Expression of α-crystallin in the retina of human sympathetic ophthalmia

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Abstract. Sympathetic ophthalmia (SO) is a bilateral, granulomatous, intraocular inflammation that occurs following a penetrating injury to one eye, and has the potential to cause blindness of both eyes. The aim of this study was to examine the expression of α-crystallin and to detect apoptotic cells in the retina of human eyes with SO. Five globes, including three with SO and two age-matched normal appearing retinae, were examined. Formalin-fixed, paraffin-embedded tissue sections were submitted to hematoxylin and eosin staining and immunohistochemistry with anti-αA and αB-crystallin antibodies. Apoptotic cells were detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method, and double-staining immunohistochemistry was conducted together with the TUNEL reaction. In normal-appearing retina, αA-crystallin immunoreactivity was predominantly detected in the cytoplasm of photoreceptors, where αB-crystallin was less marked. In SO globes, granulomatous inflammation was noted in the choroid, whereas the retina and choriocapillaris were preserved. Immunoreactivity for αA-crystallin was detected in the retina, as well as in the cytoplasm and inner/outer photoreceptor segments. By contrast, αB-crystallin was weakly noted in the SO retina. Double-staining immunohistochemistry revealed no TUNEL-positive photoreceptors in the retina displaying high immunoreactivity for αA-crystallin, but photoreceptor apoptosis was noted where expression of αA-crystallin was relatively low. The present study demonstrated that αA-crystallin was up-regulated in the cytoplasm of photoreceptors in the SO retina. This may play a protective role in the suppression of photoreceptor apoptosis associated with intraocular inflammation.

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Introduction

Crystallins were primarily identified as major structural proteins of the eye lens. The two α-crystallins, αA and αB, are the principal members of the small heat shock protein (Hsp) family (1). Although the crucial function of α-crystallins is to act as molecular chaperones, their expression and chaperone activity vary under varying pathologic conditions (2-4). Mao *et al* demonstrated the anti-apoptotic function of α-crystallins by blocking pro-apoptotic mitochondrial pathways, thereby inhibiting the activation of downstream apoptotic events (3). We recently demonstrated that αA-crystallin was up-regulated in the diabetic retina (5), which is possibly correlated with protection of retinal cells from apoptotic signals during progression of diabetic retinopathy. Moreover, a variety of experimental inflammatory models have revealed that up-regulation of αA- or αB-crystallin protected specific cells from apoptotic signals associated with inflammation (6-8).

Sympathetic ophthalmia (SO) is a bilateral, granulomatous, intraocular inflammation that occurs following a penetrating injury to one eye, and has the potential to cause blindness of both eyes (9). Although the pathogenesis of SO is not completely understood, it is believed to be caused by an altered tolerance to uveal antigen, which involves a cell-mediated immunity (9). In human eyes with SO, leukocytes initially infiltrate the uvea that is typically absent in the choriocapillaris and retina (9,10). We recently demonstrated that immune-mediated mitochondrial oxidative stress led to photoreceptor apoptosis and subsequent vision loss in SO globes (11).

Experimental autoimmune uveoretinitis (EAU) is a highly reproducible animal model for human endogenous uveitis, including SO. The rodent model of EAU has been used to study T-cell mediated inflammatory processes in the retina (12). Induction of Hsp70 leads to amelioration of EAU through suppressed antigen-specific T-cell proliferation (13). Rao *et al* demonstrated that photoreceptors selectively up-regulated αA-crystallin to protect themselves against immune-mediated mitochondrial oxidative stress in a murine model of EAU (6). These results suggest that Hsp may play an essential role in the suppression of human endogenous uveitis.

This study was conducted to examine the expression of α-crystallins in the retina by immunohistochemistry, and to detect apoptotic cells in human SO globes.

