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Vitreous Proteomics and Diabetic Retinopathy

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Abstract

Diabetic retinopathy is the major cause of acquired blindness in working age adults. Studies of the vitreous proteome have provided insights into the etiology of diabetic retinopathy and suggested potential molecular targets for treatments. Further characterization of the protein changes associated with the progression of this disease may suggest additional therapeutic approaches as well as reveal novel factors that may be useful in predicting risk and functional outcomes of interventional therapies. This article provides an overview of the various techniques used for proteomic analysis of the vitreous and details results from studies evaluating vitreous of diabetic patients using the proteomic approach.

Keywords

Diabetic retinopathy; mass spectrometry; proteomics; vitreous

Introduction

The vitreous is the largest component structure of the eye. In its normal state, it is a clear gelatinous matrix between the lens and retina that is primarily composed of water, collagen, glycosaminoglycans, and proteoglycans.¹ In addition to its optical functions, the vitreous also contains a plethora of factors that can influence retinal physiology, including growth factors, hormones, proteins with transporter activity, and enzymes. A limited number of studies have identified protein changes in the vitreous that are associated with retinal disorders. These factors may alter the physiochemical properties of this matrix and affect processes occurring in the retina as well as other structures in contact with, or adjacent to, the vitreous. Further understanding of the changes in the composition of the vitreous proteome during the course of diabetic retinopathy may provide new insights into the pathogenesis of this disease and suggest therapeutic opportunities.

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Historical Perspective

Prior to the '-omic' era, levels of candidate individual proteins in the vitreous of patients with diabetic retinopathy were compared to control subjects without diabetes using biochemical or immunological techniques, including enzymology, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), and immunoblotting. These studies have implicated numerous molecules as potential mediators of diabetic retinal vascular complications, including basic fibroblastic growth factor (bFGF)², insulin-like growth factor -1 (IGF-1),^{3,4} connective tissue growth factor (CTGF),⁵ hepatocyte growth factor (HGF),^{6,7} and vascular endothelial growth factor (VEGF)^{8,9}. Clinical trials are currently underway to investigate the effect of intravitreal injections of anti VEGF agents on proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME)^{10–13}. Current trials also include RESOLVE trial, RIDE and RISE trial, DAVINCI trial and DRCR protocol I.

Proteomic analysis of vitreous

Mass spectrometry-based proteomic technologies have facilitated the *de novo* identification and quantification of a large number of proteins within a relatively small sample. Proteomic studies encompass the identification of proteins based on amino acid sequence, measurements of protein abundance, and characterization of post-translational modifications.¹⁴ Proteomics involves a multi-step process, including sample acquisition, protein prefractionation, peptide separation and mass spectrometry, and data analysis and interpretation. Each step can utilize a variety of experimental strategies, each which provides opportunities and limitations. An example of a vitreous proteomic workflow is described below (Figure 1).

Vitreous Samples

Vitreous samples for proteomic analysis are generally obtained during pars plana vitrectomy. The major reasons for pars plana vitrectomy in patients with diabetic retinopathy include non-clearing vitreous hemorrhage, tractional retinal detachment involving or threatening the macula, or combined rhegmatogenous / tractional retinal detachment.¹⁵ The impact of these comorbidities on the vitreous proteome are not fully understood.

Prefractionation

Vitreous proteomic experiments typically start with the separation of proteins using either one-dimensional gel electrophoresis (1DE) or two-dimensional gel electrophoresis (2DE). Liquid chromatography (LC) and protein microarray can also be used.^{16,17} The purpose of gel-based separation is to fractionate and concentrate proteins based on electrophoretic mobility. After separation, the proteins within gel fractions are digested with trypsin, which cleaves at Lysine and Arginine residues. This process generates a mixture of peptides containing short segments of protein sequences. These peptide mixtures are then extracted and subjected to mass spectrometry (MS).

