Proteome Analysis of Human Aqueous Humor

Uttio Roy Chowdhury,[1] *Benjamin J. Madden,*[2] *Mary Christine Charlesworth,*[2] *and Michael P. Fautsch*[1]

Purpose. Human aqueous humor (hAH) provides nutrition and immunity within the anterior chamber of the eye. Characterization of the protein composition of hAH will identify molecules involved in maintaining a homeostatic environment for anterior segment tissues. The present study was conducted to analyze the proteome of hAH.

Methods. hAH samples obtained during elective cataract surgery were divided into three matched groups and immunodepleted of albumin, IgG, IgA, haploglobin, antitrypsin, and transferrin. Reduced and denatured proteins (20 μg) from each group were separated by gel electrophoresis. Thirty-three gel slices were excised from each of three gel lanes (n = 99), digested with trypsin, and subjected to nanoflow liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS). The protein component of hAH was also analyzed by antibody-based protein arrays, and selected proteins were quantified.

Results. A total of 676 proteins were identified in hAH. Of the 355 proteins identified by nano-LC-ESI-MS/MS, 206 were found in all three groups. Most of the proteins identified by nano-LC-ESI-MS/MS had catalytic, enzymatic, and structural properties. Using antibody-based protein arrays, 328 cytokines, chemokines, and receptors were identified. Most of the quantified proteins had concentrations that ranged between 0.1 and 2.5 ng/mL. Ten proteins were identified by both nano-LC-ESI-MS/MS and antibody protein arrays.

Conclusions. Proteomic analysis of hAH identified 676 nonredundant proteins. More than 80% of these proteins are novel identifications. The elucidation of the aqueous proteome will establish a foundation for protein function analysis and identification of differentially expressed markers associated with diseases of the anterior segment. (*Invest Ophthalmol Vis Sci.* 2010;51:4921–4931) DOI:10.1167/iovs.10-5531

Human aqueous humor (hAH) is a complex mixture of electrolytes, organic solutes, growth factors, cytokines, and additional proteins that provide the metabolic requirements to the avascular tissues of the anterior segment.[1–5] It is produced from the nonpigmented ciliary body epithelium through active transport of ions and solutes and secreted into the posterior chamber.[1,6,7] From the posterior chamber, aqueous flows between the lens and iris into the anterior chamber. hAH exits the anterior chamber via the trabecular meshwork/Schlemm's canal (conventional outflow pathway) and through the ciliary muscle bundles into the supraciliary and suprachoroidal spaces (uveoscleral pathway). A balance between the production and the drainage of hAH is important for maintaining the normal physiological intraocular pressure that is essential to maintaining the optical and refractive properties of the eye.[8]

The protein component of hAH is minimal, containing between 120 and 500 ng/μL of protein.[9,10] The proteins in hAH are thought to arise from plasma as the result of filtration through fenestrated capillaries of the ciliary body stroma via the iris root.[3] However, hAH is not a simple diffusate of plasma, since it has both qualitative and quantitative differences in protein and ion content in comparison with plasma.[9–11] Furthermore, proteins in hAH that are secreted from the anterior segment tissues may have a significant role in the pathogenesis of various eye diseases.[12] Several reports have indicated that changes in hAH proteomics can correlate with the prognosis of eye disorders.[13–15]

Identifying the protein component of tissues or fluids is vital to understanding the role these proteins have in normal physiology. Proteomic approaches have been used to identify proteins in plasma, cerebrospinal fluid, and vitreous.[16–21] An exhaustive database search and review of the protein component of plasma identified 1175 nonredundant proteins,[22] and the Human Proteome Organization (HUPO) plasma proteome project database (http://www.bioinformatics.med.umich.edu/hupo/ppp/ provided in the public domain by the University of Michigan Medical School, Ann Arbor, MI) contains more than 3000 proteins and protein isoforms.[23] In comparison, literature searches identified less than 150 proteins in hAH. Many of these proteins were identified as individual proteins based on targeted molecules of interest by Western blot analysis or enzyme-linked immunosorbent assay (ELISA). Others were identified using proteomic approaches such as Multidimensional Protein Identification Technology (MudPIT)[24] and differential protein expression.[25] Other studies using one- and two-dimensional gel electrophoresis coupled with mass spectrometry have relied mostly on comparative studies (between control and disease eyes).[25–27] Studies on rabbit aqueous humor identified 98 proteins,[28] but extrapolation to hAH is difficult due to species variation. A large variation in protein identification exists in the various studies, and only a few of the proteins have been confirmed across studies. Therefore, it is reasonable to suggest that little is known about the protein composition of hAH.

Characterization of the hAH proteome will provide new insights into the factors involved in maintaining anterior segment homeostasis and will also establish a foundation for biomarker discovery in various eye diseases of the anterior segment, such as glaucoma and corneal dystrophies. In the present study, we undertook a comprehensive nanoflow liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS) and antibody-based protein ar-

From the [1]Department of Ophthalmology and the [2]Proteomics Research Center, Mayo Clinic, Rochester, Minnesota.

Supported in part by National Institutes of Health research Grants EY07065 and EY15736; Research to Prevent Blindness, New York, New York; and the Mayo Foundation, Rochester, Minnesota.

Submitted for publication March 15, 2010; revised April 22, 2010; accepted April 23, 2010.

Disclosure: **U. Roy Chowdhury**, None; **B.J. Madden**, None; **M.C. Charlesworth**, None; **M.P. Fautsch**, None

Corresponding author: Michael P. Fautsch, Department of Ophthalmology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905; fautsch.michael@mayo.edu.

ray approach to identifying moderate to low abundance proteins in hAH.

METHODS

Collection of Human Aqueous Humor

The protocol for collection of hAH was approved by the Mayo Clinic Institutional Review Board and conforms to the Declaration of Helsinki. hAH was collected from patients undergoing elective cataract surgery, as previously described.[29,30] Briefly, a 30-gauge needle was inserted into the midanterior chamber through a paracentesis tract. hAH was slowly aspirated until the anterior chamber began to shallow. The sample was immediately snap-frozen in liquid nitrogen and stored at −80°C before screening. Patients had no other eye abnormalities except for the cataracts. A Bradford protein assay was performed on each hAH sample. A total of 155 hAH samples, each having a total protein concentration within the normal range (100–500 ng/μL),[9,10] were used in this study.

Immunodepletion and Electrophoresis

To ensure adequate volume and protein concentration for proteome analysis, 85 hAH samples were divided into three groups. Each group contained hAH acquired from individuals with similar age, sex, and protein concentrations (Table 1). Because hAH contains several abundant proteins that have been identified, an immunodepletion was performed to ensure identification of less abundant proteins. Each of the three hAH groups were run individually over a 4.6 × 50-mm commercially available protein removal system (MARS6 Multiple Affinity Removal System column; Agilent, Santa Clara, CA) and immunodepleted of albumin, transferrin, antitrypsin, haploglobin, IgG, and IgA. The nonbound flow-through fraction (depleted fraction) was collected, buffer exchanged into 20 mM ammonium bicarbonate, and assayed for protein concentration. An equivalent volume of 20 μg for each sample was concentrated to dryness in a centrifugal vacuum system. Each sample was reconstituted in SDS-PAGE sample buffer with 5% β-mercaptoethanol and electrophoresed on a 10% to 14.5% SDS-PAGE precast gel (Criterion; Bio-Rad, Hercules, CA). The gel was fixed and stained with colloidal Coomassie stain (BioSafe; Bio-Rad). Thirty-three gel slices were excised from each lane (for each group) and processed for nano-LC-ESI-MS/MS (Fig. 1).

