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Comparison of tear proteins between healthy and early diabetic retinopathy patients

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ABSTRACT

Objectives: To identify potential prognostic or diagnostic marker tear proteins for early diabetic retinopathy (DR) and to investigate the pathogenesis of this disease using proteomics techniques.

Design and methods: The tear proteins expressed in patients suffering from diabetes mellitus without the retinopathy symptoms, nonproliferative diabetic retinopathy and healthy volunteers were analyzed by 2-DE. The differentially expressed proteins in patients were identified by ESI-Q-TOF and confirmed by Western blotting.

Results: Proteins which were differentially expressed with statistical significance ($P<0.05$) in two diabetic groups as compared to those in healthy group were selected and identified by ESI-Q-TOF MS/MS. Among these proteins, three proteins (LCN-1, HSP27 and B2M) were found to exhibit a progressive reduction in two disease groups. The expression levels of which might be useful as diagnostic biomarkers of DR were verified by Western blotting

Conclusions: Proteomic analysis using tear is a novel approach that can provide insight into possible biomarker and the pathogenesis of early DR.

Introduction

Some levels of tear proteins have been shown to be associated with various pathological conditions [1,2]. Tear proteins have been investigated extensively in the past but only with the goal of identifying the relationship between the concentrations of single tear proteins and ocular surface disorders [3–5]. Recent two-dimensional gel electrophoresis (2-DE) analysis of the tear proteins has provided more dedicated information on the composition of proteins in tears [6–8]. More recently, tear sample preparation method for 2-DE analysis have optimized and protein profile of tear fluid from healthy males and females has been determined [9]. In addition, tear proteomics has been previously applied to Meibomian gland disease, dry eye symptoms [10,11] and keratoconus [12].

Diabetic retinopathy (DR) is the most common microvascular complication caused by diabetes mellitus (DM), which can eventually lead to blindness [13,14]. The initial clinical stages of DR are characterized by the development of microaneurysms, retinal hemorrhages, hard exudates, and intraretinal microvascular abnormalities [13]. Although diagnosis of DR is difficult due to a lack of noticeable symptoms in its initial stage, known as nonproliferative diabetic retinopathy (NPDR), it has been reported to breakdown the blood–retinal barrier triggering increased vascular permeability, which causes macular edema. Further progression of DR results in the development of vascular microaneurysms, deposition of lipoprotein exudates, which are known as drusen, and vascular proliferation [15].

Although patients do not notice significant vision loss during NPDR, macular edema, which occurs during NPDR, is most closely correlated with the degree of vision loss [16]. As a result of vascular permeability, water and exudates will leak out of the vessels. Although water is quickly reabsorbed into the vessels, or into the tissue under the retina, fatty materials of the exudates are absorbed slowly. These fatty exudates that are left behind form ring-like shape surrounding the leakage site [17,18]. Left over residues cause macula to swell, which can blur vision. As the disease progresses, severe NPDR enters an advanced, or proliferative diabetic retinopathy (PDR) [18], which can cause new fragile blood vessels to grow along the retina. This process results in the formation of a clear, gel-like vitreous that fills the inside of the eye. Without timely treatment, these new blood vessels can bleed, cloud vision, and destroy the retina.

Therefore, we have utilized 2-DE technique to identify human tear protein markers for the more specific and accurate prediction of progressive retinopathy in patients with diabetes. Also, differentially expressed proteins were identified by ESI-MS/MS analysis, and the proteins which might be utilized as diagnostic biomarkers for type 2 DR were confirmed via Western blotting.

Abbreviations: 2-DE, Two-dimensional electrophoresis; DMR, Diabetes mellitus without the retinopathy; NPDR, Nonproliferative diabetic retinopathy; ESI-Q-TOF, Electrospray ionization quadrupole time-of-flight; IEF, Isoelectric focusing; IPG, Immobilized pH gradient; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, Dithiothreitol; BPB, Bromophenol blue; PAGE, Polyacrylamide gel electrophoresis; pI, Isoelectric point; HbA1c, Glycated hemoglobin; LCN-1, Lipocalin-1; HSP 27, Heat shock protein 27; B2M, Beta-2 microglobulin.

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Material and methods

Subjects

This research followed the tents of the Declaration of Helsinki. The tear samples were obtained from patients with diabetes mellitus (no DMR, n = 10), NPDR patients (NPDR, n = 17) and healthy volunteers (Healthy, n = 14), respectively. They were obtained from the Department of Ophthalmology, College of Medicine, Chung-Ang University. The protocols of this study were approved by the Chung-Ang University Hospital Institutional Review Board (IRB) with the informed consent of the patients. The basal characteristics for the Healthy subjects, DM and NPDR patients are summarized in Table 1.

Sample preparation

The tear fluid was collected from the interior tear meniscus, causing the lease irritation possible, using a preweighed polyester wick (Transorb rods; American Filtrona, Richmond, VA, USA) to obtain the sample as previously described [19]. Wicks were then placed into the end of a micropipette tip located within a 1.5 mL tube and centrifuged at 10,000 rpm for 5 min. The concentration of the protein in the sample was measured using the modified Bradford method [20]. The tear samples were then stored at −70 °C and thawed only once prior to analysis.

