

Inhibitory Effect of Alpha-Crystallins on VEGF-Induced Proliferation of Retinal Microvascular Endothelial Cells

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Abstract

Purpose: Lens extraction surgery with ischemic retinopathy may trigger neovascularization, suggesting that lens removal alters the ocular concentrations of angiogenesis regulatory factors. This study aims to identify angiogenic or anti-angiogenic factors exhibiting altered expression in the vitreous after lens extraction in rabbits.

Methods: Vitreous samples after lens extraction were analyzed by two dimensional fluorescence difference gel electrophoresis (2D-DIGE) followed by mass spectrometry to identify candidate factors indicating expression changes. Western blotting and real-time polymerase chain reaction (PCR) were performed to semi-quantify protein and gene expression changes. The identified factors were tested by assessing effects on vascular endothelial growth factor (VEGF)-induced proliferation of human retinal microvascular endothelial cells (HRMECs).

Results: Three spots with reduced expression were identified as α A-crystallin by 2D-DIGE and mass spectrometry. Western blotting revealed that the protein levels in vitreous of α A- and α B-crystallin were significantly lower after lens extraction. α B-crystallin suppressed the proliferation of VEGF-stimulated HRMECs, and the combination of α A- and α B-crystallin was more effective than α B-crystallin alone.

Conclusion: The depletion of vitreal α -crystallins after lens extraction may increase VEGF-induced angiogenesis, thereby contributing to adverse outcomes such as vitreous hemorrhage, proliferative membrane, and vision loss after cataract surgery for ischemic retinopathy patients.

Keywords: Lens extraction surgery; Angiogenesis regulatory factor; Cataract; Ischemic retinopathy; Cell proliferation assay

Introduction

Regulated angiogenesis is critical for proper development and tissue repair under physiological conditions, but angiogenesis can also facilitate pathologic events such as cancer and inflammation [1]. In ocular tissue, neovascularization associated with diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration, and neovascular glaucoma causes severely deteriorated vision [2-4], and can lead to vitreous hemorrhage, proliferative membrane, and vision loss [2-5].

In eyes with ischemic oculopathy, there is growing concern that lens extraction for cataract treatment can actually make visual function worse by increasing the incidence of macular edema [6], rubeosis iridis, and neovascular glaucoma [7-9]. These clinical findings raise the possibility that lens removal suppresses the ocular expression of anti-angiogenic factors or promotes the expression of angiogenic factors. The aim of this study was to identify substances up or downregulated in the vitreous after lens removal and to directly investigate the effects of these factors on vascular endothelial cell proliferation.

Materials and Methods

Surgical procedures

Animal experiments were conducted in accordance with The Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Visual Research and with the institutional guideline of the University of Fukui on the use of animals in research. Female Japanese rabbits weighing 2.0-2.5 kg (Japan SLC, INC., Shizuoka, Japan) were acclimated to laboratory conditions for at least one week before experiments. All rabbits had ad libitum access to a standard laboratory diet of RC4 (Oriental Yeast Co., Ltd., Tokyo, Japan) and water, and were housed in pathogen-free conditions at 23°C $\pm$ 1°C and 60% $\pm$ 10% humidity under a 12 h/12 h light/dark cycle. Prior to lens removal surgery, rabbits were administered 35 mg/kg ketamine HCl (DAIICHI SANKYO COMPANY, LIMITED, Tokyo, Japan) by intramuscular injection. The same surgeon performed all internal lens removal surgeries on the right eye by phacoemulsification using a phacoemulsifier aspirator system (2000 LEGACY EVEREST, Alcon, Inc., Tokyo, Japan). First, a 2.4 mm temporal clear corneal tunnel incision was made, followed by a continuous curvilinear capsulorhexis to approximately 5.0 mm using anterior capsular forceps while preserving the posterior capsule. Hydrodissection, hydrodelineation, and phacoemulsification were then conducted as described.