Materials and methods

The Institutional Review Board of the University of Southern California (CA, USA) approved our use of human specimens obtained from the file of Doheny Eye Institute, Pathology Laboratory (CA, USA). All procedures conformed to the Declaration of Helsinki for research involving human subjects. Three formalin-fixed, paraffin-embedded human globes with a diagnosis of SO were retrieved from the archives of A. Ray Irvine Jr, Pathology Laboratory of the Doheny Eye Institute, Los Angeles, CA, USA. All patients had the traumatized eye, commonly referred to as the exciting eye, while the fellow eye was referred to as the sympathizing eye. All globes examined in this study were sympathizing eyes. The age of the patients varied between 38 and 58 years, with a mean age of 46.7. All eyes were removed since the patients had no light perception and the eyes were painful. We also analyzed two histologically normal appearing retinae obtained from age-matched patients with uveal melanoma. Prepared cut sections (5-μm) were submitted to hematoxylin and eosin staining as well as immunohistochemistry.

Immunohistochemistry. The slides were dewaxed, rehydrated and rinsed in phosphate-buffered saline (PBS) twice for 10 min. As a pretreatment, microwave-based antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). These slides were incubated with 3% hydrogen peroxide for 10 min and then with normal goat serum for 30 min. Sections were incubated with anti-αA-crystallin (1:100; Stressgen, Belgium) and αB-crystallin (1:100; Stressgen) rabbit polyclonal antibodies at room temperature for 2 h. Binding of the primary antibody was localized with the FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min. Negative controls consisted of FITC-conjugated mouse IgG incubated without treatment with the primary antibody. Slides were examined using a Zeiss LSM510 (Zeiss, Thornwood, NY, USA) confocal microscope.

Double-staining immunohistochemistry with terminal deoxy-nucleotidyl transferase dUTP nick end labeling (TUNEL) reaction. Serial sections (5-μm) were cut for TUNEL assay to evaluate distribution of the TUNEL-positive reaction in the same part as the immunoreaction with α-crystallins. An *in situ* Cell Death Detection Fluorescein kit (Roche, Indianapolis, IN, USA) was used for the TUNEL assay. The slides were dewaxed, rehydrated and rinsed twice for 10 min in PBS. These slides were incubated with 3% hydrogen peroxide for 10 min, then permeabilized with 20 μg/ml of proteinase K at room temperature for 10 min. Texas red label with enzyme solution was added to each slide and incubated in a humidified chamber at 37°C for 1 h. DNase-pretreated slides were used as positive controls and slides without added enzyme were used as negative controls. Following washing, sections were incubated with anti-αA-crystallin rabbit polyclonal antibodies at room temperature for 2 h. Binding of the primary antibody was localized with the FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min. Apoptotic cells were revealed by confocal microscopy and were defined by the presence of perinuclear chromatin condensation and apoptotic bodies.

Results

In the age-matched, normal-appearing retina, the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) were clearly identified (Fig. 1a). αA-crystallin immunoreactivity was predominantly detected in the cytoplasm of photoreceptors (Fig. 1b-d), not in the photoreceptor inner and outer segments (IS/OS) (Fig. 1b). Expression of αB-crystallin was weakly observed in the retina (Fig. 1e and f). The negative control, in which the respective antibody was omitted, displayed no staining in the retina (data not shown). The two crystallins were immunopositive in the cytoplasm of lens epithelial cells in all sections examined in this study, and served as an internal positive control (data not shown).

Immunoreactivity for α-crystallins in SO globes. All three SO globes displayed typical histologic features of SO with characteristic diffuse, non-necrotizing and granulomatous inflammation involving the uvea (Fig. 2a). The choroid was markedly thickened and infiltrated by lymphocytes associated with epithelioid and giant cells. Melanin-laden macrophages were intermingled in the choroid. No caseous necrosis was noted. The choriocapillaris was spared from inflammatory cell infiltration (Fig. 2b and c). The inflammation did not extend into the retina, where INL and ONL were clearly present (Fig. 2b and c). Immunoreactivity for αA-crystallin was highly expressed in the retina, including ONL and INL (Fig. 2e and f). Moreover, photoreceptor IS/OS displayed high immunoreactivity for αA-crystallin (Fig. 2e). αA-crystallin immunoreactivity was, however, occasionally weak in the retina. By contrast, αB-crystallin was faintly detected in almost the entire retina and Bruch's membrane (Fig. 2h and i).