Nanoflow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

The 99 gel slices obtained from the three SDS-PAGE gel lanes were subjected to in-gel trypsin digestion, and the extracted peptides analyzed by nano-ESI-LC/MS/MS with a mass spectrometer (ThermoFinnigan LTQ Orbitrap Hybrid; ThermoElectron, Bremen, Germany) coupled to a nano-LC-2D HPLC system (Eksigent, Dublin, CA). The mass spectrometer experiment was set to perform an FT full scan from 375 to 1600 m/z, with resolution set at 60,000 (at 400 m/z), followed by linear ion trap MS/MS scans on the top five $[M+2H]^{2+}$ or $[M+3H]^{3+}$ ions. All MS/MS spectra were analyzed by using Mascot (version 2.2.04;

TABLE 1. Summary of Human Aqueous Humor Samples

	Sample Size (n)	Age (Mean ± SD)	Sex (M/F)	Protein Conc. (μg/mL)
Mass Spectometry				
Group 1	30	72.7 ± 9.9	15/15	0.22
Group 2	30	72.2 ± 9.7	14/16	0.20
Group 3	25	72.7 ± 9.9	10/15	0.20
Protein array				
L-series 507				
Group 4	5	72.4 ± 9.0	3/2	0.21
Group 5	5	67.8 ± 8.7	3/2	0.20
Group 6	5	75.4 ± 7.2	2/3	0.16
Group 7	6	69.7 ± 12.5	2/4	0.17
Chemokine array				
Sample 1	1	74	1/0	0.21
Sample 2	1	76	1/0	0.19
Sample 3	1	89	1/0	0.40
Sample 4	1	76	0/1	0.23
Sample 5	1	68	0/1	0.45
Sample 6	1	70	0/1	0.11
Sample 7	3	80.7 ± 2.1	3/0	0.20
Sample 8	3	71.3 ± 5.9	0/3	0.19
Sample 9	5	73.2 ± 1.8	5/0	0.20
Sample 10	6	74.5 ± 8.4	0/6	0.22
Western Blot				
Sample 11	11	76.4 ± 7.3	2/9	0.28
ELISA				
Sample 12	1	87	0/1	0.21
Sample 13	1	71	1/0	0.19
Sample 14	1	71	1/0	0.18
Sample 15	1	86	0/1	0.12
Sample 16	1	66	0/1	0.22
Sample 17	1	60	0/1	0.19
Sample 18	1	76	0/1	0.14
Sample 19	1	72	1/0	0.40
Sample 20	1	84	1/0	0.30
Sample 21	1	78	0/1	0.10
Sample 22	1	79	1/0	0.24
Sample 23	1	52	0/1	0.13
Sample 24	1	81	1/0	0.13
Sample 25	1	91	0/1	0.48
Sample 26	1	78	0/1	0.34

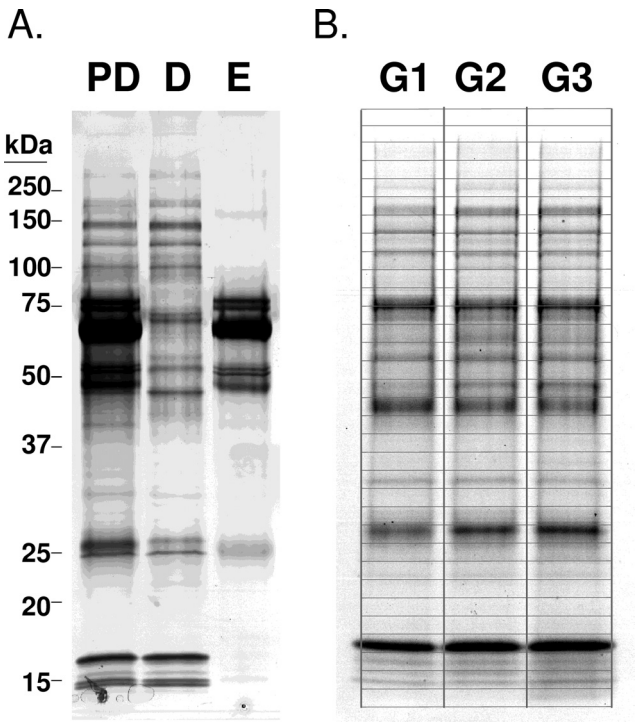

FIGURE 1. Immunodepletion of hAH. (**A**) Eighty-five hAH samples were divided into three groups and immunodepleted of albumin, transferrin, antitrypsin, haploglobin, IgG, and IgA. Groups were separated on 10% to 14.5% SDS-PAGE gradient gels. PD, predepletion; D, immunodepleted (flow-thru); E, eluted from column. (**B**) Thirty-three gel slices were isolated from each group, independently trypsinized, and processed for nano-LC-ESI-MS/MS. G1, group 1; G2, group 2: G3, group 3.

Matrix Science, London, UK;), Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12), and X! Tandem (www.thegpm.org; version 2006.09.15.3/ provided in the public domain by the Global Proteome Machine Organization, Manitoba Centre for Proteomics and Systems Biology, Winnipeg, MB, Canada). Each was set up to search the most current SwissProt database, assuming semitrypsin or full trypsin digestion with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM (SwissProt is provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland, http://www.expasy.ch/sprot/). Oxidation of methionine and iodoacetamide derivatives of cysteine was specified as variable modifications. Proteomics software (Scaffold, ver. 2.00.02; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at a higher than 95.0% probability, as specified by the peptide prophet algorithm.[31] Protein identifications were accepted if they could be established at a higher than 95% probability and contain at least two unique peptides. Protein probabilities were assigned by the protein prophet algorithm.[32] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Western Blot

hAH (300 μL) was mixed in Laemmli buffer, boiled, and separated on 4% to 15% SDS-PAGE preparative gradient gels (Bio-Rad). Proteins were transferred to a polyvinylidene diflouride membrane (Millipore, Billerica, MA) in 1× transfer buffer (50 mM Tris, 384 mM glycine, 0.01% SDS, 20% methanol). Membranes were blocked in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween, and 2% evaporated milk. Blots were probed with BigH3 (R&D Systems, Minneapolis, MN) and fibulin-3 (Chemicon International, Billerica, MA) monoclonal antibodies and with myocilin polyclonal antibody (developed in our laboratory),[33] followed by a secondary horseradish-peroxidase–linked anti-mouse or anti-rabbit antibody (GE Healthcare, Piscataway, NJ) using a multiscreen apparatus (Bio-Rad). Antibody–antigen complexes were detected using ECL Western blot signal detection reagent (GE Healthcare). Autoradiograph film (BioMax XAR; Eastman Kodak, Rochester, NY) was used to visualize the protein signals. Each film was digitized with a photo scanner (Perfection 2400; Epson, Long Beach, CA).

Protein Array

hAH from 21 individuals were divided into four groups, with each group having a similar age, sex, and protein concentration distribution (groups 4–7; Table 1). Each of the groups was assayed using four independent biotin label-based arrays (RayBiotech, Inc., Norcross, GA). Briefly, the four groups of hAH were independently dialyzed and the primary amine of the proteins in the sample was biotinylated, followed by dialysis to remove free biotin. The biotinylated samples were added to four different protein array slides (one sample group for each slide), which were prespotted in triplicate with antibodies for 507 different growth factors, cytokines and receptors. After incubation with Cy3-streptavidin, the signals were visualized by fluorescence. An internal control was included to monitor the function of the array process. Total signal strength was based on the average of the three spots. Proteins that had signal strength of more than threefold over the negative control (95% confidence) were considered positive.

Human Chemokine Array

Quantification of several proteins identified by the antibody-based protein array was performed (Quantikine Human Chemokine Array 1;

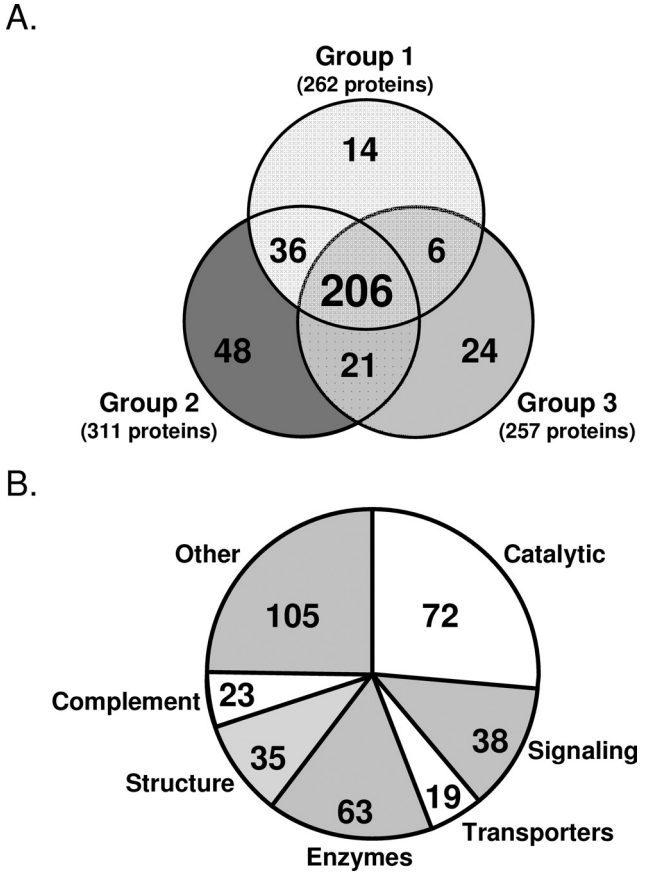

FIGURE 2. The number of hAH proteins identified by nano-LC-ESI-MS/MS. (**A**) The Venn diagram showing proteins that are common and unique between groups 1, 2, and 3. (**B**) Distribution by function of all 355 proteins identified by nano-LC-ESI-MS/MS.

