Anti-Retinal Antibodies in Serum of Laser-Treated Rabbits

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Purpose. Retinal injuries that affect the photoreceptors and/or the retinal pigment epithelium (RPE) may result in the leakage of retinal proteins into the systemic circulation. This study was designed to determine whether an immune response is elicited after an acute retinal injury resulting in circulating anti-retinal antibodies in the serum.

Methods. Fifty laser burns of different grades (minimally visible lesion [MVL], grade II [GII], or grade III [GIII] lesions) were created in the retinas of Dutch Belted rabbits. The degree of laser burns was confirmed by fundus imaging and histology. Serum samples were collected from the animals 3 months after the retinal injury. Candidate autoantigens were identified by two-dimensional (2-D) Western blots of rabbit retinal lysate probed with sera from either control or laser-treated animals. Candidate autoantigens were further characterized by immunostaining to confirm their retinal localization.

Results. Seven and 11 protein spots were selected from the MVL and GII laser-treated samples, respectively, for autoantigen identification. No protein spots were detected in the GIII laser-treated samples. Four candidate autoantigens were common to both MVL and GII lesions: dihydropyrimidinase-related protein 2, fructose-bisphosphate aldolase C, chaperonin-containing T-complex polypeptide 1 subunit zeta, and pyruvate kinase isozyme.

Conclusions. Laser-induced retinal injuries resulted in circulating anti-retinal antibodies that were detectable 3 months after the injury. The response appeared to vary with the severity of the laser retinal damage. The identification of the candidate antigens in this study suggest that this approach may permit future development of new diagnostic methods for retinal injuries. (*Invest Ophthalmol Vis Sci.* 2012;53:1764–1772) DOI:10.1167/iovs.11-7685

Retinal injuries that result from accidental or intentional laser exposure or other sources can be asymptomatic or can result in permanent vision loss. Laser retinal damage can occur by photothermal, photomechanical, photodisruptive, or photochemical mechanisms,[1] leading to varying degrees of retinal neuronal and RPE cell damage or death.[1–5] In addition, the blood-retinal barrier is disrupted, which is associated with local inflammation and the upregulation of immunoregulatory molecules, such as prostaglandin E2,[6] cellular adhesion molecules, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 in the retina.[7] Disruption of the blood-retinal barrier following laser photocoagulation may lead to the release of retinal proteins into the blood circulation. These proteins may initiate an immune response, resulting in autoantibodies that are detectable in the serum. These autoantibodies could serve as molecular biomarkers for retinal injuries caused by laser or by retinal injuries that result in similar phenotypes. The concept of a systemic immune response to retinal injuries is not new and has been described in chronic diseases that affect the retina, such as age-related macular degeneration,[8] diabetic retinopathy,[9] and glaucoma.[10] However, our knowledge of the potential systemic immune response initiated by acute retinal injuries is limited.

In this paper, we describe the identification of candidate autoantibodies detected in the sera of rabbits in response to retinal injuries induced by different grades of retinal laser photocoagulation.

Materials and Methods

Animals

A total of 25 Dutch Belted (DB) rabbits were used. Of these rabbits, 12 were laser treated, and 12 were used as controls. The eyes from the remaining animal were examined to confirm the laser lesions by histology. Animals were purchased from Myrtle's Rabbitry, Inc. (Thompsons Station, TN) and were housed at the Comparative Medicine Unit Animal Facility at the Northeastern Ohio Medical University (NEOMED). All animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experimental procedures were approved by the Institutional Animal Care and Use Committee at NEOMED.

All animal procedures were carried out under general anesthesia using a combination of subcutaneous injection of dexmedetomidine (approximately 0.25–0.5 mg/kg; Pfizer Animal Health, New York, NY) and ketamine (15–20 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). Upon completion of the procedures, atipamazole (0.5 mg/kg) was administered as a reversing agent. Euthanasia was performed using pentobarbital-containing solution (80–100 mg/kg, Fatal Plus, Butler Schein Animal Health, Dublin, OH) intravenously.

Laser Treatment

Retinal laser lesions were created after pupillary dilation with one drop each of tropicamide 1.0% (Bausch & Lomb, Tampa, FL) and phenylephrine HCl 2.5% (Alcon Laboratories, Fort Worth, TX). Using a double frequency Nd:YAG laser at 532 nm, 50 spots of mild, minimally visible laser lesions (MVL, $n = 5$), moderate, grade-II laser lesions (GII, $n = 4$), or severe, grade-III laser lesions (GIII, $n = 3$) were created in the retinas of DB rabbits. The laser settings for power (mW), duration (ms), and spot size (μm) that were used to create the three

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grades of lesions were as follows: MVL (100 mW, 100 ms, 500 μm), GII (150 mW, 200 ms, 500 μm), and GIII (300 mW, 200 ms, 500 μm). The control animals for each group were treated in the same manner, except for the laser application. Fundus photography was performed after laser treatment and in control animals following slit lamp examination. Animals were then housed for 12 weeks.

