Phosphorylation of αB-Crystallin Involves Interleukin-1β-Mediated Intracellular Retention in Retinal Müller Cells: A New Mechanism Underlying Fibrovascular Membrane Formation

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Purpose. Chronic inflammation plays a pivotal role in the pathology of proliferative diabetic retinopathy (PDR), in which biological alterations of retinal glial cells are one of the key elements. The phosphorylation of αB-crystallin/*CRYAB* modulates its molecular dynamics and chaperone activity, and attenuates αB-crystallin secretion via exosomes. In this study, we investigated the effect of phosphorylated αB-crystallin in retinal Müller cells on diabetic mimicking conditions, including interleukin (IL)-1β stimuli.

Methods. Human retinal Müller cells (MIO-M1) were used to examine gene and protein expressions with real-time quantitative PCR, enzyme linked immunosorbent assay (ELISA), and immunoblot analyses. Cell apoptosis was assessed by Caspase-3/7 assay and TdT-mediated dUTP nick-end labeling staining. Retinal tissues isolated from the Spontaneously Diabetic Torii (SDT) fatty rat, a type 2 diabetic animal model with obesity, and fibrovascular membranes from patients with PDR were examined by double-staining immunofluorescence.

Results. *CRYAB* mRNA was downregulated in MIO-M1 cells with the addition of 10 ng/mL IL-1β; however, intracellular αB-crystallin protein levels were maintained. The αB-crystallin serine 59 (Ser59) residue was phosphorylated with IL-1β application in MIO-M1 cells. Cell apoptosis in MIO-M1 cells was induced by *CRYAB* knockdown. Immunoreactivity for Ser59-phosphorylated αB-crystallin and glial fibrillary acidic protein was colocalized in glial cells of SDT fatty rats and fibrovascular membranes.

Conclusions. The Ser59 phosphorylation of αB-crystallin was modulated by IL-1β in Müller cells under diabetic mimicking inflammatory conditions, suggesting that αB-crystallin contributes to the pathogenesis of PDR through an anti-apoptotic effect.

Keywords: diabetic retinopathy, fibrovascular membrane, αB-crystallin/CRYAB, heat shock protein B5/HSPB5, interleukin-1β, Müller cell, exosomes, apoptosis

Proliferative diabetic retinopathy (PDR), the most progressive stage of diabetic retinopathy, is one of the severest vision-threatening disorders in the world. PDR is pathologically characterized by ocular neovascularization followed by the formation of epiretinal proliferative tissues called fibrovascular membranes, which consist of various types of cells, such as glial cells and endothelial cells, and of extracellular matrix.[1–3] In the pathogenesis of PDR, inflammatory conditions responding to retinal ischemia play crucial roles and proinflammatory cytokines, such as vascular endothelial growth factor (VEGF) and interleukin (IL)-1β are upregulated in ocular tissues to exacerbate ocular complications of PDR.[4,5] Among those proinflammatory cytokines which are involved in PDR, IL-1β was shown to increase in both vitreous humor and the blood of patients with PDR associated with inflammatory cytokines, and play important roles in cell death and angiogenesis by inducing ocular inflammation associated with toll-like receptors and intercellular adhesion molecule-1.[6–8]

Retinal Müller cells are retina-specific macroglia cells which span the entire neural retina, and their metabolic features are essential for homeostasis of the retina.[9] We

previously demonstrated that retinal Müller cells play crucial roles in intraocular vascular hyperpermeability and angiogenesis by macrophages/microglia-mediated IL-1β secretion in PDR.[10] Furthermore, retinal Müller cells contributed to fibrovascular membrane formation in PDR through glia-mesenchymal transition induced by transforming growth factor (TGF)-β 1/2,[11] the mechanisms of which are vital under not only physiological but also pathological conditions. However, it has not been fully documented how retinal Müller cells are involved in PDR pathogenesis, especially fibrovascular membrane formation.

The αB-crystallin, also known as HSPB5, is a member of the small heat shock protein family, which belongs to crystallin family proteins. In the ophthalmology field, αB-crystallin is expressed in various ocular tissues, such as the crystalline lens, retina, and retinal pigment epithelium. The most well-known feature of αB-crystallin is a chaperone effect of VEGF-A, but it also has anti-apoptotic effects under pathological conditions such as neural inflammation, diabetes, and tumors.[12–15] Indeed, IL-1β-mediated αB-crystallin expression is upregulated in glioblastoma cells whereas it is downregulated in chondrocytes.[16,17] Phosphorylation of αB-crystallin plays a pivotal role in its chaperone activity regulation.[18] We showed phosphorylated αB-crystallin localized with neovascular endothelial cells expressing VEGF in fibrovascular membranes obtained from patients with PDR.[19,20] Moreover, recent findings demonstrated that increased intravitreal αB-crystallin concentrations are associated with PDR and proliferative tissue formation in proliferative vitreoretinopathy with retinal detachment.[21,22] Given these studies, we hypothesized that αB-crystallin is involved in fibrovascular membrane formation in PDR. This study investigated the role of αB-crystallin to evade apoptosis and in fibrovascular membrane formation in retinal Müller cells in association with IL-1β.

Materials and Methods

Cell Line and Reagents

The human Müller cell line Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1) was obtained from the UCL Institute of Ophthalmology (London, UK) and cultured in DMEM containing 10% fetal bovine serum (FBS).[23]

Recombinant human IL-1β, IL-6, and tumor necrosis factor (TNF)-α proteins were purchased from PeproTech (Cranbury, NJ, USA). Goat anti-IL-1 receptor (IL-1R) neutralizing antibody and normal goat IgG were purchased from R&D Systems (Minneapolis, MN, USA) and pre-incubated at a dose of 10 µg/mL for 30 minutes with 10 ng/mL IL-1β. A chemical inhibitor of p38 mitogen-activated protein kinase (MAPK; SB203580; Adipogen Life Sciences, San Diego, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) and pre-incubated at a dose of 10 µM for 30 minutes with 10 ng/mL IL-1β.

