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A Decade of the Proteomics Studies of Glaucomatous Neurodegeneration

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Abstract

Glaucoma is a leading cause of blindness; however, limited understanding of the molecular mechanisms involved in optic nerve degeneration hinders the development of improved treatment strategies. Proteomics techniques that combine the protein chemistry, mass spectrometry, and bioinformatics offer the opportunity to shed light on molecular mechanisms so that new treatment strategies can be developed for immunomodulation, neuroprotection, neurorescue, neuroregeneration, and function gain in glaucoma. The proteomics technologies also hold great promise for biomarker discovery, another important goal of glaucoma research. As much as developing new treatment strategies, molecular biomarkers are strongly needed for early diagnosis of glaucoma, prediction of its prognosis, and monitoring the responses to new treatments. It is now a decade that the proteomics analysis techniques have been using to move glaucoma research forward. This review will focus on valuable applications of proteomics in the field of glaucoma research and highlight the power of this analytical toolbox in translational and clinical research towards better characterization and improved treatment of glaucomatous neurodegeneration and discovery of glaucoma-related molecular biomarkers.

Keywords

Biomarker; Glaucoma; Neurodegeneration; Proteomics

Introduction

Glaucoma is a multifactorial neurodegenerative disease, during which a stressor threshold determines the susceptibility of retinal ganglion cell (RGC) axons, somas, and synapses for injury. It has become evident that in addition to intraocular pressure-generated stress and aging, neurodegeneration in glaucoma comprises genetic [1] and epigenetic predispositions [2], compartmentalized processes [3], and secondary neurodegenerative events, such as glial activation, homeostatic dysfunction, and neuroinflammation [4-6]. Currently available treatment strategies are not sufficient to prevent progression of glaucoma that remains a leading cause of blindness. It is for this reason that a major research goal in the field of

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glaucoma has been uncovering the key molecular processes of neurodegeneration to thereby develop new and improved treatment strategies to protect and rescue neurons and provide function gain in glaucoma [4,7]. As reviewed herein, proteomics offers a powerful toolbox to accomplish this important goal. Different proteomics techniques involving the mass spectrometry as a major analysis tool [8-10] have emerged for large-scale studies of proteins, and continuous progress in mass spectrometry-based techniques and data analysis strategies have expanded the proteomics towards quantitative analysis of protein expression [11,12], post-translational modifications [13], and protein-protein interactions [9,14] in a high-throughput format. The analysis of proteome alterations that affect the functions of proteins is essential for identification of pathogenic processes that underlie the loss of RGCs and optic nerve axons in glaucoma [15,16]. Moreover, proteomics keeps great importance for development of new drugs most of which are the protein molecules targeting the disease-related proteins [17]. Identification of molecular biomarkers is another important goal of glaucoma research to detect glaucoma early, predict its prognosis, and monitor treatment responses [18], which can also be succeeded by using the proteomics techniques.

Over the last decade, proteomics has increasingly been used for large-scale analysis of molecular mechanisms, new treatment targets, and biomarkers of glaucomatous neurodegeneration. Figure 1 presents a simplified workflow for the commonly employed proteomics techniques in the field. Proteomics studies of glaucoma analysed human donor tissues as well as tissue samples from experimental models and provided lists of proteins whose expression altered in human glaucoma and animal models. The high-throughput proteomics data improved our understanding beyond that gained through earlier studies using conventional techniques and provided framework information to build new hypotheses. Besides gaining global information about altered protein expression, the proteomics data guided targeted approaches of proteomics to also analyze post-translational modifications and interaction networks of proteins. The large-scale analysis of protein expression in the whole tissue was then followed by the analysis of cell-specific proteomics responses of RGCs and glia in glaucoma. The quantitative proteomics data highlighted various molecular pathways and specific targets for new treatments. Mass spectrometry-based analysis also provided a number of candidate molecules and opened up the prospect of de novo biomarker discovery in glaucoma [19,20]. This review will highlight the valuable applications of proteomics in the field of glaucoma research with particular focus on the implications of proteomics data in translational and clinical research towards molecular mechanisms, new treatment targets, and biomarkers of “neurodegeneration”. As previously reviewed, diverse sample types have been analysed in proteomics studies of glaucoma; however, this review will not cover the studies of anterior ocular tissues which provided helpful information about intraocular pressure regulation mechanisms [15].

