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Crystallin distribution in Bruch's membrane–choroid complex from AMD and age-matched donor eyes

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Abstract

Crystallins were consistently found in a recent proteomic analysis of drusen from age-related macular degeneration (AMD) donor eyes. Here we compare the distribution of several crystallins in drusen, Bruch's membrane and choroid from AMD and non-AMD age-matched control eyes. Immunohistochemistry and Western blots of tissue samples were performed using antibodies to αA- and αB-crystallins. Bruch's membrane, drusen and the subjacent choroidal connective tissue from AMD tissues showed greater immunoreactivity for αA- and αB-crystallins than were observed in normal age-matched control tissues. Western blots also demonstrated more intense αA- and αB-crystallin signals from AMD tissues than were present in age-matched controls. These data indicate that αA- and αB-crystallins accumulate in Bruch's membrane and choroidal connective tissues to a greater degree in AMD than in normal aging. These findings suggest that the accumulation of these small heat shock proteins at this critical interface below the RPE reflects a disease-related stress response manifested during the progression of AMD.

Keywords: crystallins; drusen; Bruch's membrane; choroid; age-related macular degeneration

1. Introduction

Age-related macular degeneration (AMD) is a major cause of blindness in individuals over 55 years of age in developed countries. Between 6 and 10 million Americans are affected with AMD, and it is estimated that over 200 000 new cases are diagnosed each year (Sarks and Sarks, 1994). The cause(s) of this disease are not yet defined, however, elderly individuals that accumulate drusen in the macula are considered at high risk for progressing to AMD. Drusen are debris-like deposits that accumulate below the retinal pigment epithelium (RPE) along Bruch's membrane. Of common usage in ophthalmology clinics are the designations 'hard drusen' and 'soft drusen' (Eagle, 1984; Spraul and Grossniklaus, 1997; van der Schaft et al., 1992; Yanoff and Fine, 1992). Hard drusen refers to small spherical deposits with distinct boundaries that are common within the macular region and generally are not considered a risk factor for progressing to AMD. Soft drusen are typically larger, have shapes with indistinct borders and are considered contributors to the pathology of AMD (Abdelsalam et al., 1999; Bressler et al., 1994; Klein et al., 1997; Midena et al., 1997). However, the causal relationship between drusen and AMD is not understood.

To better understand the role of drusen in AMD we pursued proteomic analysis to define the composition of this material isolated from AMD and non-AMD donor eyes (Crabb et al., 2002). Over 120 drusen proteins were identified, with TIMP-3 (tissue metalloproteinase inhibitor-3), clusterin, vitronectin, serum albumin and crystallins being most frequently observed. Interestingly, crystallins were detected in all drusen preparations from AMD donor eyes, but inconsistently observed in non-AMD samples. The goal of this study was to establish the tissue distrubution and relative content of αA- and αB-crystallins in Bruch's membrane–choroid complex from AMD and normal age-matched donor eye tissues.

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2. Materials and methods

2.1. Donor tissues

AMD and age-matched control donor eyes were obtained from the Cleveland Eye Bank or the National Disease Research Interchange (NDRI, Philadelphia, PA). AMD donor tissues were obtained from NDRI and from the Foundation Fighting Blindness Eye Donor program. Information obtained from the consenting family member was used to initially identify the AMD tissues. Globes were enucleated within 1.5–7 hr postmortem, frozen and stored at −80°C until sample preparation. Eight AMD donor eyes (66–87 years old) and five age-matched non-AMD normal donors eyes (76–88 years old) were used in this study. To verify that the putative AMD eyes were indeed from AMD donors, care was taken at the time each eye was processed to examine the fundus. After removal of the anterior segment, the macula was viewed using an operating microscope with a magnification of 15 diameters. The presence of a fibrovascular scar, focal pigmentary loss exceeding one disc diameter consistent with geographic atrophy or dense drusen in the macula were criteria used to confirm that the tissue was indeed from an AMD donor with advanced disease. Based on the appearance of each of the AMD tissues used in this study, they can be classified as category 3 and 4 based on the recent grading system for AMD donor eyes (Olson and Feng, 2004). Each of the putative control donor eyes was identically evaluated for the presence of maculopathy, but none were observed.

2.2. Tissue preparation

Tissue samples for microscopic and protein analysis were prepared as described previously (Crabb et al , 2002). Briefly, after thawing, the anterior segment was removed and the macula examined as described above. Following removal of the vitreous and retina the macula was again examined under microscopic view for evidence of drusen or RPE changes. The RPE was gently brushed from the surface of Bruch's membrane and this cell-free surface was again examined for the presence of drusen. Some scattered drusen were present in the normal donor tissues. The Bruch's membrane–choroid complex was then isolated from the sclera. A central 10×10-mm square of Bruch's membrane-choroid was isolated for Western blot analysis and a 2×10-mm tissue strip centered on the fovea was isolated and frozen in OCT for immunohistochemistry.

2.3. Antibodies and reagents

Rabbit anti-αA and αB crystallin polyclonal antibodies were purchased from Stressgen (Victoria, Canada). Vectastain Elite ABC kit and DAB substrate kit were purchased from Vector Laboratories (Burlingame, CA).

2.4. Immunocytochemistry

Cryosections of Bruch's membrane/choroid isolates (7 μm thick) were placed on microscope slides and fixed with 4% paraformaldehyde followed by treatment with 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity. Sections were then blocked using a solution of 5% bovine serum albumin and 0.3% Triton X-100 in 1×PBS for 2 hr, followed by incubation in primary antibody overnight at 4°C. We initially tested a range of concentrations of each of the antibodies from 1:100 to 1:10 000 dilution on both AMD and normal control tissues. In tissue sections the optimal antibody dilution was found to be 1:2500 for αA- and αB-crystallin antibodies. This concentration produced clearly evident immunoreactivity in both normal and AMD tissues that was well below saturating concentrations. Concentrations of primary antibody from 1:100 and 1:500 dilutions produced fully saturating signals, but from 1:1000 to 1:10 000 dilutions produced a progressive decrease in immunoreactivity. After rinsing, sections were incubated with biotin conjugated secondary antibody following the manufacturers recommendations. Sections were rinsed in PBS, then incubated with avidin conjugated HRP in PBS for 30 min (Vectastain Elite ABC Kit), followed by color development using the DAB substrate. As a negative control, adjacent serial sections were incubated with pre-immune rabbit serum at a protein concentration equal to that used for the primary antibody. All experimental steps, except for elimination of the primary antibody, were identical between the experimental and control treatments. For positive controls, human lens tissue sections were probed with each of the antibodies.

Tissues were viewed with a Zeiss Axiophot photomicroscope with 40X plan neofluar objective. Images were digitized using a Hamamatsu CCD camera imported through Adobe Photoshop software on a PowerMac G3 computer. Care was taken to ensure that all images were recorded sequentially using alignment and illumination intensity settings. The relative density of antibody staining was established using the threshold feature in Photoshop. The original color images were converted to grayscale with a pixel intensity (*Z*-value) range from 0 (black) to 256 (white). The *Z*-value range containing the immunoreactivity signal for each treatment group was established using the threshold feature in the software. For each image, the range of threshold values was established with the lowest numerical value representing the *Z*-value at which pixels within the immunostained areas are first detectable, and the lowest threshold values represent the *Z*-value at which the pixels in the immunostained areas are fully saturated. Thus, the lower *Z*-values indicate higher intensities of immunoreactivity. A mean pixil *Z*-value for immunoreactivity was established for each field. Mean *Z*-values were measured in three different fields from each Bruch's membrane-choroid preparation, and these values were used for comparisons of the intensity of immunoreactivity between the different