

# Increased expression of $\alpha$ A-crystallin in human diabetic eye

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**Abstract.** We recently demonstrated that  $\alpha$ A-crystallin, a molecular chaperone, protected photoreceptors from apoptotic signals in intraocular inflammation. Advanced glycation end product (AGE) plays an important role in the progression of diabetic retinopathy. The aim of this study was to examine the expression of  $\alpha$ -crystallins and apoptosis in human diabetic retina, and to analyze  $\alpha$ -crystallin up-regulation in murine eyes after AGE stimulation. Eight eye globes were obtained from postmortem donors. Six out of the eight had a medical history of diabetes mellitus, while two were without diabetes. Formalin-fixed, paraffin-embedded tissue sections were subjected to H&E staining and immunohistochemistry with anti- $\alpha$ A and  $\alpha$ B-crystallins, anti-AGE and receptor for AGE (RAGE) antibodies. Apoptotic cells were detected by the TUNEL assay. Recombinant AGE protein was injected into the vitreous of adult murine eyes, and the posterior eyecups were excised 4 days after the administration. Western blot analyses and quantitative real-time PCR were performed to evaluate the alteration of  $\alpha$ -crystallin expression. Histopathology revealed no remarkable differences between diabetic and non-diabetic retinas. Immunoreactivity for  $\alpha$ A-crystallin was predominantly detected in the diabetic retina, whereas  $\alpha$ B-crystallin expression was relatively low. AGE immunoreactivity was highly detected in diabetic retina and the vitreous, whilst immunoreactivity for RAGE was less marked. TUNEL-positive apoptotic cells were occasionally observed in photoreceptors of the diabetic retina, whereas cytoplasmic immunoreactivity for  $\alpha$ A-crystallin was relatively low.  $\alpha$ A-crystallin expression was up-regulated, and  $\alpha$ B-crystallin was down-regulated in murine posterior eyecups exposed to AGE protein. The mRNA levels of  $\alpha$ A-crystallin were significantly up-regulated, whereas those of  $\alpha$ B-crystallin remained unchanged after AGE stimulation. Thus,  $\alpha$ A-crystallin and AGE were highly expressed in human diabetic retina.  $\alpha$ A-crystallin responded to AGE accumulation,

which may contribute to the protection of photoreceptors against AGE-related retinal tissue injury.

## Introduction

Diabetic retinopathy is the leading cause of blindness in the working-age population in the United States. Epidemiological studies have confirmed that hyperglycemia is the most important factor in the onset and progress of vascular complications, both in Type 1 and 2 diabetes mellitus. It is known that the formation of advanced glycation end products (AGEs) correlates with glycemic control (1). AGE may cause tissue injury both directly, through phenomena such as trapping and cross-linking and indirectly, by binding to a specific receptor for AGE (RAGE) on the surface of various cells (2). The pathological crosslink formation induced by AGEs usually affects the stable and long-lived proteins leading to increased stiffness of the protein matrix and increased resistance to removal by proteolytic means. The increase in AGE colocalized with RAGE in the diabetic retina plays an important role in the progression of retinopathy (3). Subsequent investigations have shown that the concentration of AGE was high in the vitreous and aqueous humor in patients with diabetic retinopathy (4,5). Howes *et al* have shown that immunolabeling for AGE or RAGE was low in photoreceptors in normal retinas, while the photoreceptors showed intense AGE and RAGE immunolabeling in early age-related macular degeneration (6).

The AGE hypothesis proposes that accelerated chemical modification of proteins by glucose during hyperglycemia contributes to the pathogenesis of diabetic complications including retinopathy (7). To adapt to environmental changes and survive to different types of injuries, eukaryotic cells have evolved networks of different cellular responses that detect and control different forms of stress. One of the most important families of this adaptive response is that of the heat shock proteins (HSP) (8). It was recently demonstrated that expression of HSP60 and 70 was increased in peripheral lymphocytes of patients suffering from nephropathy secondary to type 2 diabetes (9). HSP70 protein expression was significantly expressed in diabetic rat kidney tissues and in mononuclear cells from diabetic patients (10).

Chaperones (stress proteins) are essential proteins that aid in the formation and maintenance of the proper conformation of other proteins and promote cell survival after a large variety of environmental stresses. Crystallins, the major structural

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proteins of the eye lens, are primarily categorized into three distinct families:  $\alpha$ ,  $\beta$  and  $\gamma$ . The two  $\alpha$ -crystallins,  $\alpha A$  and  $\alpha B$ , are the principal members of the small HSP family acting as molecular chaperones (8). Although  $\alpha A$  and  $\alpha B$ -crystallins have related amino acid sequences with similar structural properties, they vary significantly in their tissue specificity with different functions: they may protect different proteins and they may be active under different conditions (11-15). We have previously demonstrated that the expression of  $\alpha A$  and  $\alpha B$ -crystallin in human retinoblastoma varies based on the cellular stress (16). Wang *et al* reported that  $\alpha A$ -crystallin protein expression was up-regulated in diabetic rat retina (17).

