Serum advanced glycation end-products and αB-crystallin in diabetic retinopathy patients

TAKU YAMAMOTO, SATORU KASE, MIYUKI MURATA and SUSUMU ISHIDA

Laboratory of Ocular Cell Biology and Visual Science, Department of Ophthalmology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Hokkaido 060-8638, Japan

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Abstract. αB-crystallin, one of the small heat shock proteins, which is also known as HSPB5, has cytoprotective effects under inflammatory conditions. Advanced glycation end-products (AGE) are produced through non-enzymatic glycation under conditions of hyperglycemia and they contribute to angiogenesis and inflammation. The aim of this study was to examine the levels of serum αB-crystallin and AGE concentrations in blood samples collected from proliferative diabetic retinopathy (PDR) patients. Blood samples were collected from seven diabetic patients with PDR and eight patients without diabetes mellitus who underwent vitrectomy due to PDR and idiopathic macular diseases, respectively, in a single center. The levels of serum αB-crystallin and AGE were measured by ELISA and correlations were assessed statistically. The serum levels (mean ± SEM) of AGE were significantly higher in PDR patients (28.41±0.46 μg/ml) than in patients with non-diabetic macular diseases (25.76±0.60 μg/ml; P=0.015), whereas there was no significant difference in serum αB-crystallin levels. There was one patient with an extremely high level of αB-crystallin, who was treated with systemic corticosteroid due to chronic autoimmune inflammatory diseases. The current prospective study showed that serum AGE levels were significantly higher in PDR patients; however, no correlations between serum AGE and αB-crystallin levels were identified.

Introduction

Diabetic retinopathy (DR), one of the leading causes of blindness worldwide, results from chronic hyperglycemia in patients with diabetes mellitus (DM) (1). Proliferative diabetic retinopathy (PDR), the advanced stage of DR, is characterized by severe retinal ischemia and highly upregulated expression of VEGF, resulting in the development of neovascularization in ocular tissues, vitreous hemorrhage and inflammatory fibrovascular membranes (2). Hyperglycemia also induces non-enzymatic glycation of proteins, lipids and nucleic acids, via a chemical process known as the Maillard reaction, and this promotes the formation of advanced glycation end-products (AGE) (3). AGE acts directly or indirectly via the receptor for AGE (RAGE) as a pathogenic cue in inflammation, angiogenesis and cell proliferation (4,5), and there is considerable evidence of increased serum AGE levels in DM patients with diabetic complications (6,7). In the pathogenesis of DR, AGE contributes to the expression of pro-angiogenic factors such as VEGF-A and TNF-α by means of NF-κB activation induced by RAGE-AGE interaction and the decline in the number of retinal pericytes caused by reactive oxygen species (ROS)-derived apoptosis (8,9). Furthermore, AGE-modified albumin shows pro-inflammatory effects through the induction of leukocyte adhesion in the retinal microvasculature, resulting in breakdown of the blood-retinal barrier (10).

αB-crystallin, also known as heat shock protein B5 (HSPB5), belongs to the crystallin family of proteins. It has pleiotropic roles not only in cellular protection and induction of cell proliferation, but as a molecular chaperone for VEGF-A (11,12). Blood αB-crystallin levels are reported to increase in inflammatory conditions such as multiple sclerosis and in the experimental autoimmune encephalomyelitis (EAE) mouse model (13), in which the increase may be correlated with the reduction of symptoms including paralysis (14). In ocular tissues, Kannan *et al* (15) revealed that αB-crystallin can exert cell-protective effects by inhibiting oxidative stress and endoplasmic reticulum stress in retinal pigment epithelium cells. Conversely, angiogenesis was inhibited by deletion of αB-crystallin through VEGF-A degradation in models of intraocular angiogenesis (16). Colocalization of αB-crystallin and VEGF in the endothelial cells of retinal tissues obtained from PDR patients has also been observed, suggesting that αB-crystallin is a key molecule of angiogenesis and inflammation (17).

