Comparative Proteomic Analysis of Two Uveitis Models in Lewis Rats

Kathryn L. Pepple,1 Lauren Rotkis,1 Leslie Wilson,1 Angela Sandt,1 and Russell N. Van Gelder1–3

1Department of Ophthalmology, University of Washington, Seattle, Washington, United States
2Department of Biological Structure, University of Washington, Seattle, Washington, United States
3Department of Pathology, University of Washington, Seattle, Washington, United States

Correspondence: Russell N. Van Gelder, Department of Ophthalmology, Campus Box 359608, University of Washington, 525 9th Avenue, Seattle, WA 98104, USA; russvg@uw.edu.

KLP and LR contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: June 18, 2015
Accepted: October 13, 2015

Citation: Pepple KL, Rotkis L, Wilson L, Sandt A, Van Gelder RN. Comparative proteomic analysis of two uveitis models in Lewis rats. Invest Ophthal Vis Sci. 2015;56:8449–8456. DOI:10.1167/iovs.15-17524

Purpose. Inflammation generates changes in the protein constituents of the aqueous humor. Proteins that change in multiple models of uveitis may be good biomarkers of disease or targets for therapeutic intervention. The present study was conducted to identify differentially-expressed proteins in the inflamed aqueous humor.

Methods. Two models of uveitis were induced in Lewis rats: experimental autoimmune uveitis (EAU) and primed mycobacterial uveitis (PMU). Differential gel electrophoresis was used to compare naïve and inflamed aqueous humor. Differentially-expressed proteins were separated by using 2-D gel electrophoresis and excised for identification with matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF). Expression of select proteins was verified by Western blot analysis in both the aqueous and vitreous.

Results. The inflamed aqueous from both models demonstrated an increase in total protein concentration when compared to naïve aqueous. Calprotectin, a heterodimer of S100A8 and S100A9, was increased in the aqueous in both PMU and EAU. In the vitreous, S100A8 and S100A9 were preferentially elevated in PMU. Apolipoprotein E was elevated in the aqueous of both uveitis models but was preferentially elevated in EAU. Beta-B2–crystallin levels decreased in the aqueous and vitreous of EAU but not PMU.

Conclusions. The proinflammatory molecules S100A8 and S100A9 were elevated in both models of uveitis but may play a more significant role in PMU than EAU. The neuroprotective protein β-B2–crystallin was found to decline in EAU. Therapies to modulate these proteins in vivo may be good targets in the treatment of ocular inflammation.

Keywords: S100A8, S100A9, beta-B2 crystallin, uveitis, experimental autoimmune uveoretinitis

The term “uveitis” describes a heterogeneous set of conditions that all feature intraocular inflammation.1 Ocular inflammation leads to an increase in the protein concentration in the aqueous and vitreous humors of the eye.2 The increased protein results from the influx of serum proteins during inflammation-mediated breakdown of the blood–ocular barrier, and increased local production of inflammatory mediators by resident and recruited immune cells. Normal human aqueous contains between 50 and 676 proteins, many with antioxidant, immunoregulatory, and antiangiogenic functions.3,4 Previous studies5–7 have evaluated changes in the protein concentration and composition of the aqueous humor in diseases such as glaucoma, macular degeneration, and vein occlusion with macular edema. These studies have identified changes in total protein concentration and protein composition that have correlated with disease progression and prognosis. However, none of these diseases experience the dramatic changes in intraocular protein seen as a result of the inflammation caused by uveitis.8–11