It has been demonstrated that levels of some inflammatory cytokines were significantly higher in the vitreous of patients with proliferative diabetic retinopathy (PDR) than in those of non-diabetic patients (18). This suggests that the inflammatory reaction takes place in the retina during the development of diabetic retinopathy. Indeed, Rao *et al* reported that  $\alpha A$ -crystallin was selectively up-regulated in experimental uveitis involving the retina to protect photoreceptors from apoptotic signals associated with oxidative stress (19). These results indicate that the immune response may have a role in the up-regulation of  $\alpha$ -crystallins in the retina. Mammalian Toll-like receptors (TLRs) are cellular pattern-recognizing receptors (PRRs) that recognize the molecular patterns of pathogens. After engaging the pathogenic patterned ligands, the cytosolic portion of the TLRs in monocytes and macrophages, recruits adaptor proteins, which subsequently lead to the expression of proinflammatory cytokines, and to inflammation (20). It is proposed that the possible role of RAGE as a PRR is that it may use signaling mechanisms parallel to TLRs, to solicit inflammatory reactions (20).

The aim of this study was to examine the expression and distribution of  $\alpha$ -crystallins in human diabetic and non-diabetic donor eyes. Immunoreactivity of AGE, RAGE and TLR2/4 as well as of 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine, a marker for oxidative stress (21,22), were also analyzed to evaluate the presence of inflammatory response and oxidative stress. Apoptotic cells were detected in diabetic retina by the TUNEL assay. Moreover, the expression of  $\alpha A$ -crystallin protein and mRNA was evaluated in murine posterior eyecups in which recombinant AGE protein was injected.

## Materials and methods

The institutional review board of the University of Southern California approved our use of human specimens obtained from the file of Doheny Eye Institute, Pathology Laboratory. All procedures conformed to the Declaration of Helsinki for research involving human subjects. Eight eyes were obtained from four donors who died due to cardiac-related disorders through the Doheny Eye and Tissue Transplant Bank, Los Angeles, USA. Six out of eight eyes came from donors having diabetes mellitus, while two eyes came from donors without a history of diabetes. The clinical data examined in this study are present in Table I. All eyeballs were fixed in 4% paraformaldehyde soon after enucleation.

**Immunohistochemistry.** The slides were dewaxed, rehydrated, and rinsed in phosphate-buffered saline (PBS) twice for 10 min.

As a pre-treatment, 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, then with normal goat serum for 30 min. Sections were incubated with the anti-rabbit  $\alpha A$ -crystallin (dilution 1:100; Stressgen, Ann Arbor, MI),  $\alpha B$ -crystallin (dilution 1:100; Stressgen), AGE (1:100; Abcam), RAGE (1:100; Abcam), TLR2 (1:50; Abcam), TLR4 (1:50; Abcam), 8-OHdG (1:50; Santa Cruz Biotechnology) and nitrotyrosine (1:50; Santa Cruz Biotechnology) antibodies at room temperature for 2 h. Binding of the primary antibody was detected with the FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. The negative control consisted of incubation with the FITC-conjugated mouse IgG with omission of the primary antibody. Slides were examined using a Zeiss LSM510 (Zeiss, Thornwood, NY) confocal microscope.

**Double staining immunohistochemistry with TUNEL reaction.** Serial sections of 6  $\mu$ m were used for the TUNEL assay to evaluate the colocalization of the TUNEL-positive reaction with the immunoreaction with  $\alpha$ -crystallins. An *In Situ* Cell Death Detection Fluorescein kit (Roche, Indianapolis, IN) was used for TUNEL assay. The slides were dewaxed, rehydrated, and rinsed in PBS twice for 10 min. The slides were incubated with the 3% hydrogen peroxide for 10 min, then permeabilized with proteinase K 20  $\mu$ g/ml 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-pre-treated slides were used as positive controls and slides without added enzyme served as the negative controls. After washing, sections were incubated with anti- $\alpha A$ -crystallin rabbit polyclonal antibodies at room temperature for 2 h. The localization of the bound primary antibody was identified with a FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min. Apoptotic cells were revealed by confocal microscope and were defined by the presence of perinuclear chromatin condensation and apoptotic bodies.