Correlation with αA-crystallin immunoreactivity and TUNEL-positive cells. Parikh *et al* recently demonstrated that photoreceptor apoptosis occurred on the retina of SO (11). Therefore, double-staining immunoreactivity with TUNEL reaction was conducted to determine whether immunolocalization of αA-crystallin and photoreceptor apoptotic cells are related. No TUNEL-positive cells were detected in the retina (Fig. 3a) where αA-crystallin immunoreactivity was markedly detected (Fig. 3b and c). By contrast, as shown in Fig. 3, TUNEL-positive apoptotic cells were observed in ONL (Fig. 3d, arrows), where αA-crystallin immunoreactivity was relatively weak in the cytoplasm (Fig. 3e and f, arrows).

Discussion

This study demonstrated that αA-crystallin is expressed in the cytoplasm of photoreceptors in the normal-appearing retina without inflammation. In human SO globes, granulomatous inflammation was initially present in the choroid, sparing the choriocapillaris and sensory retina. The current study revealed that αA-crystallin, although not αB-crystallin, was selectively expressed in the retina, as well as in the cytoplasm and in the IS/OS of photoreceptors. These results suggest that αA-crystallin expression is up-regulated, and that the immunoreactivity is to be found in the IS/OS of photoreceptors in SO globes.
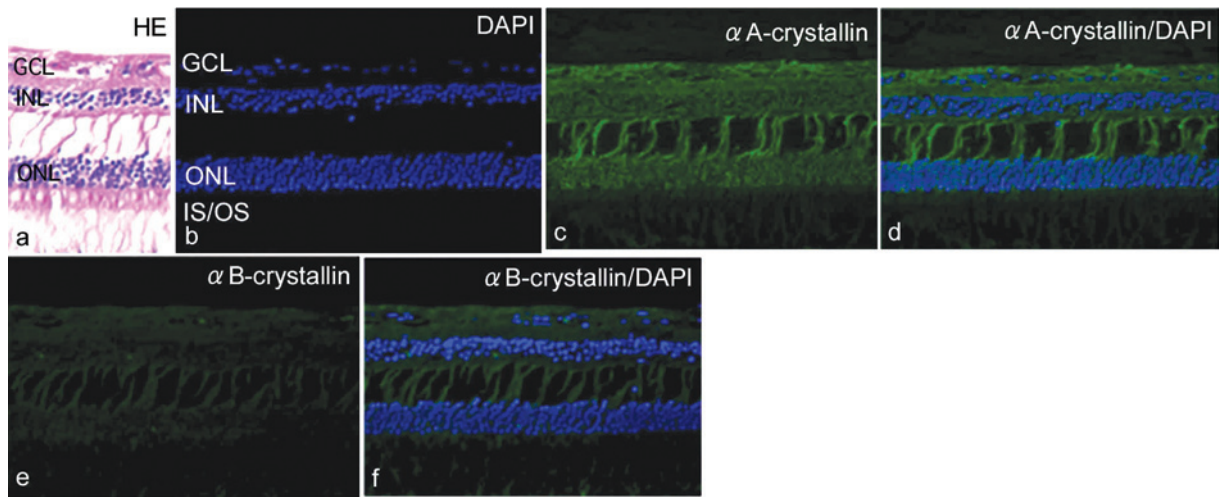
Figure 1. (a) Hematoxylin and eosin staining. (b, d and f; blue) DAPI nuclear staining. (c and d; green) Immunodetection of αA-crystallin and (e and f; green) αB-crystallin in normal appearing retina. (a and b) Three nuclear layers consisting of the ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL) and photoreceptor inner and outer segment (IS/OS) are clearly confirmed. (c and d) αA-crystallin immunoreactivity is predominantly detected in the cytoplasm of photoreceptors and is weakly noted in GCL and INL. (e and f) Immunoreactivity for αB-crystallin is barely detected. DAPI, 4',6-diamidino-2-phenylindole.
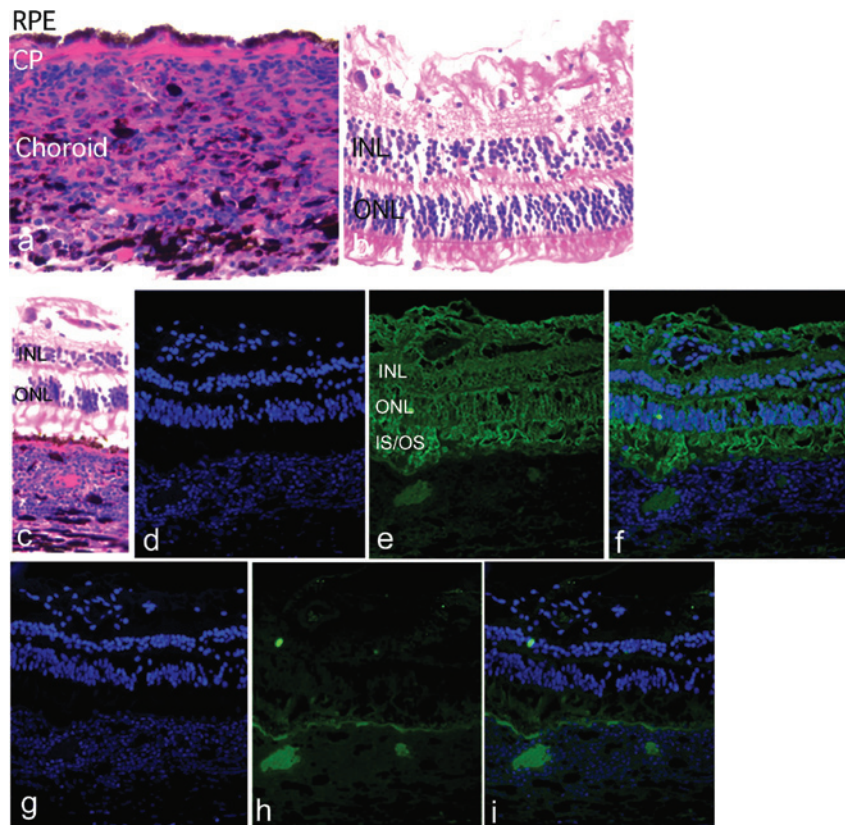