Sample preparation and determination of vitreous protein concentrations

One month after surgery, lens-extracted and control rabbit eyes were removed and frozen at -80°C . The frozen eye was cut open from post pole to corneal limbus (four directions; inferior, upper, nasal, temporal) to retrieve vitreous samples. All vitreal samples were obtained within 1 h after suppression.

For two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), vitreous samples were mixed with protein extraction buffer (7 M urea, 2 M thiourea, 3% CHAPS, 1% Triton-X-100) and total protein concentration determined using the RC DCTM protein assay kit II (BIO-RAD Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA; G-Biosciences, St Louis, MO, USA) as the standard. Protein samples (20 μg) from control eyes were labeled with 2D-DIGE Cy3 Dye while those from lens-extracted eyes were labeled with Cy5 Dye using the CyDye DIGE Fluor Minimal Labeling Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

For western blotting, samples were homogenized in radio-immunoprecipitation assay buffer by sonication. Protein concentration was determined using the BCA assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with BSA (G-Biosciences, St Louis, MO, USA) as the standard. Five micrograms of total protein was re-suspended in $6 \times$ sodium dodecyl sulfate (SDS) sample buffer (350 mM Tris-HCl at pH 6.8, 30% glycerol, 10% SDS, 9.3% dithiothreitol (DTT), and 0.1% bromophenol blue) overnight at 4°C . Vitreous was treated with $5 \times$ SDS sample.

2D-DIGE analysis of vitreous proteins

Fluorescent-labeled vitreous samples from control and lens-extracted eyes were mixed and dissolved with isoelectric focusing rehydration buffer (8 M urea, 2% w/v CHAPS, 2.8 mg/ml dithiothreitol, 0.01% w/v bromophenol blue (BPB)) and IPG buffer (pH 4-7, GE Healthcare). An Immobiline dry strip with a linear pH gradient of 4-7 (GE Healthcare) was rehydrated in rehydration solution in the Immobiline DryStrip Reswelling Cassette (GE Healthcare) and labeled samples were separated by isoelectric focusing using the Multiphor II Electrophoresis Unit (GE Healthcare). The parameters for isoelectric focusing were 200 V for 1 min, an increasing linear gradient of 200-3500 V for 1.5 h and 3500 V for 6 h.

After the first electrophoresis, the strip was equilibrated for 30 min with SDS equilibration buffer (50 mM Tris-HCl, pH 8.8; 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) DTT) to reduce disulfide bonds, then equilibrated for 30 min with DDT-free SDS equilibration buffer plus 2.5% (w/v) iodoacetamide and a few grains of BPB to alkylate cysteine residues. Second-dimension SDS-polyacrylamide gel electrophoresis (PAGE) was performed on an electrophoresis unit (BE-12R, BIO CRAFT Co., Ltd, Itabashi, Japan) using precast gradient gels (5-20%) designed with non-fluorescent glass (LDG-172, BIO CRAFT, Tokyo, Japan). The electrophoretic parameters were 40 mA for 10 min and 60 mA for 80 min. To obtain approximate molecular masses, a protein standard (Protein Ladder One, Triple-color (Broad Range), NACALAI TESQUE, INC., Kyoto, Japan) was also loaded onto the gel. Following SDS-PAGE, TyphoonTrio+(GE Healthcare) was used to acquire 2D gel patterns. The protein spots were detected using ImageQuant TL software (GE Healthcare). The spots of interest from the control eye were manually excised and digested in-gel with trypsin. Mass spectrometry was performed using a Bruker Daltonics autoflex

system (Bruker Corporation, Billerica, MA, USA) according to the manufacturer's instructions. The peptide mass data of each spot was analyzed using Mascot search (Matrix Science Inc., Boston, MA, USA).

Western blotting

Samples were separated by SDS-PAGE and the proteins transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) in a semidry transfer apparatus (BIO CRAFT Co., Ltd., Tokyo, Japan). Membranes were blocked with 5% skim milk in phosphate-buffer saline plus 0.1% Tween for 1 h and then incubated sequentially with primary antibodies at 4°C overnight and then with horseradish peroxidase conjugated secondary antibody (1/2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 h. Protein signals were detected using the ECL Western Blotting Detection Reagents (GE Healthcare Limited, Buckinghamshire, HP8 4SP, UK).

