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Periostin, discovered by nano-flow liquid chromatography and mass spectrometry, is a novel marker of diabetic retinopathy

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ABSTRACT

Diabetes can lead to serious microvascular complications including proliferative diabetic retinopathy (PDR), the leading cause of blindness in adults. Recent studies using gene array technology have attempted to apply a hypothesis-generating approach to elucidate the pathogenesis of PDR, but these studies rely on mRNA differences, which may or may not be related to significant biological processes. To better understand the basic mechanisms of PDR and to identify potential new biomarkers, we performed shotgun liquid chromatography (LC)/tandem mass spectrometry (MS/MS) analysis on pooled protein extracts from neovascular membranes obtained from PDR specimens and compared the results with those from non-vascular epiretinal membrane (ERM) specimens. We detected 226 distinct proteins in neovascular membranes and 154 in ERM. Among these proteins, 102 were specific to neovascular membranes and 30 were specific to ERM. We identified a candidate marker, periostin, as well as several known PDR markers such as pigment epithelium-derived factor (PEDF). We then performed RT-PCR using these markers. The expression of periostin was significantly up-regulated in proliferative membrane specimens. Periostin induces cell attachment and spreading and plays a role in cell adhesion. Proteomic analysis by LC/MS/MS, which permits accurate quantitative comparison, was useful in identifying new candidates such as periostin potentially involved in the pathogenesis of PDR.

1. Introduction

Both neovascular membrane formation associated with proliferative diabetic retinopathy (PDR) and relatively non-vascular epiretinal membrane (ERM) may lead to significant visual loss. ERM, first described in 1865, is a fibrocellular membrane that proliferates on the inner surface of the retina and thereby produces visual impairments of various degrees. Clinically, if thin, ERM presents as a glistening light reflex, or, if thicker, as an obvious transparent, translucent, or even pigmented membrane on ophthalmoscopic examination [1,2]. Although peripheral ERMs can exist, macular ERMs cause more visual damage. The prevalence of macular ERMs increases with age (prevalence is 2% in individuals under the age of 60 years and 12% in those over 70 years) [1]. ERMs are typically idiopathic and are usually associated with posterior vitreous detachment (PVD). They can also be associated with retinal breaks, ocular inflammation, some congenital conditions, and ocular treatments such as post-retinal detachment repair surgery and cryotherapy [3]. ERMs are composed of glial cells, retinal pigment epithelial (RPE) cells, macrophages, fibrocytes, and extracellular matrix (ECM) proteins such as collagen and fibronectin (FN) [3,4]. ERM may contain many RPE cells with phenotypic alterations. They typically have no blood vessels and, except for some ERM in young patients, have no relation with retinal vasculature [5–8]. Fibroblastic cells are present in most contractile ERMs [9]. Various etiologic factors can, however, contribute to compositional variations.

In PDR, there is angiogenic and fibrovascular proliferation leading to severe vision loss due to hemorrhage and traction retinal detachment [10,11]. In PDR, several factors may be activated in retina secondary to hyperglycemia. Ultimately, there is retinal hypoxia leading to release of angiogenic factors causing neovascularization and fibrovascular proliferation [12,13].

A number of studies have identified factors associated with the pathogenesis of PDR, e.g., angiogenic factors such as vascular endothelial growth factor [14], angiotensin-converting enzyme [15],

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insulin-like growth factor (IGF) [16], angiopoietin [17], erythropoietin [18], placenta growth factor [19] and advanced glycation end product [20], as well as antiangiogenic factors such as pigment epithelium-derived factor (PEDF) [21]. However, the majority of previous studies focused on sets of targeted proteins, particularly the molecules involved in angiogenesis and cellular proliferation, making it difficult to evaluate changes in proliferative membrane protein profiles and to identify novel markers of PDR pathogenesis.

The recent development of proteome analysis has made it feasible to analyze protein profiles in various cells, tissues and body fluids with only a small sample [22]. The aim of the present study was to compare the protein profile of neovascular membrane specimens from diabetic patients with PDR to that of non-vascular ERM. As mentioned above, ERMs are composed of glial cells, RPE cells, macrophages, fibrocytes, and ECM proteins such as collagen and fibronection [23,24]. They typically have neither blood vessels nor any relation with the retinal vasculature [25,26]. In PDR, in contrast, there is angiogenic and fibrovascular proliferation. Type II collagen and fibroblast-like cells are prominent components of PDR. Thus, ERMs are usually used as controls when proliferative vascular diseases are investigated [23]. Therefore we performed the present study using ERM as the comparative material. To carry out this comparative proteomic analysis, we used nano-flow liquid chromatography and mass spectrometry and protein identification by tandem mass spectrometry (LC/MS/MS). This technique provides an accurate quantitative comparison of the two groups of samples, allowing the identification of proteins whose levels differ significantly between the two conditions. We also measured the mRNA of proteins differentially produced in neovascular membranes and ERMs.