Global proteomics analysis of protein expression during neurodegeneration in human glaucoma and animal models

Our pioneering proteomics studies of glaucomatous neurodegeneration analyzed the retinal proteome to characterize molecular changes in human glaucoma and animal models. Comparative analysis of glaucomatous and non-glaucomatous samples using proteomics

analysis techniques provided extensive information about glaucoma-related alterations in protein expression and significantly advanced our understanding of pathogenic pathways. In our earlier studies, we employed two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) coupled with mass spectrometry. Using the gel-based proteomics technique, we could separate thousands of proteins from highly complex mixtures of retinal proteins. Through the comparison of protein staining pattern between the 2D gel images obtained with glaucomatous and control samples, we could quantify up-regulated and down-regulated proteins. This differential display analysis was followed by the analysis of excised gel spots by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry. The database analysis of peptide masses identified lists of proteins through peptide mass fingerprinting, or tandem mass spectrometry-based analysis identified proteins through peptide sequencing [21-23]. In addition to protein identity and variations in protein quantity, our gel-based proteomics studies also used specific gel staining techniques to visualize modified proteins as detailed later below [21,22,24]. The analysis of 2D gels, as well as 1D gels, was also used to similarly map retina or optic nerve proteins by other researchers and highlighted various aspects of glaucoma [25-30]. However, the gel-based proteomics analysis suffers from a number of technical limitations. Major weaknesses of gel-based analysis for peptide mass mapping include limited sensitivity and reproducibility. Due to its shortcomings, the protein expression differences detected on gels should be validated by other techniques, such as immunoblotting or tissue immunolabeling with specific antibodies. Our studies through gel-based proteomics analysis similarly complemented the proteomics data with other molecular, cellular, and pharmacological techniques, and followed the path of a number of identified molecules in focused studies as discussed in the next section.

With continuous advances in data quality and protein identification throughput, the gel-based proteomics analysis has gradually shifted towards gel-free analysis by mass spectrometry. In the gel-free approach, peptides of the enzymatically digested protein mixtures are initially separated by liquid chromatography (LC) and then subjected to mass spectrometric fragmentation and sequencing for identification of their parent proteins. This LC-tandem mass spectrometry (LC-MS/MS) approach (so called bottom-up proteomics) allows large-scale analysis of highly complex peptide mixtures and has therefore become the preferred technique for shotgun proteomics [31]. The gel-based proteomics analysis that requires 50-200 µg of protein sample per analysis, has limited in detection of 0.1-10 ng protein per spot and a narrow dynamic range of less than 10^3 . In contrast, increasing sensitivity and accuracy of the LCMS/MS systems now permits the gel-free analysis in picogram amounts of protein samples. Most of the commonly used systems require 10-50 µg protein for each run; however, modern instruments enable the analysis of nanogram quantities of samples with a dynamic range of 10^6 and protein identification sensitivity at low zeptomole levels [32]. Owing to a number of advantages, our following proteomics studies of glaucoma have increasingly focused on gel-free LC-MS/MS using MALDI-TOF/TOF or electrospray ionization (ESI)-MS/MS and resulted in protein identification with enhanced dynamic range and confidence [22,23,33-35].

Aside from protein detection and identification, quantitative analysis of protein expression can also be achieved by mass spectrometry using stable isotope labeling or label-free techniques [12,36]. Our proteomics studies employed the $^{16}\text{O}/^{18}\text{O}$ labeling technique relying on the enzymatic oxygen exchange for quantification of the resultant mass shift between differently labeled peptide ions. In addition to this highly specific, simple, and affordable technique [37,38], we also used label-free quantification as a complementary technique for estimation of protein abundances [39]. In the spectral scoring approach we used, the relative abundance of proteins can be indexed across multiple samples by counting the unique spectra and then normalizing the number of spectral counts to the predicted molecular weight of corresponding protein [22,23,33-35].

The accuracy of mass spectrometry sets 1.3-2-fold expression change as cut-off for biological significance [40]. The proteins listed through our studies using isotope labeling-based or label-free approaches of quantitative proteomics included those exhibiting an over two-fold up-regulation or down-regulation in human glaucoma and experimental models [22,23,33-35]. Similar to gel-based techniques, data verification is critical for interpretation of the proteomics data from gel-free analysis by mass spectrometry mainly due to background noise that may obscure small changes in protein expression. To fulfill this need and verify the protein expression changes detected by quantitative mass spectrometry, our studies also employed biochemistry and molecular biology techniques, as well as biological and technical replicates for proteomics data validation. Findings of these studies brought many new insights into molecular processes of glaucomatous neurodegeneration and led us to build new hypothesis which we have already started testing in additional studies.

An unexpected discovery from our retinal proteome analysis was detection of hemoglobin, an erythrocyte protein, that was also up-regulated in human glaucoma and experimental models [23]. This observation stimulated us to determine the relevance of this important oxygen-binding protein to glaucoma. To serve this purpose, we first verified the retinal hemoglobin expression by quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting of RGC and astrocyte samples and tissue immunolabeling of human and rat retinas. Our succeeding in vitro experiments then indicated that the retinal hemoglobin expression is regulated by oxygen concentration. In these experiments, RGC and astrocyte cultures were incubated under hypoxia in the presence or absence of recombinant erythropoietin (EPO) or soluble EPO receptor. We detected that hypoxia up-regulated hemoglobin expression in astrocytes through the hypoxia-inducible factor 1-alpha (HIF-1 α) and EPO signaling. In addition to this autocrine regulation, our in vitro treatment experiments revealed a paracrine mechanism through which glial EPO up-regulated hemoglobin expression in RGCs and promoted neuronal survival. Further supportive of these in vitro observations, retinal immunohistochemical analysis demonstrated that the up-regulated expression of hemoglobin is accompanied by increased expression of EPO by glia and EPO receptor on both RGCs and glia in glaucomatous eyes [23]. There is also immunohistochemical evidence of up-regulated HIF-1 α expression in the glaucomatous human retina and optic nerve [41]. These findings imply important roles for hemoglobin in regulation of oxygen homeostasis in the inner retina and astrocyte-mediated oxygen transport to RGCs. It also seems likely that an additional role for this important oxygen-binding protein in the retina could be free radical scavenging.