Two small interfering RNA (siRNA) oligos for the gene knockdown of *CRYAB* (siRNA1, s3543; siRNA2, s3544) and a negative control siRNA oligo (negative siRNA negative control no. 1 siRNA) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and used at a dose of 10 nM. Cells were transfected with siRNA using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific), according to the manufacturer's protocol.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from cells using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). Complementary DNA synthesis was performed using GoScript reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The primer sequences were as follows: human *CRYAB*, 5′-ACT TCC CTG AGT CCC TTC TAC C-3′ (forward) and 5′-GGA GAA GTG CTT CAC ATC CAG G-3′ (reverse); human *ACTB*, 5′-CAC CAT TGG CAA TGA GCG GTT C-3′ (forward) and 5′-AGG TCT TTG CGG ATG TCC ACG T-3′ (reverse). Quantitative PCR was performed using GoTaq qPCR Master mix (Promega) and StepOne plus Systems (Thermo Fisher Scientific). *ACTB* was used as the normalizing control, and gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-Linked Immunosorbent Assay

The protein levels of αB-crystallin in cell lysate and culture medium were determined with the StressXpress Alpha B Crystallin ELISA kit (StressMarq Biosciences, Victoria, Canada) per the manufacturer's instructions. The optical density was measured using a microplate reader (Tecan, Männedorf, Switzerland).

Immunoblot Analyses

Cell extracts were lysed in Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue). After quantifying protein concentrations using BCA reagent (Thermo Fisher Scientific) followed by the addition of 5% 2-mercaptoethanol to the samples, proteins were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to the PVDF membrane by electroblotting. Membranes were blocked in tris-buffered saline (TBS) containing 5% skim milk and probed with the following primary antibodies: mouse anti-αB-crystallin (Abcam, Cambridge, MA, USA), rabbit anti-phospho Ser59-αB-crystallin (Abcam), mouse anti-phospho p38 MAPK (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-p38 MAPK (Abcam), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MBL, Tokyo, Japan) antibodies. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs were used as secondary antibodies for chemiluminescence detection. Signals were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). The bands were analyzed by densitometry using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

Apoptotic Evaluations

Cell apoptosis was assessed by Caspase-3/7 assay and TdT-mediated dUTP nick-end labeling (TUNEL) staining. To measure Caspase-3 and -7 activities, MIO-M1 cells were seeded in a 96-well plate at a density of 1.5×10^4 cells per well and incubated with 100 µL of conditioned media for 9 hours before siRNA transfection. After overnight incubation with siRNA, media were changed into serum-reduced ones (1% FBS) and incubated for 48 hours, followed by 10 ng/mL IL-1β application. Twenty-four hours later, Caspase-Glo 3/7 assay system reagent (Promega) was added to each well and luminescence rates were measured using

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a microplate reader (Tecan) according to the manufacturer's instructions.

For TUNEL staining, cells were seeded on 4-well culture slides at a density of 1.2×10^5 cells per well. Following incubation in the same way as for the Caspase-3/7 assay, cells were fixed in 1% paraformaldehyde and stained for TUNEL (ApopTag fluorescein in situ apoptosis detection kit; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Lonza, Basel, Switzerland). Images were captured under a Biorevo microscope (BZ-9000; Keyence, Osaka, Japan). The number of total nuclei and TUNEL-positive cells were counted using ImageJ software (NIH) in the two views of each well. The rates of TUNEL-positive cells in total nuclei were averaged per well before statistical analysis.

Animals

Sprague-Dawley rats and Spontaneously Diabetic Torii (SDT) fatty rats were obtained from CLEA Japan Inc. (Tokyo, Japan) and 24-week-old SDT fatty rats were used for analysis. SDT fatty rat is an animal model of type 2 diabetes with obesity which shows diabetic complications, such as diabetic cataract and uveitis.[24,25] All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Ethics Review Committee for Animal Experimentation of Hokkaido University (#18-0115).

Immunofluorescence Microscopy

For SDT fatty rats' eyes, eyeballs were fixed in 4% paraformaldehyde. Paraffin sections of tissues were deparaffinized and hydrated through exposure to xylene and graded alcohols followed by water. As a pretreatment, microwave-based antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). For antibody staining, sections were washed with PBS containing 0.05% Triton X-100 and 5% FBS. Sections were probed with the following primary antibodies: mouse anti-αB-crystallin (1:200; Abcam), rabbit anti-phospho Ser59-αB-crystallin (1:100; Abcam), rabbit anti-glial fibrillary acidic protein (GFAP; 1:100; DAKO, Carpinteria, CA, USA), mouse anti-GFAP (1:100; Thermo Fisher Scientific), and rabbit anti-IL-1R (1:100; R&D Systems) antibodies. The secondary antibodies for fluorescent detection were Alexa Fluor 488 (1:200; Abcam) and 546 (1:200; Thermo Fisher Scientific). Nuclei were counterstained with DAPI (1:100; Lonza), and sections were visualized using a Biorevo microscope (Keyence).

Human Surgical Samples

Fibrovascular membrane specimens were obtained from patients with PDR who had undergone therapeutic vitrectomy in our clinic. This study was approved by the institutional review board of Hokkaido University Hospital (#021-0180) and written informed consent was obtained from the patients after an explanation of the purpose and consequences of the study.

Statistical Analysis

All results are expressed as the mean ± SEM. The Dunnett's test and Tukey–Kramer method were used for multiple comparison procedures. Differences were considered significant at $P < 0.05$.

