New Proteins as Vascular Biomarkers in Primary Open Angle Glaucomatous Aqueous Humor

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Purpose. The aim of this study was to investigate the expression level of several biomarkers in the in the aqueous humor of 14 patients with primary open angle glaucoma who underwent glaucoma surgery, and 11 nonglaucomatous normals who underwent cataract extraction surgery.

Methods. The aqueous humor proteome of 25 patients was analyzed using an antibody microarray. Fourteen patients with uncontrolled intraocular pressure—despite profound therapeutic interventions—who underwent filtering procedures and 11 control subjects who underwent surgery for senile cataracts were included in the present study. Protein expression was evaluated using Cy3/Cy5 labeling, column purification, and hybridization on antibody-spotted glass microarrays. Fluorescent signals were detected by fluorescence laser scanning.

Results. The levels of 13 proteins were significantly increased in the aqueous humor of glaucomatous patients compared with expression levels in healthy controls. One of the 13 proteins (ELAM 1) was involved in inflammation. Two of these proteins (apolipoprotein B and E) were involved in the delivery of cholesterol to cells. Five of the 13 proteins (myotrophin, myoblast determination protein 1, myogenin, vasodilator-stimulated phosphoprotein, and ankyrin-2) were involved in muscle cell differentiation and function. Three proteins (heat shock 60 kilodaltons (kDa) and 90 kDa proteins, and ubiquitin fusion degradation 1-like) were involved in stress response and the removal of damaged proteins; and two proteins (phospholipase C β and γ) were involved in signal transduction and neural development.

Conclusions. The expressions of these proteins in the aqueous humor of glaucomatous patients reflect the damage occurring in anterior chamber endothelia, mainly including the trabecular meshwork, which is the main structure of this ocular segment injured by glaucoma. (*Invest Ophthalmol Vis Sci.* 2012;53:4242–4253) DOI:10.1167/iovs.11-8902

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Glaucoma is a neurodegenerative disease that has three separate target tissues: the lateral geniculate nucleus,[1] the optic nerve,[2] and the trabecular meshwork.[3] Initially, glaucoma results in morphologic changes in all of these tissues; and in later stages, the disease results in morphological and functional atrophy of these structures. The events that compose the glaucomatous pathogenic cascade are not yet fully understood. However, it is reasonable to think that the first tissue to have disease involvement is the anterior chamber (AC). The AC is composed of three different tissues: the cornea, the iris, and the trabecular meshwork (TM), which joins the cornea and the iris along the complete 360° circumferences of the two structures. The aqueous humor leaves the AC through the TM, which is the conventional aqueous outflow pathway. Endothelial TM cells form a barrier between the aqueous humor and the lumen of Schlemm's canal and prevent the reflux of blood from the venous circulation into the aqueous-filled intraocular fluid compartment.[4] The TM plays a critical role in the regulation of aqueous outflow. TM endothelial cells control the permeability of Schlemm's canal by releasing vasoactive cytokines and other factors that are capable of increasing the permeability of the endothelial barrier.[5] In primary open-angle glaucoma (POAG) patients, the population of TM endothelial cells is markedly decreased compared with the population of TM endothelial cells in age-matched healthy controls.[6] Oxidative stress is a driving force for the loss of TM cells in POAG,[7] and for determining DNA injury, cell loss, and general malfunctioning of the TM.[8] Unlike the TM, the cornea and the iris are directly exposed to light, so they possess antioxidant defense mechanisms that are not activated in the trabecular meshwork. This situation results in the TM having a peculiar sensitivity to oxidative stress; of the tissues of the anterior chamber of the eye, the TM is the most sensitive to alterations that are induced by free radicals.[9] Recently, molecular damage to the mitochondria was tested in TM cells that were collected from glaucomatous patients and compared with molecular damage to the mitochondria in TM cells from unaffected controls. The results of this investigation indicated that the level of mitochondrial DNA deletion was dramatically increased in the TM of glaucomatous patients.[10] This same type of molecular injury is also known to occur in blood vessels during the course of atherosclerosis,[11] and it has been shown that the degree to which it occurs is related to the clinical outcome of the disease.[12] Increases in the production of reactive oxygen species in mitochondria, the degree of accumulation of mitochondrial DNA damage, and the level of dysfunction in the progressive respiratory chain are associated with atherosclerosis.[13] From a biological point of view, the AC is a space that is surrounded by an endothelium and a path by which a liquid travels, so it can be considered as being similar to a vessel.[14] It has been demonstrated that the endothelial leukocyte adhesion molecule-1 (ELAM-1), which is an oxidatively induced molecular defense mechanism that is known as the earliest marker of atherosclerotic plaque in the vasculature,[15] is present and activated in TM cells that have been collected from patients with glaucoma.[16] ELAM-1, which is

sometimes referred to as E-Selectin, is a 115-kilodalton (kDa) endothelial cell surface glycoprotein that is activated by cytokines and that mediates the adhesion of neutrophils, monocytes, eosinophils, NK cells, and T cells to activated endothelial tissues.[17] It is involved in several inflammatory pathologies including arthritis,[18] uveitis,[19] and diabetes.[20] Inflammation plays a central role in the pathogenesis of many forms of vascular disease, including atherosclerosis. Atherogenesis begins with endothelial damage, and the damaged endothelium then expresses adhesion molecules, chemokines, and proinflammatory cytokines that drive the formation of atherosclerotic plaques and spill into the blood circulation as biomarkers of atherosclerotic disease.[21] Like atherosclerosis, glaucoma presents endothelial dysfunction in the TM[22] that is accompanied by an increased production of reactive oxygen species in the mitochondria, the accumulation of damage in the mitochondrial DNA, and progressive respiratory chain dysfunction.[10,23] In both pathologies, TNF-α activates the transcription of NF-κB, which regulates the expression of genes that are involved in inflammation, oxidative stress, and endothelial dysfunction.[24]

The aim of the present study is to determine which of the proteins that are present in the aqueous humor of glaucoma patients have joint roles in the pathogenesis of glaucoma and in damaging the tissues of small vessels. This may help to identify novel molecules that play a role in the pathogenesis of glaucoma; it may also aid in the development of new diagnostic markers and therapeutic targets.