In the equine recurrent uveitis (ERU) model of uveitis, changes in the lymphocyte proteome that occur during active inflammation have been described.12 Additionally, in the rat models of endotoxin-induced uveitis (EIU) and experimental autoimmune uveitis (EAU), vitreous proteome studies have been performed on the vitreous that identified posttranslational modifications and truncations of α-crystallins in association with inflammation.13,14 However, these studies all compare one model of uveitis to the naïve condition. In this study, we compared the naïve aqueous humor proteome to two rat models of uveitis: EAU and primed mycobacterial uveitis (PMU). Experimental autoimmune uveitis is a well-established model of noninfectious T-cell–mediated autoimmune uveitis.15 Primed mycobacterial uveitis is a model of uveitis recently adapted from rabbits to the rat by our group (accompanying manuscript). Owing to the introduction of killed mycobacteria into the vitreous cavity, innate immunity plays a significant role in this form of uveitis. By using two mechanistically different forms of uveitis, comparative proteome analysis will help to identify protein changes common to both forms of uveitis, as well as proteins that may be specific to each form of inflammation.

Methods

Experimental Animals and Uveitis Induction

Female Lewis rats were purchased from Charles River (Hollister, CA, USA) or Taconic Farms (Oxnard, CA, USA).
Induction of PMU in 15 Lewis rats was initiated with a subcutaneous injection in the right hind flank of 100 µg mycobacterium tuberculosis H37Ra antigen (Difco Laboratories, Detroit, MI, USA) in 0.1 mL suspension of 50% peanut oil and 50% phosphate-buffered saline (PBS). Seven days later the right eye received a 5 µL intravitreal injection of 1 mg/mL suspension of mycobacterium tuberculosis H37Ra antigen in PBS. A second group of 15 female Lewis rats received the same subcutaneous injection of mycobacterium tuberculosis H37Ra antigen, but 7 days later received an intravitreal injection of 5 µL PBS in the right eye (sham injection group). A final group of 15 female Lewis rats received no injections or treatment and were designated as the naïve group. Induction of uveitis was confirmed by clinical examination and identification of ocular inflammation on day 2 for PMU animals and day 14 for EAU animals. On day 2, postintraocular injection animals were anesthetized and enucleated. Ten microliters of aqueous humor was aspirated and mixed with 1.1 µL 10X protease inhibitor. The vitreous was separated from the retina, homogenized with a tissue lyser in 150 µL PBS and 16 µL 10X protease inhibitor. All samples were stored at −80°C.

Induction of EAU in Lewis rats was initiated with a subcutaneous injection of 30 µg synthetic bovine IRBP peptide (residue sequence ADGSSWEGVGVVPDV) in complete Freund's adjuvant (2.5 mg/mL mycobacterium) in the right hind flank. The animal study protocol was approved by the Animal Care and Use Committee of the University of Michigan, Detroit, MI, USA. Immobile dry strips (18-cm IPG pH 3–10 nonlinear; GE Life Sciences, Pittsburgh, PA, USA) were rehydrated with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% IPG buffer, 2.8% DTT; trace bromophenol blue) and the pooled dyed samples overnight. The isoelectric focusing step was run on a Bio-Rad IEF cell at 250 V for 15 minutes, 1000 V for 1 hour, 8000 V for 3 hours, 8000 V until 25 kV-hours was reached. The second dimension was run on precast 4% to 8% Bis-Tris gels in low fluorescence glass (Jule, Inc., Milford, CT, USA) at a constant current of 24 mA for 6.5 hours at 4°C. Images were scanned on a Typhoon FLA 9000 scanner (GE Life Sciences).

**Sample Preparation for Protein Analysis**

Aqueous and vitreous were collected and pooled from 15 eyes from each treatment group. Samples were collected from naïve animals on day 0, from PMU on day 2, and from EAU on day 14. These time points were chosen for the animals with uveitis, as these times correlated with maximal inflammation observed in each model (data submitted in accompanying manuscript). From individual eyes, aqueous was aspirated and protease inhibitor added. The vitreous was separated from the retina, diluted in 150 µL PBS and 1X protease inhibitor (Sigma-Aldrich Corp., St. Louis, MO, USA), and homogenized. The sample was spun down for 1 minute at 16,000g, and supernatant was collected. All samples were stored at −80°C before testing. From half the pool, protein was precipitated by adding an equal volume of 20% trichloroacetic acid and incubating on ice for 30 minutes. The samples were centrifuged at 4°C for 15 minutes at 16,000g, and the supernatant was discarded. The pellet was washed two times with acetone and then centrifuged at 4°C for 5 minutes at 16,000g. Protein resolubilization was achieved with Ready-Prep Rehydration Sample Buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% Bio-Lyte 5/10 ampholytes, trace bromophenol blue) from Bio-Rad ( Hercules, CA, USA) for traditional 2-D gel electrophoresis and in buffer containing 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, pH 8.5, for differential gel electrophoresis (DIGE). Protein concentration was measured by Pierce 660 nm Protein Assay from Thermoscientific (Rockford, IL, USA).