2-DE

The samples (Healthy, n = 14; no DMR, n = 10; NPDR, n = 17) were not pooled and no buffer except a rehydration solution was added for IEF. In addition, no additional sample preparation steps were used for 2-DE analysis. Sufficient resolving power and reproducibility are also needed for comparative proteome analysis by 2-DE. Ready-to-use Immobiline DryStrips (24 cm, pH 3–10 NL) were used for IEF. DryStrips were rehydrated with the sample (60 μg of protein) in 450 L of the rehydration solution containing 8 M urea, 2% CHAPS, 1% IPG buffer (pH 3–10 NL), 65 mM DTT and a trace amount of BPB for 5 h without current and 5 h with a current of 80 V. IEF was carried out for a total of 100,000 Vhr using the IPGphor IEF system (GE Healthcare, Uppsala, Sweden). Following IEF separation, the IEF gel strips were equilibrated in two equilibration solutions for 15 min with gentle shaking. The first equilibration solution contained 50 mM Tris–HCl (pH 8.8) with 6 M urea, 20% glycerol, 2% SDS, and 1% DTT. In the second equilibration solution, the DTT was replaced with 2.5% iodoacetamide. The IEF strip was applied onto 11–16% gradient SDS-PAGE gel. The second-dimensional SDS-PAGE was performed using the Ettan DALT II (GE Healthcare, Uppsala, Sweden) at a constant voltage of 70 V for 1 h, 140 V 2 h and 320 V for 5 h.

Image analysis

Proteins were visualized by the silver staining method with some modifications [21]. The gels were fixed in 50% methanol, 12% acetic acid and 0.05% formaldehyde (37%) for at least 2 h. Then, the fixed gels were sensitized by incubating in 0.02% sodium thiosulfate, and subsequently, were immersed in 0.1% silver nitrate for 20 min. The development stage was carried out with 6% sodium carbonate and 0.05% formaldehyde (8 min, 4 °C). Finally, the reaction was terminated by fixing with 50% methanol and 12% acetic acid. All staining procedures were conducted at over the same time and temperature. Digitalized images of the silver stained gels were analyzed using the ImageMaster 2D Platinum version 6.0 (GE Healthcare, Uppsala, Sweden). Computer analysis of the 2-DE image was carried out using Image Master 2D Elite Software (GE Healthcare, Uppsala, Sweden). Expression levels of the spots were determined by the volume of each spot divided by the total volume of all of the spots in the gel, this technique is called Total Spot Volume Normalization by background subtraction [22]. For each protein spot, one-way analysis of variance (ANOVA) was performed with Bonferroni's test to identify the significant differences between three groups using the SPSS 12.0 for Windows computer software package (SPSS, Chicago, IL, USA).

Table 1
Clinical characteristics in the study groups.

Characteristic	Healthy group	No DMR group	NPDR group
Gender (M/F)	8/6	5/5	10/7
Age (year)	28 ± 5.12	60.7 ± 12.21	66.25 ± 6.64
BMI (kg/m^2)	21.24 ± 4.42	23.72 ± 2.89	24.19 ± 2.82
HbA1c (%)	NA	8.11 ± 1.67	9.01 ± 2.72
Diabetes (type)	0	All 2	All 2
Nephropathy (*n*)	0	3	3

Data are expressed as mean ± SD. NA, not applicable.

Mass spectrometric identification

Proteins were subjected to in-gel trypsin digestion. Excised gel spots were destained with 100 μL of destain solution with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with acetonitrile and dried in a vacuum centrifuge. The dried gel pieces were rehydrated with 50 mM ammonium bicarbonate containing 0.2 μg modified trypsin (Promega, Madison, WI) for 45 min on ice. After removal of the solution, 50 mM ammonium bicarbonate was added. A column consisting of 100–300 nL of Poros reverse phase R2 material (20–30 μm bead size, PerSeptive Biosystems, Framingham, MA, USA) was packed in a constricted GELoader tip (Eppendorf, Hamburg, Germany). Thirty microliters of the peptide mixture from the digestion supernatant was diluted in 5% formic acid, loaded onto the column, and washed with 5% formic acid. For analyses by MS/MS, peptides were eluted with 50% methanol, 49% H_2O, 1% formic acid directly into a precoated borosilicate nanoelectrospray needle (Micromass, Manchester, UK). MS/MS analysis of the peptides generated by in-gel digestion was performed using a nano-ESI on a Q-TOF mass spectrometer (Micromass, Manchester, UK). Product ions were analyzed using an orthogonal TOF analyzer that was fitted with a reflector, a microchannel plate detector and a time-to-digital converter. The data were processed using a Mass Lynx Windows NT PC system (Micromass, Manchester, UK). For identification of proteins, all MS/MS spectra recorded on tryptic peptides derived from spot were searched against protein sequences from the NCBInr databases using the MASCOT search program. The search parameters used were as follows; MS/MS Ion Search in type of search, trypsin in enzyme, carbamidomethyl (c) in fixed modifications, oxidation (M) in variable modifications, monoisotopic in mass values, ±1 Da in peptide mass tolerance, ±0.8 Da in fragment mass tolerance and 1 in max missed cleavage. The highest score identification in the mascot search results was selected, considering species.

Western blot analysis

For Western blot, 40 μg for LCN-1 and HSP27 (Healthy, no DMR and NPDR, n = 9, respectively) and 50 μg for B2M (Healthy, DM and NPDR, n = 9, respectively) of the tear protein were separated by 12% (w/v) SDS-PAGE, and then blotted onto a nitrocellulose membrane. The membrane was incubated first with a blocking solution containing a 1:1000 dilution of anti-HSP 27 antibody (ABcam, Cambridge, UK), anti-B2M antibody (Biomeda, CA, USA) and a 1:500 dilution of anti-lipocalin antibody (R & D systems, Minneapolis, MN, USA) and then with a blocking solution containing a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G secondary antibody (Santa Cruz Biotechnology, CA, USA) and a 1:5000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG secondary