**Mice.** Eight to twelve-weeks-old mice of the 129S6/SvEvTac wild-type strain (Taconic Farms, Germantown, NY) were used in this study. Mice were anesthetized and 2  $\mu$ l of human recombinant AGE-BSA protein was injected into the murine right vitreous with a 32 gauge needle. On the other hand, BSA was injected into the left vitreous. Eyeballs were enucleated 4 days after the injection. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of Southern California Animal Use Committee.

**Western blot analysis.** Murine tissues from posterior eyecups were lysed, and lysates were centrifuged at 1,200 x g for 20 min. The protein concentration was determined using the Bradford protein assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as the standard protein. Protein of 25  $\mu$ g was separated by electrophoresis on SDS polyacrylamide gel electrophoresis (PAGE; 12.5% Ready Gel, Bio-Rad Laboratories, Hercules, CA) at 110 V. The protein was then electrotransferred onto polyvinylidene difluoride (PVDF) blotting membrane (Millipore, Bedford, MA). The membranes were blocked in

Table I. Clinicopathological profiles of eye donors examined in this study.

No.	Age	Gender	Type	Duration <sup>a</sup>	Immunoreactivity							
					$\alpha$ A-crystallin		$\alpha$ B-crystallin		AGE	RAGE	TLR2	TLR4
					Center	Periphery	Center	Periphery				
1	53	M	IDDM	9	++	+	+	++	++	-	+	-
2	69	F	IDDM	10	++	++	+	++	++	+	-	-
3	39	M	NIDDM	2	+	++	+	++	++	-	-	-
4	70	F	-	0	+	+	-	+	+	-	-	-

M, male; F, female; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-IDDM; AGE, advanced glycation end product; RAGE, receptor for AGE; TLR, Toll-like receptor; +, weakly positive; ++, strongly positive. <sup>a</sup>Duration in years.

5% milk and probed with primary antibodies for 2 h at room temperature. Membranes were washed and incubated with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Images were developed by adding an enhanced chemiluminescence (ECL) detection solution (GE Healthcare, Cleveland, OH).

**Quantitative real-time RT-PCR.** Quantitative expression of mRNA was examined using real-time reverse transcriptase PCR (Applied Biosystems). The following human primer sets were designed using the Primer Express software (Applied Biosystems): L32, 5'-CTA AGG CCA ACC GTG AAA AG-3' and 5'-ACC AGA GGC ATA CAG GGA CA-3';  $\alpha$ A-crystallin, 5'-ACT GGA CCC TGG CTT TAC TG-3' and 5'-TCT GCT CTC CTT CTG TCG TG-3';  $\alpha$ B-crystallin, 5'-GCG GTG AGC TGG GAT AAT AA-3' and 5'-GCT TCA CGT CCA GAT TCA CA-3'. Product formation detection was set in the center of the linear portion of PCR amplification, and the cycle at which each reaction reached the set threshold (CT) was determined. Relative change in mRNA expression was calculated to obtain the  $2^{-\Delta\Delta CT}$  values (23). Five separate sets of RNA were isolated and examined, and each set was assayed in triplicate. Levels were normalized relative to L32 mRNA and reported as fold-change over controls.

**Statistical analyses.** For Western blot analyses, the band intensity after electrophoresis was evaluated using the ImageJ software (version 1.32j) from the National Institutes of Health, MD, and normalized to actin. Statistical evaluations were performed using the Student's t-test. The accepted level of significance for all tests was  $P < 0.05$ .

## Results

**Expression of  $\alpha$ -crystallins in the central and peripheral retinas of diabetic eyes.** Histopathology revealed that there was no neovascularization, lymphoid cell infiltration, or epiretinal membrane formation in diabetic and non-diabetic eye globes. Immunoreactivity for  $\alpha$ A-crystallin was strongly detected in the central retina including photoreceptors (Fig. 1b), whereas  $\alpha$ B-crystallin immunoreactivity was less marked (Fig. 1c). In the central retina of the eyes of non-diabetic donors,

expression of  $\alpha$ A-crystallin was weakly detected in the nerve fiber layer (Fig. 1e), while  $\alpha$ B-crystallin immunoreactivity was hardly detected in the retina (Fig. 1f). In the peripheral retina, immunoreactivity for  $\alpha$ A (Fig. 2b and c) as well as  $\alpha$ B-crystallins (Fig. 2e and f) was detected in the cytoplasm of photoreceptors.  $\alpha$ -crystallins expression was also detected in the nerve fiber layer, and in the vicinity of surface retinal vessels in diabetic eyes. In contrast,  $\alpha$ A-crystallin immunoreactivity was less marked in non-diabetic peripheral retina than in diabetic retinas.  $\alpha$ B-crystallins were expressed in lens epithelial cells in all cases examined (data not shown).