Bioinformatics analysis tools allow knowledge-based analysis of the high-throughput datasets from proteomics analysis to define functional patterns of the identified proteins and populate protein interaction networks. By the Ingenuity Pathways Analysis (Ingenuity® Systems, Mountain View, CA), a widely utilized software system for bioinformatics analysis, our recent studies revealed the canonical pathways that were found to be most significantly associated with our datasets, which included complement activation [33], toll-like receptor (TLR) signaling [34], tumor necrosis factor-alpha/tumor necrosis factor receptor (TNF- α /TNFR1) signaling, and nuclear factor-kappa B (NF- κ B) activation [35].

We detected different complement components, receptors, and regulators supportive of complement-mediated processes in human and rat glaucoma [33]. An interesting observation was related to a well-known regulatory protein, complement factor H that was down-regulated in some of our glaucomatous samples. Our following in vitro experiments with rat retinal cells indicated oxidative stress-related down-regulation of this complement regulator and thereby suggested the possibility of complement dysregulation in the glaucomatous human retina that exhibits oxidative stress [33,42]. Such a stress-related failure in the regulation of complement-mediated processes may potentially result in uncontrolled complement attack leading to collateral injury to neurons in glaucoma.

In addition, analysis of our high-throughput datasets linked a number of adaptor and kinase molecules detected in the retinal proteome to TLR signaling involved in immune responses [34]. Our accompanying in vitro experiments also disclosed that stress-related ligands, such as heat shock proteins or oxidative stress products, can up-regulate glial TLR signaling, pro-inflammatory cytokine secretion, and antigen presentation in co-cultures with T cells. When specific inhibitor treatments were used to functionally test the adaptor proteins involved in TLR signaling, such as myeloid differentiation primary response 88 (MyD88) and toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP), the stimulated glial antigen presentation was significantly decreased [34]. These findings suggest that glia can sense stressed or injured neurons and initiate inflammatory responses through the activation of TLR signaling by stress-related intrinsic ligands. Importance of these molecular responses in the regulation of immune homeostasis in glaucoma waits for additional functional testing in transgenic models.

Our retinal proteomics analysis also pointed out various molecules involved in the neurodegenerative or inflammatory pathways of TNF- α /TNFR1 signaling that are up-regulated in human glaucoma [43]. Our proteomics data indicated intricate interactions between multiple signalling pathways in determining the varied consequences of TNF- α /TNFR1 signaling [35]. The high-throughput comparative data together with the findings of immunochemical analyses, provided evidence that different apoptotic pathways, including caspases, calpains, mitochondrial and endoplasmic reticulum stress, may be reinforcing the progress of RGC death in human retina. We also detected various components of the NF- κ B activation pathway taking a key place in transcriptional activation of immune mediators. NF- κ B activation was validated by immunochemical analysis using phospho-antibodies to specific subunits. Other related components of neuroinflammatory responses we detected involved janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling and inflammasome formation (Figure 2). Furthermore, our data from proteomics analysis

provided clues about the factors that determine individual differences in TNF- α -mediated injury. For example, the expression of tumor necrosis factor- α inducing protein (TNFAIP3) that inhibits NF- κ B activation and negatively regulates TNF- α signaling exhibited individual variability among our human donors. This observation motivated us for further analysis. We studied the methylation pattern in TNFAIP3 promoter using bisulfite sequence analysis and our findings correlated protein expression differences with the promoter methylation [35]. The relevance of this observation to differences in individual susceptibility for TNF- α -mediated neuronal injury in glaucoma should be further investigated. Nevertheless, this piece of data exemplifies how the informative scope of proteomics can be expanded towards epigenetics, and how the proteomics data when integrated with the data arising from other disciplines can progress the glaucoma research even further.

In addition to proteomics analysis of human donor eyes with glaucoma, we analyzed the retinal protein expression in ocular hypertensive human donor eyes with the absence of manifest glaucoma. Comparison of the ocular hypertensive samples to glaucomatous and non-glaucomatous control samples indicated that glaucomatous neurodegeneration develops after a period of intrinsic defense response to ocular hypertension. Our proteomics data supported the notion that a threshold of alterations in protein expression which exhibits individual differences determines the switch to neurodegenerative injury in glaucoma.

In addition to retinal protein expression in human glaucoma and animal models, our current proteomics studies also analyze alterations in scleral protein expression in experimental mouse glaucoma. Our ongoing proteomics studies of the mouse sclera illuminate the molecular pathways that determine the impacts of scleral tissue properties on intraocular pressure-related stress and susceptibility for neuronal injury [45].