Real-time PCR

Complementary deoxyribonucleic acid was synthesized from vitreal total ribonucleic acid (RNA) as a template using ReverTra Ace[®] qPCR RT Kit (TOYOBO CO., LTD. Osaka, Japan) according to the manufacturer's instructions. PCR was performed with THUNDERBIRD[®] SYBR[®] qPCR Mix (TOYOBO) on the Step One Plus Real Time PCR system (Life Technologies) using the following thermal profile: 95°C for 1 min followed by 40 cycles of 95°C for 3 s and 60°C for 60 s. Target genes were αA -crystallin and αB -crystallin, while 18S ribosomal RNA (18S) was used as the internal reference gene. Primers for αA -crystallin (sense: 5'-AGATCCACGGCAAACACAAC-3' and antisense: 5'-GAAGGTGAGCATGCCGTC-3'), αB -crystallin (sense: 5'-GGGTGATGTGATTGAGGTGC-3' and antisense: 5'-GAGGACCCCATCAGATGACA-3'), and 18S (sense: 5'-AGAAACGGCTACCACATCCAA-3' and antisense: 5'-TCCAATTACAGGGCCTCGA-3') were synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan). The mRNA levels were evaluated by the $\Delta\Delta\text{CT}$ method and normalized to the reference gene.

Cell proliferation assay

Cell proliferation was performed in the following way referring to previous reports [10,11]. Human retinal microvascular endothelial cells (HRMECs: DS Pharma Biomedical Co., Ltd. Osaka, Japan) were seeded at 2×10^4 cells/mL into 96-well plates with CS-C medium, a growth medium optimized for HRMECs and culture boost (DS Pharma Biomedical Co., Ltd. Osaka, Japan) and cultured at 37°C for 24 h in a humidified atmosphere of 5% CO_2 . The medium was then changed to CS-C medium with 10% FBS and incubated for 24 h. The HRMECs were then incubated for 24 h in fresh CS-C medium containing VEGF 10 ng/mL plus various concentrations of αA -crystallin, αB -crystallin, both αA -crystallin and αB -crystallin. The number of viable cells was measured using a water soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8). Briefly, 10 μL of CCK-8 reagent was added to each well, the cells incubated at 37°C for 3 h, and the absorbance measured at 492 nm (reference wave length, 660 nm) using a spectrophotometer (Varioskan; Thermo Electron Corporation, Vantaa, Finland).

Representative 2D-DIGE protein profiles of vitreous samples from a control and lens-extracted eye are shown in Figure 1. Four spots decreased after lens extraction compared to the control eye (Figure 1, lower right image). Based on Mascot analysis, mass spectrometry showed that the proteins in spots 1-3 and 4 were rabbit α A-crystallin and bovine 40S ribosomal protein S2, respectively (Table 1). We analyzed both α A- and α B-crystallin in the following experiments since their homology is particularly high (Supplemental Figure 1).

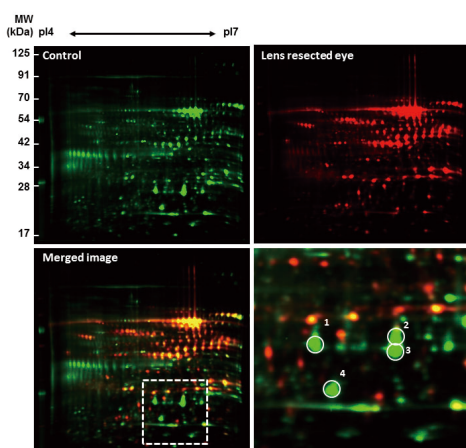


Figure 1: Lens extraction alters the vitreal protein expression profile. Proteomic analysis by 2D-gel electrophoresis was performed on vitreous samples from control (green) and lens-extracted eyes (red). Four spots were clearly decreased in sample from the lens-extracted eye compared to the control eye. Lower right panel shows high magnification image of boxed region containing these 4 spots. (MW: molecular weight, kDa; kilodalton, PI: isoelectric point).