**Correlation with  $\alpha$ A-crystallin immunoreactivity and TUNEL-positive cells.** TUNEL-positive apoptotic cells were occasionally observed in the photoreceptors of human diabetic retina. In order to determine whether there is an association between the immunolocalization of  $\alpha$ A-crystallin and the photoreceptor apoptotic cells, double staining immunoreactivity with a TUNEL reaction was conducted. TUNEL-positive cells were not detected in the majority of diabetic retinal tissues (Fig. 3a), where cytoplasmic immunoreactivity for  $\alpha$ A-crystallin in photoreceptors was strongly detected (Fig. 3b and c). In contrast, TUNEL-positive apoptotic cells were observed in photoreceptors (Fig. 3d; arrow), where cytoplasmic immunoreactivity for  $\alpha$ A-crystallin was relatively low (Fig. 3e and f; arrows).

**Immunoreactivity for AGE, RAGE and TLRs in the diabetic retina.** AGE was strongly detected in the photoreceptors, and inner nuclear layer (INL), as well as in the vitreous of all diabetic eyes (Fig. 4a and b). Furthermore, AGE immunoreactivity was strongly noted in retinal vessels (Fig. 4a and b; arrow) in the diabetic eyes. AGE expression was weakly detected in the INL and the outer nuclear layer (ONL) of the non-diabetic retina (Fig. 4c and d). In contrast, expression of RAGE was hardly detected in the diabetic retina including the photoreceptors (Fig. 4e and f), whereas RAGE immunoreactivity was detected in Bruch's membrane and large choroidal vessels (data not shown). RAGE immunoreactivity was not detected in the non-diabetic retinas (Fig. 4g and h). In one diabetic eye immunoreactivity for TLR2 was weakly detected in the inner limiting membrane of the retina (Fig. 4i and j), whereas the immunoreactivity was not observed in the ONL. Expression

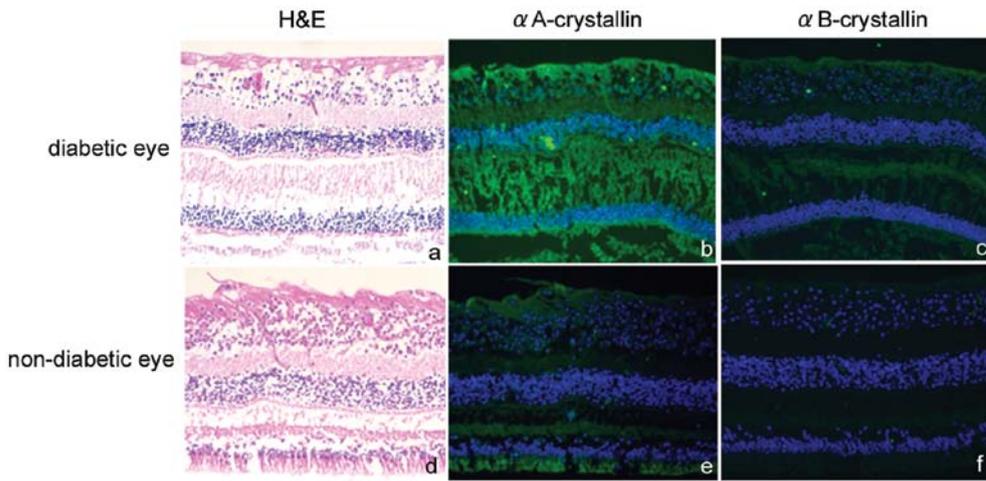


Figure 1. Hematoxylin and eosin (H&E) staining (a and d), and immunodetection of  $\alpha$ A-crystallin (b and e; green), and  $\alpha$ B-crystallin (c and f; green), and DAPI nuclear staining (b, c, e and f; blue) in the central retina of diabetic (a-c) and non-diabetic (d-f) eyes. The number of nuclei in the ganglion cell layer is increased in the central retina (a and d). Retinal neovascularization, or epiretinal membrane formation is not observed in the diabetic eye (a). In the diabetic retina, immunoreactivity for  $\alpha$ A-crystallin is strongly detected in the retina including inner limiting membrane, ganglion cell layer, inner nuclear layer, and photoreceptors (b), whereas  $\alpha$ B-crystallin immunoreactivity is less marked (c). In the central retina of non-diabetic donor eyes, expression of  $\alpha$ A-crystallin is weakly detected in the nerve fiber layer (e), whilst  $\alpha$ B-crystallin immunoreactivity is hardly detected in the retina (f).