Other researchers also used label-free quantitative LC-MS/MS to analyze the retinal proteome in non-human primates and identified over a hundred proteins exhibiting condition-specific alterations in experimental glaucoma and optic nerve transection. The early response to chronic IOP elevation in glaucoma prominently included the cellular architecture regulation [46]. Another study using LC-MS/MS and isobaric tag for relative and absolute quantification (iTRAQ) analyzed the protein expression changes in human optic nerve astrocytes and implicated transforming growth factor-beta 1 and TNF- α in the activation responses of astrocytes after exposure to in vitro biomechanical strain [47].

Targeted proteomics analysis of protein modifications and interactions linked to glaucomatous neurodegeneration

Gel-based or gel-free approaches of shotgun proteomics using LC-MS/MS provides lists of proteins and their relative expression values. However, the functional proteomics involves the enrichment of targeted proteins and isolation of protein complexes using affinity-based approaches to allow the characterization of modified proteins and their specific interactions. Since post-translational modifications of proteins are critical for regulation of intracellular processes, one of the main goals of our proteomics studies in the field of glaucoma research has been to identify modified proteins through such targeted approaches.

Protein phosphorylation, one of the most common forms of protein modifications, plays a vital role in phosphoregulation of protein function and signal transduction. A commonly used technique for analysis of phosphorylated proteins has been immunoblotting with phosphorylation site-specific antibodies, which relies on the prior knowledge of modification as well as the antibody availability. However, mass spectrometry-based analysis techniques offer a more advanced tool for identification and quantification of both the previously known and new modifications, thereby enabling the elucidation of signaling networks [8-10]. In an initial targeted proteomics study, we employed a 2D gel-based approach for global analysis of retinal phosphoproteins in experimental glaucoma. We used a fluorescence-based phosphoprotein stain (Pro-Q Diamond) to label phosphoproteins and multiplexed with Sypro Ruby to profile the total protein for spot normalization. Through the differential display analysis of 2D gel images, we compared the phosphoprotein staining at corresponding spots on gels from different samples and detected over a hundred spots that exhibited increased phosphorylation after induced ocular hypertension. The subsequent MALDI-TOF/MS and LC-MS/MS analyses of the excised gel spots identified multiple proteins. The identified phosphoproteins included a diverse set of molecules involved in stress response and cell signaling [48]. One group of proteins that exhibited increased phosphorylation in ocular hypertensive retinas was the 14-3-3 family. The gel staining-based detection of 14-3-3 phosphorylation was then verified by acidic shift and specific 14-3-3 immunolabeling of 2D gels [22]. Despite useful applications, targeted proteomics strategies face many challenges as the gel-based techniques being most vulnerable [13,49]. For example, although the phosphoprotein gel staining that we used for detection and relative quantification of phosphorylated proteins offers a simple phosphoproteomics technique, it suffers from the common problems of gel-based analysis. Besides the dynamic nature of phosphorylation, phosphorylated proteins usually have low abundance and therefore enrichment of phosphoproteins or peptides using affinity-based purification techniques is essential for their proper analysis by mass spectrometry.

Biological functions of proteins rely on interactions with other molecules for forming protein complexes and regulating intracellular signaling. Therefore, characterization of protein complexes and protein interaction networks are critical to gain insights into disease-causing molecular mechanisms [14]. As an approach towards protein interactions relevant to glaucomatous neurodegeneration, we explored the functional importance of 14-3-3 phosphorylation that we detected in the glaucomatous rat retina [22]. For targeted proteomics analysis of 14-3-3 interactions, we isolated enriched samples of 14-3-3-containing protein complexes by elution with immunoprecipitation and affinity pull-down. The LC-MS/MS analysis of co-eluted proteins identified pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family, including Bad, besides other proteins, such as calmodulin, zinc-finger proteins, ubiquitins, protein kinase C, and heat shock protein70 [48]. Based on immunoblot analysis of subcellular fractions and immunohistochemical analysis of retinas with phospho-specific antibodies, 14-3-3 phosphorylation and Bad de-phosphorylation were found to lead to mitochondrial translocation of Bad for apoptotic function in ocular hypertensive eyes. In series of in vivo treatment experiments, we also tested the association of 14-3-3 protein with RGC death in experimental glaucoma. Treatments inhibiting the 14-3-3 phosphorylation, such as a peptide inhibitor of c-jun amino(N)-terminal kinase (JNK)

called DJNKI, or Bad dephosphorylation, such as tacrolimus (FK506), maintained the cytoplasmic sequestration of Bad by 14-3-3 binding and lowered the RGC loss in ocular hypertensive eyes. These treatment responses imply the phosphorylation-dependent regulation of Bad function in controlling the mitochondria-mediated pathway of RGC death in glaucoma [50]. The 14-3-3-mediated upstream regulation mechanism may therefore be a promising treatment target for neuroprotection [22]. The protein interactions networks generated by bioinformatics tools provide guidelines for future functional studies of specific molecular pathways. Ongoing studies of protein phosphorylation and protein-protein interactions are expected to progress glaucoma research forward towards advanced understanding of pathogenic mechanisms and development of new strategies for treatment.

