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# Localization of complement 1 inhibitor (C1INH/*SERPING1*) in human eyes with age-related macular degeneration

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ABSTRACT

Age-related macular degeneration (AMD) is a common degenerative disease resulting in injury to the retina, retinal pigment epithelium and choriocapillaris. Recent data from histopathology, animal models and genetic studies have implicated altered regulation of the complement system as a major factor in the incidence and progression of this disease. A variant in the gene *SERPING1*, which encodes C1INH, an inhibitor of the classical and lectin pathways of complement activation, was recently shown to be associated with AMD. In this study we sought to determine the localization of C1INH in human donor eyes. Immunofluorescence studies using a monoclonal antibody directed against C1INH revealed localization to photoreceptor cells, inner nuclear layer neurons, choriocapillaris, and choroidal extracellular matrix. Drusen did not exhibit labeling. Genotype at rs2511989 did not appear to affect C1INH abundance or localization, nor was it associated with significant molecular weight differences when evaluated by Western blot. In a small number of eyes ($n = 7$ AMD and $n = 7$ control) AMD affection status was correlated with increased abundance of choroidal C1INH. These results indicate that C1INH protein is present in the retina and choroid, where it may regulate complement activation.



## 1. Introduction

Age-related macular degeneration (AMD) is a major cause of blindness affecting millions of individuals in the Western world (Friedman et al., 2004; VanNewkirk et al., 2000). Although prevalence rates vary among different populations and different reports, values as high as 64% of individuals over the age of 80 have been reported (de Jong, 2006). Loss of central vision in AMD can result from either atrophic changes of the retinal pigment epithelium (RPE) or aberrant growth of the vasculature of the choriocapillaris through Bruch's membrane and into the RPE and/or neurosensory retina.

Recently, considerable evidence has emerged indicating a major role of the complement system in AMD pathogenesis. Complement component C5 and the complement inhibitor vitronectin were found to be major components of drusen in human eyes (Hageman et al., 1999; Mullins and Hageman, 1997), as were the terminal complement complex C5b-9 (Johnson et al., 2000; Mullins et al., 2000), clusterin (Johnson et al., 2001) and other byproducts of complement activation (Johnson et al., 2001). The finding that eyes from patients with glomerulonephritis develop early onset drusen (Duvall-Young et al., 1989) and the elucidation of a requirement for complement activation in an animal model of choroidal neovascularization (Bora et al., 2005; Rohrer et al., 2009) provided further evidence for the role of complement activation in AMD pathogenesis.

The supposition that the complement complex participates in the development of AMD was strongly bolstered by the finding that a variant in the complement factor H gene (encoding a Tyr402His variant) is a major risk factor for the development of AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). This finding has been subsequently replicated by a number of groups, and variations in other genes with products involved in the complement system have also been found to affect AMD risk, including C2 (Gold et al., 2006), complement factor B (Gold et al., 2006), and complement factor 3 (Yates et al., 2007).

Ennis et al. recently described a single nucleotide polymorphism (rs2511989) in *SERPING1* to be associated with AMD in two independent cohorts (Ennis et al., 2008), although a second report did not replicate this association (Park et al., 2009). The *SERPING1* gene

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encodes C1INH, a glycoprotein that inhibits complement activation by interfering with the proteolytic activity of C1r/C1s in the classical pathway and mannose binding protein-associated serine proteases in the lectin pathway (Wagenaar-Bos and Hack, 2006). *SERPING1* mRNA is present in human retina and RPE/choroid (Ennis et al., 2008). In the current report we evaluated the expression of C1INH protein in unaffected eyes, eyes from patients with AMD, and patients with the high or low risk *SERPING1* genotypes. We found consistent labeling of photoreceptor cells and variable labeling of the choriocapillaris. Eyes from donors homozygous for either phenotype were compared, and no obvious differences in localization were noted. In addition, AMD and control eyes were compared and AMD eyes showed more C1INH labeling in the choroid than controls. These results are discussed in the context of the complement system in AMD.

## 2. Materials and methods

### 2.1. Donor eyes

Human donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA) following informed consent from the donors' families. Eyes were processed immediately on receipt. Macular and extramacular tissues were punched using disposable trephines, and punches were either fixed (4% paraformaldehyde in phosphate buffered saline, for 2 h) or divided into retinal and RPE/choroidal layers which were flash frozen separately in liquid nitrogen. For biochemical studies all samples were preserved within 8 h of death, which is within a time frame during which protein composition of ocular tissues is well preserved (Ethen et al., 2006). In some cases, ophthalmic records were available, and retinal diagnoses were recorded.

### 2.2. Genotyping

Either postmortem blood samples or small fragments of ciliary body were used for DNA extraction. For tissue, the DNeasy Blood and Tissue Kit (Qiagen; Valencia, CA) was utilized, according to the manufacturer's instructions. Donors were genotyped for the intronic SNP (rs2511989) in *SERPING1* using the Taqman assay, as described previously for the U.S. cohort (Ennis et al., 2008).