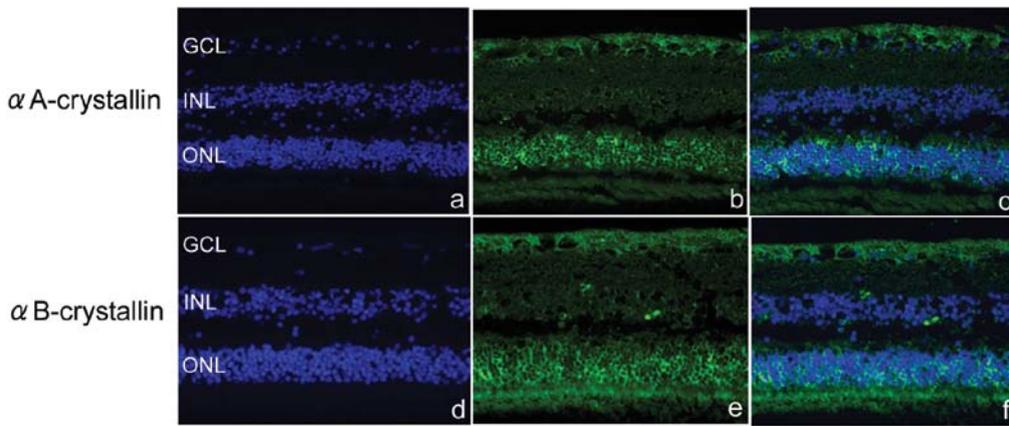


Figure 2. DAPI nuclear staining (a-f; blue), and immunodetection of  $\alpha$ A-crystallin (b and c; green), and  $\alpha$ B-crystallin (e and f; green) in the peripheral retina of human diabetic eyes. In the peripheral retina, the ganglion cell layer shows a single nuclear layer in diabetic eyes (a and d). Immunoreactivity for  $\alpha$ A- (b and c) as well as  $\alpha$ B- (e and f) crystallins is detected in the cytoplasm of photoreceptors and in the nerve fiber layer in diabetic eyes.

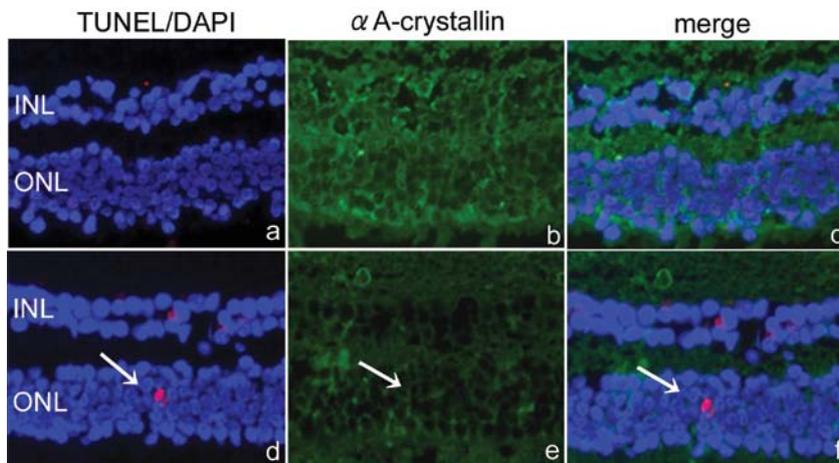


Figure 3. Double staining immunohistochemistry with TUNEL reaction in the retina of diabetic eyes. TUNEL-positive cells are not detected in major parts of the diabetic retina including the inner (INL), and outer nuclear layer (ONL) (a), where cytoplasmic immunoreactivity for  $\alpha$ A-crystallin in photoreceptors is strongly detected (b and c). TUNEL-positive apoptotic cells are occasionally observed in photoreceptors (d, arrow), where cytoplasmic immunoreactivity for  $\alpha$ A-crystallin is weakly detected (e and f; arrows).