Western blotting of vitreous samples obtained from control and lens-extracted eyes confirmed the results of 2D-DIGE (Figure 2). The vitreal expression levels of α A- and α B-crystallin were lower in lens-extracted eyes compared to controls eyes. On the other hand, β - and γ -crystallin were not detected in the vitreous samples from either control or lens-extracted eyes, but were detected together with α A- and α B-crystallin in lens extract (positive control). We also investigated the gene expression levels of α A- and α B-crystallin by RT-PCR but found no significant difference between lens-extracted and control eyes (Figure 3).

Spot number	Protein Name	Mascot Score [*]	Peptide Count [†]
1	Alpha-crystallin A (CRYAA_RABIT)	127	10

2	Alpha-crystallin (CRYAA_RABIT) A	151	11
3	Alpha-crystallin (CRYAA_RABIT) A	247	15
4	40S ribosomal protein S2 (RS2_BOVIN)	56	5

Table 1: Mass spectrometry based on mascot analysis (*Score greater than 54 was considered significant ($p < 0.05$) in the Mascot search using the database SwissProt 2014_07, †Number of matched peptides).

Supplementary Fig. 1

Alignment of rabbit α A- and α B-crystallins

aA MDTVIQHFWFKRTLGPFY-PSRLFDQFEGGELFYEDLLPFLSSTISPYR--QSLFRT-- 55
aB MDIAIHFWRIWRRPFFHSPSRLEDFQFEGGHEHLSLEDFT-TSTLSFPFLRPLSPFAPS 59
 :::*::*:*:*: *****::*:*: *:::*:*: *:*:

aA VLDSGISEVSRDRDKFVIFLDVKHFSPEDLTKVKQEDFVEIGHKNRQDDHGYISREFH 115
aB WIDTGLSEMRLEKDFSRVNLVDKHSPEELKVLWGLGVIEVGHKEHRQDEHGFIISREFH 119
 ::***::*:*: *: *****::*:*: *: *****:*****:

aA RRYRLSPNVQDQSALSCLSADGMLTFSQPKVQSGLDAGHERAIPVSRKEEP--SSAPSS 173
aB RKYRIAPADVPLITITSSLSGGVLTVPGRKQ---AGPGETIPTREEKPAVTAAPKK 175
 ::*:*:*:*: ***:*****: *: *****:*****:

Identity(*): 55%
Positives(:): 75%

Supplemental Figure 1: Alignment of rabbit α A- and α B-crystallins.

Effects of α -crystallins on proliferation of human retinal vascular endothelial cells

To examine the effects of the α -crystallin family on angiogenesis, we examined whether crystallins affected VEGF-induced proliferation of HRMECs *in vitro*. At 10 ng/ml, VEGF enhanced cell proliferation by about 2.2–2.3-fold compared to controls (no VEGF) ($P<0.01$). α B-crystallin dose-dependently inhibited VEGF-induced cell proliferation (by 88.5%, $P<0.05$; 85.6%, $P<0.01$ and 85.4%, $P<0.01$, respectively), while α A-crystallin alone showed no inhibitory effect at any concentration (0.01, 0.1, and 1 μ g/ml) compared to VEGF alone (Figure 4). However, α A-crystallin promoted the anti-proliferative effect of low-dose α B-crystallin on VEGF-treated HRMECs (0.01 μ g/ml; 79.6% and 0.1 μ g/ml; 81.8%, $P<0.01$, respectively) compared to low-dose α B-crystallin alone (Figure 5). In the absence of VEGF, neither α A-crystallin alone, α B-crystallin alone, nor their combination influenced the growth of HRMECs (data not shown).