Methods

Patients

This study had a case-control design. Both patients and controls underwent ocular surgery for therapeutic purposes. Aqueous humor samples were obtained from clinically uncontrolled primary open-angle glaucoma patients (cases) and patients with senile cataracts (controls) prior to undergoing trabeculectomies and cataract surgeries, respectively.

Case samples were collected from 14 POAG patients (6 males, 8 females) with no tonometric compensation as established by both clinical and instrumental examinations in the same manner as reported elsewhere.[25] The main elements of POAG diagnosis were the morphology of the ocular papillae, IOP values, and visual field analysis. Patients were excluded if they met the exclusion criterion of the presence of any ocular, systemic, or neurological diseases other than POAG and POAG-related optic nerve damage. An additional exclusion criterion was the presence of glaucoma of any type other than POAG. The mean age of the POAG patients was 73.7 ± 3.22 years (means ± SE).

All of the glaucomatous patients were treated with maximal therapy that included: topical beta-blockers (100% of patients; n = 14/14); prostanoids (92.85% of patients; n = 13/14); topical carbonic anhydrase inhibitors (100% of patients; n = 14/14); and systemic carbonic anhydrase inhibitors (42.85% of patients; n = 6/14). In three patients (21, 42%) study authors suspended pharmacological anti-glaucoma therapy during the 3 days before AH collection.

The inclusion criteria for controls included the following: an open anterior chamber angle; no history of previous filtration surgery; pupil size >5 mm after dilatation; the absence of pseudoexfoliation syndrome, diabetes, uveitis, systemic collagenopathy, and objective neurological signs; no history of using systemic antihypertensive drugs; and no history of using corticosteroids or systemic or topical antiglaucomatous drugs during a period of at least 32 days[25] immediately preceding their enrollment in the study.

All of the patients underwent Humphrey 30-2 computerized visual field examinations (750 Humphrey Field Analyzer II; Humphrey Ind, San Leandro, CA) 11 to 28 days prior to surgery. To evaluate visual field losses, the glaucoma staging system (GSS 2)[26] was used. All of the patients underwent tonometry evaluations during the week before surgery; these evaluations were conducted by the same physician and used Goldmann tonometry. The average values of the last six measurements that were obtained in the mornings (between the hours of 8 and 10) during the last week prior to surgery are reported in Table 1.

Table 1. Characteristics of Controls and POAG Patients Enrolled in this Study

Pathological Status	Sex	Age (years)	IOP (mmHg)	VFD (GSS)
Controls	5 males 6 females	71.0 ± 3.88	14.1 ± 2.0	0.0 ± 0.00
POAG	6 males 8 females	73.7 ± 3.22	27.7 ± 1.61	2.9 ± 1.15

GSS, glaucoma staging system.
Values are mean ± SE.

Samples were collected from 11 age- (72.9 ± 11.8 years) and sex-matched controls (5 males, 6 females) prior to surgical interventions to remove cataracts. The IOPs of the control subjects were measured by mean standard tonometry, and a mean value of 14.1 ± 2.0 mmHg (mean ± SD) was detected.

Clinical characteristics of the glaucomatous patients are reported in Table 1.

All of the enrolled subjects provided informed written consent and were treated in accordance with the Declaration of Helsinki.

Prior to surgery, a 100-mL intravenous injection of Mannitol was administered to both cases and controls, patients' eyes were treated with a peribulbar anesthetic (bupivacaine hydrochloride 5%), and papillary dilatation was induced using a solution containing 10% phenylephrine and 1% tropicamide. The eyelids and the surrounding skin were swabbed with disinfectant. AH sampling was performed in the frame-shift of trabeculectomy surgery for POAG patients. A corneal tunnel was established using a 19 scalpel (blade), and aqueous humor (100–200 μL) was aspirated from the AC by inserting a 26-gauge needle just before surgery. The same surgical procedure (corneal tunneling and needle AH sampling) was used in controls (corneal patients undergoing cataract intervention). Primary AH was collected performing AH sampling immediately after corneal tunneling (i.e., before the occurrence of any variation inside anterior chamber). Anterior chamber never collapsed in any of the examined subjects.

In order to avoid any influence of circadian variables[27] all AH samplings were performed on Wednesday from 10 to 12 AM.

AH sample aliquots were immediately stored in a deep freezer (−80°C) until proteome analysis was performed. Analyses of each sample were performed within 3 months of collecting the samples.

Detection of Aqueous Humor Proteins Using an Antibody Microarray

Due to the small amount of protein that was available in the aqueous humor samples that were collected, standard Western blotting methods could not be used to analyze the protein expression levels in the samples. Accordingly, study authors decided to use the antibody microarray method instead.[28] The analysis was conducted using two antibody microarrays (Explorer Antibody Microarrays; Full Moon BioSystems Inc., Sunnyvale, CA; and Clontech Ab Microarray TM 500s; Clontech, Mountain View, CA) that were spotted with direct antibodies against a total of 1264 proteins. Two aliquots from each sample that contained 20 μg of protein (quantified using the bicinchoninic acid method) were diluted 1:1 μg/μL in Extraction/Labeling Buffer (Clontech) and labeled via a 2-hour incubation at 4°C with 9.1 μg of either Cy3 or Cy5 (GE Healthcare, Genoa, Italy). The

labeled proteins were purified via elution in 100 μL of 1× desalting buffer in protein desalting spin columns (Pierce Biotechnology, Rockford, IL) and hybridized for 40 minutes onto glass antibody microarrays. The slides were washed, dried, and analyzed by laser scanning and fluorescence detection (ScanArray Lite; Packard Bioscience, Waltham, MA). Signal quantification was performed by microarray analysis software (QuantArray; GSI Lumonics, Bedford, MA), which subtracted the local background fluorescence from the signal intensities. An internal standard of albumin was used in each of the samples to perform quantitative standardization of the results obtained. Protein functions were inferred from the Swiss protein database (in the public domain at http://expasy.org/sprot/) and from the available scientific literature.