Previous studies showed that AGE are likely to regulate αB-crystallin expression. In fact, in our previous study, it was demonstrated that αB-crystallin protein is downregulated while αA-crystallin protein is upregulated in wild-type murine posterior eyecups exposed to AGE protein (18). Moreover,

Correspondence to: Dr Satoru Kase, Laboratory of Ocular Cell Biology and Visual Science, Department of Ophthalmology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, N-15, W-7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan
E-mail: kaseron@med.hokudai.ac.jp

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αB-crystallin protein expression is noted and AGE accumulates in the retinal tissues of human cadaver diabetic eyes without retinopathy (18,19). Other reports showed that AGE concentrations are elevated in the vitreous fluid and aqueous humor of DR patients compared with age-matched controls (20,21), and the AGE concentration was higher in PDR patients' blood than in the control group (22,23). Conversely, the αB-crystallin protein concentration increased in the vitreous fluid of PDR patients compared with eyes without DR (24). Another report showed increased intravitreal αB-crystallin levels in rhegmatogenous retinal detachment patients (25). Given this evidence, it is possible that αB-crystallin and AGE proteins may be released from somatic cells, including retinal pigment epithelium cells, and eventually be secreted into humoral fluids such as vitreous fluids and sera. However, there are no reports on concentrations of αB-crystallin and AGE in sera of the same PDR patients. In this study, the correlation between αB-crystallin and AGE in serum samples were explored, which were prospectively collected from PDR patients.

Patients and methods

Study population. This study was performed according to the tenets of the Declaration of Helsinki and approved by the institutional review board of Hokkaido University Hospital (approval no. 019-0186; December 2019). Patients who came to the Department of Ophthalmology of Hokkaido University Hospital (Hokkaido, Japan) and underwent *pars plana vitrectomy* were enrolled after providing written informed consent. Patients' sera were collected between December 2019 and December 2020 prior to vitrectomy. Grading of DR was performed according to the literature (26) by retina specialists in our hospital who have >3 years of experience as ophthalmologists, and PDR was defined as proliferative tissue caused by neovessels. Serum samples and matching clinical data were obtained from 7 PDR patients (5 females and 2 males, referred to as the PDR group) and 8 non-diabetic patients with idiopathic macular diseases including an epiretinal membrane and a macular hole (3 females and 5 males, non-DM group). The random blood sugar level, estimated glomerular filtration rate (eGFR), aspartate aminotransferase and alanine transaminase were routinely measured in patients' blood. Patient backgrounds and demographics are described in Table I. The median age of the seven enrolled PDR patients and eight non-DM patients was 62 years (age range, 34-78 years) and 72 years (age range, 59-83 years), respectively.

ELISA. The protein levels of αB-crystallin and AGE were determined using an αB-crystallin ELISA kit (cat. no. SKT-123, StressMarq Biosciences Inc.) and an AGE assay kit (cat. no. ab238539, Abcam) according to the manufacturers' instructions. αB-crystallin and AGE were measured by sandwich ELISA and competitive ELISA, respectively. The optical density was determined at 450 nm using a microplate reader (Sunrise absorbance reader, Tecan Group, Ltd.). For AGE measurement, the standard curve was calibrated using AGE-BSA (μg/ml) and the serum AGE concentration is expressed in terms of AGE-BSA.

Statistical analysis. All results are presented as the mean ± SEM. A Mann-Whitney U test was used to assess differences in serum concentrations between PDR and non-DM. Spearman's rank correlation analysis was used to analyze the correlation between serum AGE and αB-crystallin concentrations. A Fisher's exact test was used to examine if there were any sex-based differences. $P<0.05$ was considered to indicate a statistically significant difference. A Smirnov-Grubbs test was used to exclude outlier data in the determination of the normal ranges. Correlation analysis of αB-crystallin and AGE was performed after excluding any outliers.

