Elevation of Apolipoprotein A-I and Apolipoprotein H Levels in the Vitreous Fluid and Overexpression in the Retina of Diabetic Patients

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Objectives: To determine levels of apolipoprotein (apo) A-I and apo H in the vitreous fluid of patients with proliferative diabetic retinopathy (PDR) and to examine whether apo A-I and apo H messenger RNA (mRNA) levels are overexpressed in the diabetic retina.

Methods: Vitreous samples from 4 diabetic patients with PDR and 8 nondiabetic patients with macular hole were selected for proteomic analysis. Fourteen additional samples (7 from patients with PDR and 7 from patients with macular hole) were used for Western blot analysis. Fourteen postmortem eyes (7 from diabetic and 7 from nondiabetic donors) were used to perform quantitative real-time polymerase chain reaction analysis.

Results: Intravitreous apo A-I and apo H levels were significantly higher in patients with PDR than in the control group. The apo A-I and apo H mRNA levels obtained from the retinas of diabetic donors were significantly higher than those obtained from nondiabetic donors. Retinal pigment epithelium was the main contributor to the differences.

Conclusions: Levels of apo A-I and apo H are elevated in the vitreous fluid of diabetic patients with PDR. In addition, we provide the first evidence, to our knowledge, that a higher expression of apo A-I and apo H mRNAs exists in the diabetic retina.

Clinical Relevance: The results of this study may be relevant to new treatment strategies aimed toward reducing the development of diabetic retinopathy.

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Proliferative diabetic retinopathy (PDR) remains the leading cause of new blindness among working-age individuals in developed countries. Vitreous fluid obtained from diabetic patients with PDR undergoing vitreoretinal surgery is currently used in clinical research as a surrogate for the events that are taking place in the retina. Nondiabetic eye donors or nondiabetic patients in whom vitrectomy is indicated for conditions in which the retina is not directly affected by neovascularization, such as idiopathic macular hole (MH), could serve as a control group. However, 3 confounding factors could lead to misinterpretation of the results. First, vitreous hemorrhage, which often occurs in PDR, can produce a massive influx of serum proteins into the vitreous, thus precluding the usefulness of the vitreous fluid when studying the intraocular production of a particular protein. Second, the disruption of the blood-retinal barrier that occurs in diabetic retinopathy increases protein levels in the vitreous body of diabetic patients. Therefore, the elevated intravitreous level of any particular protein does not necessarily suggest intraocular production, and might reflect only the nonspecific increase in protein levels that results from serum diffusion. Third, serum levels of a specific protein could influence their vitreous concentrations and, in consequence, this event should be considered in the design of the study and in the analysis of the results. Taking all these confounding factors into account, we have recently compared the protein profiles of human vitreous from diabetic patients with PDR with the vitreous fluid obtained from nondiabetic patients with MH using the fluorescence-based difference gel electrophoresis (DIGE) strategy. This method provided an accurate quantitative comparison between the 2 groups of samples, with apolipoprotein (apo) A-I and apo H levels among the highly expressed proteins in the vitreous fluid of patients with PDR.

In the present study, apart from confirming the higher abundance of apo A-I and apo H in the vitreous fluid of pa-
tients with PDR, we provide evidence that levels of apo A-I and apo H messenger RNA (mRNA) are higher in the retina of diabetic patients who did not have clinically detectable diabetic retinopathy. These findings point to intracellularly produced lipoproteins as new factors involved in the physiopathologic mechanisms of diabetic retinopathy.

**METHODS**

**SUBJECTS AND SAMPLE SELECTION**

Samples of vitreous from 4 patients with type 1 diabetes mellitus and active PDR and from 8 nondiabetic patients with MH (control group) who were closely matched by age (mean [SD], 48.7 [11.2] vs 47.8 [10.4] years; P = .76) were selected from our vitreous bank for proteomic analysis. Among the patients with MH, the condition was of traumatic origin in 2 and idiopathic in the others. Because protein content in patients with MH is lower than in patients with PDR, the control samples had to be prepared as pools of 2 vitreous samples each to provide enough protein for the analysis.

