Intraocular Expression of Serum Amyloid A and Interleukin-6 in Proliferative Diabetic Retinopathy

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• PURPOSE: **Because serum amyloid A can regulate angiogenesis, we searched for an association between serum amyloid A and interleukin-6 (IL-6), as proinflammatory factors, and proliferative diabetic retinopathy (PDR).**
• DESIGN: **Retrospective, comparative study.**
• METHODS: **Seventy-six patients (76 eyes) with PDR and 31 patients (31 eyes) with nondiabetic ocular disease (control group), including idiopathic epiretinal membranes (8 eyes) and idiopathic macular holes (23 eyes), were enrolled. Enzyme-linked immunosorbent assay, dual-color immunofluorescence staining, and semiquantitative reverse-transcription polymerase chain reaction were used to examine the serum amyloid A and IL-6 levels in vitreous and plasma, expression of protein and mRNA of serum amyloid A in the excised membranes, respectively.**
• RESULTS: **Vitreous serum amyloid A and IL-6 levels in the study group were significantly higher than those in the control group (both $P < .001$), whereas the plasma concentrations of serum amyloid A and IL-6 did not vary significantly between the groups ($P = .555$ and $P = .621$, respectively). A significant correlation was observed between the vitreous and plasma levels of serum amyloid A in subjects with PDR ($r = 0.525$; $P < .001$). In fibrovascular membranes of the study group, colocalization of endothelial marker CD31 with serum amyloid A and colocalization of fibrillar structure markers fibronectin with serum amyloid A were observed. Expression of serum amyloid A mRNA was significantly higher in fibrovascular membranes with PDR than in idiopathic epiretinal membranes ($P = .004$).**
• CONCLUSIONS: **Serum amyloid A and IL-6 may be involved with the inflammatory process in the development of PDR. Local expression of serum amyloid A may exist in PDR. (Am J Ophthalmol 2011;152:678–685.**

DIABETIC RETINOPATHY, ESPECIALLY PROLIFERATIVE diabetic retinopathy (PDR), has been observed as one of the major causes of blindness in people of working age in the industrialized world.[1] Blindness caused by PDR can be the result of abnormal fibrovascular proliferation with subsequent intravitreal hemorrhage and tractional retinal detachment.[2] Although the precise mechanisms of PDR have not yet been proved fully, mounting evidence suggests that PDR may be associated with inflammatory processes.[3]

Serum amyloid A is one of the classic acute phase proteins that responds to injury, infection, inflammation, and neoplasia.[4] Although serum amyloid A is produced mainly by hepatocytes under the appropriate stimulation, extrahepatic serum amyloid A synthesis also has been implicated in the pathogenesis of several chronic inflammatory diseases, including atherosclerosis, Alzheimer disease, inflammatory arthritis, and several cancer variants by monocytes, endothelial cells, fibroblasts, and so forth.[5,6] In diabetes, as well as in other chronic inflammatory conditions, the basal level of the plasma serum amyloid A concentration is elevated persistently.[7] Recent studies have shown that serum amyloid A can increase angiogenesis via the induction of endothelial proliferation, migration, tube formation, and sprouting activity in vitro.[8]

Interleukin-6 (IL-6) is an inflammatory cytokine with a variety of biological effects, such as participating in immune response and host defense mechanisms.[9,10] Studies have reported that the advanced glycation end products in the vitreous fluid may be involved in the development of diabetic retinopathy by inducing the production of IL-6 from retinal Müller cells.[11] IL-6 in the vitreous fluid and retinal tissue plays a critical role in the pathophysiologic process of diabetic retinopathy.[12] Recent studies have shown that IL-6 induces the expression of vascular endothelial growth factor, which has been considered the most important primary mediator of retinal angiogenesis.[13,14] In addition, IL-6 plays an essential role in the synergistic induction of the human serum amyloid A gene when stimulated with proinflammatory cytokines.[15]

Because both serum amyloid A and IL-6 have been recognized as factors involved in angiogenesis and inflammation, which are crucial in the development of PDR, and because the roles of serum amyloid A and IL-6 in PDR have not been elucidated, we conducted the present study to examine the concentration of serum amyloid A and IL-6 in the plasma and vitreous fluid of patients with PDR. The relationships between the concentration of serum amyloid A and IL-6 and the expression of serum amyloid A in fibrovascular membranes in subjects with PDR also were investigated.

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METHODS

• **SUBJECTS:** Undiluted vitreous fluid and blood samples of 76 eyes of 76 diabetic patients (study group) and 31 eyes of 31 nondiabetic patients (control group), including idiopathic macular holes (23 eyes) or preretinal membranes (8 eyes), were collected. Exclusion criteria included (1) treatment with corticosteroids or immunosuppressive agents, (2) previous ocular surgery, (3) a history of uveitis, (4) iris rubeosis, and (5) a history of systematic immune diseases.