Statistical Analyses

Proteome expression profiles were analyzed using gene expression analysis software (GeneSpring 7.3; Agilent Technologies, Santa Clara, CA). Raw data from which the backgrounds have been subtracted were log transformed and normalized both per chip and per protein by median centering. Proteins identified as POAG hallmarks were identified on the basis of their ability to discriminate between pathological status and controls basing on: support vector machine analysis, k-nearest neighbor analysis, statistical significance $P < 0.05$, and >2-fold variation between POAG patients and controls as evaluated by volcano plot analysis. Only in the case of convergence of all criteria, the proteins were identified as POAG-related.

Selection of Proteins Involved in Vascular Lesions

Proteins that were involved in vascular lesions were selected on the basis of having biological functions that matched the composition of a functional categorization in the gene-spider function of the gene expression analysis software (Agilent Technologies). The categorization of each selected protein was confirmed by examining the Swiss protein database and by analyzing the available scientific literature by entering each protein name into the PubMed database (in the public domain at http://www.ncbi.nlm.nih.gov/).

Results

Proteome profile well distinguished between POAG cases and controls as evaluated by PCA (Fig. 1A). This discrimination was independent of patient number and was obtained also using four POAG patients and three controls only (Fig. 1B), but dependent on the number of analyzed protein. Indeed, best diagnostic performances (reported as percentage of correct prediction using computerized support vector machine analysis) were obtained using 100 or more proteins (Fig. 1C). Study authors specifically evaluated the relationship between the number of analyzed protein and the diagnostic efficacy (i.e., the test ability to discriminate between controls and POAG patients).

In order to evaluate any possible effect of drug administration on AH proteome 3, POAG cases refrain from pharmacological therapy for 72 hours before AH sampling. Proteome profile of these subjects is highlighted by green circles in Fig. 1A. As inferred from principal component analysis, no influence of pharmacological therapy suspension was detected (Fig. 1A).

Among the 1264 proteins that were tested, 12 that were related to endothelial damage were expressed in the aqueous humor and had statistically significant increases in their expression levels in samples from POAG patients compared with expression levels in control samples. The expressions of these proteins were barely detectable in the aqueous humor samples that were taken from the control patients, yet these proteins were highly expressed in the samples from patients with POAG. Indeed, when the intensity of fluorescence was expressed using a color scale on which blue represents the minimum expression level and red represents the maximum expression level; the observed intensity of fluorescence was low in control samples and high in POAG case samples. This difference in intensity was clear from an evaluation of each one of the two protein spots that were included in each antibody microarray (Fig. 2). From a quantitative standpoint, there was a significant increase in the quantities of these 12 proteins, which could be determined by calculating the ratios of the log-transformed normalized data regarding the protein expression intensities in samples from POAG patients and controls. Indeed, the levels of protein expression in the aqueous humors of POAG patients were increased by a minimum of 2.0-fold up to a maximum of 2.54-fold relative to expression levels in the samples from the control patients (Fig. 3).

The identities, fold variations, and biological functions of these 12 proteins are reported in Table 2.

Among these 12 proteins, two proteins (apolipoprotein B and E) are involved in the delivery of cholesterol to cells. Five of the proteins (myotrophin, myoblast determination protein 1, myogenin, vasodilator-stimulated phosphoprotein, and ankyrin-2) are involved in muscle cell differentiation and function. Three of them (heat shock 60 kDa and 90 kDa, and ubiquitin fusion degradation 1-like) are involved in stress responses and the removal of damaged proteins; and 2 of the 12 proteins (phospholipase C beta and gamma) are involved in signal transduction and neural development.

Discussion

AH proteome analyses well discriminate between the 14 POAG and 11 control subjects. This result could not be achieved using a single-marker endpoint. Indeed, the antibody microarray method relies on the great number of proteins analyzed (1200) for each subject, allowing study authors to obtain significant results with a sample size lower than those used in standard trials. Indeed, proteomic studies analyze expression of many proteins in few subjects, at variance with standard clinical studies, analyzing one or few endpoints in many subjects ($n = 30$). This feature explains why proteomic studies performed in the ophthalmologic area typically recruit 5–16 samples per experimental group.[29–32]

In principle, the observed expression levels of AH proteins might be related to either the occurrence of POAG or the chronic administration of antiglaucomatous drugs. Every available treatment for preventing progressive glaucomatous optic neuropathy has potential adverse effects.[33] However, the hypothesis that antiglaucomatous therapy may be the factor that determines the heightened expressions of these proteins in the aqueous humor of patients with POAG seems to be absolutely false. Both timolol and the topical carbonic anhydrase inhibitors that are used in glaucoma treatment regimens are substances that have the ability to protect trabecular endothelial cells.[34] Oxidative stress is able to induce characteristic glaucomatous TM changes in vitro[35] and in vivo,[7,36] but the oxidative stress-induced changes in the TM can be minimized by the use of prostaglandin analogues.[35] In addition, inflicting oxidative stress on TM cells induces changes that are typical of POAG, such as ECM accumulation, cell death, disarrangement of the cytoskeleton, advanced senescence, and the release of inflammatory markers. However, these effects are markedly reduced when the TM cells are pretreated with antioxidants, prostaglandin analogues, beta-blockers, or local carbonic anhydrase inhibitors.[37]