TABLE 2. Aqueous Humor Proteins Identified by Nano-LC-ESI-MS/MS

Found in 3 of 3 Groups

Acid ceramidase (Q13510)
Actin, cytoplasmic 1 (P60709)
Afamin (P43652)
Agrin (O00468)
Aldehyde dehydrogenase (P30838)
Alpha-1-acid glycoprotein 1 (P02763)
Alpha-1-acid glycoprotein 2 (P19652)
Alpha-1-antichymotrypsin (P01011)
Alpha-1B-glycoprotein (P04217)
Alpha-2-plasmin inhibitor (P08697)
Alpha-2-HS-glycoprotein (P02765)
Alpha-2-macroglobulin (P01023)
Alpha-enolase (P06733)
Alpha-N-acetylglucosaminidase (P54802)
Amyloid beta A4 (P05067)
Amyloid-like 2 (Q06481)
Angiotensinogen (P01019)
Antithrombin-III (P01008)
Apolipoprotein A-I (P02647)
Apolipoprotein A-II (P02652)
Apolipoprotein A-IV (P06727)
Apolipoprotein D (P05090)
Apolipoprotein E (P02649)
Apolipoprotein H (P02749)
Attractin (O75882)
Autotaxin (Q13822)
Beta crystallin B2 (P43320)
Beta crystallin S (P22914)
Beta-2-microglobulin (P61769)
Beta-Ala-His dipeptidase (Q96KN2)
Biotinidase (P43251)
Calgranulin-A (P05109)
Calsyntenin-1 (O94985)
Carbonic anhydrase 1 (P00915)
Carbonic anhydrase 2 (P00918)
Carbonic anhydrase-related 10 (Q9NS85)
Carboxypeptidase B2 (Q96IY4)
Carboxypeptidase E (P16870)
Cartilage acidic 1 (Q9NQ79)
Catalase (P04040)
Cathepsin B (P07858)
Cathepsin D (P07339)
Cathepsin L (P07711)
Cathepsin Z (Q9UBR2)
CD59 glycoprotein (P13987)
Ceroid-lipofuscinosis neuronal 5(O75503)
Chitinase-3-like 1 (P36222)
Clusterin (P10909)
Coagulation factor V (P12259)
Coagulation factor II (P00734)
Collagen alpha-1(VI) chain (P12109)
Collagen alpha-2(IX) chain (Q14055)
Complement C1r subcomponent (P00736)
Complement C1s subcomponent (P09871)
Complement C2 (P06681)
Complement C3 (P01024)
Complement C5 (P01031)
Complement component C6 (P13671)
Complement component C7 (P10643)
Complement component C8 α (P07357)
Complement component C8 β (P07358)
Complement component C8 γ (P07360)
Complement component C9 γ (P02748)
Complement factor B (P00751)
Complement factor H (P08603)
Complement factor I (P05156)
Connective tissue growth factor (P29279)
Contactin-1 (Q12860)
Contactin-2 (Q02246)
Corticosteroid-binding globulin (P08185)
Cystatin A (P01040)
Cystatin C (P01034)
Decorin (P07585)
Dermcidin (P81605)
Dickkopf-related 3 (Q9UBP4)
Dipeptidyl-peptidase 2 (Q9UHL4)
Dystroglycan (Q14118)
EGF-like domain-containing protein 4 (Q7Z7M0)
Epididymal secretory protein E1 (P61916)
Extracellular matrix protein 1 (Q16610)
Extracellular superoxide dismutase [Cu-Zn] (P08294)
Ferritin heavy chain (P02794)
Ferroxidase (P00450)
Fetuin-B (Q9UGM5)
Fibrillin-1 (P35555)
Fibrinogen alpha chain (P02671)
Fibrinogen beta chain (P02675)
Fibrinogen gamma chain (P02679)
Fibronectin precursor (P02751)
Fibulin-1 (P23142)
Fibulin-3 (Q12805)
Follistatin-like 5 (Q8N475)
Galectin-3-binding protein (Q08380)
Gamma crystallin D (P07320)
Gamma-Glu-X carboxypeptidase (Q92820)
Gelsolin (P06396)
Glucose-6-phosphate isomerase (P06744)
Glutaminyl-peptide cyclotransferase (Q16769)
Glutathione peroxidase 3 (P22352)
Glutathione S-transferase P (P09211)
Glyceraldehyde-3-phosphate dehydrogenase (P04406)
Heat shock 70 kDa protein 13 (P48723)
Hemoglobin alpha chain (P69905)
Hemoglobin beta chain (P68871)
Hemoglobin delta chain (P02042)
Hemopexin (P02790)
Heparin cofactor 2 (P05546)
Hevin (Q14515)
Hexosaminidase B (P07686)
Histidine-proline-rich glycoprotein (P04196)
Hyaluronan-binding protein 2 (Q14520)
Ig gamma-1 chain C region (P01857)
Ins (1,3,4,5)P(4) 3-phosphate (Q9UNW1)
Insulin-like growth factor-binding protein 6 (P24592)
Insulin-like growth factor-binding protein 7 (Q16270)
Insulin-like growth factor-binding, acid labile (P35858)
Inter-alpha-trypsin inhibitor heavy chain H1 (P19827)
Inter-alpha-trypsin inhibitor heavy chain H4 (Q14624)
Inter-alpha-trypsin inhibitor heavy chain H2 (P19823)
Inter-alpha-trypsin inhibitor heavy chain H3 (Q06033)
Inter-alpha-trypsin inhibitor light chain (P02760)
Interphotoreceptor retinoid-binding protein (P10745)
Kallistatin (P29622)
Kininogen-1 (P01042)
Latent-TGF beta-binding 2 (Q14767)
Leucine-rich alpha-2-glycoprotein (P02750)
Limbic system-associated membrane (Q13449)
Lipocalin-1 (P31025)
Lipocalin-2 (P80188)
L-lactate dehydrogenase A chain (P00338)
L-lactate dehydrogenase B chain (P07195)
Lumican (P51884)
Malate dehydrogenase, cytoplasmic (P40925)
Matrix metalloproteinase-2 (P08253)
Megalin (P98164)
Metalloproteinase inhibitor 1 (P01033)
Metalloproteinase inhibitor 2 (P16035)
Monocyte differentiation antigen CD14 (P08571)
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase (P20933)
N-acetylglucosamine-6-sulfatase (P15586)
N-acetyllactosaminide β-1,3-N-acetylglucosaminyltransferase (O43505)
N-acetylmuramoyl-L-alanine amidase (Q96PD5)
Neural cell adhesion molecule 1, 140 kDa (P13591)
Neural cell adhesion molecule L1-like protein (O00533)
Neurexin-3-alpha (Q9Y4C0)
Neuronal cell adhesion molecule (Q92823)
Neuronal growth regulator 1 (Q7Z3B1)
Neuroserpin (Q99574)
Neurotrimin (Q9P121)
Nucleobindin-1 (Q02818)

(*continues*)

TABLE 2 (*continued*). Aqueous Humor Proteins Identified by Nano-LC-ESI-MS/MS

Found in 3 of 3 Groups (*continued*)