**Two-Dimensional Polyacrylamide Gel Electrophoresis and Mass Spectrometry**

Thirty micrograms of the pooled and resolubilized protein was added to rehydration buffer and incubated overnight with an 11-cm immobilized pH gradient strip (3–10 pH IPG strip; Bio-Rad) at room temperature. Isoelectric focusing was performed on a Bio-Rad IEF cell at 8000 V until 20,000 V-hours was reached. The second dimension was performed by using 10% to 20% Tris-HCl gels. Gels were stained with Bio-Safe Coomassie Stain (Bio-Rad). Spots of interest from 2-D gels of naïve and PMU samples were excised. Protein spots were double washed in a solution of 50% methanol, 5% acetic acid and then reduced in 10 mM dithiothreitol and alkylated in 100 mM iodoacetamide. Finally, the protein spots were digested in trypsin and submitted for analysis by liquid chromatography–tandem mass spectrometry.

**Western Blot**

Samples were loaded in equal volume of original pooled sample (0.7 µL aqueous per lane or 5 µL vitreous per lane) or in equal protein concentration (5 µg total protein per lane). All samples were loaded in a final volume of 40 µL in PBS. For Supplementary Figure S1, the aqueous humor was centrifuged at 250g at room temperature for 7 minutes. The supernatant fraction was collected and an appropriate volume of 10X protease inhibitor was added for a final concentration of 1X (1–1.5 µL). The cell pellet was washed with 1X PBS three times and then resuspended in 15 µL 1X PBS containing 1X protease inhibitor. Samples were separated on 4% to 12% Bis-Tris Gels from Invitrogen (Grand Island, NY, USA) on a NuPage MES SDS running buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.5) at 200 V for 40 minutes and then transferred to Pierce Nitrocellulose membrane (Thermoscientific) overnight at 14 V at 4°C. Membranes were blocked with 5% milk 1% BSA in Tris-buffered saline, 0.05% Tween 20 for 1 hour at 4°C. Blots were incubated overnight with primary antibodies at 4°C with shaking. Primary antibodies included goat anti-S100A8/calgranulin A (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-S100A9/calgranulin B (1:200; Santa Cruz Biotechnology), and rabbit anti-albumin (1:60,000; Abcam, Cambridge, MA, USA). Blots were then washed three times in 1X TBS with 0.05% Tween-20 at room temperature and then incubated at room temperature for 1 hour with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies (1:10,000, goat anti-rabbit HRP conjugated; Sigma-Aldrich Corp.), donkey polyclonal anti-goat HRP conjugated (1:20,000 for anti-S100A8 and 1:40,000 for anti-S100A9; Abcam). Blots were then washed five times in 1X TBS with 0.05% Tween-20 and once in 1X TBS at room temperature and then secondary antibody was detected by using chemiluminescent substrate (Thermoscientific) for 5 minutes at room temperature.
RESULTS

Inflammation Increases Protein Concentration but Decreases Protein Complexity

After induction of uveitis, the protein concentration of the aqueous and vitreous were measured and compared to control eyes. The protein levels were the lowest in naïve eyes with total protein concentrations of 1.18 µg/µL in the aqueous (AqH) and 1.17 µg/µL in the vitreous (Table). The EAU eyes had the highest protein concentration in the AqH (22.17 µg/µL) and vitreous (43.50 µg/µL). Protein concentration was also increased in PMU eyes, to 11.56 µg/µL in the AqH and 10.2 µg/µL in the vitreous. Sham injection (3.41 µg/µL) increased the AqH protein nearly 3-fold when compared to naïve condition (1.18 µg/µL), but vitreous protein concentration was not affected (naïve, 1.70 µg/µL).