Mass spectrometry

There are a number of different types of MS systems current in use (see ref 18 for review). Two of the most widely used systems are matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and LC-tandem mass spectrometry (MS/MS). Moreover, there are a number of variations in the configuration and technologies for both of the proteomic platforms. Proteomics using one of these systems, LC-MS/MS, will be briefly described to illustrate an example of mass spectrometry-based protein identification. Tryptic digests from isolated proteins are separated by capillary LC and subjected to electron spray ionization prior to mass spectrometry. The detector in the mass spectrometer measures the abundance of peptides that are resolved according to a ratio of mass over charge (m/z). Selected peptides are then isolated and fragmented by collision-induced dissociation (CID) and the abundance of peptide fragments are resolved and detected according to their m/z . (Figure 1)

Data Analysis

The m/z of the precursor ion as well as the m/z of fragments of this precursor provide information that is used to derive its amino acid sequence. Algorithms that match mass spectral data to available protein amino acid sequences are used to perform this protein assignment process (Reviewed in Ref 19). These algorithms perform a comparison of the experimentally determined MS peak mass values with the predicted molecular mass values of the peptides generated in silico by a theoretical digestion of each protein in a database. Comparisons are also made between the experimentally observed CID fragment ions and predicted fragments for peptides of the appropriate m/z , based on defined fragmentation parameters. This algorithm can generate both false negative and false positive assignments, which are influenced by the stringency of spectra to sequence matching criteria. An estimate of the rate of false positive assignments using specific matching criteria can be estimated using decoy database.²⁰ The level of false negative is determined by a variety of factors, including sample composition, peptide abundance, and the analysis algorithm and criteria.

There are a number of methods used to compare the relative abundance of proteins for comparison among samples. The most widely used methods to quantify protein abundance include measurement of protein staining intensity in SDS-PAGE and measurements of the abundance of tryptic peptides using isotope-labeling or label-free techniques (reviewed in ref 21).

The availability of vitreous protein inventories and changes in abundance associated with diabetic retinopathy creates opportunities for large-scale analyses of these data to further characterize the properties and functions of the vitreous proteome. Bioinformatics concentrates on techniques facilitating the acquisition, storage, organization, archiving, analysis and visualization of biological and medical data.^{23, 24} Identified proteins can be grouped and analyzed according to a variety of annotations. The gene ontology provides a systemic language, or ontology for the consistent description of attributes of genes and gene products, in three key biological domains – molecular function, biological process and cellular component²². Bioinformatic tools that have been applied to the vitreous proteomics for diabetic retinal disease include the Database for Annotation, Visualization and Integrated Discovery (DAVID).^{20,25}

The vitreous proteome in Diabetic Retinopathy

One of the first studies using mass spectrometry-based proteomics to characterize human vitreous proteome in diabetic retinopathy was reported by Shimizu et al.²⁶ This study, using 2D-PAGE, silver staining to evaluate protein abundance and MS-based identification of selected proteins, identified 35 proteins in vitreous which had not been reported in plasma. This study reported increased levels of pigment epithelial-derived factor (PEDF) in the vitreous of diabetic patients with proliferative angiogenesis. Gao et al^{20,27} used 1D-SDS-PAGE and LC-MS/MS to characterize and compare the vitreous proteomes from people without diabetes (NDM), people with diabetes but no diabetic retinopathy (noDR), and people with PDR. These studies identified 252 proteins in the vitreous from the 3 groups of patients and fifty-six proteins were found to be differentially abundant in the diabetic vitreous as compared to the non-diabetic vitreous. Kim et al²⁸ used several proteomic methods to identify 531 proteins in the vitreous from PDR (415 proteins) and non-diabetic (346 proteins) samples. (Table 1)