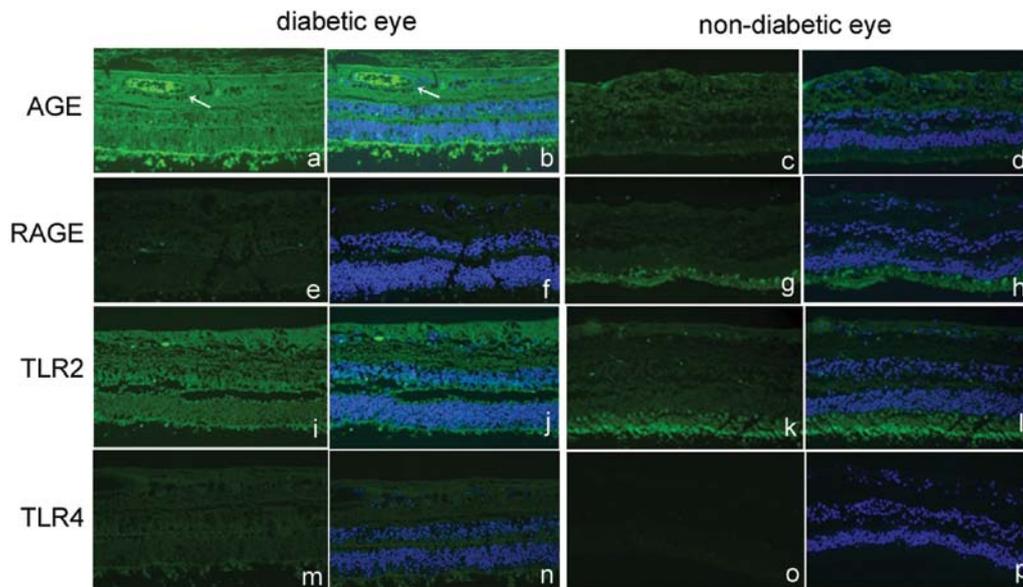


Figure 4. Expression of advanced glycation end product (AGE) (a-d; green), receptor of AGE (RAGE) (e-h; green), Toll-like receptor (TLR) 2 (i-l; green), and TLR4 (m-p; green), and DAPI nuclear staining (b, d, f, h, j, l, n and p; blue) in human diabetic (a, b, e, f, i, j, m and n) and non-diabetic (c, d, g, h, k, l, o and p) eyes. AGE is strongly detected in the photoreceptors, and inner nuclear layer (INL) as well as in the vitreous of all diabetic eyes. In contrast, AGE expression is weakly detected in the INL of non-diabetic retina (c and d). In addition, AGE immunoreactivity is strongly noted in retinal vessels (a and b; arrow) in diabetic eyes. Expression of RAGE is hardly detected in the diabetic retina including retinal vessels, nerve fiber layer, INL or photoreceptors (e and f). In contrast, RAGE immunoreactivity is not detected in the non-diabetic retinas (g and h). In one out of the five diabetic eyes immunoreactivity for TLR2 was weakly detected in the inner limiting membrane of the retina (i and j), whereas the immunoreactivity was not observed in photoreceptors. Expression of TLR2 is not detected in non-diabetic eyes (k and l). TLR4 immunoreactivity is hardly detected in the diabetic (m and n) and non-diabetic (o and p) retinas.