The protein constituents of the aqueous humor in naïve and inflamed (EAU and PMU) eyes were then compared by using DIGE (Fig. 1). Proteins from naïve eyes were labeled with Cy2 (Fig. 1A, blue), PMU with Cy5 (Fig. 1C, red), and EAU with Cy3 (Fig. 1B, green). Albumin was identified as a major component of the aqueous in all conditions (Fig. 1D, asterisk). More individual spots were identified in the naïve aqueous than in either inflamed condition. For example, a large number of spots located in the 20- to 25-kDa range with an isoelectric focusing point of pH of 5 to 6.6 were detected in the naïve aqueous, but not in PMU or EAU aqueous (Fig. 1D, bracket). This suggests there is a decreased protein complexity in the inflamed aqueous. Some of the proteins found in the naïve aqueous were still present in the inflamed conditions (Fig. 1D, white spots). However, differential concentrations are indicat-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Concentration, µg/µL</th>
<th>Fold Induction Compared to Naïve</th>
</tr>
</thead>
<tbody>
<tr>
<td>AqH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>3.41</td>
<td>2.9</td>
</tr>
<tr>
<td>PMU</td>
<td>11.56</td>
<td>9.8</td>
</tr>
<tr>
<td>EAU</td>
<td>22.17</td>
<td>18.8</td>
</tr>
<tr>
<td>Vitreous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>1.60</td>
<td>1.0</td>
</tr>
<tr>
<td>PMU</td>
<td>10.20</td>
<td>5.9</td>
</tr>
<tr>
<td>EAU</td>
<td>43.50</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Figure 1. Differential gel electrophoresis analysis identifies aqueous humor protein profiles specific for PMU and EAU inflammation. Aqueous humor protein from pooled eyes (A) naïve in blue, (B) EAU in green, (C) PMU in red, (D) merged. Proteins common to all conditions appear white. Asterisk (*) identifies serum albumin. Bracket indicates proteins increased in or specific to the naïve aqueous. Open arrow indicates an example of proteins common to EAU and PMU but not present in naïve aqueous. Closed arrow indicates EAU-specific protein. Closed arrowhead indicates a PMU-specific protein. Open arrowhead indicates proteins elevated in both PMU and EAU that appear green in the merge image due to increased signal from the EAU channel (Cy3).
ed for many of these proteins by the relative fluorescent intensities on the merged image.

Some proteins are specific for inflammation and were present in both PMU and EAU, but not in naïve samples (Fig. 1D, open arrow). Rare proteins were specific to EAU (Fig. 1D, closed arrow) or PMU (Fig. 1D, closed arrowhead), but many of the proteins that were expressed in inflammation were expressed in both models. The EAU spot intensity was often qualitatively increased compared to PMU spot intensity, leading to green spots on the merged image (Fig. 1D, open arrowheads).

Identification of Differentially-Expressed Aqueous Proteins During Inflammation

To further identify the differentially-expressed proteins, two-dimensional gel electrophoresis was performed on PMU and naïve aqueous, and spots of interest were excised and identified by mass spectrometry (Fig. 2). By comparing the two gels, three categories of proteins could be identified: proteins that were present in PMU and not naïve aqueous (group 1), proteins present in naïve aqueous and not PMU (group 2), and proteins present in both (group 3). Seventeen spots that demonstrated differential expression between PMU and naïve eyes were identified by mass spectrometry and grouped by expression pattern (table in Fig. 2). Group 1 (spots 1–4), consists of three proteins: S100A9 (14 kDa), protein S100A8 (10.8 kDa), and apolipoprotein E (ApoE; 36 kDa). S100A9 was identified twice at the same molecular weight, but at two different isoelectric focusing points. Group 2 proteins include lacrimal gland protein (9.8 kDa), α₂u-globulin (13 kDa), and a number of α-, β- and γ-crystallin proteins in the 20-kDa range. Group 3 proteins were identified in both conditions and include β-crystallin B2 (23.4 kDa), albumin (69 kDa), vitamin D-binding protein (54 kDa), and actin (41.7 kDa).