Opticin (Oculoglycan) (Q9UBM4)
Osteopontin (P10451)
Pappalysin-2 (Q9BXP8)
PEBP family protein (Q96S96)
Perlecan (P98160)
Peroxiredoxin-2 (P32119)
Phosphatidylethanolamine-binding 1 (P30086)
Pigment epithelium-derived factor (P36955)
Plasma protease C1 inhibitor (P05155)
Plasma retinol-binding (P02753)
Plasma serine protease inhibitor (P05154)
Plasminogen (P00747)
Proactivator polypeptide (P07602)
Procollagen C-proteinase enhancer 1 (Q15113)
Prolactin-inducible (P12273)
Prostaglandin-D2 synthase (P41222)
Protein CutA (O60888)
Protein FAM3C (Q92520)
Protein kinase C-binding NELL2 (Q99435)
Protein-tyrosine-protein phosphatase zeta (P23471)
Reelin (P78509)
Retinal dehydrogenase 1 (P00352)
Retinoic acid receptor responder 2 (Q99969)
Retinoschisin (O15537)
RNase A (P07998)
Ribonuclease T2 (O00584)
Secreted frizzled-related 3 (Q92765)
Selenium-binding 1 (Q13228)
Selenoprotein P (P49908)
Semaphorin-4B (Q9NPR2)
Semaphorin-7A (O75326)
Serum albumin (P02768)
Serum paraoxonase/arylesterase 1 (P27169)
Sialate O-acetylesterase (Q9HAT2)
SPARC (P09486)
Spondin-1 (Q9HCB6)
Superoxide dismutase [Cu-Zn] (P00441)
Target of Nesh-SH3 (Q7Z7G0)
Tenascin-R (Q92752)
Testican-1 (Q08629
Testican-2 (Q92563)
Tetranectin (P05452)
Thyroxine-binding globulin (P05543)
Transforming growth factor-β-induced ig-h3 (Q15582)
Transketolase (P29401)
Transthyretin (P02766)
Triosephosphate isomerase (P60174)
Tripeptidyl-peptidase 1 (O14773)
Ubiquitin (P62988)
Vasorin (Q6EMK4)
Vesicular integral-membrane VIP36 (Q12907)
Vitamin D-binding (P02774)
Vitamin K-dependent S (P07225)
Vitronectin (P04004)
Zinc-alpha-2-glycoprotein (P25311)

Found in 2 of 3 Groups

14–3-3 protein zeta/delta (P63104)
Alpha-1-antitrypsin (P01009)
Alpha-L-fucosidase 2 (Q9BTY2)
Amyloid-like 1 (P51693)
Angiogenin (P03950)
Aspartate aminotransferase, cytoplasmic (P17174)
Beta-1,4-galactosyltransferase 1 (P15291)
Beta-mannosidase (O00462)
Calcyclin (P06703)
Calgranulin-B (P06702)
Calreticulin (P27797)
Calsyntenin-3 (Q9BQT9)
Coagulation factor X (P00742)
Complement C1q TNF-related 3 (Q9BXJ4)
Complement C4-A (P0C0L4)
Complement factor H-related 1 (Q03591)
C-reactive protein (P02741)
Delta-aminolevulinic acid dehydratase (P13716)
Dermatopontin (Q07507)
Desmocollin-1 (Q08554)
Di-N-acetylchitobiase (Q01459)
Ferritin light chain (P02792)
Follistatin-related protein 1 (Q12841)
Gamma crystallin C (P07315)
Gamma-enolase (P09104)
Growth-arrest-specific protein 6 (Q14393)
Heat shock 70 kDa protein 1 (P08107)
Heat shock 70 kDa protein 1L (P34931)
Heat shock 70 kDa protein 8 (P11142)
Hornerin (Q86YZ3)
Hypoxanthine-guanine phosphoribosyltransferase (P00492)
Iduronate 2-sulfatase (P22304)
Insulin-like growth factor-binding protein 2 (P18065)
Interleukin-6 receptor beta chain (P40189)
Kininogenin (P03952)
Lactotransferrin (P02788)
Leukocyte elastase inhibitor (P30740)
Lysosome-associated membrane glycoprotein 2 (P13473)
Lysozyme C (P61626)
Lysyl hydroxylase 1 (Q02809)
Macrophage colony-stimulating factor 1 receptor (P07333)
Macrophage migration inhibitory factor (P14174)
Macrophage stimulatory protein (P26927)
Major prion protein (P04156)
Mammalian ependymin-related protein 1 (Q9UM22)
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA (P33908)
N-cadherin (P19022)
Neurexin-2-alpha (Q9P2S2)
Nucleoside diphosphate kinase A (P15531)
Peptidyl-glycine alpha-amidating monooxygenase (P19021)
Peptidyl-prolyl cis-trans isomerase A (P62937)
Protein C7orf24 (O75223)
Seizure 6-like protein (Q9BYH1)
Semaphorin-3A (Q14563)
Serum amyloid P-component (P02743)
Sex hormone-binding globulin (P04278)
Soluble calcium-activated nucleotidase 1 (Q8WVQ1)
Stromal cell-derived factor 4 (Q9BRK5)
Transaldolase (P37837)
Transferrin (P02787)
TGF-beta-2 (P61812)
VPS10 domain-containing receptor SorCS1 (Q8WY21)
Wnt inhibitory factor 1 (Q9Y5W5)

(*continues*)

TABLE 2 (*continued*). Aqueous Humor Proteins Identified by Nano-LC-ESI-MS/MS

Found in 1 of 3 Groups

14–3-3 protein epsilon (P62258)
14–3-3 protein sigma (P31947)
40S ribosomal protein S5 (P46782)
6-phosphogluconate dehydrogenase, decarboxylating (P52209)
Adipocyte-derived leucine aminopeptidase (Q9NZ08)
Aldose reductase (P15121)
Alpha-mannosidase 2 (Q16706)
Alpha-N-acetylgalactosaminidase (P17050)
Angiopoietin-like 7 factor (O43827)
Annexin A1 (P04083)
Annexin A2 (P07355)
Annexin A5 (P08758)
Apolipoprotein B-100 (P04114)
Arginase-1 (P05089)
ATP synthase subunit beta, mitochondrial (P06576)
Beta crystallin B1 (P53674)
Beta-hexosaminidase alpha chain (P06865)
Cadherin-6 (P55285)
Calsyntenin-2 (Q9H4D0)
Carbonic anhydrase 3 (P07451)
Carboxypeptidase A4 (Q9UI42)
Caspase-14 (P31944)
CD44 antigen (P16070)
CD98 antigen (P08195)
Coagulation factor XII (P00748)
Collagen alpha-3(VI) chain (P12111)
Complement C1q subcomponent subunit A (P02745)
Complement C1q subcomponent subunit B (P02746)
Complement C1q subcomponent subunit C (P02747)
Complement C4-B (P0C0L5)
Complement factor D (P00746)
Complement factor H-related 2 (P36980)
Cystatin S (P01036)
Desmocollin-3 (Q14574)
Desmoglein-2 (Q14126)
Desmoplakin (P15924)
Desmoplakin-3 (P14923)
Elongation factor 1-alpha 1 (P68104)
Elongation factor 2 (P13639)
Fatty acid-binding protein, epidermal (Q01469)
Fibroblast growth factor-binding 2 (Q98YJ0)
Fructose-bisphosphate aldolase A (P04075)
Galectin-7 (P47929)
Ganglioside GM2 activator (P17900)
Glucosidase 2 subunit beta (P14314)
Glutamate receptor 4 (P48058)
Glutathione reductase, mitochondrial (P00390)
Glutathione synthetase (P48637)
Glutathione transferase omega-1 (P78417)
Hemoglobin subunit gamma-1 (P69891)
Hepatocyte growth factor activator (Q04756)
Ig gamma-2 chain C region (P01859)
Ig gamma-4 chain C region (P01861)
Ig kappa chain C region (P01834)
Insulin-like growth factor-binding 4 (P22692)
Insulin-like growth factor-binding 5 (P24593)
Kallikrein-11 (Q9UBX7)
Keratocan (O60938)
Leucine-rich repeat-containing 4B (Q9NT99)
Lipophilin-B (O95969)
Microfibril-associated glycoprotein 4 (P55083)
Neogenin (Q92859)
Neutrophil defensin 1 (P59665)
Nidogen-1 (P14543)
Peroxiredoxin-1 (Q06830)
Peroxiredoxin-6 (P30041)
Phosphoglycerate mutase 1 (P18669)
Plexin-B2 (O15031)
Polypeptide N-acetylgalactosaminyltransferase 2 (Q10471)
Proteasome subunit alpha type 5 (P28066)
Protein S100-A7 (P31151)
Protein Z-dependent protease inhibitor (Q9UK55)
Protein-glutamine gamma-glutamyltransferase E (Q08188)
Purine nucleoside phosphorylase (P00491)
Pyruvate kinase isozymes M1/M2 (P14618)
Retinoic acid receptor responder 1 (P49788)
Salivary alpha-amylase (P04745)
S-arrestin (P10523)
Secretogranin-1 (P05060)
Serpin B12 (Q96P63)
Serpin B3 (P29508)
Serpin B6 (P35237)
Serum amyloid A-4 (P35542)
Thrombospondin-4 (P35443)
Tropomyosin alpha-3 chain (P06753)
Vacuolar ATP synthase subunit S1 (Q15904)

The UniProt accession number is shown in parentheses for each protein (UniProt is provided in the public domain by The UniProt Consortium; http://www.uniprot.org).