Results

αB-Crystallin/*CRYAB* Expression Changes and Intracellular Retention With IL-1β

Because we estimated that pro-inflammatory cytokines affect αB-crystallin expression in retinal Müller cells, this study used representative cytokines, including IL-1β, IL-6, and TNF-α, which were reported to show increases in the vitreous humor of patients with PDR.[5] Among them, IL-1β (fold change = 0.46 [10 ng/mL]; $P < 0.01$) and high-dose TNF-α (fold change = 0.41 [100 ng/mL]; $P < 0.01$) suppressed *CRYAB* mRNA expression (Fig. 1A, Supplementary Fig. S1A, S1B). As we previously revealed that IL-1β contributed to the pathogenesis of PDR in retinal Müller cells,[10] we focused on IL-1β and showed suppression of *CRYAB* mRNA expression in a time-dependent manner (Fig. 1B). In protein analysis by ELISA, protein levels of αB-crystallin in culture media decreased corresponding to *CRYAB* mRNA in cell lysates, whereas the αB-crystallin protein levels were maintained in cell lysates treated with IL-1β (Figs. 1C, 1D). Moreover, we investigated intracellular αB-crystallin quantities by immunoblot over a longer time and found that αB-crystallin expression was still maintained around 48 hours, whereas the repression tends to be reduced afterward (Figs. 1E, 1F). These data demonstrated that IL-1β suppressed *CRYAB* mRNA synthesis but intracellular protein levels of αB-crystallin were relatively maintained, suggesting the regulatory mechanism underlying intracellular retention of αB-crystallin in retinal Müller cells.

IL-1β-Induced Phosphorylation of αB-Crystallin Ser59 Residue in Retinal Müller Cells

D'Agostino et al. showed exosome secretion suppression via phosphorylation of the Ser59 residue in αB-crystallin.[26] In order to determine the cause of the difference between intra- and extracellular αB-crystallin protein levels, we next investigated the phosphorylation of Ser59 mediated by IL-1β, IL-6, and TNF-α, the pro-inflammatory cytokines which were also used in *CRYAB* mRNA expression analysis. This study demonstrated that only IL-1β promoted Ser59 phosphorylation between the three cytokines (Figs. 2A, 2B). Based on this finding, we demonstrated Ser59 phosphorylation with IL-1β in a dose-dependent manner, which was inhibited by IL-1R neutralizing antibody (Figs. 2C–2F). Given that αB-crystallin Ser59 phosphorylation has been shown to be promoted by p38 MAPK,[27] we assessed signal pathways of Ser59 phosphorylation. The phosphorylation of p38 MAPK was detected along with αB-crystallin Ser59 phosphorylation by IL-1β stimulation (see Fig. 2C) and inhibited by SB203580, a chemical inhibitor of p38 MAPK (Figs. 2G, 2H). These results showed that IL-1β-driven αB-crystallin Ser59 phosphorylation was inducted through IL-1R activation and phosphorylation of p38 MAPK.

Induction of Apoptosis by *CRYAB* Knockdown in Retinal Müller Cells

One of the pivotal roles of αB-crystallin is an anti-apoptotic function under neuronal inflammatory conditions.[28] To