Blood Collection

Twelve weeks after laser treatment, blood was collected by cardiac puncture from both laser-treated and control animals. Blood collection was performed under deep anesthesia using a combination of ketamine (50–70 mg/kg) and dexmedetomidine (1–2 mg/kg). The collected blood was incubated at room temperature for approximately 15 minutes to allow clotting, then centrifuged for 15 minutes before serum collection. Contact with the blood cells (bottom layer) was avoided to prevent possible contamination. The serum was aliquoted and stored at −80°C until processing.

Histology

Eyes that were enucleated 24 hours after laser treatment were fixed in formalin and embedded in paraffin. Paraffin sections (5 μm) of whole retinas were stained with hematoxylin and eosin (H&E) for visualization of the laser burns.

Retinal Protein Preparation

Normal DB rabbit retinal proteins were prepared as previously described[11] with some modifications. Briefly, whole retinas containing sensory retina, RPE, and choroid were homogenized in 0.5 mL of homogenization buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 200 mM sucrose, 1 mM phenylmethylsulfonyl fluoride), and cell debris was removed by centrifugation at 1000*g* for 5 minutes at 4°C. The supernatant was centrifuged at 16,000*g* for 30 minutes at 4°C. The resulting supernatant was solubilized in Triton X-100 solubilization buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.05% SDS, 2.5% glycerol, 1.0 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) for at least 4 hours at 4°C. Protein concentration was measured using the bicinchoninic acid Protein Assay Kit (Thermo Scientific, Rockford, IL). Proteins were concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA).

Two-Dimensional (2-D) PAGE

Retinal proteins (80 μg/strip) were diluted in ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% bromophenol blue; Bio-Rad, Hercules, CA), loaded onto 11-cm Immobiline Dry Strips, pH 3 to 11 nonlinear (GE Healthcare, Piscataway, NJ), and rehydrated for 16 hours at room temperature. The first-dimension separation was performed in a Protean isoelectric focusing cell (Bio-Rad) at 250 V for 15 minutes, followed by an increase to 8000 V for 30 kVh. After the first-dimension separation, gel strips were rinsed in 1X Tris-glycine (TG) running buffer (25 mM Tris, 192 mM glycine + 0.1% SDS) for 10 minutes, equilibrated for 15 minutes in equilibration buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 2% dithiothreitol [DTT]), followed by 15 minutes in equilibration buffer II (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 2.5% iodoacetamide), then loaded onto 12.5% criterion Tris-HCl gels (Bio-Rad, Hercules, CA). Proteins were subjected to electrophoresis at 175 V in 1X TG running buffer for the second-dimension separation.

Western Blot

Following 2-D PAGE, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane overnight at 10 V in 1X TG transfer buffer (25 mM Tris, 192 mM glycine + 0.05% SDS + 20% methanol). Membranes were blocked with 5% BSA in Tris-buffered saline + 0.1% Tween 20 (TBS-T) for 8 hours at 4°C, then probed with sera from the 12-week laser-treated or control animals. Sera from each rabbit were tested individually and not pooled. The serum was applied at a 1:100 dilution in 2% BSA in TBS-T overnight at 4°C, followed by three washes with TBS-T for 10 minutes each. Goat anti-rabbit IgG-horseradish peroxidase (HRP)–conjugated secondary antibody (SantaCruz Biotechnologies, Santa Cruz, CA) was applied at a 1:25,000 dilution in 2% BSA in TBS-T for 1 hour at room temperature, followed by four washes with TBS-T for 10 minutes each. The signal was visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

Protein Spot Analysis

Western blot images were scanned and analyzed using Ludesi Redfin software basic analysis (Ludesi, Malmö, SE-211 17, Sweden; http://www.ludesi.com/redfin/).Western blot images probed with sera from laser-treated animals were compared with those probed with sera from control animals. Spots that were present in the blots from at least two laser-treated animals and absent in all of the control blots within a group were selected for protein identification. The membranes were matched using all-to-all spot matching. The integrated intensity of each spot was measured, background corrected, and normalized. The normalization removes differences originating from variations in staining, scanning time, and protein loading by mathematically minimizing the median expression difference between the matched spots.[12] For spot excision, gels were run with 11-cm Immobiline Dry Strips, pH 3–11 nonlinear, rehydrated with 120 μg of protein as described above, then silver stained as previously described.[13,14] Briefly, gels were fixed in 45% methanol + 7% acetic acid for at least 30 minutes and washed three times for 5 minutes each to remove the fixer. Gels were sensitized by incubation in 0.02% sodium thiosulfate pentahydrate for 90 seconds, followed by three washes with water for 30 seconds each. Gels were incubated for a minimum of 25 minutes in 0.2% silver-nitrate solution, followed by three washes with water for 1 minute each. Gels were then developed by incubation in 6% sodium carbonate + 0.018% formaldehyde + 0.0004% sodium thiosulfate pentahydrate until stained. Development was terminated by incubation in 45% methanol + 7% acetic acid. The selected spots were excised from the silver-stained gels for analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Liquid Chromatography Tandem Mass Spectrometry