Upregulation of Protein S100A8 and Protein S100A9

Western blot analysis was used to confirm the differential expression of proteins identified by mass spectrometry. Naïve, sham injection, PMU, and EAU aqueous and vitreous were tested to determine dependency of protein changes on experimental condition. Samples were tested in parallel by loading equal volume or equal protein concentration per well. Equal volume samples reflect the concentration of the protein found inside the eye, while equal protein samples reflect the relative abundance of the protein in relation to all other proteins in the sample. Serum albumin was used as loading control for these experiments. In the equal volume experiments, serum albumin was markedly increased in the inflamed conditions (PMU and EAU) compared to naïve and sham injection.

Both S100A8 and S100A9 were identified by 2-D gel electrophoresis and mass spectrometry (spots 1–3) as present in PMU and not naïve aqueous. These results were confirmed by Western blot analysis in the aqueous humor (Figs. 3A, 3C) and vitreous (Figs. 3B, 3D) humor in both EAU and PMU (Fig. 3). Qualitatively, both S100A8 and S100A9 are more abundant in the aqueous than the vitreous. The different information provided by the equal volume and equal protein is most striking in the vitreous experiment (Fig. 3B). Although the S100 proteins were present in the EAU vitreous at equal volume, when an equal protein concentration was compared, the amount of the S100 proteins was nearly undetectable in EAU while still present in PMU.
To determine if S100A9 in the PMU aqueous is present primarily in an extracellular secreted form, or within the cells of the inflammatory infiltrate, we separated the cellular and supernatant fractions of the aqueous humor by centrifugation and tested each fraction for the presence of S100A9 protein (Supplementary Fig. S1). S100A9 protein was only detected in the cellular fraction.

A second protein from group 1 (present in PMU not naïve aqueous) is ApoE. In the aqueous, low levels of ApoE were found in naïve eyes in the equal protein condition, but it was undetectable in the equal volume condition. Apolipoprotein E was present in the aqueous of both PMU and EAU. Beta-B2–crystallin was present in the aqueous of naïve and sham, but was increased in PMU. In EAU, β-B2–crystallin was decreased in comparison with naïve and sham. In the vitreous, β-B2–crystallin was present in all conditions, but relative concentration decreased in EAU more than PMU when compared to naïve.

A second protein from group 1 (present in PMU not naïve aqueous) is ApoE. In the aqueous, low levels of ApoE were found in naïve eyes in the equal protein condition, but it was undetectable in the equal volume condition. Apolipoprotein E was present in the aqueous of both PMU and EAU. Beta-B2–crystallin was present in the aqueous of naïve and sham, but had higher protein levels in EAU than in PMU. Vitreous levels of ApoE were much lower than aqueous levels in all conditions. Beta-B2–crystallin was identified among the group 3 proteins (present in naïve and PMU). Beta-B2–crystallin was notably decreased in the EAU aqueous when compared to PMU, sham, and naïve. In the vitreous, β-B2–crystallin was present in all conditions by equal volume, but the equal protein comparison identified a similar marked decrease in EAU when compared with naïve.

**DISCUSSION**

Uveitis encompasses a heterogenous group of diseases. Mechanisms driving the underlying ocular inflammation are likely shared in most diseases, but as evidenced by variable responses to therapy there must also be disease-specific mediators. In this study, we explored the proteome of two models of uveitis to identify proteins that may be common to most forms of inflammation, and those that show model or mechanism specificity.