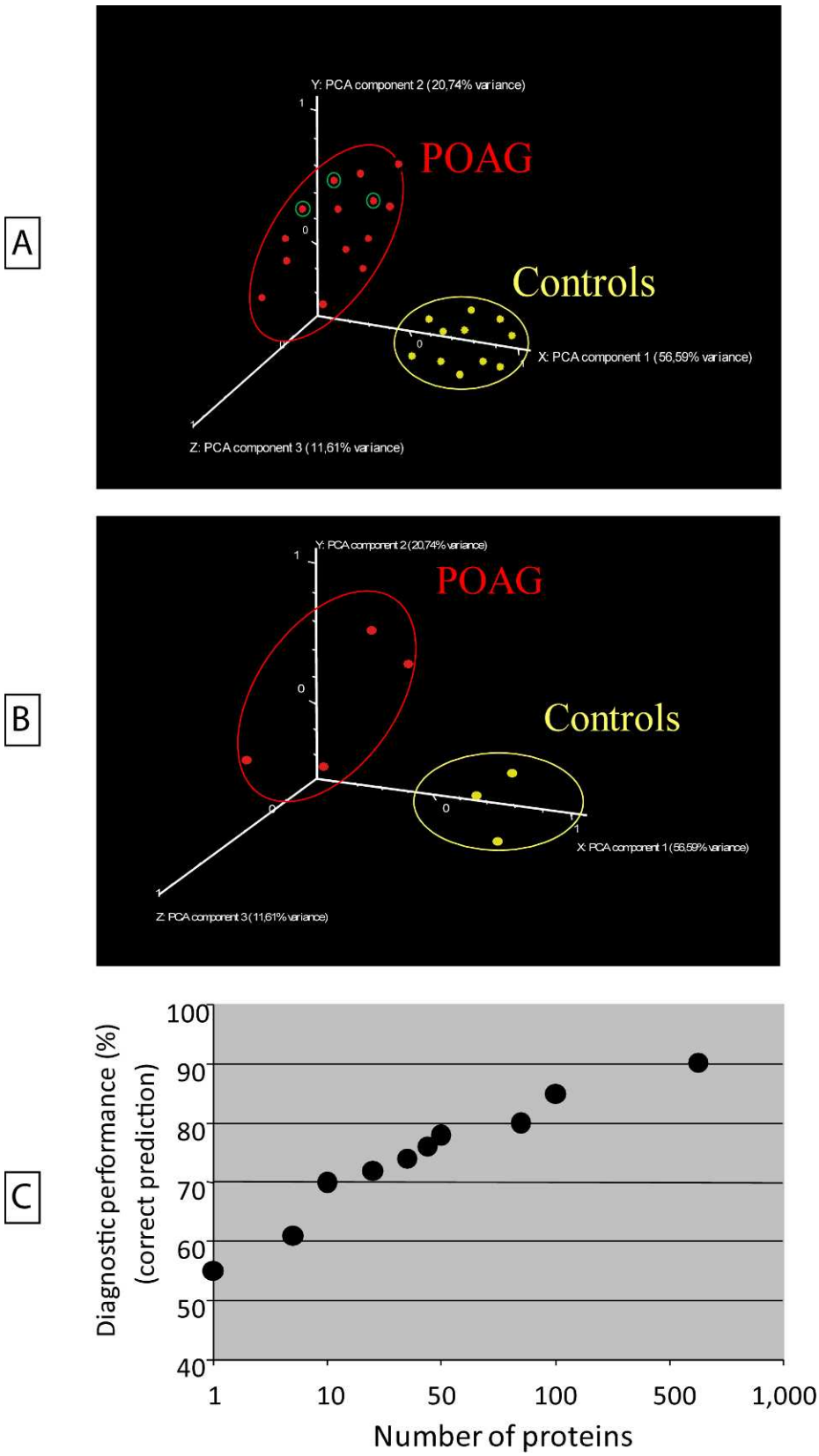

Figure 1. Proteome profile well distinguished between POAG cases and Controls as evaluated by principal component analysis (PCA) of variance. Each *dot* represent one subject (*red*, POAG; *yellow,* controls) located in a 3D space according to the variances of data obtained for the 1264 AH proteins analyzed (**A**). Green circles indicate three POAG patients suspending pharmacological therapy 72 hours before AH sampling. POAG-control discrimination was independent of patient number and was obtained also using four POAG subjects and three controls only (**B**), but dependent on the number of analyzed protein. Indeed, best diagnostic performances (reported as percentage of correct prediction using computerized support vector machine analysis) were obtained using 100 proteins or more (**C**).

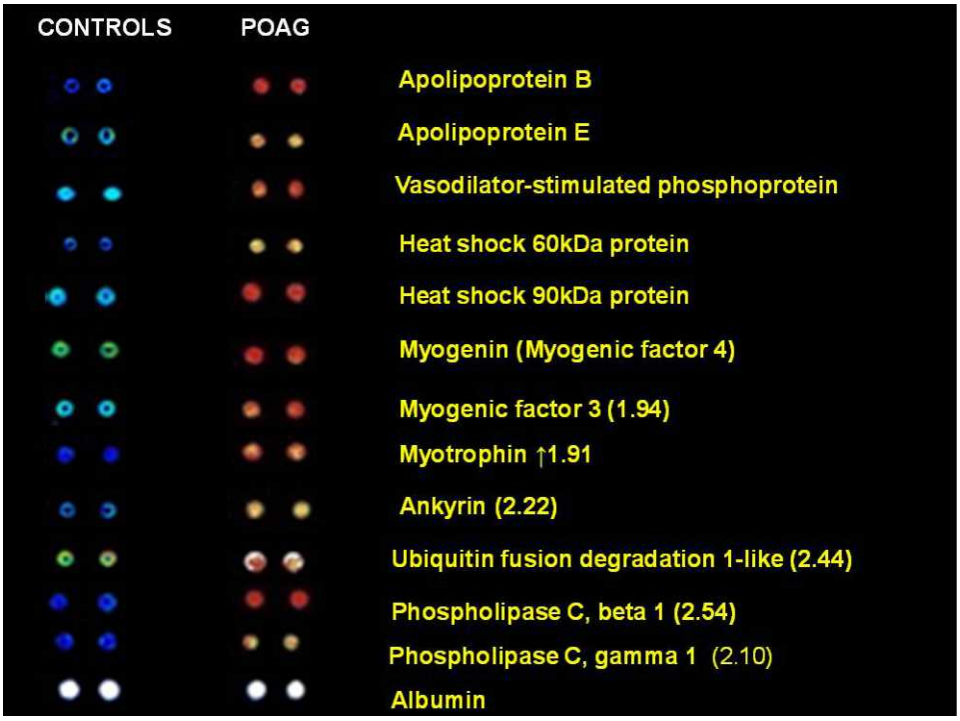

Figure 2. Intensity of protein expression as inferred from the fluorescence detected by antibody microarray techniques in the aqueous humor of control (*left column*) and POAG (*right column*) patients. The figure shows the expression levels that were obtained for each of the two protein spots that were included in the antibody microarray according to a color scale in which blue implies minimal protein expression and red corresponds to maximal protein expression.

Accordingly, the observed presence of a specific set of proteins in the AH samples from POAG patients is most likely related to the disease. The proteins that were detected are known as hallmarks of the vascular atherosclerotic process, which indicates that the anterior chamber of the eye not only has morphological similarities to blood vessels (i.e., both are formed by walls covered by endothelium), but also has functional and pathological similarities.