Fourteen additional samples (from 7 patients with PDR and 7 with MH) were used for Western blot analysis. The exclusion criteria were previous vitreoretinal surgery, photoacoagulation in the preceding 6 months, recent vitreous hemorrhage (<3 months before vitrectomy) or intravitreous hemoglobin level higher than 5 mg/mL, renal failure (creatinine level ≥ 1.4 mg/dL [to convert to micromoles per liter, multiply by 88.4]), and other chronic diseases apart from diabetes. Details of the vitrectomy and sample collection have been previously described elsewhere. Briefly, a classic 3-port pars plana vitrectomy was performed. Undiluted vitreous samples (approximately 1 mL total) were obtained at the onset of vitrectomy by means of aspiration into a 1-mL (20-gauge) syringe attached to the vitreous cutter (Ten-Thousand Ocutome; Alcon, Irvine, California) before starting an intravitreous infusion of balanced salt solution. The vitreous samples were transferred to a tube, immediately placed on ice, and centrifuged at 16,000g for 5 minutes at 4°C. Supernatants were frozen at −80°C until assayed. For serum determinations, blood samples were collected simultaneously with the vitreous, then centrifuged at 3,000g for 10 minutes at 4°C to obtain serum, divided into aliquots, and stored at −80°C until assayed.

Seven postmortem eyes were obtained from diabetic donors who were free of funduscopic abnormalities in the ophthalmologic examination performed during the preceding 2 years. Seven eye cups obtained from nondiabetic donors were used as the control group. Both groups were matched by mean (SD) age (diabetic donors, 72.2 [2.9] years, and nondiabetic donors, 70.6 [5.7] years; P = .63). The time elapsed from death to eye enucleation was less than 6 hours. After enucleation, eyes were snap frozen in liquid nitrogen and stored at −80°C.

The protocol for sample collection was approved by the hospital ethics committee, and informed consent was obtained from patients. The study was conducted under the auspices of the human studies review board of our institution and in accordance with the principles expressed in the Declaration of Helsinki.

**VITREOUS HEMOGLOBIN LEVELS**

Vitreous hemoglobin levels were measured with a spectrophotometer (Uvikon 860; Kontron Instruments, Zurich, Switzerland) using the classic method of Harboe5 for measuring plasma hemoglobin levels in micromolar concentration. This method has been further validated, and in our studies, the lowest limit of detection was 0.03 mg/mL.

**SAMPLE PREPARATION, DIGE PROTEOMIC ANALYSIS, AND PROTEIN IDENTIFICATION**

Sample preparation and the details of DIGE proteomic analysis and protein identification by mass spectrometry have been described elsewhere. Briefly, samples for proteomic analysis were concentrated by ultrafiltration. Affinity chromatography was used to remove albumin and IgG from samples, which were then further purified by acetone precipitation.

The protein concentration was adjusted to 2 mg/mL (to convert protein to grams per liter, multiply by 1) by the addition of a DIGE labeling buffer. A pool consisting of equal amounts of each of the samples analyzed in the experiment was prepared to be used as an internal standard for quantitative comparisons. To avoid any possible bias derived from labeling efficiency, half of the samples of each group were labeled with Cy3 dye and the other half with Cy5 dye. A third fluorescent dye, Cy2, was used to label the internal standard sample. One sample from a patient with PDR and 1 from a patient with MH, together with an aliquot of the internal standard pool, were then separated by means of 2-dimensional electrophoresis in each of the gels (Figure 1). This experimental design allows the accurate quantification and statistical assessment of the differences in protein abundances observed between the 2 sample groups.

We performed 2-dimensional electrophoresis using commercially available reagents and equipment, including fluorescence images of the gels obtained on a scanner (Typhoon 9400; GE Healthcare, Fairfield, Connecticut). Image analysis and statistical quantification of relative protein abundances were performed using software from the manufacturer (DeCyder, version 5.0; GE Healthcare).

