rupturing Bruch's membrane (e.g., Nozaki et al., 2006). This injury triggers CNV, which can be visualized by imaging techniques (e.g., Campa et al., 2008). Although mouse CNV has been investigated by a number of investigators, it is still not clear how the complement cascade is initiated; and hence which pathways are required for pathology. The strongest argument in support of the involvement of the AP comes from both animal and human data (e.g., Bora et al., 2006; Rohrer et al., 2009; Edwards et al., 2005; Gold et al., 2006; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). Conflicting evidence has been published on the involvement of the CP. CP involvement is suggested by the human data, as C2 polymorphisms correlate with AMD (Gold et al., 2006), and autoantibodies are present in patients (Gu et al., 2003; Patel et al., 2005). In the mouse model, blocking C1qα by siRNA does not appear to interfere with CNV development (Bora et al., 2006); however, negative results using *in vivo* siRNA may be difficult to interpret, and should be repeated using $C1q\alpha^{-/-}$ mice. B- and T-cell infiltrations in mouse CNV appear to be minimal or absent (Tsutsumi-Miyahara et al., 2004), and removing T-cells using antibodies does not interfere with CNV development (Tsutsumi-Miyahara et al., 2004), suggesting that autoantibody formation and cellular immunity do not contribute. No data are available on LP involvement in mouse CNV. Finally, and most importantly, as no information is published on the mechanism underlying activation of the C3 convertase in CNV, it is unclear how the AP is initiated. Here, we use a combination of knockout strategies, to analyze the role of complement activation pathways in CNV development.

2. Materials and methods

2.1. Animals

$CFB^{-/-}$ (Matsumoto et al., 1997), $MBL\text{-}A/C^{-/-}$ (referred to as $MBL^{-/-}$) (Takahashi and Ezekowitz, 2005), $C1q\alpha^{-/-}$ (referred to as $C1q^{-/-}$) (Stuart et al., 2005), and $C1q\alpha^{-/-}$ $MBL\text{-}A/C^{-/-}$ (referred to as $C1q^{-/-}$ $MBL^{-/-}$) mice on a C57BL/6 background were generated from homozygous breeding pairs; C57BL/6 mice also generated from breeding pairs (Harlan Laboratories, Indianapolis, IN) were used as controls. All animals are on a C57BL/6 background and have been backcrossed >7 generations. Relevant serum complement protein levels are not changed in the different knockout strains used here, eliminating the possibility that the complement deficiency states of these animals might complicate data interpretation (Banda et al., 2007). Mice were housed in the Medical University of South Carolina animal care facility under a 12:12 h light:dark cycle with access to food and water *ad libitum*. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee.

CNV lesions were generated as described previously (Rohrer et al., 2009). In short, 3-month-old mice were anesthetized (xylazine and ketamine, 20 and 80 mg/kg, respectively) and pupils dilated (2.5% phenylephrine HCl and 1% atropine sulfate). Argon laser photocoagulation (532 nm; 100 μm spot size; 0.1 s duration; 100 mW) was used to generate four laser spots in each eye surrounding the optic nerve, while using a handheld coverslip as a contact lens. Bubble formation at the laser spot indicated the rupture of Bruch's membrane.

2.2. Assessment of CNV lesions

Relative CNV size was determined in flat-mount preparations of RPE–choroid stained with isolectin B (which binds to terminal β-D-galactose residues on endothelial cells and selectively labels the murine vasculature; (Nozaki et al., 2006; Rohrer et al., 2009)). In brief, eyes were collected and immersion-fixed in 4% paraformaldehyde (PFA) for 2 h at 4 °C after which the anterior chamber, lens and retina were removed. The eyecups were incubated in blocking solution (3% bovine serum albumin, 10% normal goat serum, and 0.4% Triton-X in Tris-buffered saline) for 1 h. Isolectin B (1:100 of 1 mg/mL solution; Sigma–Aldrich, St. Louis, MO) was applied to eyecups overnight at 4 °C in blocking solution. Following extensive washing, eyecups were flattened using four relaxing cuts, coverslipped using Fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL), and examined by confocal microscopy (Leica TCS SP2 AOBS, Leica Bannockburn, IL). Fluorescence measurements, taken from 2 μm sections using confocal microscopy (40× oil lens), were used for size determination. A Z-stack of images through the entire depth of the CNV lesion was obtained, using the same laser intensity setting for all experiments. For each slice the overall fluorescence was determined to obtain pixel intensity against depth, from which the area under the curve (indirect volume measurement) was calculated (Rohrer et al., 2009). Data are expressed as mean ± SEM per eye.

2.3. Electroretinography

ERG recordings and data analyses were performed as previously detailed (Gresh et al., 2003; Richards et al., 2006) using the EPIC-2000 system (LKC Technologies, Inc., Gaithersburg, MD). Stimuli to determine overall retinal responsiveness consisted of 10 μs single-flashes at a fixed intensity (2.48 cd s/m^2) under scotopic conditions. Measurements were performed prior to performing the CNV lesion (baseline ERG) and after the CNV lesion period. Peak *a*-wave ampli-