Sample Preparation. The excised 2-D spot gel pieces were processed individually as previously described[14] with minor modifications to the gel washing steps. The gel pieces were fixed in 1 mL of 40% methanol, 7% acetic acid, for 30 minutes and destained by washing with water once, followed by washing for 30 minutes with 50 mM sodium thiosulfate and 15 mM potassium ferricyanide until the silver color disappeared. Pieces were then washed four times with water, once with 200 mM ammonium bicarbonate, and twice with 100 mM ammonium bicarbonate in 50% acetonitrile, then dried under vacuum using a Savant SpeedVac (Instruments Inc., Farmingdale, NY). To each gel piece, 200 μL of 100 mM ammonium bicarbonate (pH 8.0) containing 0.5 μg trypsin (Promega, Madison, WI) was added and incubated overnight at 37°C. Peptides in each gel piece were extracted with three washes of 70% acetonitrile and 0.1% formic acid. The extracts were then pooled together and dried. Twenty microliters of 6 M guanidine-HCl in 5 mM potassium phosphate and 1 mM DTT (pH 6.5) was added to each dried sample and sonicated. Peptides were then extracted using a C18 ZipTip (Millipore) and subjected to nanospray LC-MS/MS analysis. Each sample was run once on the mass spectrometer using an extended linear gradient as described below.

Nanospray LC-MS/MS Spectrometry. Automated nanospray LC-MS/MS was performed using an LTQ-LC/MS (ThermoFisher Scientific, Waltham, MA). Peptide mixtures were separated using a C18 reverse-phase column (0.75-Å internal diameter at a flow rate of 1 μL/min) in

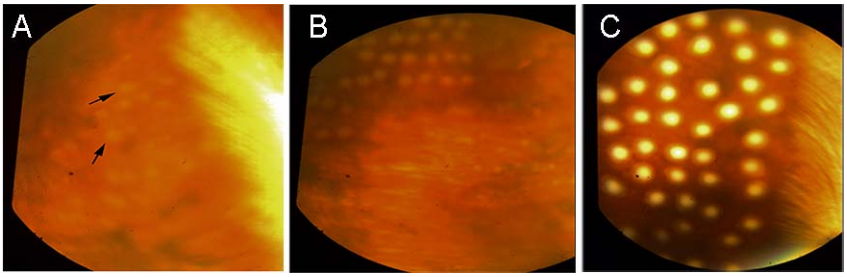

Figure 1. Fundus pictures of laser lesions in the DB rabbit retina 24 hours after laser treatment. Note the light-gray retinal lesions produced by MVL laser burns (*arrows*, **A**), the grayish-white lesions produced by GII laser burns (**B**), and the intense white lesions produced by the GIII laser burns (**C**).

line with the mass spectrometer. The mobile phases consisted of 0.1% formic acid containing 5% acetonitrile (A) and 0.1% formic acid in 95% acetonitrile (B), respectively. A 180-minute linear gradient was used, followed by 60 minutes of equilibration in solvent A. The ions eluted from the column were electrosprayed at a voltage of 1.75 kV. The LC-MS/MS cycle was six MS/MS scans per full MS scan, with dynamic exclusion enabled, +/− 1.5 Da tolerance, and 12-second exclusion duration.

Data Analysis. The collected MS data were analyzed using the SEQUEST algorithm (Thermo Fisher Scientific), searched against the mammalian including the rabbit subset of the UniProt database,[15] using a peptide mass tolerance of 2.5 Da, a fragment mass tolerance of "zero," which is effectively 1 Da, and monoisotopic masses.

Expression of the Candidate Autoantigens in the Rabbit Retina

Immunohistochemistry. Deparaffinized slides from normal DB rabbit eyes were rehydrated using graded ethanol. Antigen retrieval was performed using 1X antigen retrieval solution (Dako, Carpinteria, CA) per the manufacturer's instructions. The sections were blocked for 45 minutes using 10% normal goat or donkey serum and subsequently labeled by overnight incubation at 4°C with antibodies to the following proteins: dihydropyrimidinase-related protein 2 (DRP-2; 1:100; LifeSpan BioSciences, Seattle, WA), fructose-bisphosphate aldolase C (ALDC; 1:100; SantaCruz Biotechnologies), pyruvate kinase isozyme (PKM2; 1:100; LifeSpan BioSciences), and chaperonin containing T-complex polypeptide 1 subunit zeta (TCP-1-zeta; 1:100; Abnova, Taipei, Taiwan). The sections were then incubated with Texas Red–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature. Blocking and washing steps were performed in a humid chamber at room temperature. All washes were performed three times for 5 minutes each in PBS. Antibody binding was detected by immunofluorescence and visualized using an Olympus BX51 fluorescent microscope (Olympus, Tokyo, Japan).