Protein oxidation appears to be another important protein modification in glaucoma. Although reversible redox reactions are part of physiological processes, irreversible modifications under pathological conditions may affect neuronal survival and immune homeostasis in glaucoma similar to other neurodegenerative diseases or aging [5,51]. Protein carbonylation is a common form of oxidative protein modifications and addition of carbonyl groups leads to further reaction resulting in generation of advanced lipoxidation end products and advanced glycation end products (AGEs) also increased in glaucoma [42]. Due to chemical instability, rather than reactive oxygen species, their stable end products, such as protein carbonyls, are quantitated for targeted proteomics analysis of protein oxidation [52]. Despite evolving techniques for redox proteomics [53], a convenient immunochemical approach, called oxyblot analysis, is widely used to detect the dinitrophenyl derivatives of carbonyls. This gel-based technique detects protein carbonyls reacting with 2,4-dinitrophenylhydrazine and the following analysis by mass spectrometry identifies proteins in detected spots. Through the oxyblot analysis, we detected a significant increase in the number and intensity of carbonylated spots that reflect increased protein oxidation in glaucomatous retinas relative to controls. The MALDI-TOF/MS and LC-MS/MS analysis then identified a number of proteins targeted by carbonyls. In addition, Western blots analysis of retinal proteins and immunolabeling of retinal tissue sections demonstrated increase in anti-carbonyl reactivity in the inner retina of glaucomatous eyes, including RGCs [21]. Similar to experimental glaucoma, increased protein carbonyls [42] and 4-hydroxy-2-nonenal adducts [33] that also reflect protein modification caused by oxidative stress by-products were predominantly detected in the inner retinal layers of glaucomatous human donor eyes. Oxidative protein modifications can also be distinguished by a specific shift in the mass spectra [54,55] as we more recently used to determine protein oxidation in serum samples [56]. The observation of increased protein oxidation led further studies highlighting the oxidative stress-mediated component of glaucomatous neurodegeneration. Evidently, oxidative stress reduces the ability of RGCs and axons to deal with intraocular pressure-related stress in glaucoma and facilitates neurodegenerative injury by generating direct neurotoxicity and also secondary neurodegenerative consequences, such as glial dysfunction, immune system dysregulation, neuroinflammatory processes [5,51,57]. With respect to increased protein oxidation in the glaucomatous retina [21] and increased reactivity of the glaucomatous sera to oxidatively stressed retina [56], oxidized epitopes of proteins may serve as the signal in stimulating the immune response to self-proteins in glaucoma [5]. Our ongoing studies using animal models aim to further determine the

pathogenic importance and time course of oxidative stress-related processes during glaucomatous neurodegeneration and the potential of antioxidant treatment as an immunomodulation and neuroprotection strategy in glaucoma.

The increased accumulation of AGEs in the retina and optic nerve of glaucomatous eyes, which involves both protein oxidation and glycosylation [42], motivated us to also analyze retinal protein glycosylation in rats with experimental glaucoma in comparison to rats with streptozosin-induced diabetes [24]. Through a 2D gel-based approach using the Pro-Q Emerald glycoprotein gel staining, we detected a significant increase in glycosylation at multiple spots on gels derived from diabetic samples relative to non-diabetic controls. Unlike non-diabetic controls, intensity of glycoprotein staining in 2D gels obtained with ocular hypertensive samples was also increased at many shared spots with diabetics.

Some other researchers also analyzed the modified proteins, such as citrullinated [25] or isolevuglandin-modified proteins [58], in glaucomatous samples through immunoprecipitation followed by gel-based mass spectrometry.

Cell-specific proteomics analysis of cellular responses in experimental glaucoma

Apart from the broad dynamic range in protein abundance, complex cellular composition of the retina and optic nerve makes the analysis of protein expression even more challenging for glaucoma research. It is undoubted that cell-specific analysis is essential to speed the characterization of pathogenic processes in glaucomatous neurodegeneration, because data interpretation is more complicated after analysis of the whole retina or optic nerve that contain many different cell types. Isolation of cell-specific samples for proteomics analysis reduces the sample complexity, increases the sensitivity of detection, and improves the informative value of the mass spectral data. Obviously, RGCs, including their axons, somas, and synapses, are victims in glaucomatous neurodegeneration and should constitute the major focus of cell-specific analysis in glaucoma. With respect to glia-mediated neurodegenerative processes in glaucoma, better characterization of glial responses is also equally important in the field [4,5]. Consequently, our cell-specific proteomics studies that began with the analysis of RGC samples in 2004 [19,22,23,59,60], continued with the analysis of glial cells in more recent studies to also illuminate the regulation of glial homeostatic functions and neurodegenerative consequences of glial activation responses in glaucoma [60].