2. Materials and methods

2.1. Subjects and sample collection

Thirteen patients with PDR and 13 with idiopathic ERM underwent vitrectomy during the period from May 2008 through October 2009 at Showa University Hospital or Kozawa Eye Hospital and Diabetes Center. The specimens obtained from patients with idiopathic ERMs secondary to DR, ocular sarcoidosis, retinal vein occlusion and other ocular abnormalities were excluded from this study. The clinical data of the patients are summarized in Table 1. All diabetic patients fulfilled the World Health Organization criteria for Type 2 diabetes. The duration of diabetes in all of these patients exceeded 6 years. In all samples, we used three cases for each LC/MS/MS analysis and the remainder for real-time (RT)-PCR analysis. All patients provided informed consent before enrollment in the study, in accordance with the protocol approved by the Institutional Review Board at Showa University Hospital.

2.2. Ophthalmologic examination

Fundoscopy, retinal photograph and/or fluorescein angiography after pupillary dilation were performed in all subjects. The diagnosis of DR was made by experienced ophthalmologists based on the presence of one or more of the following clinical features in the fundus: hemorrhages, hard or soft exudates, venous beading, intra-retinal microvascular abnormalities, cotton-wool spots, pre-retinal new vessels, fibrous proliferation and photo-coagulation scars. DR was graded based on the Davis classification as follows: no retinopathy, simple (background) retinopathy, and pre-proliferative and proliferative retinopathy [27].

2.3. Peptide extraction for LC/MS/MS analysis

Proteins from samples for LC/MS/MS were extracted using 10 μl of 8 M Urea solution with an ultrasonic homogenizer. After homogenization, 90 μl of 90% 100 mM Ammonium Bicarbonate Buffer (ABB: pH 8.0)/10% Acetonitrile were added, followed by the addition of 4 μl of 100 mM dithiothreitol in ABB. The samples were incubated at 37 °C for 60 min and returned to room temperature. Next, 10 μl of 100 mM iodoacetamide in ABB were added and the samples were incubated at 37 °C for 30 min in the dark. Finally, proteins in the samples were digested with Trypsin (15–18 units) by overnight incubation at 37 °C. After extraction, all samples were stored at −20 °C until LC/MS/MS analysis.

2.4. Shotgun liquid chromatography (LC)/tandem mass spectrometry (MS/MS)

Peptide-mixture samples processed from each tissue were used for nano-flow reverse phase liquid chromatography followed by tandem MS, using an LTQ linear ion-trap mass spectrometer (Thermo Fischer, San Jose, CA, USA). A capillary reverse phase HPLC–MS/MS system (ZAPLOUS System™; AMR, Tokyo, Japan) composed of a Paradigm MS4 dual solvent delivery system (Michrom BioResources, Auburn, CA, USA), an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), and Finnigan LTQ linear ion-trap mass spectrometers (ITMS; Thermo Fischer, San Jose, CA, USA) equipped with an XYZ nanoelectrospray ionization (NSI) source (AMR, Tokyo, Japan). All samples were evaporated, and peptides were redissolved with MS-grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent A). Aliquots of 10 μL (equivalent to 1 μg of proteins) were automatically injected into a peptide Captrap column (Michrom BioResources) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with solvent A, the peptides were loaded into a separation capillary reverse phase column (Mono Cap 150 x 0.2 mm, GL Sciences) by switching the valve. The following eluents used were: A, 98% H_2O /2% acetonitrile/0.1% formic acid, and B, 10% H_2O/90% acetonitrile/0.1% formic acid. The column was developed at a flow rate of ~1 μL/min with the concentration gradient of acetonitrile, as follows: first, from 5% B to 55% B in 100 min, then from 55% B to 95% B in 1 min, maintenance at 95% B for 9 min, then from 95% B to 5% B in 3 min, and finally re-equilibration with 5% B for 15 min. Effluents were introduced into the mass spectrometer via the NSI interface that had a separation column outlet connected directly with an NSI needle (150 μm OD/20 μm ID FortisTip; OmniSeparo-TJ, Hyogo, Japan) [28]. The ESI voltage was 2.0 kV and the transfer capillary of the LTQ inlet was heated at 200 °C. No sheath or auxiliary gas was used. The mass spectrometer was operated in the z range of 450–1800 in a data-dependent acquisition mode, in which detecting the most abundant ions at a retention time automatically acquires MS/MS scans for those ions

Table 1
Collection of membrane samples from PDR and ERM patients and clinical characteristics of the patients.

Sample set (patient numbers)	Mean age (range)	Number of females	BMI (kg/m^2)	HbA1c (%)	FPG (mg/dl)
PDR for LC/MS/MS (n = 3)	58.0 ± 7.0 (50–63)	1	25.2 ± 3.2	8.26 ± 1.22	189 ± 45.0
ERM for LC/MS/MS (n = 3)	67.6 ± 12.5 (56–81)	3	22.5 ± 3.0	–	93.3 ± 8.7
PDR for RT-PCR (n = 10)	46.6 ± 14.3 (30–64)	3	26.7 ± 4.3	8.1 ± 2.3	201.5 ± 83.0
ERM for RT-PCR (n = 10)	65.2 ± 9.9 (48–74)	6	23.7 ± 3.4	–	96.1 ± 14.6

BMI: body mass index, HbA1c: glycosylated hemoglobin, FPG: fasting plasma glucose.