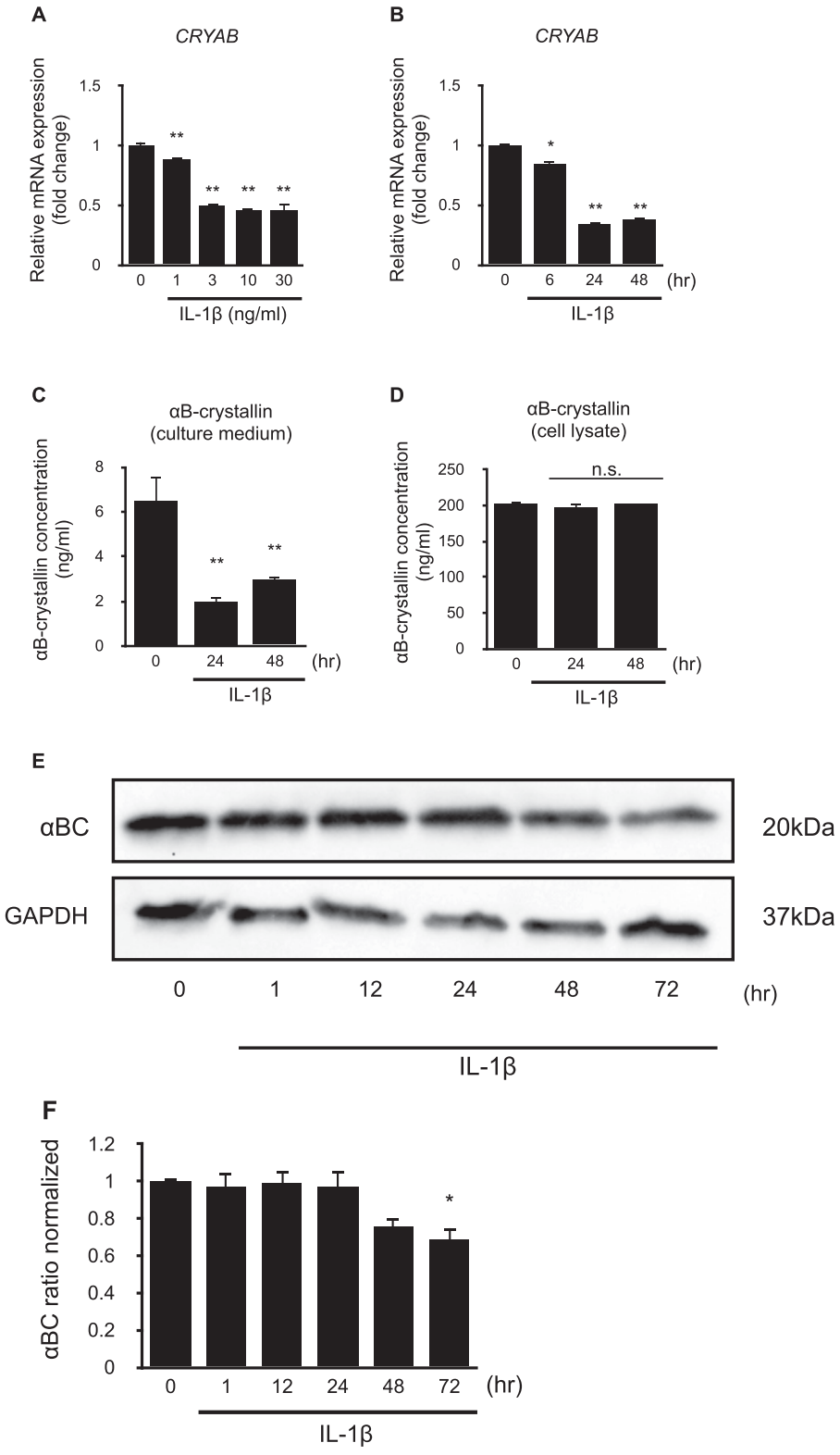

Figure 1. Intracellular αB-crystallin protein level was maintained regardless of IL-1β-induced synthesis suppression in retinal Müller cells. (**A, B**) Dose-dependency (**A**) and time-dependency (**B**) of *CRYAB* mRNA levels under IL-1β treatment were analyzed by reverse transcription qPCR analyses. (**C, D**) Extracellular αB-crystallin protein level in 2 mL culture medium (**C**) and intracellular αB-crystallin protein level in 100 µL cell lysates (**D**) under 10 ng/mL IL-1β stimulation were measured by ELISA. (**E, F**) αB-crystallin protein expression changes under 10 ng/mL IL-1β stimulation were confirmed by immunoblotting (**E**). Densitometric values are presented as ratio normalized to control (**F**). There were 3 to 4 per group in RT-qPCR and ELISA, $n = 3$ in densitometry, $^{*}P < 0.05$, $^{**}P < 0.01$.

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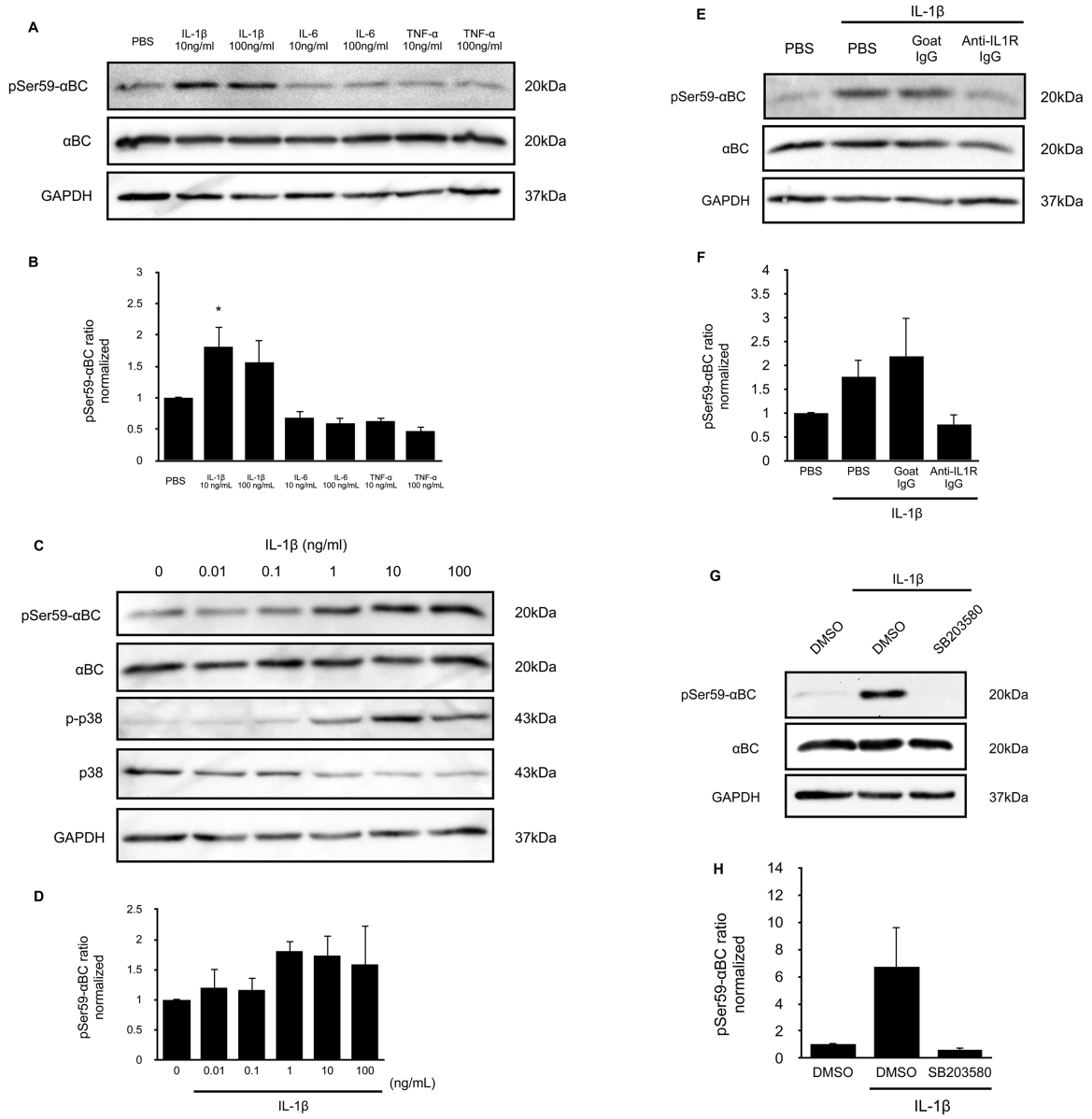

Figure 2. Ser59 phosphorylation of αB-crystallin was induced by IL-1β via p38 MAPK activation. (**A-D**) The αB-crystallin Ser59 phosphorylation was induced by IL-1β among three cytokines involved in PDR (**A, B**), and dose-dependency of phosphorylation with IL-1β stimulation was analyzed (**C, D**). (**E-H**) IL-1β-induced Ser59 phosphorylation was attenuated by both 10 µg/mL IL-1R neutralizing antibody (**E, F**) and 10 µM p38 MAPK inhibitor (SB203580) (**G, H**) for 30 minutes followed by stimulation with IL-1β (10 ng/mL) for 24 hours. Densitometric values are presented as ratio normalized to the control group (**B, D, F, H**). There were 3 in densitometry, $^*P < 0.05$, Dunnett's test was used to compare to the control group.

investigate the pathogenetic aspect of IL-1β-induced intracellular retention of αB-crystallin in PDR, we analyzed anti-apoptotic effects in retinal Müller cells. Based on the literature which showed apoptosis acceleration with αB-crystallin short hairpin RNA in rats' neural cells under serum deprivation,[13] we induced apoptosis by serum reduction with 1% FBS contained in media after *CRYAB* siRNA transfection and assessed apoptotic activities with IL-1β application.