For cell-specific analysis of the proteome, we isolated enriched samples of RGCs and astrocytes by immunomagnetic cell selection as we originally described for in vitro culturing of RGCs [61]. This simple process that needs approximately one hour to complete is advantageous over alternative techniques that require longer and more difficult protocols. The most important advantage presented by the immunomagnetic cell isolation is the protein yields allowing the proteome analysis with or without sample pooling [20]. We verified our immunomagnetically isolated cell-specific samples by back labeling of RGCs [61], cellular morphology and immunolabeling pattern in culture [61], and RT-PCR [23] or Western blot analysis of isolated cells for specific cell markers [19,60]. Analysis of the isolated samples

of RGCs [19,23,60] or astrocytes [60] in multiple studies employed gel-based or gel-free proteomics techniques using isotope labeling-based or label-free LC-MS/MS. We detected and quantitated thousands of proteins with high confidence (with 0.1% protein false-discovery-rate), including the proteins with up-regulated or down-regulated expression characterizing the distinct responses of ocular hypertensive RGCs and astrocytes in experimental glaucoma [60]. Figure 3 shows functional groups of the up-regulated proteins in ocular hypertensive RGCs and astrocytes relative to normotensive controls. Our high-throughput proteomics data indicated a prominent stress response and cell death signaling activated in glaucomatous samples of RGCs; however, cellular activation and immune/inflammatory responses were predominant in astrocytes isolated from glaucomatous eyes.

Up-regulated immune mediators/regulators in ocular hypertensive astrocytes included a number of proteins linked to TNF- α /TNFR1 signaling, JAK/STAT signaling, TLR signaling, and inflammasome formation. NF- κ B, as being a common regulator of these molecular pathways involved in astrocyte-mediated neuroinflammatory responses, appears to be a key treatment target for immunomodulation in glaucoma (Figure 4). In addition, our cell-specific proteomics analysis disclosed up-regulation of autophagy signaling in glaucoma and activation of its upstream negative regulator, mTOR, in ocular hypertensive astrocytes. In conjunction with the supportive findings of immunochemical analysis, the proteomics data delivered clues for cell-specific regulation of autophagy and stimulated further research to determine whether this cytoplasmic homeostasis mechanism may also contribute to induced autoimmunity in glaucoma [60].

Another group of researchers also verified the feasibility of RGC-specific proteomics analysis. They isolated enriched samples of RGCs by immunopanning and LC-MS/MS iTRAQ analysis identified 268 proteins some of which were differentially expressed in experimental rat glaucoma [62].

Our studies currently pursue the major proteomics outcomes in focus-in studies for testing their functional importance in cell-specific transgenic models. These studies are awaited to translate the cell-specific proteomics data into molecular processes underlying the glaucomatous neurodegeneration, so that new treatment targets can be identified to provide immunomodulation and enhance neuronal survival and visual function in glaucoma.

Proteomics analysis for biomarker discovery in glaucoma

Molecular biomarkers are deeply needed for early diagnosis of glaucoma and assessment of risk profiles for disease progression. Not only glaucoma prediction, but also monitoring the treatment responses is a growing need in clinical trials of forthcoming neuroprotective treatments. Identification of clinically applicable molecular biomarkers is an area of active research, and proteomics takes its place at the center of ongoing studies [18].

Most of the work that assists glaucoma-related biomarker development included the analysis of serum antibodies and their target antigens in ocular tissues. This seems to be a favorable strategy for molecular biomarker screening in glaucoma, since many patients with glaucoma present an increase in serum immunoreactivity to a list of ocular proteins. Indeed, protein arrays containing a panel of antigenic proteins provided useful information about the

molecular signature of autoantibody responses in glaucoma [63,64]. The unique serum antibody pattern among patients with glaucoma [64] and similarities in complex antibody profiles among various ethnic populations [65,66] support the potential of these antibodies for clinical screening. Targeting the antibody response seems particularly advantageous for signal amplification in discovering the glaucoma-related biomarkers, because the limited dynamic range and detection threshold of proteomics techniques create an important challenge in detecting the low abundant biomarkers within complex pool of the human blood. However, while the protein arrays used for profiling the antibody response are limited to a number of pre-chosen antigenic proteins, mass spectrometry-based techniques present a high-throughput analytical approach for de novo discovery of biomarker candidates and also enable the analysis of modified proteins as potential biomarkers [67-69].