Results

Study population. There was no significant difference in age between the two groups (P=0.082). None of the male-to-female ratio, levels of hepatic enzymes, or eGFR showed significant differences between the two groups. The random blood sugar level was significantly higher in the PDR group (non-DM, 5.92±0.29 mmol/l; PDR, 9.21±1.20 mmol/l; $P<0.05$; Table I). Therefore, the PDR patients enrolled in this study were characterized by hyperglycemia without renal dysfunction.

Correlation between serum AGE and αB-crystallin. Serum levels of AGE were significantly higher in the PDR group than in the non-DM group (PDR, 28.41±0.46 μg/ml; non-DM, 25.76±0.60 μg/ml; P=0.015). In contrast, serum αB-crystallin levels did not differ significantly between the non-DM and PDR groups (1.58±0.26 and 1.66±0.23 ng/ml, respectively; P=0.949; Fig. 1). There was no significant correlation between serum αB-crystallin and AGE ($\rho=-0.276$, P=0.340). The existence of outlier data from one patient was confirmed by the Smirnov-Grubbs test, and these were excluded from this analysis; the serum αB-crystallin levels of this patient in the non-DM group was extremely high (123.9 ng/ml). In fact, this patient was treated with oral prednisolone for 2 decades, with a total dose of ~10,000 mg, due to rheumatoid arthritis and interstitial pneumonia, whereas no other patients had received systemic corticosteroid therapy.

Discussion

The present study was a prospective observational study with PDR patients' sera, and the results demonstrated significantly higher AGE levels in the PDR group, which is consistent with the report of a case controlled study (22). Blood-retinal barrier breakdown occurs in patients with DR, and AGE induces microvascular hyperpermeability in vascular endothelial cells via RAGE (10,27). Prolonged hyperglycemia causes AGE accumulation in various tissues, and it has been reported that intravitreal AGE elevation is correlated with serum AGE and hemoglobin A1c (HbA1c) levels in diabetic patients (28,29). HbA1c is one of the non-enzymatic glycation products as well as AGE; however, AGE reflects longer-term cumulative diabetic exposure as they are harder to degrade than HbA1c (30). AGE immunoreactivity was strongly noted in cadaver diabetic retinal tissues including central retinal arteries/veins (19). Therefore, the AGE concentration is likely to be higher in sera of PDR patients with a long-term DM.

Our previous report revealed that αB-crystallin is expressed in neovessels within PDR membranes (17), where αB-crystallin

Table I. Clinicopathological characteristics of participating patients.

Factor	Non-diabetes mellitus	Proliferative diabetic retinopathy	P-value
Number of patients	8	7	
Ages, years[b]	72 (range 59-83)	62 (range 34-78)	0.118
Sex, %			0.315
Male	63	29	
Female	37	71	
Blood sugar level, mmol/l[c]	5.92±0.29	9.21±1.20	0.011[a]
Estimated glomerular filtration rate, ml/min[c]	58.05±4.49	61.80±9.13	0.418
Aspartate aminotransferase, U/l[c]	20.50±0.96	21.14±2.20	0.861
Alanine transaminase, U/l[c]	22.25±3.86	20.86±3.64	0.728

[a]P<0.05. [b]Median. [c]Mean ± SEM.