RayBiotech). Using a glass chip–based multiplex sandwich ELISA system, we quantified proteins in 10 independent hAH samples (Table 1). Briefly, 100 μL of sample diluent was added to each well of a 16-well gasket that was fitted onto a glass slide. After an initial 30-minute incubation, 50 μL of sample was added to the well and left on a rotator for 1 hour at room temperature. After incubation, the samples were decanted from the wells and rinsed with wash buffer (supplied with the kit). Cy3-equivalent dye-conjugated streptavidin was added to each well and incubated for 1 hour at room temperature. The wells were washed, and signal intensity was captured with a laser microarray scanner (GenePix 4000B; Molecular Devices, Sunnyvale, CA) at excitation 555 nm, emission 565 nm, and resolution 10 μm. The fluorescence intensity from the array dots corresponded to the concentration of the respective cytokine in the sample. Absolute quantification of the cytokines was calculated (RayBio Q Analyzer software ver. 5.40; RayBiotech, Inc.). Concentration of protein was established after analysis of a serial diluted five-point standard curve for each cytokine.

Enzyme-Linked Immunosorbent Assay

A sandwich ELISA kit was used to quantify eotaxin-2 in 15 hAH samples (Table 1, samples 11–25; RayBio Human Eotaxin-2; RayBiotech, Inc.). Briefly, 50 μL of hAH was mixed with 50 μL sample dilution buffer, incubated for 2.5 hours in a microtiter plate, rinsed with wash buffer (four times), and incubated with 100 μL of biotinylated antibody for 1 hour. The solution was discarded, rinsed, incubated with 100 μL of streptavidin for 45 minutes, rinsed again, and incubated with 100 μL of substrate reagent (TMB One-Step; DakoUSA, Carpinteria, CA) for 30 minutes and read at 450 nm on a microplate reader (Infinite M200; Tecan Systems, Inc., San Jose, CA). The final concentration of eotaxin-2, expressed in pg/mL was determined by using the intersect of the standard curve prepared by assaying known concentrations of recombinant eotaxin-2.

Results

Analysis of Aqueous Humor Proteome by Nano-LC-ESI-MS/MS

Abundant proteins, such as albumin which constitutes nearly 50% of the protein composition of hAH, tend to diminish the characterization of less abundant proteins. To remove abundant proteins and facilitate identification of intermediately expressed proteins, we immunodepleted groups 1 to 3 of hAH (Table 1) of albumin, transferrin, antitrypsin, haploglobin, fibrinogen, IgG, and IgA and separated them by gel electrophoresis (Fig. 1). By means of gel slice extraction and trypsin digestion, nano-LC-ESI-MS/MS identified 355 proteins, of which 206 were in all three groups (Fig. 2A, Table 2). An additional 63 proteins were identified in two of the three groups, whereas another 86 were found in only one of the three groups (Table 2). Most the nano-LC-ESI-MS/MS identified proteins could be classified as having structural, enzymatic or catalytic properties (Fig. 2B). More than than 80% of the 355 proteins identified by nano-LC-ESI-MS/MS have not been reported in hAH. Comparison of our hAH protein dataset with two independent plasma proteome databases[22,23] showed that only 58% of the hAH proteins have also been identified in human plasma.

To verify our mass spectrometry results, we analyzed two random proteins, BigH3 (kerato-epithelin) and fibulin-3, by Western blot for their presence in an independent hAH sample (Table 1). Antibodies against both proteins confirmed the presence of BigH3 and fibulin-3 in hAH (Fig. 3). A third protein, myocilin, which has been reported in hAH[34,35] and confirmed by our nano-LC-ESI-MS/MS study, was also identified in hAH by Western blot (Fig. 3).

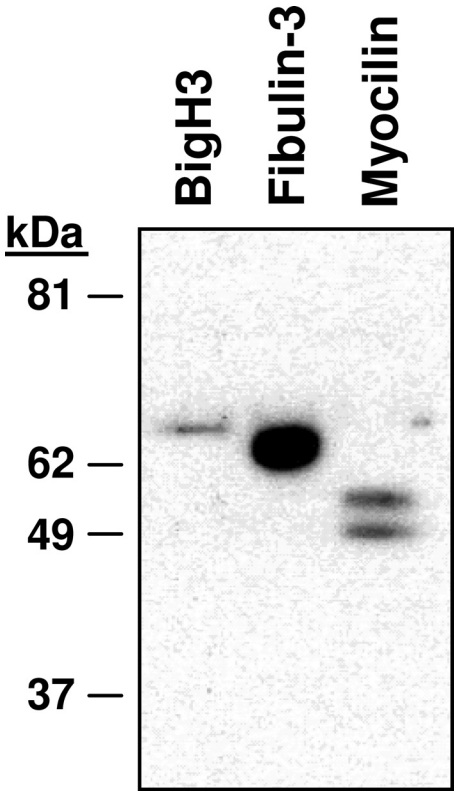

Figure 3. Verification of BigH3, fibulin-3, and myocilin in hAH. Three proteins—BigH3, fibulin-3, and myocilin—were assessed by Western blot for their presence in hAH. All three proteins were determined to be present in the three groups by nano-LC-ESI-MS/MS and were confirmed by Western blot analysis.

Growth Factor, Cytokine, and Receptor Identification in Aqueous Humor

Of the 355 proteins identified by mass spectrometry, only a small percentage of the proteins were growth factors, cytokines, or receptors, which is not surprising considering their relative low abundance. We implemented a more targeted approach to identify growth factors, cytokines, and receptors in hAH, where 21 hAH samples were divided into four groups (groups 4–7, Table 1) and used to independently probe antibody-based protein arrays (RayBiotech). Of the 507 proteins on the arrays, 328 were identified in hAH. A total of 217 proteins were identified in at least three of four groups, whereas another 111 proteins were found in only one or two groups (Table 3). An illustration of the results for some of the TGFβ family members is shown in Figure 4. TGFβ2 and -3 and TGFβ type I and II receptors were identified in all four groups. TGFβ1 was identified in two groups (groups 4 and 5) and TGFβ5 was not present in any of the four groups. Only 10 proteins identified by the antibody-based protein array were also detected in nano-LC-ESI-MS/MS studies.