We excised protein spots of interest from the gel using an automated system (Spot Picker; GE Healthcare). In-gel trypsin digestion was performed as described using autolysis-stabilized trypsin (Promega, Madison, Wisconsin). Tryptic digests were purified using microtiter plates (Ziptip; Millipore Corporation, Billerica, Massachusetts). We used peptide mass fingerprint by matrix-assisted laser desorption ionization—mass spectrometry for protein identification.

**WESTERN BLOT ANALYSIS**

Vitreous and serum samples were transferred into a lysis buffer (1% Triton X-100, 100 mM Tris hydrochloride [pH, 7.5], 150 mM sodium chloride, 1.5 mM EDTA [pH, 8.0], 0.1 mM phenylmethylsulfonyl fluoride, and 1 tablet of protease inhibitors per 10 mL of buffer) (Complete Mini protease inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany) and then homogenized by means of sonication for 30 seconds. The homogenates were incubated on ice for 30 minutes and centrifuged at 15,000 rpm at 4°C for 10 minutes. The protein concentration of the supernatant was determined using Bradford assay (Bio-Rad Laboratories GmbH, München, Germany). For normalizing the protein levels, the same amounts (10 µg for apo A-I and 20 µg for apo H) of soluble proteins from vitreous fluid were loaded into the gel.

Samples and recombinant human apo A-I (PeproTech EC, London, England) and apo H (Calbiochem, Darmstadt, Germany) protein were mixed with Laemmli sample buffer (Bio-Rad Laboratories GmbH) and boiled for 10 minutes. Protein samples were resolved by 12% sodium dodecyl sulfate—polyacrylamide gel electrophoresis. After electrophoretic separation, the proteins were transferred onto polyvinylidene fluoro-
mRNA ISOLATION AND COMPLEMENTARY DNA SYNTHESIS

Neuroretina and retinal pigment epithelium (RPE) were harvested under the microscopic dissection of isolated right eye cups from the donors. Poly A⁺ mRNA from tissue samples was isolated using a magnetic bead-based separation technology (Dynabeads oligo(dT)₃₀; Invitrogen, Eugene, Oregon). The mRNA concentration was determined by spectrophotometric measures at 260 and 280 nm. Poly A⁺ mRNA was divided into 200-ng aliquots that were reverse transcribed using the cloned avian myeloblastosis virus first-strand complementary DNA (cDNA) synthesis kit (Invitrogen) following the manufacturer’s protocol for oligo(dT)₃₀ priming. The resulting cDNA was then used in semiquantitative and real-time polymerase chain reaction (PCR) analyses.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from isolated retinal tissues using a commercially available kit (RNasyMini Kit with DNase digestion; Qiagen Distributors, IZASA, Barcelona, Spain) according to the manufacturer’s instructions. A 1-µg aliquot of total RNA was used directly for reverse transcription, which was performed using random hexanucleotide priming and reverse transcription reagents (TaqMan; Applied Biosystems, Madrid, Spain) in a 50-µL reaction according to the protocol provided by the manufacturer.

Quantitative real-time PCR analysis using specific primers (TaqMan premade gene expression assay Hs0017267 [Gene Bank RfSeq NM 000799.2]; Applied Biosystems) was performed taking 2 µL of the reverse transcription reaction product as a template in a PCR setup with a PCR mix (TaqMan Universal MasterMix; Applied Biosystems). The reaction was conducted as follows: 95°C for 10 minutes, 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C in a sequence detection system (Applied Biosystems). Each sample was assayed in duplicate and control negative samples were included in each experiment. Automatic relative quantification data were obtained using the manufacturer’s software (ABI Prism 7000 SDS; Applied Biosystems), with β-actin as an endogenous gene expression control (Hs9999903_m1; Applied Biosystems).

STATISTICAL ANALYSIS

We used the Kolmogorov-Smirnov test to confirm the assumption of the normality of the variables. The unpaired (2-tailed) t test was used to compare continuous variables that were expressed as mean (SD). Because of their skewed distribution, mRNA values were displayed as median and range, and the statistical comparisons were performed using a nonparametric test (Mann-Whitney test). Levels of statistical significance were set at P < .05.