Firstly, we confirmed that IL-1β application did not compensate for the reduction of intracellular αB-crystallin under *CRYAB* knockdown conditions (Fig. 3A, Supplementary Fig. S2). We simultaneously verified that *CRYAB* knockdown did not inhibit p38 MAPK phosphorylation which was mediated by IL-1β (see Fig. 3A). Caspase-3/7 activities, which are essential processes for the cell apoptosis signal cascade, were promoted by *CRYAB* siRNA transfection compared with the inactive siRNA construct (Fig. 3B, Supplementary Fig. S3). In the TUNEL assay, the rate of TUNEL-positive Müller cells significantly increased in *CRYAB* knockdown groups compared with both non-treated and negative-siRNA-treated group after IL-1β application (Figs. 3C, 3D). These data indicate that αB-crystallin has an anti-apoptotic effect in retinal Müller cells under diabetic mimicking inflammatory conditions; therefore, *CRYAB* knockdown induces apoptosis via caspase-3/7 activation under IL-1β stimulation.

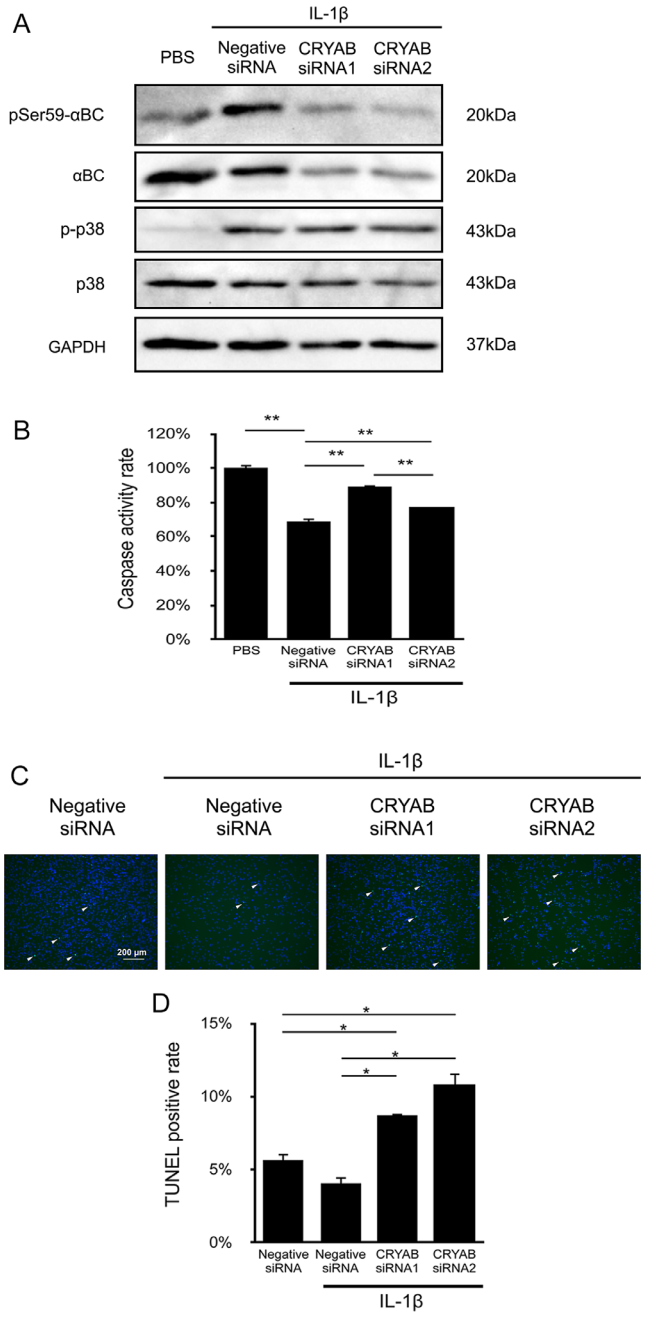

Figure 3. The αB-crystallin reduction led to apoptosis in retinal Müller cells. (**A**) *CRYAB* siRNA transfection caused αB-crystallin protein downregulation but did not affect p38 MAPK activation. (**B-D**) The αB-crystallin knockdown impaired IL-1β-mediated Caspase-3/7 activity deterioration (**B**) and increased TUNEL positive cells (**C, D**) under 10 ng/mL IL-1β treatment for 24 hours. The percentage of TUNEL-positive cells were 5.656%, 4.023%, 8.744%, and 10.854% in negative siRNA without IL-1β, and negative siRNA, *CRYAB* siRNA1, and *CRYAB* siRNA2 under IL-1β stimulation, respectively. There were 3 to 4 per group, $^{*}P < 0.05$, $^{**}P < 0.01$.

Histological Distribution of αB-Crystallin in the Retinas Obtained From Type 2 Diabetes Model Rats

SDT fatty rat is an animal model of type 2 diabetes with obesity which shows diabetic complications such as diabetic cataract and uveitis.[24,25] Although there is no appropriate experimental animal model for PDR with fibrovascular membrane formation, we found that SDT fatty rats showed the upregulation of some pro-inflammatory cytokines observed in the vitreous humor and mimicked an early phase of diabetic retinopathy (manuscript in preparation). Thus, retinal Müller cells in the retinas of SDT fatty rats maintained their original histological positions while GFAP, the activated glial marker, was positive. Additionally, this study demonstrated that the localization of αB-crystallin has not been clarified in retinal Müller cells which keep their histological characters as it stands. We demonstrated that both αB-crystallin and Ser59 phosphorylated αB-crystallin were localized in retinal Müller cells obtained from SDT fatty rats (Fig. 4, Supplementary Fig. S4). We verified αB-crystallin localization in the retinas of Sprague-Dawley rats as the non-diabetic retinal tissue, and found that αB-crystallin distribution did not markedly change compared with SDT fatty rats, whereas GFAP was not clearly determined in retinal Müller cells (Supplementary Fig. S5).