• **PHYSICAL AND OCULAR EXAMINATIONS:** All patients underwent an ophthalmic and medical examination. The ophthalmic examination included the history of previous ocular treatments, slit-lamp biomicroscopy, gonioscopy, ophthalmoscopy, fluorescein angiography, and fundus color photography, which was performed with a fundus camera (TRC-50EX; Tokyo Optical Co, Ltd, Tokyo, Japan). The severity of diabetic retinopathy was assessed on the standardized fundus color photographs and on the fluorescein angiograms, or by ocular ultrasound if a vitreous hemorrhage or opacity lens prevented an ophthalmoscopic examination of the ocular fundus. The medical examination included a blood examination for the fasting plasma concentrations of blood glucose and glycosylated hemoglobin. Systolic and diastolic blood pressures were measured with a mercury sphygmomanometer with the patient in the sitting position, after the patient had rested for at least 15 minutes. Arterial hypertension was defined as a systolic blood pressure of 140 mm Hg or higher, a diastolic blood pressure of 90 mm Hg or higher, or treatment with antihypertensive medication.

• **SAMPLE COLLECTION:** Vitreous samples were collected in sterile tubes at the time of the pars plana vitrectomy with 3 pars plana sclerotomy incisions and were frozen immediately at −80 C. At the start of the removal of the vitreous body, approximately 1 mL undiluted vitreous gel was aspirated through the vitreous cutter under simultaneous inflation of the vitreous cavity with air through the infusion canula. Blood samples were collected from the corresponding patients. Those samples were placed on ice rapidly and then centrifuged at 3000 revolutions per minute for 10 minutes at 4 C, after which the liquid component without sediment was immediately stored at −80 C until the assay was performed.

• **ENZYME-LINKED IMMUNOSORBENT ASSAYS:** Both serum amyloid A and IL-6 were measured in vitreous fluid samples from the same eye as well as in the plasma. The concentrations of serum amyloid A and IL-6 were measured by ELISA using kits for human serum amyloid A and IL-6, respectively (human serum amyloid A ELISA Kit; Invitrogen Corporation, Camarillo, California, USA; and human IL-6 ELISA kit; Invitrogen Corporation, Camarillo, California, USA). The minimum detectable doses of human serum amyloid A and human IL-6 were < 4 ng/mL and 2 pg/mL, respectively. Each assay was performed according to the manufacturer's instructions. Because of the sample volume, serum amyloid A levels were measured in 81 plasma samples (57 samples from study group, 24 samples from the control group) and in 72 vitreous fluid samples (50 samples from the study group, 22 samples from the control group); the IL-6 concentrations were detected in all samples.

• **DUAL-COLOR IMMUNOFLUORESCENCE STAINING:** Dual-color immunofluorescence staining was performed on the frozen sections of the fibrovascular membranes and idiopathic epiretinal membranes by staining with mouse antiserum amyloid A monoclonal immunoglobulin G (IgG; 1:200 dilution, no. ab55720; Abcam, Cambridge, Massachusetts, USA) and with rabbit anti-CD31 polyclonal IgG (1:100 dilution, no. bs-0468R; Biosynthesis Biotechnology Co, Ltd, Beijing, China) or rabbit antifibronectin (FN) polyclonal IgG (1:200 dilution, no. bs-0666R; Biosynthesis Biotechnology Co, Ltd). Serum amyloid A was examined with rhodamine B isothiocyanate conjugated goat antimouse IgG (1:200 dilution, no. bsR-0296G; Biosynthesis Biotechnology Co, Ltd), and CD31 or FN was examined with fluorescein isothiocyanate (FITC) conjugated goat antirabbit IgG (1:100 dilution, no. bsF-0295G; Biosynthesis Biotechnology Co, Ltd). The samples were counterstained with 4,′ 6′-diamino-2-phenylindole (DAPI) (1:1000 dilution, no. D9542; Sigma-Aldrich, St. Louis, Missouri, USA). All sections were examined with a fluorescence microscope (DS-Ril-U2; Nikon, Tokyo, Japan) and photographed (DS-U2; Nikon).

• **REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION:** After RNA extraction and reverse transcription, reverse-transcription polymerase chain reaction was conducted for the cDNA samples in fibrovascular membranes from 6 patients with PDR and in membranes from 6 patients with idiopathic epiretinal membranes using sequence-specific primers for glyceraldehydes phosphate dehydrogenase and serum amyloid A. Glyceraldehydes phosphate dehydrogenase, one of the housekeeping genes, served as the positive control. The following primers were used: for glyceraldehydes phosphate dehydrogenase (120 bp): forward primer, 5′-GAGTCCACTGGCGTCTTCAC-3′; reverse primer, 5′-GTTCACACCCATGACGAACA -3′; serum amyloid A1 (121 bp): forward primer, 5′-TGGGGCTCGGGACATGTGGA-3′; reverse primer, 5′-GGCACCCCCAGGTCCCCTTT-3′. The polymerase chain reaction products were visualized on ethidium bromide-stained 2% agarose gels under ultraviolet light. Quantification of the polymerase chain reaction products was carried out with BandScan software (version 4.5; Glyco Inc., San Leandro, California, USA).