RESULTS

DIGE AND WESTERN BLOT ANALYSES

The results of apo A-I and apo H proteomic analysis by DIGE are summarized in Figure 1. To further validate these results, apart from samples used in DIGE, we used Western blot to assess the additional sets of 7 PDR and 7 MH vitreous samples. Intravitreous mean levels of apo A-I and apo H were significantly higher in the samples from patients with PDR than in the control group (0.29
The expression of β-actin mRNA was similar in the RPE and the neuroretina \( (P = .94) \). In addition, no differences were observed in β-actin mRNA expression between retinas from diabetic and nondiabetic donors \( (P = .88) \). Thus, we have calculated apo A-I and apo H mRNA gene expression after normalizing with β-actin.

Expression of apo A-I and apo H mRNA was detected in the retinas from both nondiabetic and diabetic donors. The comparative apo A-I and apo H mRNA expression in retinas from diabetic and nondiabetic donors is shown in Figure 3. A higher expression of both apo A-I and apo H mRNA was found in the RPE than in the neuroretina in diabetic donors \( (P = .04) \). No differences in serum abundance were observed for apo A-I \( (3.09 \pm 2.46) \) vs \( 3.10 \pm 2.48 \) \( (P = .99) \) or apo H \( (0.39 \pm 0.08) \) vs \( 0.38 \pm 0.13 \) \( (P = .77) \). St indicates internal standard.

In recent years, accumulating evidence has indicated that lipoprotein deposition plays an essential role in the pathogenesis of age-related macular degeneration.6-14 However, little is know about the origin of lipoproteins in the retina of diabetic patients and their potential role in the pathogenesis of diabetic retinopathy. In the present study, after considering the confounding factors that could lead to misinterpretation of the results when vitreous fluid is used as a surrogate for the events that are taking place in the retina, we have found that apo A-I and apo H levels are significantly increased in the vitreous fluid of patients with PDR. In this regard, apo A-I and apo H have also been characterized as major lipoproteins in the human cerebrospinal fluid.13 In the proteomic analysis of the vitreous fluid, apart from apo A-I and apo H, we also found significantly increased levels of the following proteins in patients with PDR: fibrinogen A, zinc α2-glycoprotein, complement factor B, complement C3, complement C9, and complement C4b. In contrast, levels of pigment epithelial derived factor, interstitial retinol-1-binding protein, and inter-α-trypsin inhibitor heavy chain-2 were significantly decreased in patients with PDR.3

In addition, and more important, we provided, to our knowledge, the first evidence that a significant increase of both apo A-I and apo H mRNA levels exists in the diabetic retina. It must be emphasized that the diabetic donors were found to be free of microvascular abnormalities in the ophthalmologic examinations performed in the 2 years before death, thus suggesting that the enhancement of apo A-I and apo H is an early event in the eyes of diabetic patients. Furthermore, we found RPE to be an important source of apo A-I and apo H and the main contributor to the differences observed between diabetic and nondiabetic donors.

The major apoprotein of high-density lipoprotein, apo A-I, has been classically described as being synthesized only by the liver and intestine.10 However, in recent years, apo A-I has been found in the vitreous fluid17 and in human...
man RPE cells. In addition, apo A-I mRNA has been recently detected in the RPE and retina in humans. In the present study, we have shown that apo A-I levels were significantly higher in the vitreous of patients with PDR than in nondiabetic patients with MH, and a higher expression of mRNA was also detected in diabetic retinas.

The transcriptional control of apo A-I gene expression in diabetes has been intensively studied in the liver of rats with streptozotocin-induced diabetes and in cultures of the human hepatoma cell line HepG2, and there is agreement that hyperglycemia downregulates apo A-I mRNA expression. However, the effect of hyperglycemia on apo A-I expression in other tissues such as the RPE and the neuroretina is still unknown. In this regard, it should be mentioned that Kawai et al observed an increased secretion of native apo A-I from the main lacrimal gland in patients with diabetic retinopathy, but it was not detected in healthy subjects. In addition, the concentration of apo A-I in the tears was significantly correlated with the stage of diabetic retinopathy. Therefore, it could be postulated that the diabetic milieu could stimulate apo A-I expression by the retina. Because apo A-I is a potent scavenger of oxygen-reactive species, it may play an important role in protecting the retina from oxidative stress due to diabetes.