Western Blot. DB retinal protein lysates (20 ug/lane) were separated by SDS-PAGE and then transferred to PVDF membrane overnight. Membranes were blocked in 2% BSA in TBS-T for 2 hours and then incubated with the primary antibody: DRP2 (1:1000; LifeSpan BioSciences), ALDC (1:500; SantaCruz Biotechnologies), PKM2 (1:1000; LifeSpan BioSciences), and TCP-1-zeta (1:200; Abnova) for 2 hours, followed by HRP-conjugated secondary antibody for 1 hour. All washes were performed three times for 10 minutes each in 1X TBS-T. Signal was detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

Functional Categories

Autoantigens were analyzed using Pathway Studio 6.0 software (Ariadne, Rockville, MD). Using the human homolog gene identifiers, the autoantigens were analyzed for their molecular function and biological process.

Results

Fundus Imaging and Histology

MVL laser burns caused light-gray retinal lesions, and GII burns produced grayish-white lesions, while GIII burns produced lesions with distinct white centers surrounded by grayish-white rings (Figs. 1A, 1B, and 1C, respectively). H&E staining of the retina treated with MVL lesions showed minimal disruption of the retinal pigment epithelium with intact overlying retina (Fig. 2A). GII lesions showed disruption of the sensory retina with mummification of the outer segments and disruption of the RPE (not shown), and GIII lesions showed RPE disruption with inflammatory reaction in the underlying choroid, mummification of the outer and inner segments, and disruption and edema of the outer nuclear layer (Fig. 2B).

Candidate Autoantibody Detection by 2-D Western Blot

Sera from GII laser-treated animals had 11 reactive protein spots (Fig. 3), while sera from MVL-treated animals had seven reactive protein spots (data not shown). Sera from GIII laser-treated animals did not show any reactive protein spots (data not shown). The spot patterns were similar among animals within a group.

Identification of the Candidate Autoantigens by LC-MS/MS

A total of 18 protein spots from the 2-D Western blot were selected for analysis by LC-MS/MS from the 7 MVL-treated and 11 GII-treated animals (Fig. 4, Table 1). Four of these spots yielded the same protein. The analysis of these spots revealed a total of 14 proteins. Four proteins, DRP-2, ALDC, TCP-1-zeta, and PKM2, were common to both MVL- and GII-treated animals, while the remaining 10 proteins were seen in either the MVL- or GII-treated animals. Of those 10 proteins, 3 were detected in MVL-treated rabbits, and 7 were detected in GII-treated animals. Varying numbers of autoantigens were detected in different animals (Table 1).

The four autoantigens present in both the MVL- and GII-treated samples were further characterized to confirm their expression in the retina using immunohistochemistry and Western blot.

Expression of the Identified Proteins (Candidate Autoantigens) in the Retina

To confirm the possibility that the candidate autoantibodies following laser injury, identified in this study, could have been developed against antigens expressed in the rabbit retina, we performed immunohistochemistry and Western blot using

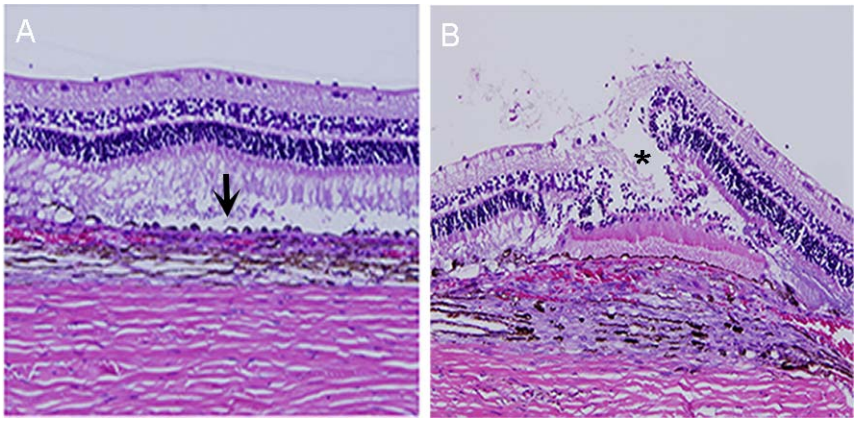

Figure 2. Histopathological confirmation of laser lesions in DB rabbits. (**A**) MVL lesion showing retinal pigment epithelial vacuolization, mildly disrupted outer segments and an intact but artifactiously detached sensory retina. This change was determined after serial sections through the block and the lesions were identified in the retinal pigment epithelium by gross microscopy. (**B**) GIII lesions (*) demonstrating disruption of the sensory retina and the retinal pigment epithelium, retina edema with a mild inflammatory reaction, and a vascular engorgement in the underlying choroid (H&E; original magnification ×10).

normal DB retina probed with commercially available antibodies to the four autoantigens that were common between the MVL- and GII-laser treated samples. We confirmed that these four common candidate autoantigens (DRP-2, ALDC, PKM2, and TCP-1-zeta) were expressed in the normal DB retina (Fig. 5A–E). The proteins were mainly cytoplasmic and were expressed diffusely in all retinal layers. Except for DRP2, all antibodies were specific and did not cross-react with other proteins in the retina as shown in the Western blot (Fig. 5F).