Figure 2. (a-c) Hematoxylin and eosin staining. (d, f, g and i; blue) DAPI nuclear staining. (e and f; green) Expression of αA- and αB- (h and i; green) crystallin in the retina of sympathetic ophthalmia. (a) Inflammatory cells made up of lymphocytes, epithelioid cells and melanin-laden macrophages infiltrate the choroid. (b and c) Retinal pigment epithelium (RPE), choriocapillaris (CP) and retina are preserved, and the inner nuclear layer (INL) and outer nuclear layer (ONL) are clearly confirmed in the retina. (e and f) Immunoreactivity for αA-crystallin is highly expressed in the retina including photoreceptors and INL. (e) Moreover, photoreceptor inner and outer segments (IS/OS) reveal high immunoreactivity for αA-crystallin. (h and i) By contrast, αB-crystallin is faintly detected in the retina, while Bruch's membrane displays weak expression of αB-crystallin. (e, f, h and i) Neither αA- nor αB-crystallin expression is observed in choroidal inflammation. DAPI, 4',6-diamidino-2-phenylindole.

Apoptotic cell death of photoreceptors occurs in the retina of SO without inflammatory cell infiltration and may cause a loss of vision (11). We clearly demonstrated that photoreceptor apoptosis could be observed in the retina, where αA-crystallin immunoreactivity was relatively low. On the other hand, in the retina where αA-crystallin immunoreactivity was markedly detected, no TUNEL-positive signals were observed. Rao *et al* found that αA-crystallin was expressed in the photoreceptors
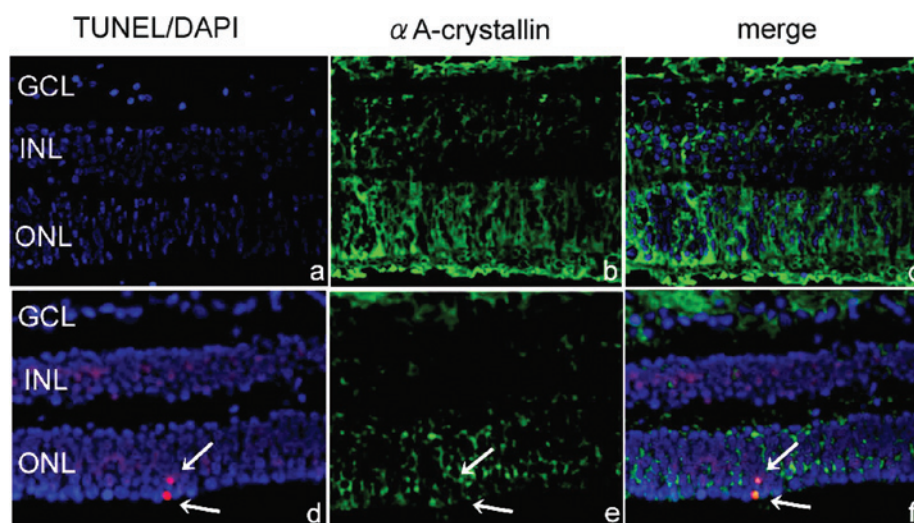
Figure 3. Double staining immunohistochemistry with TUNEL reaction in the retina of sympathetic ophthalmia. (a) TUNEL-positive cells are not detected in this part of the retina including the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL), where (b and c) αA-crystallin immunoreactivity is markedly detected. (b and c) Immunoreactivity for αA-crystallin is also detected in photoreceptor segments. (d, arrows) TUNEL-positive apoptotic cells are observed in ONL, (e and f; arrows) where αA-crystallin immunoreactivity is weakly detected. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

and protected them from apoptotic signals in an EAU model (6). In actuality, αA-crystallin exerts an anti-apoptotic function by blocking pro-apoptotic mitochondrial pathways, thereby inhibiting the activation of downstream apoptotic events (3). Therefore, strong expression of αA-crystallin in photoreceptors may have a protective role against apoptotic signals in SO.

Recently, studies have evaluated the pathogenesis of retinal damage in intraocular inflammation using an EAU model. Increased generation of reactive oxygen and nitric oxide species was observed in the photoreceptor mitochondria, indicating oxidative stress, prior to inflammatory cell infiltration in EAU (14). Such oxidative damage in the mitochondria may be the initial event leading to retinal degeneration in EAU (15). Oxidative stress leads to up-regulation of a host of Hsp, including α-crystallins (16). Rao *et al* demonstrated that photoreceptors selectively up-regulated αA-crystallin against immune-mediated mitochondrial oxidative stress by EAU involving the retina (6). We recently demonstrated that mitochondrial oxidative stress occurs in the photoreceptor inner segment in SO globes of humans (11). These results suggest that the αA-crystallin up-regulation observed in this study may result from photoreceptor oxidative stress in SO.

The potential role of αB-crystallin in the pathology of various inflammatory models has been extensively studied (17). αB-crystallin is believed to have a suppressive effect on immunity and an anti-apoptotic role in glia and endothelial cells in experimental inflammatory and angiogenesis models, respectively (7,18). In fact, recombinant αB-crystallin protein reduced the number of inflammatory foci and apoptotic glial cell death in the model (7). Whiston *et al* identified the critical role of αB-crystallin in protecting the retina during inflammation using a murine model of *Staphylococcus aureus*-induced endophthalmitis (8). These results suggest αB-crystallin may be a therapeutic target for human inflammatory diseases. In contrast to past publications, this study demonstrated that αA-crystallin, although not αB-crystallin, responded to the inflammation in human SO globes; these findings have been supported by EAU animal models (6). Therefore, the overexpression model of αA-crystallin and application of the recombinant protein into the EAU model should prove the therapeutic role of αA-crystallin in intraocular inflammation.

Acknowledgements

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