Apolipoprotein H, also known as β2-glycoprotein I, is a soluble 54-kDa plasma glycoprotein, primarily synthesized in the liver. The physiological role of apo H is still uncertain. However, apo H inhibits the activation of the intrinsic pathway of blood coagulation and the prothrombinase activity of activated platelets by covering negatively charged surfaces necessary for both activities. It is also thought to be involved in several thrombosis-related diseases, in particular the antiphospholipid syndrome where apo H is a target of a specific autoantibody response. A substantial amount of apo H is bound to lipoprotein particles and has been suggested to be involved in triglyceride metabolism. Apolipoprotein H participates in autoimmune diseases, thrombosis, atherosclerosis, and inflammatory processes. Expression of apo H has been identified in the photoreceptor layer of dog retina. Recently, by means of proteomic analysis, Ouchi et al have found that apo H was expressed in the vitreous fluid of patients with PDR with concomitant macular edema. Patients with PDR included in the present study did not have macular edema, but optical coherence tomography was not performed and, therefore, we cannot rule out the existence of a discrete, nonclinically significant macular edema in these patients.

To our knowledge, no experimental studies have evaluated the effect of glucose on apo H expression, and only 1 study has shown a direct relationship between apo H and blood glucose levels in healthy individuals. The role of apo H in the pathogenesis of diabetic retinopathy remains to be elucidated, but it could be related to complement activation or its potential role in the recognition of dying cells. In this regard, apo H contains short consensus repeats that are essential for complement activation, which is involved in diabetic retinopathy development. Colocalization of activated complement and apo H exists in the human myocardium, and apo H has been proposed as a new player in the inflammatory changes ensuing in infarcted myocardium. In addition, apo H binds apoptotic cells in vivo, and this might trigger or enhance complement activation, thus facilitating the recognition and clearance of apoptotic cells. Furthermore, apo H inhibits the apoptosis-related events in vascular cells. Given that apoptosis is an early event in the pathogenesis of diabetic retinopathy, the elevated apo H expression detected in the retinas from diabetic patients could be involved not only in the clearance of retinal apoptotic cells but also in preventing apoptosis.

Although lipoprotein assembly and secretion is a constitutive retinal function and the role and the significance of apo A-I and apo H in the setting of diabetic retinopathy require further investigation. The neuroretina is rich in lipids and, therefore, one must consider the possible role of these lipoproteins in supplying the needs of neurons for lipids, especially the long axonal projection neurons such as retinal ganglion cells, which are vulnerable to metabolic mediators related to diabetes. Another point of interest could be the potential relationship between the increase in apolipoprotein levels and the presence of hard exudates. It has been suggested that elevated lipid levels in systemic circulation constitute a risk factor for diabetic retinopathy. However, results have been inconsistent, there are few prospective studies, and it is unclear to what extent some previously observed relationship may be confounded by the degree of hyperglycemia. In a recent prospective study, Miljanovic et al found a relationship between serum lipid levels and increased risk of clinically significant macular edema and hard exudates but no relationship between serum lipid levels and progression of diabetic retinopathy or the development of PDR. In another prospective study, Leung et al concluded that serum lipid levels are not related to microvascular disease in the retina. Our findings allow us to hypothesize that intraocular rather than bloodborne lipids might be more important in the pathogenesis of diabetic retinopathy. However, experimental studies are needed to address this hypothesis.

In conclusion, levels of apo A-I and apo H are elevated in the vitreous fluid of diabetic patients with PDR. In addition, a higher expression of apo A-I and apo H exists in retinas from diabetic donors who did not have clinically detectable retinopathy compared with retinas from nondiabetic donors. Further studies to investigate the role of apo A-I and apo H not only in the pathogenesis of diabetic retinopathy but also as new therapeutic strategies are warranted.

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