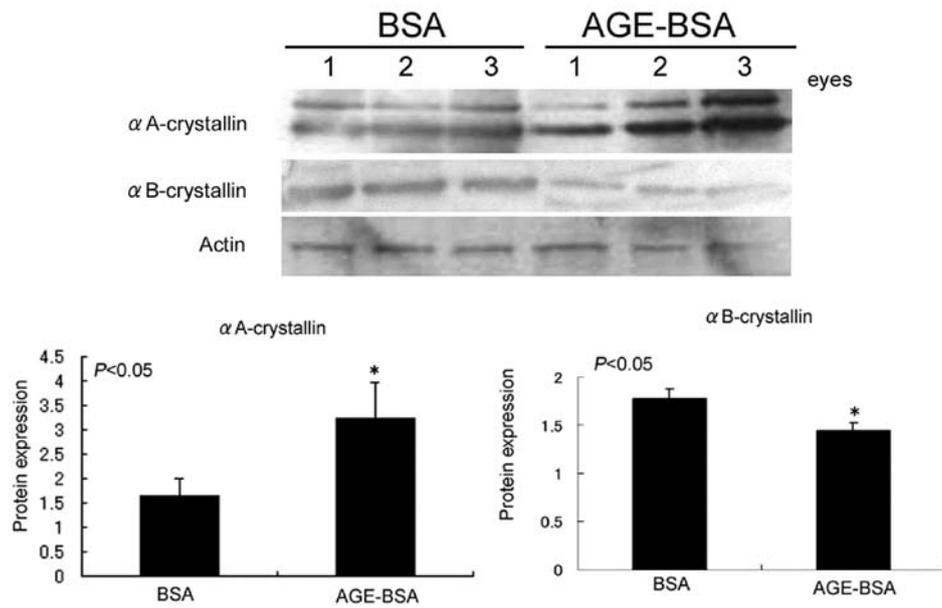


Figure 5. Expression of  $\alpha$ A- and  $\alpha$ B-crystallin protein in murine posterior eyecups using Western blot analysis. Recombinant AGE protein was injected into the vitreous of adult mice, and then total protein was extracted from posterior eyecups to analyze the expression of  $\alpha$ -crystallin proteins 4 days later. Western blot analysis reveals that protein expression of  $\alpha$ A-crystallin is up-regulated after AGE protein injection, whereas that of  $\alpha$ B-crystallin is down-regulated (upper panel). Densitometric analysis shows a significant increase in  $\alpha$ A-crystallin, and a decrease in  $\alpha$ B-crystallin expression (lower panel;  $P < 0.05$  for both).

of TLR2 was not detected in non-diabetic eyes (Fig. 4k and l). Immunoreactivity for TLR4 was hardly detected in the diabetic (Fig. 4m and n) and non-diabetic (Fig. 4o and p) retinas. TLR2 and TLR4 expressions were weakly detected in the corneal epithelium of diabetic eyes. Neither 8-OHdG nor nitrotyrosine immunoreactivity was detected in diabetic and non-diabetic retinas (data not shown).

*$\alpha$ A-crystallin protein and gene expression up-regulation after recombinant AGE protein stimulation.* Recombinant AGE protein was injected into the vitreous of adult mice, and then posterior eyecups were extracted to analyze the protein and mRNA expression of  $\alpha$ -crystallins. Western blot analysis revealed that the expression of  $\alpha$ A-crystallin protein was up-regulated after AGE protein injection, whereas  $\alpha$ B-crystallin

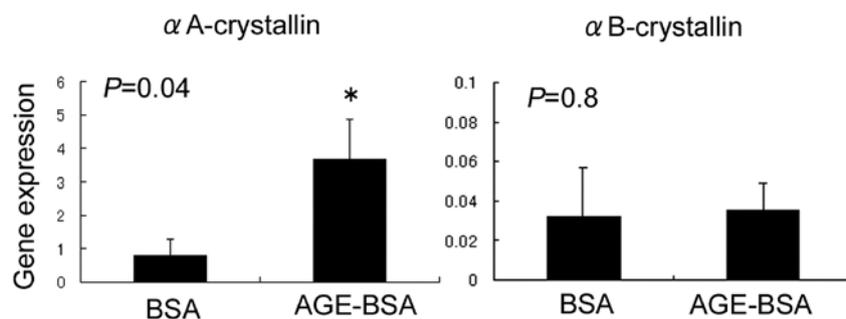


Figure 6. Expression of  $\alpha$ A- and  $\alpha$ B-crystallin mRNA in murine posterior eyecups by quantitative real-time PCR. Recombinant AGE protein was injected into the vitreous of adult mice, and then mRNA was extracted from posterior eyecups to analyze the expression of the  $\alpha$ -crystallins gene. Real-time PCR demonstrates that  $\alpha$ A-crystallin mRNA expression is significantly up-regulated after AGE stimulation (left panel;  $P=0.04$ ). In contrast,  $\alpha$ B-crystallin gene expression remains unchanged after the treatment (right panel;  $P=0.8$ )

was down-regulated (Fig. 5a). Densitometric analysis showed a significant increase in  $\alpha$ A-crystallin expression ( $P<0.05$ ), and a decrease in  $\alpha$ B-crystallin ( $P<0.05$ ). Quantitative real-time PCR demonstrated that  $\alpha$ A-crystallin mRNA expression was significantly up-regulated after AGE treatment (Fig. 6) ( $P=0.04$ ). In contrast,  $\alpha$ B-crystallin gene expression showed no significant change after the treatment (Fig. 6) ( $P=0.8$ )

## Discussion

Rao *et al* recently reported that  $\alpha$ A-crystallin up-regulation protected photoreceptors from apoptotic signals in experimental uveitis (19). In the current study, we demonstrated that immunoreactivity  $\alpha$ A-crystallin was strongly detected in the central retina including INL and ONL. Moreover, expression of  $\alpha$ A-crystallin was clearly observed in the cytoplasm of photoreceptors located in the peripheral retina of human diabetic eyes. Although  $\alpha$ B-crystallin immunoreactivity was also detected in the peripheral retina of diabetic eyes, the immunoreactivity was hardly detected in the central retina. It has been demonstrated that  $\alpha$ A-crystallin protein expression is up-regulated in the diabetic rat retina (17), indicating that immunohistochemical findings obtained in this study are consistent with those in animal model. It has been shown that photoreceptor apoptosis takes place in the retina at the early onset of diabetes in rats (24). In the present study, TUNEL-positive apoptotic cells were noted in photoreceptors of the human diabetic retina. Furthermore, double staining immunohistochemistry revealed that photoreceptor apoptosis was found, where cytoplasmic immunoreactivity for  $\alpha$ A-crystallin was relatively low. These data suggest that  $\alpha$ A-crystallin was up-regulated to protect photoreceptors from apoptotic signals associated with the onset of diabetic retinopathy.