Molecular Function/Biological Process for the Candidate Autoantigens

The 14 candidate autoantigens identified in this study as potential biomarkers for laser-induced retinal injuries were analyzed for their molecular function and biological process (Table 2) using Pathway Studio 6.0. Eleven of these autoantigens appeared to have a protein-binding function. Four were involved in glycolysis. Two autoantigens had an anti-apoptotic role, while others were involved in actin filament depolymerization, the glyceraldehydes-3-phosphate metabolic process, neural fold formation, cell size, or regulation of neurotransmitter levels with cofilin 1 being involved in most of these processes.

Discussion

In this paper, we describe the identification of candidate anti-retinal antibodies that were detected in sera of laser-treated rabbits. Detection of these antibodies in the serum indicates that a systemic immune response is elicited following retinal laser injuries.

The eye is a specialized compartment with respect to the immune response and is sequestered from the rest of the immune system unless its protective barriers are impaired. Disruption of the blood-retinal barrier caused by laser in this study likely resulted in leakage of retinal proteins from the site of injury into the systemic circulation. These proteins may have resulted in the immune response that was detectable after 12 weeks.

It is important to note that even the MVL retinal laser lesions, which represent a mild response to laser-retina interaction, initiated an autoantibody response. Clinically, these lesions are often transient and may not be detected after a few weeks.[15] A serum autoantibody response could potentially serve as an indicator of a transient retinal injury, a possibility that needs to be further investigated.

The immune response appeared to be more robust after the MVL and GII laser lesions than after the severe GIII lesions, which resulted in a poor response. The more severe GIII lesions may have led to protein degradation at the site of injury,

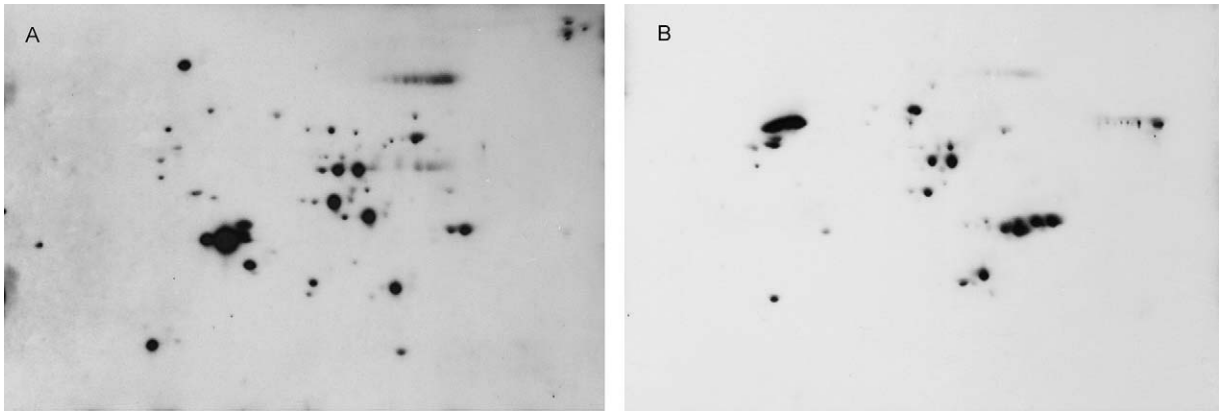

Figure 3. An example of a 2-D Western blot showing the pattern of the candidate autoantibody using GII laser-treated serum as primary antibody (**A**) and control serum (**B**).