Co-Localization of αB-Crystallin and IL-1R With GFAP in Retinal Müller Cells in Fibrovascular Membranes Derived From Patients With PDR

To investigate whether the findings we showed in the in vitro experiments are applicable to retinal Müller cells in PDR pathogenesis, we analyzed the localization of αB-crystallin in fibrovascular membranes derived from human PDR patients. We previously showed that αB-crystallin localized with CD31-positive neovascular endothelial cells in fibrovascular membrane specimens.[19,20] In this study, we newly demonstrated that all αB-crystallin, Ser59 phosphorylated αB-crystallin, and IL-1R were co-localized with GFAP (Fig. 5). These findings suggest that the αB-crystallin regulation mechanism we showed in in vitro experiments involves retinal Müller cells of PDR fibrovascular membranes.

Discussion

This study revealed that αB-crystallin in retinal Müller cells contributes to fibrovascular membrane formation via a change in IL-1β-induced molecular dynamics in PDR pathogenesis. The regulatory mechanisms of αB-crystallin differ in the cellular characters, and both upregulation and downregulation of *CRYAB* mRNA expression on treatment with IL-1β were reported depending on the cell lines.[16,17] The current study demonstrated that *CRYAB* mRNA expression decreased with IL-1β in retinal Müller cells, whereas intracellular αB-crystallin protein levels were maintained (see Fig. 1). Ser59 phosphorylation was shown to decrease the extracellular αB-crystallin level by suppressing an unconventional secretion pathway which involves multivesicular bodies.[26] We indicated that phosphorylation in this regulatory system altered αB-crystallin exosome secretion, resulting in the difference between intra- and extracellular levels of αB-crystallin protein (see Fig. 2). As a lack of intracellular αB-crystallin leads to apoptosis (see Fig. 3), we suggested that the Ser59-phosphorylation-mediated intracellular retention of αB-crystallin contributes to the pathogenesis of fibrovascular membrane formation by escape from apoptosis in retinal Müller cells (Fig. 6). Taken together, the schema summarized IL-1β-p38 MAPK axis under diabetic

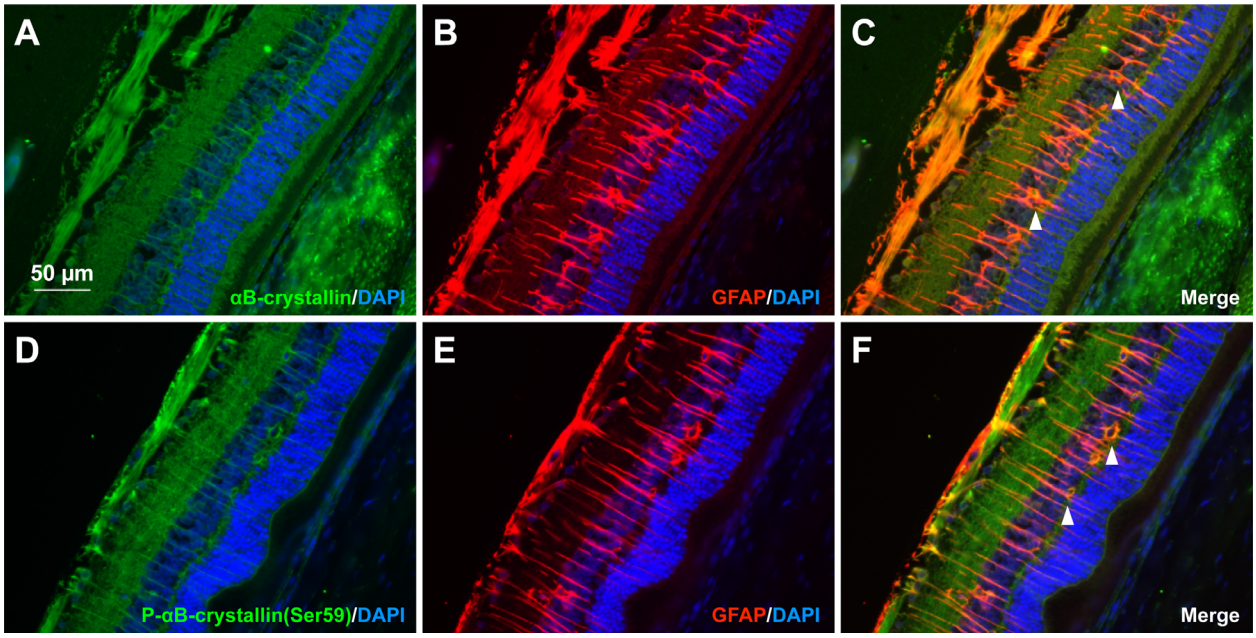

Figure 4. Co-localization of αB-crystallin and Ser59-phosphorylated αB-crystallin with GFAP positive retinal Müller cells in the retinas of type 2 diabetes model rats. (**A-F**) Double labeling of αB-crystallin (*green*), GFAP (*red*), and DAPI (*blue*) (**A-C**); Ser59-phosphorylated αB-crystallin (*green*), GFAP (*red*), and DAPI (*blue*) (**D-F**) in the retina sections of SDT fatty rats.