Quantification of Growth Factors and Cytokines

Using independent hAH samples (Table 1, samples 1–10) and a chemokine array kit (Quantibody Human Chemokine Array 1; RayBiotech), we quantified 25 proteins (Table 4). Most of the growth factors and cytokines quantified had concentrations between 0.1 and 2.5 ng/mL. Osteopontin, a member of the matricellular protein family, originally identified in hAH by the nano-LC-ESI-MS/MS studies had exhibited levels near 70 ng/mL. Three additional proteins, IL-18 BPA, IL-28A, and IL-29, that

were analyzed on the chemokine array, but were not included in the antibody-based protein array containing 507 proteins, were also identified in hAH. Six additional quantified proteins (IL-17P, MCP-3, MIP-3a, MPIF-1, TARC, and TSLP) that were not present in the antibody-based protein array were confirmed to be absent by the cytokine array. Eotaxin-2, which was not one

Table 3. Growth Factors, Cytokines, and Receptors in Aqueous Humor

In 4 of 4 Groups

Activin RIA/ALK-2	CXCL11	GASP-1/WFIKKNRP	Leptin (OB)	Osteocrin
Activin RIB/ALK-4	CXCL13	G-CSF R/CD 114	LIF R alpha	OX40 ligand/TNFSF4
Angiopoietin-2	CXCL14/BRAK	GFR alpha-2	LIGHT/TNFSF14	PD-ECGF
Angiopoietin-4	CXCR2/IL-8 RB	GFR alpha-3	Lipocalin-1	PF4/CXCL4
Angiopoietin-like 1	CXCR3	GFR alpha-4	L-Selectin (CD62L)	Pref-1
APRIL	CXCR4 (fusin)	Glucagon	Luciferase	RELM beta
AR (Amphiregulin)	CXCR5/BLR-1	Glut1	Lymphotoxin beta R	S100 A8/A9
Artemin	DcR3/TNFRSF6B	Glut3	MCP-1	Smad 8
Axl	Dkk-3	Glut5	MCP-2	Soggy-1
B7-1/CD80	Dkk-4	Glypican 3	M-CSF	SPARC
BD-1	DR3/TNFRSF25	ICAM-2	M-CSF R	Spinesin
beta-NGF	Dtk	IFN-alpha/beta R1	MFG-E8	TACI/TNFRSF13B
BMP-5	EGF	IFN-gamma R1	MFRP	TCCR/WSX-1
BMPR-IA/ALK-3	Epiregulin	IGFBP-1	MIF	TGF-beta 2
CCL28/VIC	ErbB3	IGFBP-2	MIP-1b	TGF-beta 3
CCR1	Fas Ligand	IGFBP-3	MMP-10	TGF-beta RI/ALK-5
CCR2	FGF Basic	IL-1 R9	MMP-11/Stromelysin-3	TGF-beta RII
CCR3	FGF R3	IL-12 p70	MMP-13	Thrombospondin-1
CCR4	FGF R4	IL-12 R beta 1	MMP-15	Thrombospondin-2
CCR7	FGF-11	IL-17E	MMP-24/MT5-MMP	TLR1
CCR8	FGF-18	IL-21 R	MMP-7	TLR4
CCR9	FGF-4	IL-23	MMP-8	TRAIL R4/TNFRSF10D
CD14	FGF-5	IL-23 R	MMP-9	TREM-1
CD117	FGF-9	IL-3 R alpha	NAP-2	TROY/TNFRSF19
CD154	FGF-BP	IL-31	NCAM-1/CD56	TSG-6
CD163	Follistatin	IL-31 RA	Neuropilin-2	VCAM-1 (CD106)
Chordin-Like 1	Follistatin-like 1	IL-6 R	Neurturin	VE-Cadherin
Chordin-Like 2	Fractalkine	Inhibin B	NGF R	VEGF-B
CLC	Frizzled-3	Kremen-2	NOV/CCN3	VEGI
CNTF R alpha	Frizzled-4	LBP	Orexin B	WIF-1
CRTH-2				

In 3 of 4 Groups

6Ckine	EDA-A2	Glut2	IL-20 R beta	Osteoprotegerin
Activin RII A/B	EDG-1	HRG-beta 1	IL-24	PDGF-D
Adiponectin	Eotaxin/CCL11	ICAM-1	Inhibin A	Persephin
AgRP	Eotaxin-2/MPIF-2	IGFBP-rp1/IGFBP-7	IP-10	PIGF
Angiopoietin-like factor	ErbB4	IL-1 F7/FIL1 zeta	Kininostatin	RELT/TNFRSF19L
Angiostatin	Erythropoietin	IL-1 R6/IL-1 Rrp2	Kremen-1	SDF-1/CXCL12
BCMA/TNFRSF17	E-Selectin	IL-1 F10/IL-1HY2	Latent TGF-beta bp1	sFRP-1
beta-Catenin	FAM3B	IL-13 R alpha 2	Lck	Siglec-9
BIK	FGF-20	IL-17F	Lefty - A	SLPI
CCL14	FGF-7/KGF	IL-17RC	LRP-6	Thrombopoietin (TPO)
CCR6	Frizzled-1	IL-18 R beta/AcPL	Lymphotoxin beta	Thrombospondin-4
Cerberus 1	GASP-2/WFIKKN	IL-20	MCP-4/CCL13	TLR3
Chem R23	GCP-2/CXCL6	IL-20 R alpha	MDC	VEGF-D
Decorin				

In 2 of 4 Groups

Activin A	EDAR	GDF1	HGFR	MMP-14
Activin B	EGF R/ErbB1	GDF3	IGF-II	MMP-16/MT3-MMP
Activin C	EG-VEGF/PK1	GDF5	IL-1 F6/FIL1 epsilon	MSP alpha chain
Activin RIIA	Endoglin/CD105	GDF8	IL-1 F9/IL-1 H1	MSP beta-chain
Angiogenin	Endostatin	GDF9	IL-17RD	NT-4
Angiopoietin-like 2	EN-RAGE	GDF11	IL-18 R alpha/IL-1 R5	PLUNC
APJ	Eotaxin-3/CCL26	GDF-15	IL-8	P-selectin
BDNF	Fas/TNFRSF6	GFR alpha-1	IL-9	sgp130
beta-Defensin 2	FGF-12	GITR Ligand	Insulin R	TFPI
BMP-2	FGF-19	GM-CSF	Leptin R	TGF-beta 1
BMP-6	FGF-21	GREMLIN	LIF	Thrombospondin
BMP-15	FGF-8	GRO-a	Lymphotactin/XCL1	TMEFF2
CTACK/CCL27	GCSF	Hepassocin	MIP-1a	

(*continues*)

TABLE 3 (*continued*). Growth Factors, Cytokines, and Receptors in Aqueous Humor

In 1 of 4 Groups				
BMP-7	Galectin-3	IL-11	Insulin	Siglec-5/CD170
BMP-8	Granzyme A	IL-12 p40	LRP-1	SMDF/NRG1Isoform
BTC	HCR/CRAM-A/B	IL-12 R beta 2	MAC-1	Tissue Factor
CD40/TNFRSF5	HGF	IL-17B R	MIP-3 beta	TNF-beta
CTLA-4/CD152	HRG-alpha	IL-19	MMP-3	TRAIL R3/TNFRSF10C
CXCR1/IL-8 RA	IGFBP-6	IL-2 R gamma	MMP-12	TWEAK/TNFSF12
D6	IGF-I SR	IL-21	Musk	Ubiquitin + 1
Endocan	IL-1 alpha	IL-22 BP	Neuregulin	VEGF
FGF-6	IL-1 beta	IL-7 R alpha	PARC/CCL18	VEFR R3
Frizzled-7	IL-1 ra			

of the cytokines spotted on the cytokine array, was assayed by ELISA (Table 1, samples 11–25). Eotaxin-2 was confirmed to be present in hAH with a concentration of 49 ± 31 pg/mL (mean ± SD, $n = 15$).

DISCUSSION

Avascular tissues of the anterior chamber receive their external cues from components of aqueous humor. Changes in protein or ionic concentrations within aqueous humor may have significant effects on cellular function and cell–matrix communication. However, only approximately 150 proteins have been identified in hAH. Using an approach that included multiple proteomic techniques and multigroup comparisons, we have identified 676 nonredundant proteins in hAH. More than 80% of the proteins are novel identifications for aqueous humor. To date, this study provides the most comprehensive list of proteins present in hAH.

Aqueous humor maintains a normal homeostatic environment and is essential to the proper functioning of anterior chamber tissues. Therefore, it is not surprising that most of the proteins identified by nano-LC-ESI-MS/MS have catalytic and enzymatic functions. For example, one of the functions of aqueous humor is to maintain a pathogen-free environment. Being immunoprivileged, the anterior chamber relies on complement to successfully rid the chamber of pathogens. Our proteomic data supports the presence of both the classic and alternative complement pathways. In all, 23 complement proteins were identified in hAH including C1q, C1r, C1s, C2–9, and complement regulatory molecules such as CD59; complement B, D, H, and I; and C1 inhibitor. The balance between complement activation molecules and complement regulatory molecules is important in maintaining healthy anterior segment tissues and avoiding autoimmune reactions that would significantly alter the function of these tissues.