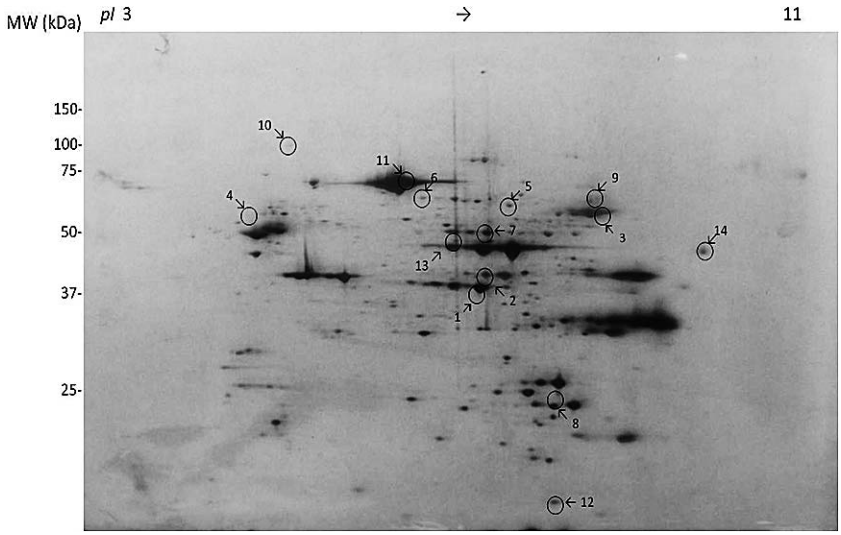

Figure 4. Silver staining of DB rabbit retinal lysate probed with serum from a GII laser-treated animal. Numbers on the gel represent the protein spots that were selected for further analyses.

to the extent that they did not appear in the serum and therefore did not result in an immune reaction similar to MVL and GII lesions that could be detected in serum. Antibodies against some proteins such as serotransferrin and ubiquilin were detected in only one group for reasons that are not clear. It could be that these responses are specific to the laser grade used for each group.

Although the autoantigens identified in this study are not retinal-specific proteins, immunohistochemistry and Western blot demonstrated that at least four antigens that were common to MVL and GII lesions were expressed in the retina in a diffuse fashion including the outer retina. Other autoantibodies detected in this study were raised against proteins that were previously known to be expressed in the retina.[16,17] Furthermore, this observation suggests that only retinal proteins that are abundant and possibly upregulated following retinal damage (as described with DRP-2 below) leak into the systemic circulation, act as autoantigens, and result in an immune response. The detection of autoantigens in the serum that are not specific to the eye raises the question of tissue specificity, a question raised by Adamus and Wilson,[18] when testing for autoantibodies in autoimmune retinal diseases (AIRs). It is, however, unlikely that the autoantigens detected in our study play a role in autoimmunity within the eye, but they could be used as a marker(s) for retinal tissue damage.

Table 1. Candidate Autoantigens Identified by LC-MS/MS in Each Animal Following Varying Degrees of Laser Photocoagulation (MVL, GII, and GIII, + present, − absent)

Uniprot Accession	Protein Description	MVL ($n = 5$)					GII ($n = 4$)				GIII ($n = 3$)		
		2T	3T	192	193	194	4T	5T	130	131	6T	7T	8T
O02675	Dihydropyrimidinase-related protein 2	−	−	+	−	+	+	+	+	+	−	−	−
P00939	Triosephosphate isomerase	−	−	−	−	−	+	−	+	+	−	−	−
Q9GKW3	Fructose-bisphosphate aldolase C	+	+	−	−	−	+	+	−	−	−	−	−
Q6B855	Transketolase	+	+	−	−	−	−	−	−	−	−	−	−
P03974	Transitional endoplasmic reticulum ATPase	−	−	−	−	−	−	−	+	+	−	−	−
P19134	Serotransferrin	−	−	−	−	−	−	−	+	+	−	−	−
Q5E9F7	Cofilin-1	+	+	−	−	−	−	−	−	−	−	−	−
Q9XSJ4	α-enolase	+	+	−	−	−	−	−	−	−	−	−	−
O77622	Chaperonin containing T-complex polypeptide 1 subunit zeta	−	−	+	−	+	+	+	−	−	−	−	−
P11974	Pyruvate kinase isozymes M1/M2	−	−	+	+	+	+	+	−	−	−	−	−
P68105	Elongation factor 1-α 1	+	+	−	−	−	−	−	−	−	−	−	−
P15103	Glutamine synthetase	−	−	+	+	+	−	−	−	−	−	−	−
Q9UMX0	Ubiquilin-1	−	−	+	−	+	−	−	−	−	−	−	−
P69895	Tubulin β-2 chain	−	−	−	+	+	−	−	−	−	−	−	−