Discussion

Both 2D-DIGE and Western blotting demonstrated that α A- and α B-crystallin proteins were reduced in the vitreous after lens extraction, consistent with reports that α -crystallins are secreted into the human aqueous humor from both clear and cataractous lenses [12,13]. Alternatively, vitreal mRNA expression levels of α -crystallins did not change significantly after lens extraction, indicating that the protein decrease was due to absence of the lens rather than decreased

protein synthesis in the vitreous. We also found that α B-crystallin, either alone or in the presence of α A-crystallin, inhibited VEGF-induced proliferation of HRMECs. Taken together, these results suggest that the reduction of vitreal α -crystallins following lens extraction may contribute to retinal neovascularization.

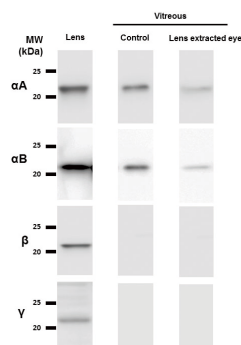


Figure 2: Western blots showing decreased α A- and α B-crystallin protein levels in vitreous from lens-extracted eyes. Immunoreactivity for crystallin family proteins was detected in vitreous samples from control and lens-extracted eyes. Immunoreactive bands corresponded to the putative molecular masses of each crystallin protein (each monomer is approximately 20 kDa). α A-, α B-, β - and γ -crystallins were detected in lens extract (positive control), while only α A- and α B-crystallin were detected in the vitreous. Vitreal levels of α A- and α B-crystallin proteins were markedly reduced in lens-extracted eyes (MW: molecular weight, kDa: kilodalton).

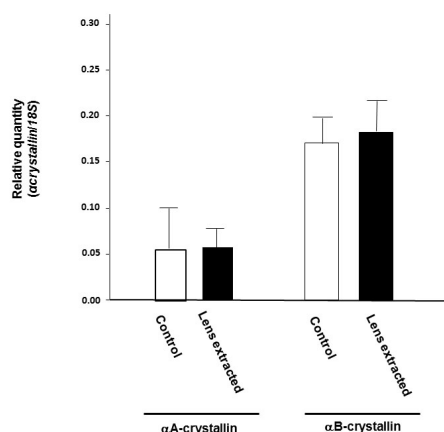


Figure 3: Lens extraction does not alter the mRNA expression levels of α A- and α B-crystallin in vitreous. Results of real time RT-PCR showing no significant difference in vitreal α A- and α B-crystallin mRNA levels between control and lens-extracted eyes (Mann-Whitney nonparametric test). The relative quantity of each gene is normalized to 18S mRNA expression (internal control) and expressed relative to expression in lens extraction (Relative value in lens=1).

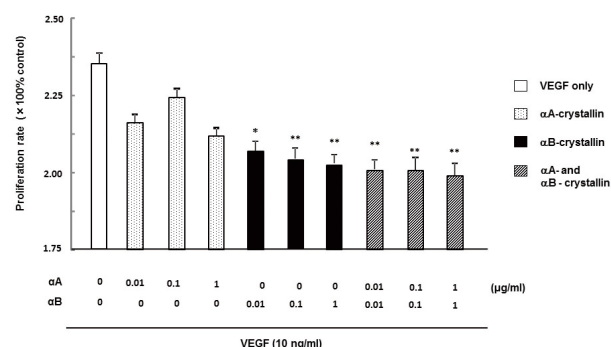


Figure 4: AlphaB-crystallin suppresses vascular endothelial growth factor (VEGF)-induced proliferation of human retinal microvascular endothelial cells. Human retinal microvascular endothelial cells (HRMECs) were incubated with the indicated concentration of α A- and/or α B-crystallin (0, 0.01, 0.1, or 1 μ g/ml) in the presence of VEGF (10 ng/ml) for 24 h. α B-crystallin alone and the combination of α A-crystallin and low-dose α B-crystallin reduced proliferation rate. Proliferation rate is shown as mean $\pm$ S.E.M (n=6) relative to control cultures without VEGF (rate=1), (*; $P < 0.05$ vs. vehicle (VEGF only), **; $P < 0.01$ vs. vehicle (Dunnett's test)).