This finding was first demonstrated for ELAM-1, which was the earliest established marker of atherosclerotic plaque in the vasculature. The consistent presence of ELAM-1 was demonstrated in the trabecular meshwork of glaucomatous subjects.[16] Study results detected the presence of ELAM-1 in the AH of patients with POAG, thereby providing a new source for the detection of this molecular marker of glaucoma. Furthermore, our study provides evidence that the expression levels of other proteins that have roles in vascular endothelium damage are also altered in the ACs of glaucoma patients. Apolipoprotein B (ApoB) is the main apolipoprotein in chylomicrons and low-density lipoproteins.[39] ApoB proteins have roles in the pathogenesis of coronary disease and have a role in the progression of atherosclerosis. The ApoB gene is a predictive factor of cardio-cerebrovascular diseases in the elderly.[40] The role of ApoB in glaucoma is still unknown, even though it is possible to imagine a relationship between ApoB activity and topical beta-blocker therapy. Indeed, topical application of timolol adversely affects the plasma levels of HDL, cholesterol, and triglycerides, even though the total cholesterol and LDL cholesterol levels are not significantly affected.[41] Another possible explanation for the presence of ApoB in the aqueous humor of glaucoma patients could be that chronic inflammation is characterized by the production of cytokines such as the NF-κB transcription factor, TNF-alpha, which has been identified as being involved in inflammatory, anti-apoptotic, and immune responses.[42] TNF-alpha leads to the production of ApoB.[43] It is unlikely that heightened ApoB expression levels originate from extracellular distribution of ApoB in the peripheral cornea.[44]

Apolipoprotein E is also expressed at high levels in the glaucomatous aqueous humor. Apolipoprotein E production is upregulated in response to oxidative stress, and Apolipoprotein E itself is endowed with antioxidant properties.[45] Therefore, its presence in the aqueous humor of glaucoma patients confirms both the pathogenic role of oxidative stress in this disease and the occurrence of TM endothelium dysfunction.

Another protein that can be used to characterize the glaucomatous aqueous humor is vasodilator-stimulated phosphoprotein (VASP). VASP is associated with filamentous actin formation, and it plays a major role in cell adhesion and motility. VASP is also involved in the intracellular signaling pathways that regulate integrin-extracellular matrix interactions.[46] It plays an important role in the formation of adherens-junctions, and it is important for actin cytoskeleton remodeling events that are involved in the maintenance of functional endothelia.[47] Thus, the presence of this protein in the glaucomatous aqueous humor indicates that the endothelial barrier of the TM is altered in glaucoma. This occurs as a result of three distinct interrelated mechanisms: decreases in the endothelial cellularity that are related to aging[48] and glaucoma[6]; elevated intraocular pressure that increases the tension across adherens junctions and thereby alters the maintenance of the polarity and function of the endothelial barrier,[47,49] in addition to altering cell survival and proliferation[50,51]; and immune system mechanisms in which endothelial barrier function is altered by the presence of soluble polymorphonuclear leukocyte-derived mediators that are released during inflammatory states. In the human brain, activated human