Many studies followed a traditional approach for analysis of autoantibodies which involved the experimental steps of retina or optic nerve protein separation by 1D or 2D SDS-PAGE, probing the transferred membranes with glaucomatous sera, and then analysis of the immunoreactive bands or spots for identification of autoantigens by mass spectrometry [70-72]. In a recent study, we aimed for high-throughput characterization of autoantigens through an immunoproteomics approach. In this antibody-based approach towards potential biomarker candidates, we initially analyzed five randomly selected samples from our blood sample pool that includes blood samples from different patient groups with glaucoma and age-matched controls. We eluted serum immunoglobulin G (IgG) from these samples using Melon gel spin columns or protein G-Dynabeads. After verification of the serum IgG elutes, co-eluted proteins were analysed by mass spectrometry that listed hundreds of proteins. We then employed a narrowing down strategy to lessen the number of autoantigens by determining the overlap between the proteomics data collected from the glaucomatous IgG elutes in this study and from the glaucomatous human retina in our previous studies [23,33-35]. Among the proteins overlapped between serum and retina proteomes (Figure 5), we selected ten candidate biomarkers for initial validation analysis. We measured serum titers of these ten candidates in all of our blood samples from 111 patients with glaucoma and 49 age-matched healthy controls without glaucoma by specific enzyme-linked immunosorbent assays (ELISAs). We found that the serum ELISA titers for four of these candidate molecules, including apoptosis-inducing factor, cyclic AMP-responsive element binding protein (CREB)-binding protein, ephrin type-A receptor, and huntingtin protein, were greater in glaucomatous samples compared to age-matched non-glaucomatous controls [56]. However, serum ELISA titers exhibited prominent variability between individual samples, which is not unexpected owing to characteristic complexity in etiology and inter-patient heterogeneity of glaucoma. This observation strengthens the view that a range of biomarkers, rather than a single molecule, would be more useful to provide the biomarker sensitivity and specificity needed for clinical utility in glaucoma.

Our immunoproteomics study also determined the oxidation status of serum proteins. By searching a 16 Da mass shift reflecting the addition of oxidation moiety, we detected a greater abundance of methionine oxidation in IgG elutes from glaucomatous sera compared to age-matched healthy controls. The methionine oxidation counts normalized to mass spectra counts were two or more in 23% and 11% of the proteins in glaucomatous and control samples, respectively, while the methionine oxidation in 27% of proteins was

detectable only in glaucomatous samples. This observation of increased protein oxidation in IgG elutes from glaucomatous samples was also parallel to the findings of accompanying in vitro experiments that indicated a greater immunoreactivity of the glaucomatous serum against oxidatively stressed retinal proteins in cell cultures compared with immunoreactivity to control proteins [56]. These findings, along with the increased protein oxidation in the glaucomatous retina [21], suggest that the modified serum proteins, as opposed to native (unmodified) proteins, are most valuable as glaucoma-related biomarkers.

Although serum antibodies were widely studied by different research groups and a list of autoantigens led the path to serum biomarker discovery in glaucoma, except for our unpublished work, glaucoma-related biomarkers have not been studied by direct proteomics analysis of blood samples. We analyzed the serum samples collected from different groups of 196 patients with glaucoma (including patients with primary glaucoma or pseudoexfoliative glaucoma) and 92 healthy controls without glaucoma. The LC-MS/MS analysis of sera identified over 300 proteins with high confidence (0.2% protein and 5.0% peptide false discovery rates), including various immune mediators and molecular components of cell death signaling. As shown in Figure 6, we identified 248 proteins common to all sample groups, 22 proteins detected only in glaucomatous samples [73]. The identified serum proteins may serve as potential biomarkers to be validated in patient populations for future clinical applications as a diagnostic and/or prognostic tool in glaucoma.

Because of the easy sampling of blood, most of the biomarker studies of glaucoma aimed to develop biomarkers that can be measured in blood samples. However, it should be emphasized that analyzing the more proximal fluids, such as tear or aqueous humor, may in fact present a higher sensitivity and specificity for screening and monitoring the glaucoma-related biomarkers. More recent studies using LC-MS/MS iTRAQ or label-free techniques analyzed tear samples and detected anti-glaucoma medication-related alterations in the tear protein profile in patients with glaucoma [74,75]. The aqueous humor samples collected from glaucomatous patients and controls were also analyzed using protein chip array followed by surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF), 2D gel-based MALDI-TOF/MS [76,77], or gel-free LC-MS/MS analysis [78] and identified a number of molecules as potential biomarker candidates to be validated. More work is demanded to validate the feasibility of glaucoma-related biomarker analysis in tear and aqueous humor samples.

Our work is ongoing to identify clinically useful biomarkers that can predict the initiation and/or progression of neuronal injury in glaucoma. We hope that continuously increasing availability of improved protein separation strategies and growing capabilities of mass spectrometry for proteome coverage, accurate quantification, and data analysis will help further progress the ongoing efforts towards the identification of glaucoma-related biomarkers in blood samples. However, despite quickly evolving techniques that increase the throughput and confidence of proteomics analysis for biomarker discovery, the translation of biomarker candidates into clinical applications remains highly challenging. Following the discovery phase, the biomarker validation phase is critical for sensitivity and specificity calculations to thereby value biomarker candidates for clinical prediction. Thus,

although blood sampling from a small cohort is sufficient for the discovery phase, the biomarker validation phase requires the studies of larger and heterogeneous cohorts, including patients with different demographics and comorbidities, different types and stages of glaucoma under different treatments, and disease-free controls, for appropriate statistical power. ELISA is a highly specific technique with low pg/mL sensitivity; however, more advanced analysis techniques, including the selective reaction monitoring that offers a multiplexed platform using stable isotope dilution-MS [79], also promise for protein biomarker measurements for clinical testing.