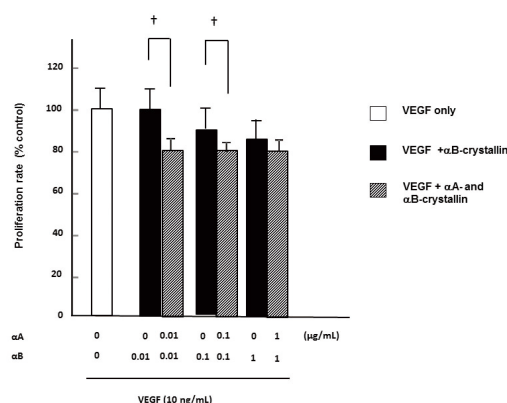


Figure 5: Synergistic effect of crystallins on cell proliferation. HRMECs were incubated with the indicated crystallin concentration (0.01, 0.1, or 1 μ g/ml) plus VEGF (10 ng/ml) for 24 h. The combination of α A- and low-dose α B-crystallin (0.01 and 0.1 μ g/ml) significantly reduced proliferation compared to α B-crystallin alone. Proliferation rate shown as mean $\pm$ S.E.M (n=6) relative to vehicle plus VEGF (100%). (†: $P < 0.01$ vs. vehicle (Dunnett's test)).

Rubeosis and vitreous hemorrhage are occasionally observed after cataract surgery in patients with severe ischemic retinal diseases [7-9]. In the current animal study, the inhibitory effect of extracellular α -crystallins on HRMEC proliferation was only observed when cells were exposed to VEGF, indicating that this anti-proliferative effect is due to interference with VEGF-activated signaling pathways.

α -Crystallins are molecular chaperones[14] with diverse functions in apoptosis [15,16], inflammation [17], and angiogenesis[18,19]. In our study, extracellular α B-crystallin exhibited much stronger inhibitory action on VEGF-induced HRMEC proliferation than α A-crystallin (Figure 4), although α A-crystallin potentiated the effects of low-dose α B-crystallin (Figure 5). In contrast, a previous study found that subconjunctival injection of extracellular α A-crystallin significantly attenuated suture-induced corneal neovascularization [20]. As molecular chaperones, α -crystallins stabilize other proteins [21] and the interaction between α A- and α B-crystallin is required to form structurally stable complexes [22]. Thus, α A-crystallin may facilitate α B-crystallin activity against VEGF through protein stabilization.

In contrast to these anti-angiogenic effects of extracellular α -crystallins, several reports suggest that endogenous intracellular α -crystallins promote angiogenesis. Pathologic neovascularization was attenuated in α A-crystallin knockout mice subjected to laser-induced choroidal neovascularization and oxygen-induced retinopathy [23]. In addition, α B-crystallin was shown to stabilize pro-angiogenic VEGF [19] and fibroblast growth factor [24]. This promotion of angiogenesis was supported by phosphorylation of intracellular α -crystallins [25]. It is currently unclear how extracellular α -crystallins regulate angiogenesis.

In conclusion, we demonstrated that α B-crystallin and the combination of α A- and α B-crystallin inhibit the proliferation of HRMECs. In light of clinical studies showing that vitreal α -crystallin protein levels are reduced in angiogenic diseases [26], it is possible that reduced vitreal α -crystallins following lens removal promotes pathological angiogenesis. Although further study is needed, application of exogenous crystallins may be an effective therapeutic strategy to inhibit aberrant neovascularization following cataract surgery.

Author's Contributions

All the authors were involved in the concept and design of the study, data acquisition, data analysis and interpretation, drafting manuscript, technical support and final review of the manuscript.

Compliance with Ethical Standards

Funding

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Ethical Approval

Animal experiments were conducted in accordance with The Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Visual Research and with the institutional guideline of the University of Fukui on the use of animals in research. This article does not contain any studies with human participants performed by any of the authors.

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