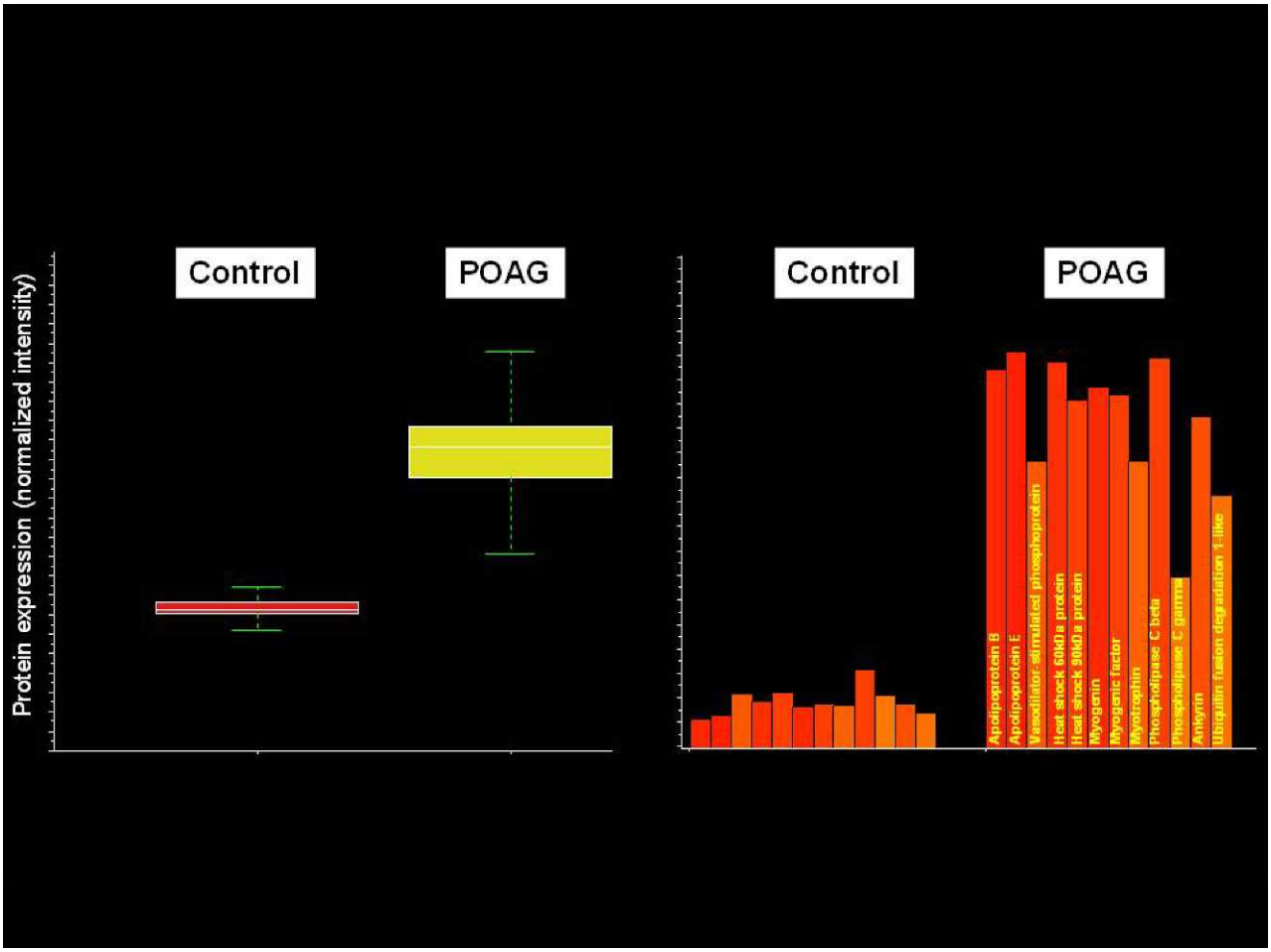

Figure 3. Quantitative analysis of the expression of 13 proteins that are related to vascular alterations that exhibited significant increases in the aqueous humor of POAG patients. Relative protein expression levels were evaluated by calculating the ratio between the expression intensities in POAG and control samples based on log-transformed, normalized data. The *left* panel shows the means and confidence intervals of the average expression levels of the 13 proteins for the control and POAG groups. The *right* panel shows the expression levels of each protein in control and POAG patients.

PMNs release glutamate and induce the endothelial expression of group I or group III glutamate receptors. These in turn function to decrease endothelial VASP phosphorylation and barrier functions.[52] Recently, in confirmation of this mechanism, Alvarado et al.[53] verified that the innate immune system in general, and monocytes in particular, play an important role in maintaining aqueous outflow homeostasis. The recruitment of increased numbers of monocytes following a selective laser trabeculoplasty most likely plays a role in lowering the IOP.[53] The level of VASP expression is closely related to changes in endothelial function and vascular oxidative stress, and VASP represents a biochemical marker for monitoring both endothelial integrity and activity in the nitric oxide (NO)-stimulated soluble guanylyl cyclase/cGMP-dependent protein kinase type I pathway.[54]

Heat shock 60 kDa protein 1 (Hsp60) is a mitochondrial chaperonin involved in the removal and repair of damaged proteins. Although heat shock proteins are typically regarded as being exclusively intracellular molecules, they can be released from several cell types, including endothelial cells, in the absence of cellular necrosis.[55,56] The mitochondrial chaperonin Hsp60 is one of the most important components of the protein-folding system inside the mitochondrial matrix.[57] Hsp60 also has both anti-apoptotic[58] and pro-apoptotic roles.[59] Hsp60 mediates NF-κB-dependent survival signaling in cells, and the cytosolic fraction of Hsp60 serves as a means of survival guidance by controlling mitochondria-derived ROS via the expression of NF-κB target genes.[60] Hsp60 has also been implicated in T-lymphocyte activation[61] and in causing inflammatory reactions.[62] High levels of Hsp60 are associated with early atherosclerosis,[63] serum concentrations of the pro-inflammatory cytokine tumor necrosis factor-alpha and other markers of inflammation in overtly healthy individuals.[64] The presence of Hsp60 in the glaucomatous aqueous humor is logical, given the fact that there is a clear mitochondrial dysfunction in glaucoma[10] and, being a regulator of transitions in the permeability of mitochondrial membranes, Hsp60 is mainly located in the mitochondria.[65]

Like Hsp60, heat shock protein 90 kDa (Hsp70) is also present in glaucomatous aqueous humor. This protein is overexpressed in both carotid atherosclerotic plaques and in serum from patients with atherosclerosis; strong Hsp70 immunoreactivity is also detected in the muscles, endothelial cell layers, and inflammatory infiltrates of carotid plaques.[66]

In addition to endothelial cells, the contractile component of muscle tissue plays a fundamental role in the proper functioning of the TM. Myoblast differentiation is a multistep process characterized by permanent exit from the cell cycle, maturation into mononucleated myocytes, and fusion into multinucleated myotubes. This differentiation program is

Table 2. Identities and Biological Functions of Protein Markers of Endothelial Damage Detected in the Aqueous Humors of POAG Patients and Controls by Antibody Microarray Analysis

NAME	Swiss Protein Code	Fold Variation (POAG/Control Ratio)	Function
Apolipoprotein B	P04114	2.44	Primary apolipoprotein of low-density lipoproteins that is responsible for carrying cholesterol to tissues. Major protein constituent of LDL (ApoB100) and VLDL (ApoB100). ApoB100 functions as a recognition signal for the cellular binding and internalization of LDL particles via the apoB/E receptor.
Apolipoprotein E	P02649	2.07	Primary apolipoprotein of low-density lipoproteins that is responsible for carrying cholesterol to tissues. Mediates the binding, internalization, and catabolism of lipoprotein particles, and is a ligand for the LDL (ApoB/E) receptor.
Myotrophin	P58546	2.00	Primary role in muscle and neural development.
Myoblast determination protein 1	P15172	2.02	Myogenic factor involved in muscle differentiation. Induces the differentiation of fibroblasts into myoblasts. Activates muscle-specific promoters.
Myogenin (myogenic factor 4)	Q9HCA4	2.04	Muscle differentiation, development, and function
Vasodilator-stimulated phosphoprotein	P50552	2.17	Controls remodeling of the actin cytoskeleton and plays a substantial role in cell adhesion and motility. It is an actin-associated protein that is involved in a range of processes that are dependent on cytoskeleton remodeling and cell polarity, such as axon guidance, lamellipodial and filopodial dynamics. Promotes actin filament elongation. Stimulates actin filament elongation by promoting the transfer of profilin-bound actin monomers to the barbed end of growing actin filaments. Plays a role in actin-based mobility.
Heat shock 60 kDa protein	P10809	2.20	Intracellular chaperone that assists in mitochondrial protein folding, unfolding, and degradation. Apoptosis regulator. Stress response protein. Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides that are generated under stress conditions in the mitochondrial matrix.
Heat shock 90 kDa protein	P08238	2.04	Molecular chaperone. Has ATPase activity. Involved in the stress response, axon growth guidance, and atherosclerosis. Acts as a nitric oxide synthetase regulator.
Ubiquitin fusion degradation 1-like	Q541A5	2.44	Signaling and energy-metabolism proteins that regulate the endoplasmic reticulum–associated degradation pathway. Involved in the removal of damaged proteins and in ubiquitin-dependent protein catabolism.
Phospholipase C, beta	Q9NQ66	2.54	Mediates the production of the second messenger molecules diacylglycerol and inositol 1,4,5-trisphosphate. Involved in signal transduction, neural development, the glutamate signaling pathway, and inflammation.
Phospholipase C, gamma	P19174	2.10	Plays a role in actin reorganization and cell migration. Mediates the production of the second messenger molecules diacylglycerol and inositol 1,4,5-trisphosphate. Involved in signal transduction, neural development, and inflammation.
Ankyrin-2	Q01484	2.22	Neural protein. Attaches integral membrane proteins to cytoskeletal elements. Required for the coordinated assembly of Na/Ca exchanger, Na/K-ATPase and inositol 1,4,5-trisphosphate receptors at sarcoplasmic reticulum sites in myocytes. Required for the coordinated expression of the Na/K ATPase, the Na/Ca exchanger, and beta-2-spectrin in the inner segments of rod photoreceptors. Expressed in the plasma membrane of the inner segments of the photoreceptors in retina. Required for the growth and contraction of myocytes. Involved in neural axon growth and guidance.
ELAM-1	P16581	2.00	Cell-surface glycoprotein that has a role in immunoadhesion. Mediates the adhesion of neutrophils from the blood in the cytokine-activated endothelium by means of interaction with PSGL1/SELPLG. A polymorphism in position 149 is associated with an increased risk of coronary artery disease. Involved in inflammation, cell adhesion, the activation of phospholipase C, calcium signaling, and actin filament-based processes.