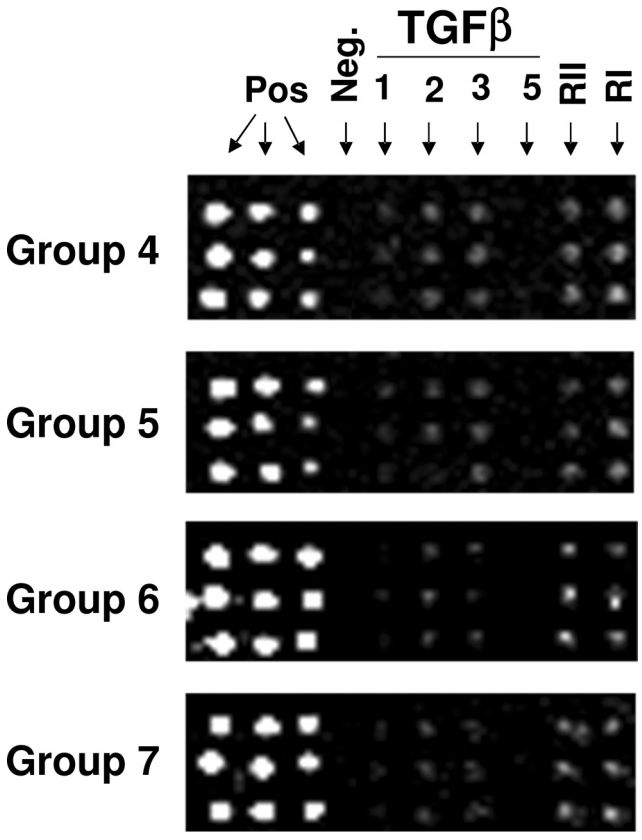

FIGURE 4. Presence and absence of several TGFβ family members in hAH. Representative images of several TGFβ-family members and their corresponding fluorescent signal intensity from antibody-based protein arrays. TGFβ2, TGFβ3, type I, and type II receptors were present in all four groups (groups 4–7). TGFβ1 was present in groups 4 and 5, but was "absent" in groups 6 and 7 because of low signal strength of less than threefold above background. TGFβ5 was negative in all groups.

TABLE 4. Quantification of 25 Aqueous Humor Proteins

Protein	hAH (pg/mL)
6Ckine	713 ± 186
CTACK	1259 ± 330
CCL28	220 ± 56
Eotaxin-2	49 ± 31*
Eotaxin-3	484 ± 111
GCP-2	129 ± 48
GRO	239 ± 52
HCC-1	2507 ± 843
IL-9	6881 ± 2585
IL-17P	NP
IL-18 BPA	689 ± 409
IL-28A	317 ± 118
IL-29	2205 ± 672
IP-10	225 ± 277
LIF	133 ± 59
MCP-2	10 ± 7
MCP-3	NP
MDC	24 ± 23
MIP-3a	NP
MPIF-1	NP
NAP-2	1261 ± 851
OPN	69813 ± 21625
PARC	1656 ± 1101
TARC	NP
TSLP	NP

Data are expressed as the mean ± SD ($n = 10$). NP, not present (below assay's sensitivity).

* Analyzed by ELISA.

Catalytic proteins such as type IV collagen are principal components of basement membranes[36] and are one of the major extracellular matrix proteins upregulated during glaucoma. Other catalytic enzymes found in hAH, such as lactate dehydrogenase, have already been suggested to have a role in hAH outflow regulation, while showing a strong presence in the uveoscleral tissue.[37] Several respiratory pathway catalytic enzymes such as aldolase and ketolase were also found in hAH and may have been secreted into the hAH by cells bathed by the fluid. The presence of strong angiogenic inducers as angiogenin and angiogenic inhibitors PEDF,[38] type IV collagen,[39] and vitamin D binding protein,[40] suggests the presence of an equilibrium within hAH between proangiogenic and antiangiogenic molecules. The balance between angiogenic and antiangiogenic proteins may be important in the pathogenesis of anterior segment diseases.

Our antibody-based protein array study served the purpose of identifying proteins in the hAH that are present in low abundance and therefore are difficult to identify by nano-LC-ESI-MS/MS. Several members of the transforming growth factor β, tumor necrosis factor, fibroblast growth factor, interleukin, and growth differentiation factor families were identified. In addition, numerous growth factor and cytokine receptors were found in hAH, including receptors of the C-C chemokine, tumor necrosis factor, and interleukin superfamilies. In other biological fluids, circulating and soluble receptors play an important role in regulating growth factor and cytokine activity. Soluble receptors are normal components of body fluids in healthy individuals and levels of these receptors can modulate various growth factor and cytokine activities.[41] For example, ligand binding to soluble receptors can protect the ligand from degradation, inhibit the ligand from initiating a signaling cascade or act in an agonistic manner. In the case of IL-6, the binding of IL-6 to its soluble receptor can stimulate cells that do not normally express an IL-6 receptor.[42,43] Analysis of soluble receptors in hAH isolated from anterior chamber disorders, such as corneal dystrophies and glaucoma, may serve, not only as markers for the disease but as therapeutic targets in treating the disorder.

An important question in the midst of the description of the hAH proteome is "where are all the proteins found in hAH coming from?" Although hAH is considered to be a plasma filtrate, there are considerable differences between plasma and hAH that suggest several cells and tissues from the anterior segment may be involved in active secretion of ions/proteins into hAH.[9–11] Bovine and primate studies suggest that hAH proteins originate in the ciliary body capillaries and move via a protein gradient toward the iris root where they diffuse through the ciliary epithelium into hAH.[2,44,45] However, other studies have suggested that the ciliary epithelium and the pigmented and nonpigmented cells of the ciliary body are actively involved in pumping out regulatory proteins into the hAH possibly in conjunction with tissues surrounding the anterior/posterior chambers.[46,47] These proteins include hemopexin, ceruloplasmin, ferroxidase, and glutathione *S*-transferase, enzymatic proteins involved in detoxification and oxidative damage protection.[48] Although many of the proteins identified may come from blood, cDNAs encoding plasma proteins have also been identified in the ciliary body.[46] Among these are complement component C4, α2-macroglobulin, and the plasma form of glutathione peroxidase.[46] Our study confirmed their presence in human hAH and suggests that the ciliary body may be one of the tissues in the anterior chamber that has the ability to produce and secrete traditional plasma proteins into hAH.

hAH samples obtained from patients undergoing cataract removal has been the traditional control in studies of hAH from patients with anterior chamber disorders, such as uveal melanoma, myopia, corneal rejection, and glaucoma.[25–27,49,50] Although considered normal, the presence of a cataract affects the concentration and components of hAH as suggested by the increase in α-antitrypsin, α2-macroglobulin, and β-crystallin proteins.[51,52] Although hAH collected from normal healthy adults would have been ideal, such samples cannot be obtained ethically.[53] Therefore, it is possible that some of the proteins identified in our study are present because of the underlying cataract condition. Another limitation of this study was that our growth factor, cytokine, and receptor analysis was limited to the proteins that were present on the protein array. Therefore, not all growth factors, cytokines, and receptors in hAH were identified. Although our study was thorough, 676 proteins are probably only a fraction of the overall protein profile within hAH. Nevertheless, our study provides a comprehensive list of the hAH proteins. This list may be considered as a reference to look for differences in protein expression in various pathologic conditions of the anterior segment with the possibility of identifying novel biomarkers for the disease and possible targets for novel therapeutic treatments.