### 2.3. Histology and immunohistochemistry

Tissues were cryopreserved in sucrose solution and embedded in Optimal Cutting Temperature Compound (Ted Pella, Redding, CA) using the methods of Barthel and Raymond (Barthel and Raymond, 1990). Immunohistochemical and lectin histochemical labeling was performed as described previously (Mullins et al., 2005, 2006). A monoclonal antibody directed against C1INH (Abcam, monoclonal antibody raised against full length C1INH protein) was used at a concentration of 2 μg/mL and detected with Alexa-488-conjugated goat anti-mouse antibody (Invitrogen; Carlsbad, CA). In order to confirm the specificity of this antibody, for some experiments antibody dilutions were preincubated with a 10 fold excess of purified C1INH protein (R&D Systems, Minneapolis, MN), as described previously for intercellular adhesion molecule-1 (ICAM1) (Mullins et al., 2006).

Dual labeling was also performed with anti-C1INH and biotinylated *Ulex europaeus* agglutinin-I (UEA-I; Vector Laboratories, Burlingame CA), visualized with avidin-Texas red (Vector Laboratories) as described previously (Mullins et al., 2005). Antibodies directed against the bipolar cell marker PKC-alpha (1 μg/mL, Santa Cruz; SC-208) were also used in conjunction with C1INH antibodies, and were detected with Alexa-546-conjugated goat anti-rabbit antibodies (Invitrogen). For some experiments, adjacent tissue sections were labeled with either C1INH antibody or with monoclonal antibodies directed against the neoepitope in complement C9 that is exposed during formation of the terminal complement complex (15 μg/mL; clone aE11, DAKO, Carpinteria, CA). Sections were counterstained with 100 μg/mL 4′,6-diamidino-2-phenylindole (DAPI).

For studies on the effect of AMD on C1INH localization, superotemporal-to-macular wedges of 7 AMD eyes and 7 control eyes were labeled with anti-C1INH (2 μg/mL). The retina and choroid were evaluated and patterns were recorded in a masked fashion. The 7 affected eyes (mean age 78.3 years) had either atrophic AMD ($n = 6$), characterized by RPE mottling and atrophy and/or macular drusen, or choroidal neovascularization (one case). The unaffected eyes had a mean age of 80.4 years.

### 2.4. Western blot analysis

In order to evaluate C1INH protein in retinal and RPE/choroidal tissues, Western blots were performed as described previously (Mullins et al., 2006). Briefly, punches of extramacular retina and RPE/choroid were homogenized with a Kontes pestle (Kimble Chase; Vineland, NJ) in ice cold protease inhibitors (Complete Mini Tablets; Roche, Indianapolis, IN) and 10–20 μg of total protein were separated on either 10% or 4–15% gradient gels, transferred to polyvinylidene difluoride (PVDF) membrane (BioRad; Hercules, CA), and blocked with filtered 5% nonfat dry milk in PBS with 0.1% Tween-20. Blots were then incubated with anti-C1INH antibody (130 ng/mL), washed, probed with peroxidase conjugated anti-mouse antibody (Amersham), washed, and bands were visualized with the ECL plus kit (GE Life Sciences).

## 3. Results

### 3.1. Immunohistochemistry

In order to evaluate the specificity of commercially available C1INH antibodies, purified human C1INH protein was preincubated with diluted antibody solutions. A monoclonal antibody (Abcam, Ab54898) showed an almost complete loss of binding on tissue sections following preadsorption with C1INH (Fig. 1A and B); this antibody was used for the remainder of experiments described in this report. This antibody also recognized purified C1INH protein on Western blot (data not shown). A second polyclonal antiserum (Santa Cruz Biologicals, SC46297) did not show decreased labeling on tissue sections following preadsorption and was not used for additional studies.

Tissue sections of human retina labeled with antibodies directed against human C1INH revealed reactivity with cone photoreceptor cells, including the inner and outer segments, cell bodies, axons and pedicles (Fig. 1A). Rod inner segments showed some reactivity, although this was less notable than cone inner segments. In addition, inner nuclear layer neurons corresponding to bipolar cells appeared positive when labeled with anti-C1INH. Dual labeling with anti-PKC antibody showed colocalization of this bipolar cell antigen with C1INH (Fig. 1C). Variable labeling was also noted in the ganglion cell layer.

Choroidal labeling revealed that blood vessel lumens were typically immunoreactive, presumably due to circulating C1INH adherent to the vessel walls. The walls of choroidal capillaries were also labeled in some donors (Fig. 2). Dual labeling with the endothelial cell binding lectin UEA-I (Mullins et al., 2005) (Fig. 2A and B) revealed most of the vascular labeling to be within or around the choriocapillaris. The choroidal extracellular matrix including choriocapillary pillars in some donors showed reactivity.