Conclusions

Proteomics provides a powerful toolbox for quantitative analysis of protein expression, modifications, and interactions in a high-throughput format. Increasing applications of proteomics over the past decade have provided with the lists of proteins differently expressed in human glaucoma and animal models compared to controls. The high-throughput proteomics data and the findings of targeted proteomics analysis of protein modifications and interaction networks advanced our comprehension of molecular mechanisms in glaucomatous neurodegeneration. With the growing capability of mass spectrometry for protein analysis and continuous advancements in the analysis and computational integration of protein modifications and interactions, the applications of proteomics in the field of glaucoma research will progress even further. Ongoing studies for functional testing will translate the proteomics data into new treatment possibilities for patients with glaucoma, and clinical validation studies will move the identified biomarker candidates from bench to bedside.

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Abbreviations

AGEs	advanced glycation end products
Bcl-2	B-cell lymphoma 2
CREB	cyclic AMP-responsive element binding protein
ELISA	enzyme-linked immunosorbent assays
EPO	erythropoietin
FK506	tacrolimus
HIF-1α	hypoxia-inducible factor 1-alpha
IgG	immunoglobulin G
iTRAQ	isobaric tag for relative and absolute quantification
JAK	janus kinase

JNK1	c-jun amino(N)-terminal kinase
LC	liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MALDI-TOF/TOF	matrix-assisted laser desorption/ionization time-of-flight/time-of-flight
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor-kappa B
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
RGC	retinal ganglion cell
STAT	signal transducer and activator of transcription
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF	surface-enhanced laser desorption/ionization-time of flight
TIRAP	toll/interleukin-1 receptor domain-containing adaptor protein
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TNFAIP	tumor necrosis factor-alpha inducing protein
TNFR	tumor necrosis factor receptor
2D	two-dimensional

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Figure 1.

Workflow for proteomics analysis of molecular pathways, treatment targets, and biomarkers of glaucomatous neurodegeneration. Illustrated are the proteomics techniques employed for high-throughput characterization of protein expression, modifications, and interactions in human glaucoma and experimental models.

Figure 2.

Global proteomics analysis of the glaucomatous human retina. The quantitative LCMS/MS analysis of retinal protein samples isolated from human donor eyes provided list of proteins differentially regulated in human glaucoma. The knowledge-based analysis of the proteomics datasets established extended networks of protein interactions. Shown is a simplified network of protein interactions that regulate the death- or survival-promoting pathways of TNF- α /TNFR1 signaling and mediation of immune response. In this network generated by the Ingenuity Pathways Analysis (recently published [35]), red color codes for proteins with significantly increased expression and yellow color codes for proteins with no significantly increased expression in glaucomatous versus non-glaucomatous samples. Uncolored proteins were undetectable in our samples, and the expression of TNFAIP3 (shown in blue) exhibited inter-individual differences.

Figure 3.

Cell-specific proteomics analysis of glaucomatous neurodegeneration. Astrocyte and RGC samples were isolated by immunomagnetic cell selection and analyzed by quantitative LCMS/MS. The Ingenuity Pathways Analysis of the cell-specific proteomics data supported distinct responses of these two important cell types in experimental rat glaucoma. Graphs show the functional groups of up-regulated astrocyte and RGC proteins in rat retinas with experimental glaucoma compared with controls.

This figure has recently been published [60].

Figure 4.

Astroglial inflammatory responses in experimental glaucoma. This network that was generated by The Ingenuity Pathways Analysis illustrates the significantly up-regulated astrocyte proteins in ocular hypertensive versus normotensive rat retina samples in red color. The high-throughput proteomics data as well as the findings of Western blot analysis and immunohistochemical labeling indicated that TNF- α /TNFR1 signaling, JAK/STAT signaling, TLR signaling, and inflammasome are co-players in the mediation of neuroinflammation by ocular hypertensive astrocytes, and NF- κ B is a common regulator of these pathways. This figure has recently been published [60].

Figure 5.

Potential biomarker candidates of glaucoma. Immunoproteomics analysis of isolated serum IgG elutes by LC-MS/MS identified 231 proteins co-eluted with IgG. Ninety-six proteins were common in the proteomics data from serum IgG elutes and our previous proteomics data from the glaucomatous human retina. By specific ELISAs, we then measured the levels of selected ten proteins in all of our blood samples collected from glaucomatous patients and controls (including non-glaucomatous healthy controls and a group of patients with diabetic retinopathy). The bar graph shows the mean values and the table summarizes the overall data from ELISA for the four biomarker candidates (ng/ml). The underlined p values indicate the statistical difference in glaucomatous versus non-glaucomatous samples, and other p values indicate the statistical difference between glaucomatous and diabetic samples (Mann-Whitney rank sum test). This table has recently been published [56].

Figure 6.

Potential biomarker candidates of glaucoma. Direct LC-MS/MS analysis of serum samples identified a list of 325 proteins in different groups of patients with primary open-angle glaucoma (POAG) or pseudoexfoliative glaucoma (XFG) and healthy controls (Ctrl) without glaucoma. As recently presented [73], 248 proteins were common to all three sample groups, while the listed 22 were detected only in glaucomatous samples.