The total protein content in the PDR vitreous has been shown to be higher as compared to nondiabetic samples. It has been postulated that immunoglobulin, alpha1-antitrypsin, alpha2-HS glycoprotein, PEDF, apolipoprotein A1, complement C3 and albumin may be the predominant contributors to the increased protein content of the PDR vitreous.²⁷ These proteins have been reported to be up-regulated in the diabetic vitreous by other authors.²⁹ Apo A 1 is a potent scavenger of oxygen-reactive species, and may have a role in protecting retina from the oxidative stress due to diabetes.³⁰ Garcia-Ramirez et al identified several components of the complement factor (C4b, factor B, C3 and C9) in PDR vitreous.³¹ These authors postulated that activation of the complement cascade leads to initiation and progression of thrombosis, leucostasis and apoptosis, causing the vascular lesions in diabetic retinopathy. The levels of PEDF, a potent inhibitor of angiogenesis, have not been found to be consistent across different studies, some reporting it to be down-regulated³¹; and others as up-regulated^{27,29} in the PDR vitreous. Other proteins that have been found to be increased in abundance in the diabetic vitreous include apo H³⁰, prostaglandin –D2 synthetase, plasma glutathione peroxidase, intra retinol binding protein, catalase,³² enolase,³² prostaglandin-H2 D isomerase,³³ serine protease inhibitor,³³ ankyrin repeat domain 15 protein,³³ angiotensinogen²⁷, prothrombin²⁷, antithrombin III²⁷, Factor XII²⁷ and peroxiredoxin-1.²⁷

Proteins with decreased concentration in the vitreous proteome include A-IV precursor,³³ alpha1-antitrypsin,³³ beta V spectrin,³³ superoxide dismutase³³, calsyntenin-1²⁷, interphotoreceptor retinoid-binding protein²⁷ and neuroserpin²⁷.

In patients with diabetic macular edema, Ouchi et al reported increased expression of PEDF, ApoA-4, ApoA-1, Trip-11, PRBP and vitamin D binding protein, and absence of Apo H.³⁴ Simo R et al performed quantitative real-time polymerase chain reaction analysis using donor eyes from diabetic patients and controls. They found higher expression of apo A-1 and apo H mRNAs in the diabetic retina which was in concordance with their findings on the vitreous proteome.³⁰

Translation of proteomic findings to further understanding of ocular physiology

The characterization of the vitreous proteome in patients with diabetic retinopathy has suggested novel factors and pathways that may contribute to this disease. While previous reports have demonstrated certain biochemical and biological functions that are intrinsic to many of the proteins identified in the vitreous the functions of most of these proteins in ocular physiology have not yet been determined. To elucidate the potential role of specific proteins pathophysiology of the disease, our group has performed intravitreal injections of purified proteins, identified by vitreous proteomics, into rodent models and followed by analyses of retinal responses. In a report by Gao et al 2007, we identified increased abundance of carbonic anhydrase-1 (CA-1) in the vitreous of diabetic patients compared with both nondiabetic subjects and diabetic patients without diabetic retinopathy. Since diabetic retinopathy is associated with increased retinal vascular permeability, we examined the effect of introducing CA-1 into the vitreous on retinal vascular permeability in rats. Using fluorescein angiography, we showed that CA-1 induced vascular leakage, which was not present in the sham-injected control group. Co-injection of acetazolamide and CA-1 decreased the vascular leakage. We showed that CA-1 increases retinal vascular permeability by inducing alkalization of the vitreous which increases kallikrein activity and the generation of factor XIIa. Complement 1 inhibitor, neutralizing antibody to prekallikrein and bradykinin receptor antagonist all decreased the retinal edema produced by CA-1.²⁷ These results revealed a new pathway involving CA-1 and the kallikrein-kinin system that may contribute to the increase in retinal vascular permeability in advanced diabetic retinopathy. Moreover, these results show that a functional analysis can reveal novel intraocular actions of proteins identified by vitreous proteomics, which not be readily predicted based on existing protein function annotations.

Limitations of vitreous proteomics

The vitreous used in proteomic studies is obtained from patients undergoing pars plana vitrectomy. This limits the amount and number of samples that can be obtained for various disease states. The surgical procedure in diabetic retinopathy is usually performed for complications arising from later stages of disease, which may induce changes in addition to those specific to diabetes or diabetic retinopathy. The cross-sectional design of vitreous proteomic studies suggest that in general a large numbers of samples would be needed to evaluate associations between various therapeutic interventions and the composition of the vitreous proteome in humans.