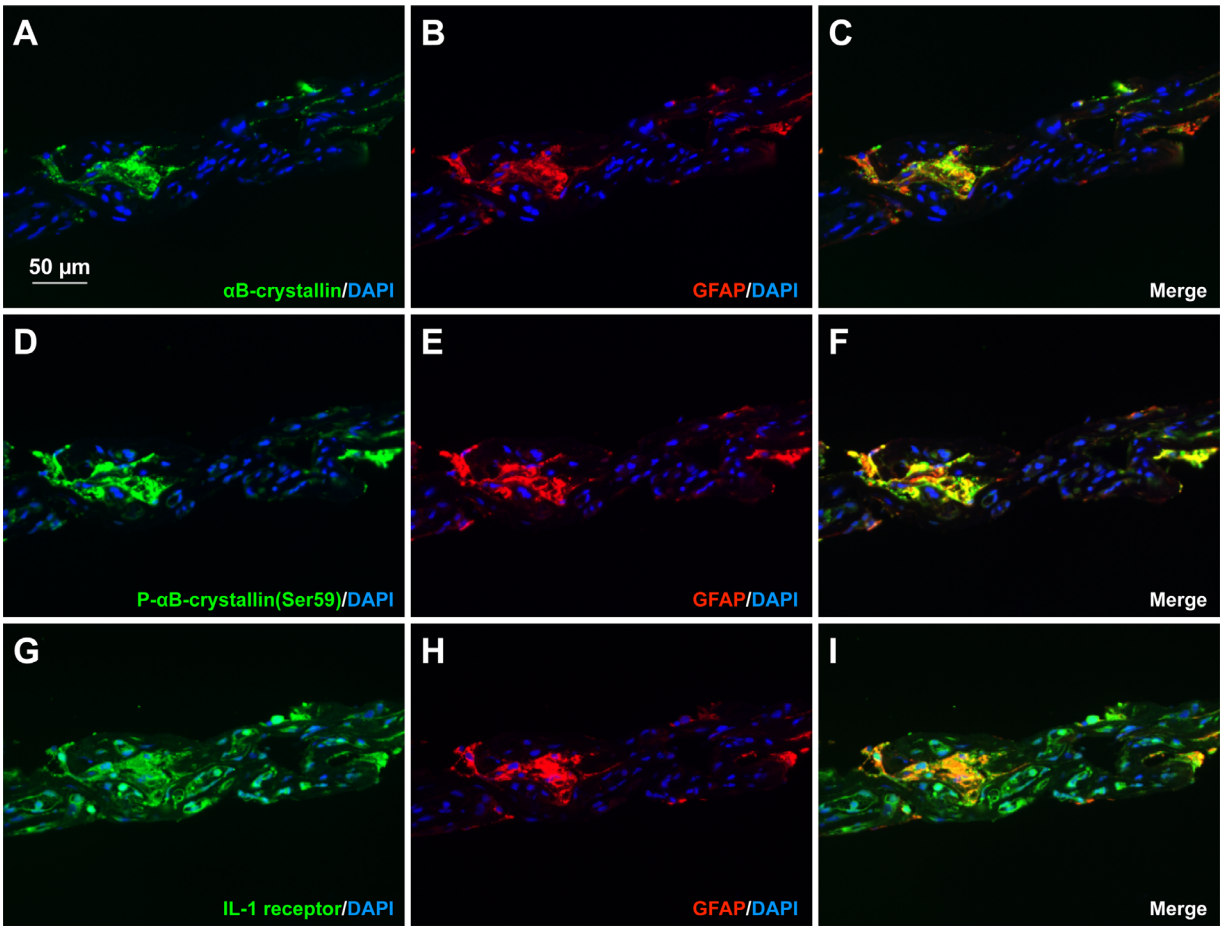

Figure 5. Co-localization of αB-crystallin, Ser59-phosphorylated αB-crystallin, and IL-1R with GFAP positive cells in the fibrovascular membrane obtained from a patient with PDR. (**A-I**) Double labeling of αB-crystallin (*green*), GFAP (*red*), and DAPI (*blue*) (**A-C**); Ser59-phosphorylated αB-crystallin (*green*), GFAP (*red*), and DAPI (*blue*) (**D-F**); IL-1R (*green*), GFAP (*red*), and DAPI (*blue*) (**G-I**) in diabetic fibrovascular tissue specimens.

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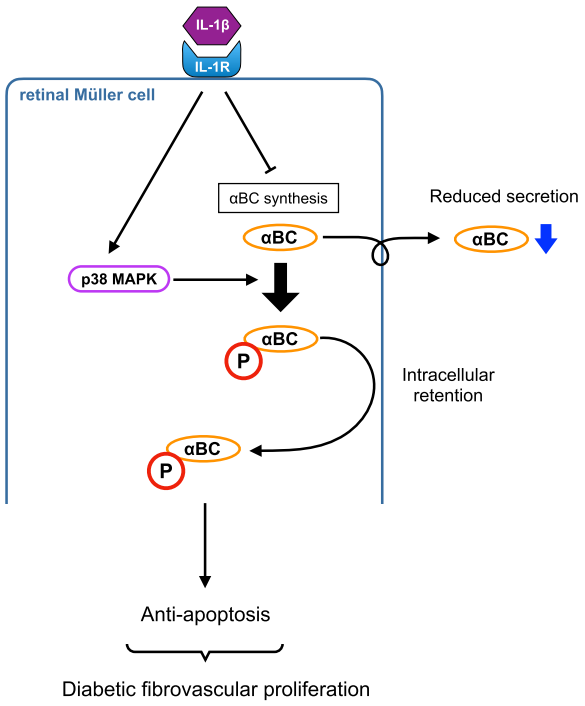

Figure 6. Schema showing αB-crystallin secretion reduction and intracellular retention of Ser59-phosphorylated αB-crystallin under IL-1β stimulation in retinal Müller cells. Our current data suggest that these mechanisms are associated with the pathogenesis of fibrovascular membrane formation in PDR.

mimicking inflammatory conditions. IL-1β-p38 MAPK axis induced Ser59-phosphorylation of αB-crystallin, whereas IL-1β suppressed αB-crystallin synthesis. The phosphorylation of αB-crystallin caused the divergence of intra- and extracellular αB-crystallin levels, which could eventually lead to escape from apoptosis in retinal Müller cells (see Fig. 6).