The reported proteins undergo significant increases ($P < 0.05$) as evaluated by volcano plot analysis and a k-nearest neighbor algorithm. Fold variation increases are reported as the ratio between the expression intensity of each protein in POAG samples vs. control samples.

controlled by a basic helix-loop-helix family of myogenic regulatory factors (MRFs) that includes MyoD and myogenin.[67] The results that we have reported in the current study indicate that these proteins are present in glaucomatous AH samples. The most frequently represented MRF is myogenin or myogenic factor 4, which belongs to a family of myogenic proteins that are regulators of muscle-specific gene expression, and which is known for its role in embryonic myogenesis, adult muscle growth, and regeneration.[68] Myogenin expression is activated upon muscle injury to help facilitate muscle regeneration and repair.[69]

Myogenin expression causes inflammatory macrophages to stimulate myogenic cell proliferation[70] and induces myogenic cell growth.[71] A similar phenomenon occurs in the TM; after a selective laser trabeculoplasty, monocytes are recruited to the trabecular meshwork in humans. Indeed, the number of monocytes/macrophages in the TM increases substantially following a selective laser trabeculoplasty, and monocytes

augment both the outflow facility and the conductivity of Schlemm's canal endothelial cells.[53]

The second myogenic protein that was detected in glaucomatous aqueous humor is myoblast determination protein 1 or myogenic factor 3. This protein regulates the determination and differentiation of skeletal muscles.[72] Myogenic factor 3 is a master regulator of skeletal myogenesis due to its ability to initiate the myogenic program in a number of cell types, including myoblasts, fibroblasts, and others.[73] Myogenic factor 3 also acts as a proapoptotic factor.[74] Therefore, as it does in old muscle tissue, apoptosis in the TM may play a causative role in the depletion of satellite cells by impairing the regenerative response to injury and the contractile cellular component. Indeed, myogenic factor 3 is involved in aging-related cell apoptosis during muscle regeneration.[74]

Another protein that is linked to muscle cells that is also present in the glaucomatous aqueous humor is myotrophin, which is a myocyte-specific protein.[75] Neonatal myocytes that were treated with myotrophin displayed an accelerated myofibrillar growth pattern and organized into sarcomeres.[76] Increased levels of myotrophin have also been correlated with the onset of muscle hypertrophy in humans.[77] The stimulatory activity of myotrophin on the synthesis of muscle proteins is mediated through the protein kinase C (PKC) signaling pathway.[78] PKC levels are significantly increased in the aqueous humors of patients with POAG.[27] PKC influences aqueous humor outflow, and it affects cellular relaxation, contraction, and morphological changes in TM and sclerocorneal cells.[79] Myotrophin exerts its action by activating the NF-κB pathway[80]; in the TM, this pathway is activated by oxidative stress.[81]

The alterations in concentrations of muscle cell proteins that occur in conjunction with glaucoma resemble the early changes that occur during the atherosclerotic process in blood vessels. Indeed, smooth muscle cells change their phenotypes by losing their contractile function and by migrating from the middle layers to the inner layers of a muscle in an attempt to remove lipid deposits. This mutation of smooth muscle cells is related to alterations in cellular DNA, and it triggers the whole atherosclerotic process.[82]

The involvement of the muscular component of the TM in glaucoma is confirmed by the presence of high levels of ankyrin in the glaucomatous aqueous humor. Ankyrin anchors cytoskeletal components to the intracellular machinery in muscle tissues, and its presence is particularly prominent in contractile tissues.[83,84]