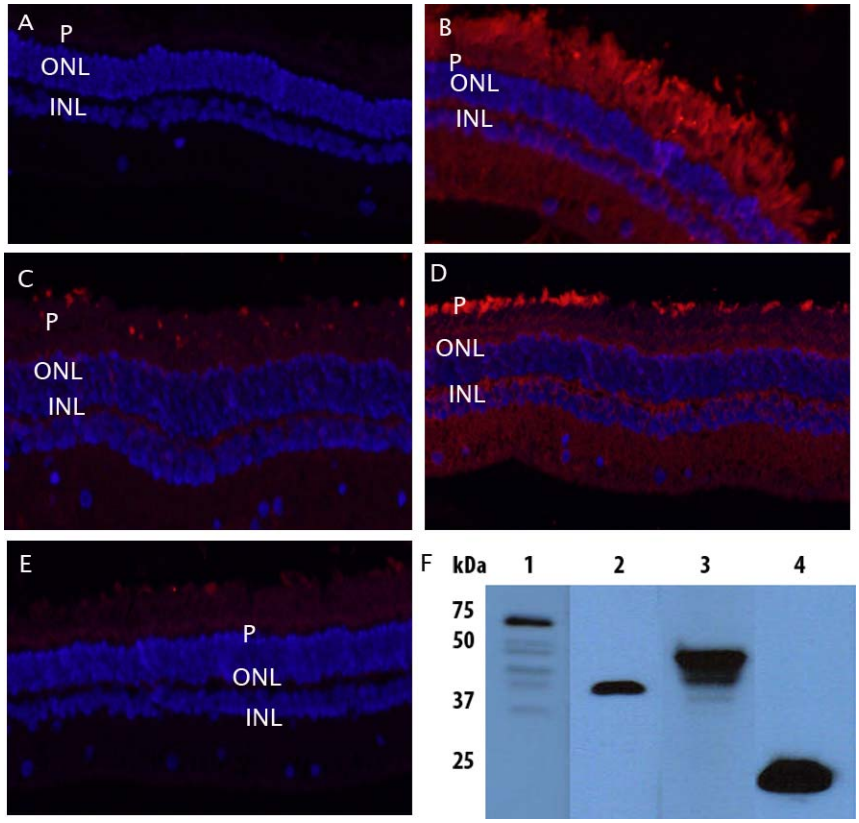

Figure 5. Immunohistochemistry showing (**A**) negative control; (**B**) DRP-2, note moderate intracytoplasmic labeling of all the retinal layers including outer and inner segments; (**C**) ALDC, faint intracytoplasmic labeling is noted in the inner and outer retinal structures; (**D**) PKM2, faint intracytoplasmic labeling is noted in the inner and outer retinal structures but the tips of the outer segments show staining that is likely edge artifact; (**E**) TCP-1-zeta, faint staining is noted mainly in the inner and outer segment region. The negative control did not show immunoreactivity in any of the layers (original magnification ×10, Texas-Red [red], DAPI [nuclear stain]), and (**F**) Western blot analysis of DB retinal lysates probed with antibodies against DRP-2 (*lane 1*, 62 kDa), ALDC (*lane 2*, 40 kDa), PKM2 (*lane 3*, 58 kDa), and TCP-1 (*lane 4*, 26 kDa).

Anti-retinal antibodies have been previously described in many ocular diseases,[19] including AMD,[20,21] glaucoma,[22–24] diabetic retinopathy (DR),[9] RP,[25] cancer-associated retinopathy (CAR), and melanoma-associated retinopathy (MAR),[26,27] and the immune responses appear distinct between the conditions. For example, higher titers of antibodies to retinal S-antigen were reported in patients with proliferating DR after argon laser photocoagulation, reaching its maximum at 8 weeks and declining at 12 weeks post laser.[28] We did not detect antibodies to S-antigen in our study. It is possible that the differences could be related to a species-specific response or might be related to the timing at which we examined the serum, which was at 12 weeks after the injury, a point at which the serum titers of S-antigen were noted to decline. Additional studies looking at the immune response at different time points after injury may help determine when a peak immune response is likely to be detected. Using Western blot, anti-retinal antibodies are also reported in the serum of normal human patients. The autoantigens that result in this response are, however, not identified.[29] On the other hand, in autoimmune retinopathies, the autoantibodies detected are directed against a variety of different antigens,[9,19,30–37] including recoverin, a-enolase, heat-shock protein 70, carbonic anhydrase II, collapsin response-mediating protein-5 (CRMP-5), lens epithelium-derived growth factor (LEDGF), tubby-like protein 1, transducin α, and aldolase C. Other candidate proteins with known molecular sizes have

Table 2. Molecular Functions/Biological Processes of the Autoantigens as Analyzed by the Pathway Studio

Molecular Function/Biological Process	Protein
Protein binding	ALDC, TCP-1-zeta, cofilin-1, DRP-2. elongation factor 1-α 1, α-enolase, PKM2, transketolase, tubulin β-2 chain, ubiquilin, transitional endoplasmic reticulum ATPase
Glycolysis	ALDC, PKM2, α-enolase, triosephosphate isomerase
Anti-apoptosis	Cofilin-1, elongation factor 1-α 1
Positive regulation of actin filament depolymerization	Cofilin-1
Glyceraldehyde-3-phosphate metabolic process	Triosephosphate isomerase
Neural fold formation	Cofilin-1
Negative regulation of cell size	Cofilin-1
Regulation of neurotransmitter levels	Glutamine synthetase

also been detected but not identified. Except for a-enolase and ALDC, none of the anti-retinal antibodies identified in this study was previously reported.