Subsequent investigations have addressed that concentration of AGE was high in the vitreous and aqueous humor in patients with diabetic retinopathy (4,5). In addition, AGE concentration was already increased in the aqueous humor in diabetic patients without retinopathy (4). These results imply that AGE is significantly accumulated in the onset of diabetic microangiopathy. AGE accumulation was significantly noted in the retina including photoreceptors and retinal vessels of diabetic eyes by immunohistochemistry, suggesting that AGE accumulation may cause diabetic microangiopathy. AGE has been found to be a product of oxidation glycation, indicating

that it may be a marker of oxidative stress (1). On the other hand, it has been reported that AGEs are formed by non-oxidative reactions (25). In this study, immunoreactivity for 8-OHdG or nitrotyrosine was not detected in diabetic eyes, suggesting that accumulation of AGE in the diabetic intraocular tissues may be caused by non-oxidative reaction.

We have demonstrated that  $\alpha$ A-crystallin was strongly expressed in the diabetic retina, where AGE was highly accumulated in this study. In order to examine the alteration of the expression of  $\alpha$ -crystallins by AGE protein exposure, we injected recombinant AGE protein into murine vitreous, and collected the posterior eyecups.  $\alpha$ A-crystallin protein expression was significantly up-regulated, and  $\alpha$ B-crystallin expression was down-regulated after administration of AGE protein (Fig. 5). Furthermore,  $\alpha$ A-crystallin mRNA expression was also significantly up-regulated after the treatment; however,  $\alpha$ B-crystallin mRNA remained unchanged. These results strongly suggest that  $\alpha$ A-crystallin primarily responded to accumulation of AGE protein in the diabetic retina. In fact, the chaperone systems allow recognition of the incompletely folded protein and subsequent targeting of the misfolded proteins for degradation by the proteasome (26,27). It is reported that AGE-modified proteins undergo physicochemical changes that alter charge, solubility, and conformation, resulting in an altered protein and in increased resistance to removal by proteolytic means (2,9,28). Therefore, the up-regulation of  $\alpha$ A-crystallin may reflect an increased chaperone activity to recognize the altered proteins modified by AGE in diabetic retinopathy.

Rao *et al* reported that  $\alpha$ A-crystallin was up-regulated in photoreceptors to protect against apoptotic signals in experimental intraocular inflammation (19). It is likely that RAGE is a major amplification factor in the inflammatory response (29). Pachydaki *et al* demonstrated that RAGE expression was detected in the glial cells, and vascular endothelial cells intermingled in the epiretinal membrane of PDR (29). In this study, strong expression of RAGE or TLRs was not observed in the retinal tissues of diabetic eyes, where AGE was highly accumulated. This suggests that interaction of RAGE and TLR may play an important role in the progression and advanced stage of diabetic retinopathy. On the other hand, AGE may cause tissue injury both directly, and indirectly by binding to RAGE on the surface of various cells (2). These results support the hypothesis that the pathogenesis of diabetic retinopathy is

caused by direct tissue injury through accumulated AGE rather than binding to the receptor.

This study demonstrated that AGE was highly accumulated in the diabetic retina, which would contribute to acceleration of microvasculopathy (30). Inhibition of the AGE pathway including AGE formation, AGE-RAGE interaction, and RAGE expression has been considered a therapeutic molecular target (30). In this study, we found that  $\alpha$ A-crystallin was strongly expressed in the diabetic retina of humans, which was possibly induced by AGE accumulation using a murine model. It has been demonstrated that  $\alpha$ A-crystallin has a protective role in photoreceptors involved in retinal inflammation (19). Therefore, up-regulation of  $\alpha$ A-crystallin is suggested to be beneficial for prevention of photoreceptor loss associated with the onset of diabetic retinopathy, although further studies are needed to analyze  $\alpha$ A-crystallin expression in the progression to PDR. Taken together, interaction with  $\alpha$ A-crystallin and AGE may be a novel therapeutic target for patients with diabetic retinopathy.

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