Another one of the proteins found in glaucomatous aqueous humor is ubiquitin. The ubiquitin-proteasome system is the major nonlysosomal proteolytic pathway used for degrading intracellular proteins, and it is of vital importance for maintaining homeostasis and normal function in eukaryotic cells.[85] It is also essential for modulating the cellular response to stress,[86,87] the endothelial cell cycle,[88] the regulation of cell migration, and motility in the endothelium.[89] The ubiquitin-proteasome system is also involved in the regulation of endothelial nitric oxide synthase activity. Endothelial nitric oxide synthase is a key enzyme of vascular homeostasis that influences the oxidative stress response in the vasculature and thereby contributes to endothelial dysfunction.[90] A balance between vasoconstrictors and vasodilators is necessary for maintenance of the physiological structure and function of the endothelium,[91] and ubiquitin is implicated in modulating the permeability of the endothelial barrier and the release of endothelins and nitric oxide. Whenever this balance is disrupted, as it is in glaucoma, the outcome is endothelial dysfunction and injury.[92] The TMs in glaucoma patients exhibit cytoskeletal changes in cells,[93] altered cellularity,[6] and changes in the extracellular matrix.[94] From a molecular standpoint, the ocular AC is similar to other vessel tissues and its behavior also resembles that of a vessel. This observation is supported by other proteins that were detected in the glaucomatous AH samples (i.e., phospholipase C beta 1 and gamma 1). These proteins generate two intracellular products: inositol 1,4,5-trisphosphate, which is a universal calcium-mobilizing second messenger, and diacylglycerol, which is a protein kinase C (PKC) activator.[95] Both of these molecules have been detected in glaucomatous AH samples.[28] Calcium mobilization plays a pivotal role in muscle contraction, and the ciliary muscle plays an important role in the regulation of aqueous humor outflow in the mammalian eye. The ligamentous insertions of the ciliary muscle in the TM modulate the permeability of the TM tissue to the AH.[96] When the ciliary muscle contracts, its insertions widen, and the intercellular spaces in the TM and the permeability of its tissues increase due to increases in the total area of endothelial cell surfaces that are exposed to the AH. Furthermore, the TM itself is a contractile tissue that has properties that are similar to the properties of smooth muscle, and its contractility is linked to Rho kinase A, which can be regulated by PKC isoforms.[97,98] The presence of PKC isoforms in glaucomatous AH samples is most likely correlated with the TM motility damage that occurs during the course of glaucoma.[28] PKC activation triggers regulatory mechanisms that are involved in cytoskeletal reorganization and intercellular adhesion. These regulatory mechanisms then influence the shapes of the TM cells.[79] PKC has also been implicated in the transcriptional regulation of matrix metalloproteinases. These proteins maintain normal AH outflow rates.[99] Elevated levels of matrix metalloproteinases in the AHs of glaucomatous eyes may be produced by both inflammatory cells and cells of the TM.[100] Phospholipase C beta and gamma mobilize internal calcium stores by means of PKC activation, and they engage a number of protein kinase pathways that control or modulate many cellular activities, including cell division, transformation, differentiation, shape, motility, and apoptosis.[95]

On the whole, AH protein alterations detected indicate that a remarkable endothelial damage affects the anterior chamber in glaucoma. The most likely source of such damage is the trabecular meshwork, which is the main tissue undergoing severe molecular, histological, and functional alteration in the anterior chamber during POAG. Indeed, since the AH outflow is severely altered in advanced glaucoma, the possibility that TM endothelial derived proteins remain in the AH exists and is even increased during the inversion of the flow as occurring during AH aspiration by corneal puncture. Obtained results indicate that molecular damage is not limited to endothelium but also involves the muscle components of the TM structure (Fig. 4).

Conclusions

The validity of using an antibody microarray to analyze a proteome has previously been established under a wide variety of experimental and clinical conditions.[101–113] In the present study, study authors used this method to analyze the aqueous humor proteome for the first time. The main relevant attribute of this method of analysis is that it allows for a global overview of the profile of the proteome. This goal cannot be achieved by methods that are targeted at analyzing single proteins such as immunohistochemistry, ELISA, GC/MS, or HPLC. Furthermore, antibody microarray analysis results in direct and immediate identification of the analyzed proteins using a minimal amount of specimen, such as the amount of aqueous humor that could be collected via anterior chamber puncture. This characteristic

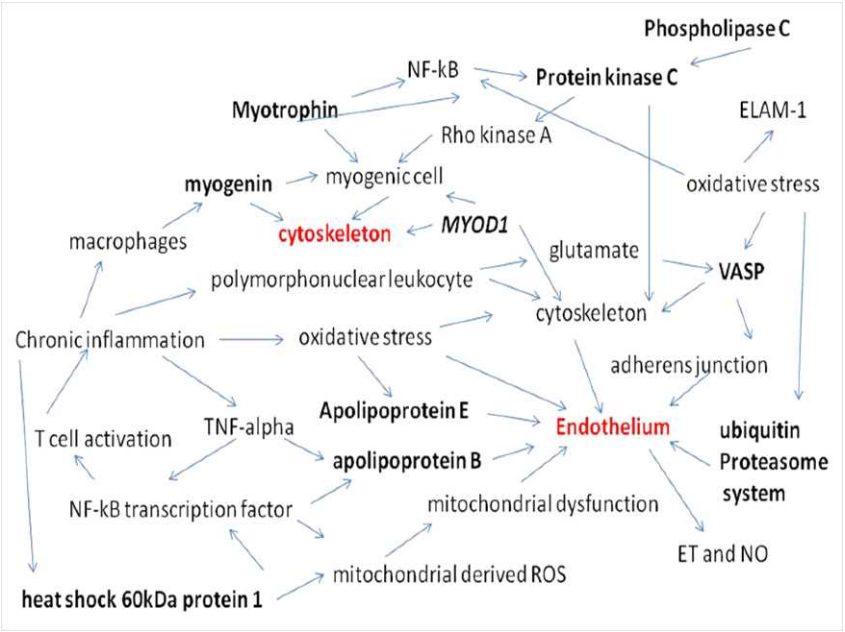

Figure 4. Functional interactions of aqueous humor proteins associated with POAG glaucoma and involved in vascular damage as identified in the herein presented study (*bold characters*). Regulated functions are shown in *grey*, and cellular, and subcellular targets, are shown in *red*. The speculative reciprocal relationship (*arrows*) are based on cited bibliography (see "Discussion") and Swiss-protein database information available for each protein.

will favor the future application of this ready-to-use biochip in human clinical studies.

In total, the proteome alterations that were detected in glaucomatous AH suggest that POAG is associated with altered expression levels of adhesion molecules that are produced by endothelial cells, the recruitment of inflammatory cells and the recruitment cytokines that target endothelial cells, vascular smooth muscle cells, the extracellular matrix, and the mitochondria. These proteomic changes ultimately lead to apoptosis and degeneration of the TM.[3] From anatomical, physiological, and pathological points of view, the AC is a vessel and behaves like a vessel. During the course of POAG, the changes that occur in the AC are characterized by mechanisms and molecular events that resemble the events that occur during atherosclerosis. These mechanisms include endothelial dysfunction, lipoprotein alteration, modification of smooth muscle cell functions, oxidative damage, inflammation, loss of intercellular adhesion, mitochondrial failure, and apoptosis.

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