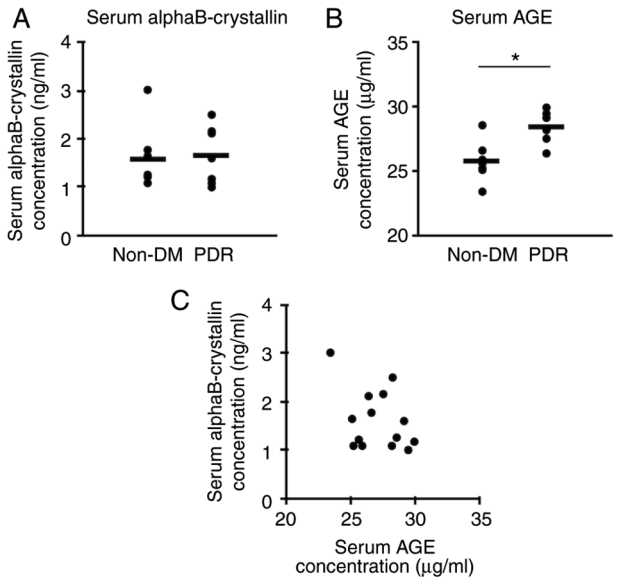

Figure 1. Correlation between serum αB-crystallin and AGE in non-DM and PDR patients. Protein levels of (A) αB-crystallin and (B) AGE in the sera of non-DM and PDR patients. Black symbols indicate individual samples. *P<0.05. (C) There was no correlation between αB-crystallin and AGE levels in the sera of patients. AGE, advanced glycation-end products; DM, diabetes mellitus; PDR, proliferative diabetic retinopathy.

is phosphorylated on serine 59 through upregulation of phosphorylated p38 mitogen-activated protein kinase (31). αB-crystallin showed cytosolic localization (15), but it could be secreted through an unconventional secretion pathway via exosomes following phosphorylation on serine 59 (32). Although there are no reports on the relationship between AGE and αB-crystallin concentrations in vitreous fluid, it was reported that αB-crystallin protein concentrations increased in vitreous fluids of PDR patients compared with those without DR in proportion to VEGF concentrations (24). Therefore, it was hypothesized that αB-crystallin may also be secreted into the sera together with AGE in PDR patients. However, the current prospective study found neither αB-crystallin elevation in the PDR group nor a significant correlation between serum αB-crystallin and AGE concentrations. Further studies are needed to elucidate the mechanisms underlying extracellular secretion of αB-crystallin from intraocular tissues, such as from the retina of diabetic patients.

It should be noted that the serum αB-crystallin levels were ~80X higher than in the non-DM group in one patient with systemic inflammatory diseases who had been treated with oral corticosteroids. Among patients with multiple sclerosis, a chronic neuroinflammatory disease characterized by demyelination of the central nerve system, blood αB-crystallin levels were higher than those of healthy controls (13). Furthermore, it has been shown that proinflammatory cytokines including IL-1β and TNF-α promote αB-crystallin mRNA upregulation in a glioblastoma cell line (33). As indicated in the literature, inflammatory conditions can induce αB-crystallin expression both intra- and extracellularly, and this is involved in the protection of targeted cells from apoptosis. While inflammation can affect αB-crystallin expression, it has been shown that dexamethasone increases the amount of αB-crystallin protein in glomerular podocytes (34), which indicates that therapeutic glucocorticoid is also capable of elevating αB-crystallin expression. Additional research using DR models is needed to further elucidate how glucocorticoid treatment changes the expression levels of αB-crystallin.

The present study has some limitations. First, patients without ophthalmic diseases and diabetic and non-diabetic patients who have not undergone vitrectomy were not enrolled in this study. Therefore, further studies are required to confirm whether serum levels of αB-crystallin or AGE differ between subjects without any ocular diseases and with idiopathic macular diseases. Next, although the number of patients examined was limited, this study prospectively enrolled only patients who consented to participation in the IRB-approved study based on written informed consent during designated study periods. Furthermore, the novel coronavirus disease (COVID-19) pandemic spread in our province in Japan from March 2020, in the middle of the recruitment of participants. In fact, it has been reported that surgical activity was significantly

lower during the COVID-19 pandemic than before (35). Thus, we encountered similar social challenges in Japan, and this severe pandemic prevented the collection of a larger number of samples. Further studies with a larger cohort will be needed to confirm the results.

In summary, the current prospective study revealed that serum AGE levels were significantly higher in diabetic patients, while serum αB-crystallin levels in diabetic patients did not differ from those of non-diabetic subjects.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TY contributed to data curation, analysis, experimentation and writing the manuscript. SK designed the study, collected the samples and edited the manuscript. MM and SI interpreted the data and edited the manuscript. TY and SK confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed according to the tenets of the Declaration of Helsinki and approved by the institutional review board of Hokkaido University Hospital (Hokkaido, Japan; approval no. s019-0186; December 2019). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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