Retinal Müller cells show both proliferation and apoptosis which affect the pathogenesis of diabetic retinopathy.[29,30] In terms of apoptosis, small heat shock proteins, including αB-crystallin, have been shown to attenuate apoptosis by the inhibition of mitochondria-derived reactive oxygen species and the blockade of caspase signal pathways.[31] Another study demonstrated that αA-crystallin, allied small heat shock protein to αB-crystallin, prevented apoptosis induced by serum deprivation in retinal neuronal cells.[32] We also adopted serum reduction as an apoptosis inducer in this study, and it is reasonable because one of the important factors in PDR pathogenesis is ischemia which can promote apoptosis in ocular tissues.[33] We reported, for the first time, that αB-crystallin was located in both the diabetic retinopathy model rats' retinal Müller cells and GFAP-positive cells (see Figs. 4, 5). The former revealed that anatomic positions were correctly maintained in the retina, the latter of which means the translocated Müller cells in the PDR patient-derived fibrovascular membranes. According to these findings, it is indicated that αB-crystallin aids escape from apoptosis in retinal Müller cells during the pathogenesis of PDR, characterizing fibrotic features by subsequent production of intermediate filaments, such as GFAP within the fibrovascular membranes.

The phosphorylation of αB-crystallin alters its molecular activity in terms of both quantity and quality. As with the retinal pigment epithelial cells under diabetic conditions,[34] we found αB-crystallin synthesis attenuation in retinal Müller cells; however, this study simultaneously elucidated the quantitative difference between intra- and extracellular αB-crystallin accompanied by Ser59 phosphorylation. The αB-crystallin is known to be secreted via exosomes into ocular tissues, and another report showed that phosphorylation and/or O-linked-β-N-acetylglucosamine modification can be key regulators of this process.[35,36] Three serine residues in αB-crystallin protein: Ser19, Ser45, and Ser59, can each be phosphorylated by different signal molecules each,[27,37] whereas Ser59, the most essential regulator of αB-crystallin-containing exosome secretion, is phosphorylated by p38 MAPK.[26] IL-1β was shown to activate p38 MAPK in retinal Müller cells,[38] thereby altering intra- and extracellular αB-crystallin increased by the Ser59-phosphorylation-induced inhibition of the exosome-mediated protein secretion pathway. On the other hand, the phosphorylation of αB-crystallin modifies its chaperone activity depending on the phosphorylation sites, and it is related to alteration of oligomer formation via phosphorylation-induced destabilization of intersubunit interactions.[18,39] In terms of neuroglia, it is suggested that αB-crystallin phosphorylation leads to the gliosis of astrocytes in the demyelinated area of the brain under inflammatory conditions, which changes depending on binding partners.[40] According to these, it is suggested that the multifaced functions of the Ser59 phosphorylation of αB-crystallin contribute to fibrovascular membrane formation, although we focused on the anti-apoptotic effect of the intracellular retention of αB-crystallin in this study.

The fibrovascular membrane consists of various types of cells and extracellular matrix, including not only retinal Müller cells but also retinal pigment epithelial cells. The essential process of pathogenetic fibrosis by retinal pigment epithelial cells is epithelial-mesenchymal transition (EMT), and αB-crystallin is known to be involved this mechanism through the TGF-β1-induced SMAD/Snail axis.[41,42] In addition, the lack of αB-crystallin attenuates TGF-β1 signaling by the inhibition of SMAD4 nuclear export in idiopathic pulmonary fibrosis,[43] and these findings support the profibrotic function of αB-crystallin in EMT. We proposed glial-mesenchymal transition (GMT) as a concept similar to EMT in retinal Müller cells, which contributes to fibrovascular membrane formation via the TGF-β-Snail axis associated with the SMAD pathway.[11,44] As shown in the literature, Wu and Kanda, and our colleagues confirmed the localization of components of GMT, such as α-SMA and SM22 in fibrotic tissues obtained from patients with an idiopathic epiretinal membrane or PDR.[11,44] Fibrovascular tissue formation by glia cells has been shown to be related to the inflammatory condition,[45] and we demonstrated co-localization of IL-1R and αB-crystallin in fibrovascular specimens obtained from patients with PDR in this study. Hence, αB-crystallin might have a potential role in fibrovascular membrane formation under inflammatory conditions of PDR via GMT, which would be a future issue that needs to be clarified.

This study has limitations, such as that we did not discuss extracellular αB-crystallin functions because we focused on intracellular αB-crystallin. Retinal Müller cells contribute to maintenance of the neural retina with the secretion of neurotrophic factors and have crucial roles as a major cellular source of survival signals for retinal neurons under diabetic conditions.[9,46] Recent studies indicated that extracellular αB-crystallin secreted from retinal Müller cells had neuroprotective effects in retinal ischemia

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model mice and glaucoma model mice via the regulation of neurotrophin.[47,48] Furthermore, retinal Müller cell-derived αA-crystallin was shown to have neuroprotective functions by anti-apoptotic effects in a paracrine manner.[49] Therefore, it is estimated that extracellular αB-crystallin from retinal Müller cells acts on adjacent cells to serve neuroprotective effects by paracrine, and IL-1β-mediated secretion reduction of αB-crystallin damages in the neural retina under diabetic conditions. As impairment of the neural function, such as the reduction of oscillatory potential amplitudes in electroretinograms, can be confirmed in patients with early diabetic retinopathy,[50] the reduction of extracellular αB-crystallin by IL-1β may have the potential to deteriorate the pathogenesis of diabetic retinopathy. Further studies on extracellular αB-crystallin from retinal Müller cells will be needed to gain deeper insights.

In summary, the present findings indicate that phosphorylation of the αB-crystallin serine 59 residue was modulated by IL-1β in Müller cells under diabetic conditions, suggesting that αB-crystallin contributes to the pathogenesis of PDR through an anti-apoptotic effect and fibrovascular membrane formation.

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