References

1. To CH, Kong CW, Chan CY, Shahidullah M, Do CW. The mechanism of aqueous humour formation. *Clin Exp Optom.* 2002;85:335–349.
2. Barsotti MF, Bartels SP, Freddo TF, Kamm RD. The source of protein in the aqueous humor of the normal monkey eye. *Invest Ophthalmol Vis Sci.* 1992;33:581–595.
3. Freddo TF, Bartels SP, Barsotti MF, Kamm RD. The source of proteins in the aqueous humor of the normal rabbit. *Invest Ophthalmol Vis Sci.* 1990;31:125–137.
4. Freddo TF. The Glenn A. Fry Award Lecture 1992: aqueous humor proteins: a key for unlocking glaucoma? *Optom Vis Sci.* 1993;70:263–270.
5. McLaren JW, Ziai N, Brubaker RF. A simple three-compartment model of anterior segment kinetics. *Exp Eye Res.* 1993;56:355–366.
6. Fitt AD, Gonzalez G. Fluid mechanics of the human eye: aqueous humour flow in the anterior chamber. *Bull Math Biol.* 2006;68:53–71.
7. Murray DL, Bartels SP. The relationship between aqueous humor flow and anterior chamber protein concentration in rabbits. *Invest Ophthalmol Vis Sci.* 1993;34:370–376.
8. Mark HH. Aqueous humor dynamics in historical perspective. *Surv Ophthalmol.* 2010;55:89–100.
9. Cole DF. Comparative aspects of the intraocular fluids. In: Davson H, Graham LT Jr, eds. *The Eye*. New York: Academic Press; 1974:71–121.
10. Tripathi RC, Millard CB, Tripathi BJ. Protein composition of human aqueous humor: SDS-PAGE analysis of surgical and post-mortem samples. *Exp Eye Res.* 1989;48:117–130.
11. Kinsey VE. The chemical composition and the osmotic pressure of the aqueous humor and plasma of the rabbit. *J Gen Physiol.* 1951;34:389–402.
12. Klenkler B, Sheardown H. Growth factors in the anterior segment: role in tissue maintenance, wound healing and ocular pathology. *Exp Eye Res.* 2004;79:677–688.
13. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci.* 2006;47:226–234.
14. Maatta M, Tervahartiala T, Harju M, Airaksinen J, Autio-Harmainen H, Sorsa T. Matrix metalloproteinases and their tissue inhibitors in aqueous humor of patients with primary open-angle glaucoma, exfoliation syndrome, and exfoliation glaucoma. *J Glaucoma.* 2005;14:64–69.
15. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol.* 2004;25:19–22.
16. Garcia-Ramirez M, Canals F, Hernandez C, et al. Proteomic analysis of human vitreous fluid by fluorescence-based difference gel elec-

trophoresis (DIGE): a new strategy for identifying potential candidates in the pathogenesis of proliferative diabetic retinopathy. *Diabetologia.* 2007;50:1294–1303.
17. Goldman D, Merril CR, Ebert MH. Two-dimensional gel electrophoresis of cerebrospinal fluid proteins. *Clin Chem.* 1980;26: 1317–1322.
18. Kim T, Kim SJ, Kim K, et al. Profiling of vitreous proteomes from proliferative diabetic retinopathy and nondiabetic patients. *Proteomics.* 2007;7:4203–4215.
19. Sickmann A, Dormeyer W, Wortelkamp S, Woitalla D, Kuhn W, Meyer HE. Towards a high resolution separation of human cerebrospinal fluid. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2002;771:167–196.
20. Yuan X, Desiderio DM. Proteomics analysis of human cerebrospinal fluid. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2005; 815:179–189.
21. Yuan X, Desiderio DM. Proteomics analysis of prefractionated human lumbar cerebrospinal fluid. *Proteomics.* 2005;5:541–550.
22. Anderson NL, Polanski M, Pieper R, et al. The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol Cell Proteomics.* 2004;3:311–326.
23. Omenn GS, States DJ, Adamski MR, et al. Overview of the HUGO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboritories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics.* 2005;5:3226–3245.
24. Richardson MR, Price MO, Price FW, et al. Proteomic analysis of human aqueous humor using multidimensional protein identification technology. *Mol Vis.* 2009;15:2740–2750.
25. Rohde E, Tomlinson AJ, Johnson DH, Naylor S. Comparison of protein mixtures in aqueous humor by membrane preconcentration-capillary electrophoresis-mass spectrometry. *Electrophoresis.* 1998;19:2361–2370.
26. Duan X, Lu Q, Xue P, et al. Proteomic analysis of aqueous humor from patients with myopia. *Mol Vis.* 2008;14:370–377.
27. Funding M, Vorum H, Honore B, Nexo E, Ehlers N. Proteomic analysis of aqueous humour from patients with acute corneal rejection. *Acta Ophthalmol Scand.* 2005;83:31–39.
28. Stastna M, Behrens A, Noguera G, Herretes S, McDonnell P, Van Eyk JE. Proteomics of the aqueous humor in healthy New Zealand rabbits. *Proteomics.* 2007;7:4358–4375.
29. Fautsch MP, Johnson DH. Characterization of myocilin-myocilin interactions. *Invest Ophthalmol Vis Sci.* 2001;42:2324–2331.
30. Fautsch MP, Vrabel AM, Peterson SL, Johnson DH. In vitro and in vivo characterization of disulfide bond use in myocilin complex formation. *Mol Vis.* 2004;10:417–425.
31. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem.* 2002;74:5383–5392.
32. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 2003;75:4646–4658.
33. Fautsch MP, Bahler CK, Jewison DJ, Johnson DH. Recombinant TIGR/MYOC increases outflow resistance in the human anterior segment. *Invest Ophthalmol Vis Sci.* 2000;41:4163–4168.
34. Rao PV, Allingham RR, Epstein DL. TIGR/myocilin in human aqueous humor. *Exp Eye Res.* 2000;71:637–641.
35. Russell P, Tamm ER, Grehn FJ, Picht G, Johnson M. The presence and properties of myocilin in the aqueous humor. *Invest Ophthalmol Vis Sci.* 2001;42:983–986.
36. Kefalides NA. Structure and biosynthesis of basement membranes. *Int Rev Connect Tissue Res.* 1973;6:63–104.
37. Cavallotti C, Pescosolido N, Artico M, Cavallotti D. Uveoscleral outflow in dog's eye: role of several enzymes. *Int Ophthalmol.* 1998;22:233–238.
38. Dawson DW, Volpert OV, Gillis P, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science.* 1999; 285:245–248.
39. Colorado PC, Torre A, Kamphaus G, et al. Anti-angiogenic cues from vascular basement membrane collagen. *Cancer Res.* 2000;60: 2520–2526.
40. Kalkunte S, Brard L, Granai CO, Swamy N. Inhibition of angiogenesis by vitamin D-binding protein: characterization of anti-endothelial activity of DBP-maf. *Angiogenesis.* 2005;8:349–360.
41. Heaney ML, Golde DW. Soluble receptors and human disease. *J Leukocyte Biol.* 1998;64:135–146.
42. Mackiewicz A, Schooltink H, Heinrich PC, Rose-John S. Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins. *J Immunol.* 1992;149:2021–2027.
43. Taga T, Hibi M, Hirata Y, et al. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell.* 1989;58:573–581.
44. Allansmith MR, Whitney CR, McClellan BH, Newman LP. Immunoglobulins in the human eye: location, type, and amount. *Arch Ophthalmol.* 1973;89:36–45.
45. Radius RL, Anderson DR. Distribution of albumin in the normal monkey eye as revealed by Evans blue fluorescence microscopy. *Invest Ophthalmol Vis Sci.* 1980;19:238–243.
46. Coca-Prados M, Escribano J, Ortego J. Differential gene expression in the human ciliary epithelium. *Prog Retin Eye Res.* 1999;18:403–429.
47. Ortego J, Escribano J, Coca-Prados M. Gene expression of proteases and protease inhibitors in the human ciliary epithelium and ODM-2 cells. *Exp Eye Res.* 1997;65:289–299.
48. Ahmad H, Singh SV, Srivastava SK, Awasthi YC. Glutathione S-transferase of bovine iris and ciliary body: characterization of isoenzymes. *Curr Eye Res.* 1989;8:175–184.
49. Missotten GS, Beijnen JH, Keunen JEE, Bonfrer JMG. Proteomics of uveal melanoma. *Melanoma Res.* 2003;13:627–629.
50. Howell KG, Vrabel AM, Roy Chowdhury U, Stamer WD, Fautsch MF. Myocilin levels in primary open-angle glaucoma and pseudoexfoliation glaucoma human aqueous humor. *J Glaucoma.* Published online February 22, 2010.
51. Nakamura M, Russell P, Carper DA, Inana G, Kinoshita JH. Alteration of a developmentally regulated, heat-stable polypeptide in the lens of the Philly mouse. Implications for cataract formation. *J Biol Chem.* 1988;263:19218–19221.
52. Ando H, Twining SS, Yue BY, et al. MMPs and proteinase inhibitors in the human aqueous humor. *Invest Ophthalmol Vis Sci.* 1993; 34:3541–3548.
53. Escoffier P, Paris L, Bodaghi B, Danis M, Mazier D, Marinach-Patrice C. Pooling aqueous humor samples: bias in 2D-LC-MS/MS strategy? *J Proteome Res.* 2010;9:789–797.