Functionally, the proteins/autoantigens identified in this study fall under two predominant categories, namely, protein binding and glucose metabolism (Table 2). The retina is a metabolically active structure, and the presence of a large number of proteins related to protein binding and glucose metabolism is not surprising. Proteins related to glucose metabolism are upregulated after brain injury.[38,39] It could be that these proteins are also upregulated in the retina in response to laser injury.

Anti-enolase and anti-ALDC antibodies that were detected in laser-treated rabbits are also described in CAR, MAR, RP, and DR.[9,40] RP, CAR, and DR are all characterized by a breakdown in the blood-retinal barrier, similar to this study. It is likely that enolase and ALDC both leak through the blood-retinal barrier in these cases and are likely general markers of both acute damage to the retina, as demonstrated in the rabbit, and chronic damage, as represented by the chronic retinal diseases. Below is a description of the characteristics of the four autoantigens (DRP-2, ALDC, PKM2, and TCP-1-zeta) that were detected in the sera of both MVL and GII laser-treated rabbits.

The protein DRP-2 is involved in neuronal differentiation and axonal guidance.[41] DRP-2 may play a role in replacement and regeneration of neurons.[42] DRP-2 protein and mRNA levels are upregulated following cerebral ischemia in the rat brain[43,44] and in the adult cat visual cortex following retinal photocoagulation.[45] Because DRP-2 is expressed in the retina,[46] these studies suggest that DRP-2 upregulation may also occur in the retina following laser photocoagulation.

ALDC is expressed in the brain[47] and the retina.[48] Anti-ALDC autoantibodies have been detected in the sera of patients with DR because of the loss of the blood-retinal barrier in this disease.[9] As in DR, ALDC antibodies that were detected in the sera of laser-treated rabbits in this study may have resulted from the leaking of the protein from the retina to the serum because of the breakdown of the blood-retinal barrier caused by laser photocoagulation.

TCP-1-zeta is a subunit of the eukaryotic chaperonin CCT (chaperonin containing T-complex polypeptide 1). CCT contains 8 different subunits and is required for the folding of an estimated 15% of newly translated proteins, including actin and tubulin.[49] CCT is expressed in the retina, with a higher abundance in the photoreceptors and bipolar cells.[50] Suppression of CCT activity in mouse photoreceptors results in malformation of the outer segment and triggers rapid retinal degeneration.[51] Because CCT is one of the more abundant proteins in the retina, disruption of the photoreceptors by laser photocoagulation, as shown by histology, may have led to the release of this protein into the serum through the blood-retinal barrier.

Pyruvate kinase is a glycolytic enzyme that catalyzes the formation of pyruvate and adenosine triphosphate (ATP) from phosphoenolpyruvate and adenosine diphosphate (ADP) and exists in four isoforms.[52] The M1-type is found in the skeletal muscle, the heart, and the brain during development[53] and in the retinal pigment epithelium.[54] PKM2 autoantibodies have been detected in the sera of patients with post-streptococcal CNS syndromes,[55] suggesting that this protein could be antigenic.

Serum reactivity with purified proteins further validated the LC-MS/MS results. The only protein seen in both control and treated serum was TCP-1. Autoantibodies recognizing TCP-1 (CCT) in serum of healthy and rheumatoid arthritis patients are cross-reactive with Hsp60, GroEL, and Hsp65,[56] suggesting that CCT-reactive autoantibodies recognize conformational epitopes that are conserved among CCT and other Hsp60 family members. The reactivity of the control serum to CCT protein in this study was similar to that seen in the laser-treated serum. It is possible that the reactivity seen in our control serum is a cross-reactivity to other Hsp60 proteins, since these proteins are also present in the systemic circulation.[57]

One limitation of this study was that the use of 2-D PAGE may have restricted our ability to identify a wider range of proteins, especially proteins that are hydrophobic or have a very high or low molecular weight.[58,59] However, 2-D PAGE followed by mass spectrometry used in this study is the most common approach used in proteomics studies. To increase the sensitivity of 2-D PAGE, one could (1) enrich the retinal protein lysates for detection of the lower abundant proteins by prefractionation of the sample, (2) focus on a specific range of isoelectric point, (3) deplete the highly abundant proteins such as albumin from the serum, or (4) use a larger volume of sample (which would require using larger gels and equipment for electrophoresis and transfer, and then confirmation of the candidate autoantibodies by immunoprecipitation or ELISA).

Conclusion

Retinal injuries affecting the photoreceptors and/or the RPE may result in leakage of retinal proteins into the systemic circulation. These proteins may initiate an immune response and result in detectable autoantibodies that could serve as molecular biomarkers for retinal injuries. The autoantibody response may vary with the degree of the laser injury. As mentioned by Adamus et al.[18] and Hooks et al.,[19] the identification of anti-retinal antibodies is neither specific nor sensitive enough to be used for diagnostic purposes but may be helpful markers for retinal diseases including retinal injuries caused by laser described in this study.

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