In uveitis, the immunologic and physical barriers breakdown between the eye and the blood, leading to an increase in protein content of intraocular fluids. In humans this process is recognized clinically as flare. Previous studies in the rat EIU and EAU models, as well as in ERU, have looked for proteome changes in the vitreous during inflammation. For this study, we decided to look at protein changes in the aqueous of two rat models of uveitis by using DIGE and matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF). We found that the aqueous protein composition during inflammation is distinct from the naïve composition and that select proteins appear to be differentially upregulated in each uveitis model. These data support the previous studies identifying a different aqueous proteome in an inflamed eye when compared to naïve aqueous. Differential gel electrophoresis also identified an overall decrease in the complexity of the protein constituents in the aqueous of inflamed eyes when compared to naïve. In the inflamed eye, the blood–retinal barrier is disrupted and major serum proteins such as albumin and IgG are known to increase in the vitreous. This can make detection of less prevalent proteins difficult. However, in our case we found that the proteins S100A8, S100A9, and ApoE were still detectably increased in the inflamed condition, suggesting a significant increase in the levels of these proteins.

In both the PMU and EAU models of uveitis, S100A8 and S100A9 were found in high levels in both the aqueous and vitreous of the inflamed eyes when compared to naïve and sham.
Proteomic Analysis in Two Rat Models of Uveitis

We identified differential expression of many crystallin proteins between the inflamed and naive conditions. Many of the crystallins were not detectable in the inflamed aqueous when compared with the naive condition. These findings are consistent with previous reports of changes in crystallin expression detected in models of ocular inflammation.13,14,49–52 Crystallins are the major structural proteins of the lens and are grouped into three families: the α-, β-, and γ-crystallins.53 However, crystallins are not lens-exclusive proteins and are also expressed in the neural retina54,55 and in select extraocular tissues.56–58 The αA and αB-crystallins are members of the small heat shock family of proteins and have been shown to play a role in preventing neuronal apoptosis in response to multiple forms of cell stress.59 The β-crystallins are Greek key motif-containing proteins. This motif is composed of antiparallel β pleated sheets that allows for optically transparent protein packing in the lens60 and Ca+ binding.61 Like α-crystallins, members of the β-crystallin family are also upregulated in response to cellular stresses, but rather than performing an antiapoptotic role, they seem to be important in neural regeneration.62

In this study, we found that β-B2-crystallin levels decreased in the AqH and vitreous in the EAU uveitis model at the time of peak inflammation (day 14). This is in contrast to previous work that has reported the upregulation of αA- and β-B2-crystallin in mitochondria during the early stages of EAU.69 However, these results could be reconciled by considering that EAU is an overwhelming panuveitis that leads to significant retinal necrosis, such that by the time of peak inflammation, any increased expression of β-B2-crystallin may not be detectable above the background loss of the protein as retinal cells are destroyed. This temporal association of changes in β-B2-crystalline, together with the data identifying a neuroprotective and antiapoptotic role for crystallins in the eye, suggests that supplementing crystallin function during active inflammation could be an adjunctive therapy for patients with uveitis.

Both models tested in this study were relatively acute (2 days for PMU and 2 weeks for EAU) and are self-limited. We chose to study the rat because of the relatively larger amounts of ocular fluid available than with mouse. At present, there are no validated chronic uveitis models or spontaneous uveitis models in the rat. It is possible the proteome of chronic inflammation differs substantially from that seen in the acute condition and may suggest a different set of molecular targets for therapy. Further work on development of tractable animal models of uveitis is needed to address this and other important questions.
Proteomic Analysis in Two Rat Models of Uveitis

Acknowledgments

Supported by a grant from Alcon Research Laboratories. KLP is supported by National Institutes of Health (NIH) Award K08EY025998. RVG is supported by the Burroughs-Wellcome Translational Scientist Award, an unrestricted award from Research to Prevent Blindness, and NIH P30 EY001730. Disclosure: K.L. Pepple, None; L. Rotkis, None; L. Wilson, None; A. Sandt, None; R.N. Van Gelder, None

References