The vitreous proteome in diabetic patients is also altered by intraocular hemorrhage and increased permeability of the blood retinal barrier. Although retinal and vitreous hemorrhages are associated with advanced diabetic retinopathy, and these hemorrhages alter the vitreous proteome, the contribution of these proteomic changes in the pathogenesis of this disease is not fully understood. The isolation of a subset of less abundant proteins is difficult as the vitreous contains a high concentration of albumin and immunoglobulin that may overlap with the less abundant proteins involved in the pathogenesis of the disease.

ELISA and western blot analysis provide opportunities for independent confirmation of protein identification and for quantifying levels of candidate proteins that may be associated with disease pathogenesis.

Future directions

Additional cross-sectional studies comparing proteomic changes in the vitreous that are associated with different stages of diabetic retinopathy will likely provide further insights into disease pathogenesis and may suggest new treatment and diagnostic opportunities. Treatment strategies may be based on down regulating or inhibiting proteins involved in disease progression and/or up regulating proteins with a protective function. Further characterization of the vitreous in relation to other clinical information may reveal makers associated with disease progression or the clinical response to therapeutic interventions. Comparison of the proteomic profile to the genomic profile may provide information regarding the probable genetic component of the disease.

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Figure 1.
Steps involved in the proteomic analysis of the vitreous.

Table 1

Review of studies on the vitreous proteome in diabetic patients

	Methodology	Case	Control	No. of proteins identified	Proteins in vitreous with DR	Role of proteins in pathogenesis of DR
Nakanishi et al. 2002	2-DE+MALDI-MS/ 2-DE+ESI-MS/MS	Vitreous from eyes with DR	Vitreous from eyes with MH	51 proteins identified	-	-
Yamane et al.2003	2-DE + ESI-MS, 2-DE+MALDI-MS, Western blot	Vitreous and serum from PDR eyes	Vitreous and serum from MH eyes	18 proteins identified in MH and 38 proteins in PDR	18 proteins differentially expressed in PDR Vitreous	Catalase and Enolase were upregulated in the vitreous of patients with PDR
Kim SJ et al, 2005	2-DE + MALDI-TOF, 2-DE + MS/MS	Vitreous from eyes with PDR	Vitreous from eye with MH	-	5 proteins were upregulated and 3 down regulated in diabetic vitreous	Increased levels of acute phase reactant proteins and PEDF and decreased levels of α1-antitrypsin precursor protein in PDR vitreous
Garcia-Ramirez, 2007	DIGE + MALDI-MS	Vitreous from type 1 diabetic patients with PDR	Vitreous from eyes with MH	-	11 proteins were differentially identified in vitreous humor of diabetic patients	Factors involved in classical pathway activation were upregulated and PEDF was down regulated in diabetic vitreous.
Gao et al, 2007	1-DE + MS	Vitreous from eyes with PDR and diabetic patients with no DR	Vitreous from eyes without diabetes	117	31 proteins were differentially detected among the 3 groups	Carbonic anhydrase –1 was upregulated in diabetic vitreous, involved with increasing retinal vascular permeability
Gao et al, 2008	1-DE + nano-LC/MS/MS	Vitreous from eyes with PDR and diabetic patients with no DR	Vitreous from eyes without diabetes	252	56 proteins were differentially identified among the three groups	Factors involved in complement activation, coagulation pathway and kallikrein-kinin system were increased in PDR vitreous
Ouchi et al, 2005	LC-MS/MS	Vitreous from eyes with pre-proliferative DR+DME	Vitreous from eyes with pre-proliferative DR, no DME	14 proteins identified in DME group and 15 in non-DME group	-	PEDF, ApoA-4, Apo-A-1, Trip-11, PRBP and VDBP may have a role in DME pathogenesis
Simo et al, 2008	DIGE + MALDI-MS	Vitreous from PDR eyes with PDR	Vitreous from eyes with MH	-	-	ApoA- 1 and Apo H upregulated in PDR vitreous
Kim et al, 2007	IS/2-DE/MALDI-MS, nano-LC-MALDI-MS/MS, nano-LC/ESI-MS/MS	Vitreous from PDR eyes with PDR	Vitreous from eyes with MH	531	415 proteins identified in PDR VH and 346 in non diabetic VH	Several factors involved in angiogenesis identified including IGFBP-2, CA, osteopontin, angiotensinogen, clusterin