Western blot patterns of serum autoantibodies against optic nerve antigens in dogs with goniodysgenesis-related glaucoma

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Objective—To investigate whether differences existed between clinically normal dogs and dogs with goniodysgenesis-related glaucoma (GDRG) in serum autoantibodies against optic nerve antigens.

Animals—16 dogs with GDRG, 17 healthy dogs with unremarkable pectinate ligament and iridocorneal angle morphology, and 13 euthanized dogs with no major ocular abnormalities or underlying diseases.

Procedures—Western blotting was performed with optic nerve extracts from the euthanized dogs as an antigen source and serum from clinically normal dogs and dogs with GDRG as a primary antibody (autoantibody) source. Blots were evaluated for presence and density of bands.

Results—Multiple bands were identified on western blots from all dogs with GDRG and all clinically normal dogs, with a high degree of variability among individual dogs. Dogs with GDRG were significantly more likely than healthy dogs to have bands present at 38, 40, and 68 kDa. Dogs with GDRG had significant increases in autoreactivity at 40 and 53 kDa and a significant decrease in autoreactivity at 48 kDa.

Conclusions and Clinical Relevance—Significant differences in serum autoantibodies against optic nerve antigens were found in dogs with versus without GDRG. Although it remains unclear whether these differences were part of the pathogenesis of disease or were sequelae to glaucomatous changes, these findings provide support for the hypothesis that immune-mediated mechanisms play a role in the development or progression of GDRG. However, the high degree of variability among individual dogs and the considerable overlap between groups suggest that the clinical usefulness of this technique for distinguishing dogs with GDRG from clinically normal dogs is likely limited. (Am J Vet Res 2013;74:621–628)
performed to evaluate changes in serum autoantibody profiles against retinal and optic nerve antigens in glaucomatous humans, through use of a western blot–based technique with retinal or optic nerve digests as antigen sources. These studies have revealed significant differences, involving both increases and decreases in au-toreactivity, between healthy people and people with glaucoma. It remains unclear, as the investigators in these studies have pointed out, whether changes in au-toreactivity are part of the underlying pathogenesis of disease or are sequelae to the damage caused by glaucoma. However, the apparent consistency of findings to date supports the legitimacy and potential usefulness of the described method. Similar methodology has been used in studies of other human immune-mediated disorders, with equally promising results.

We hypothesized that use of a similar western blot technique to evaluate dogs with GDRG would reveal key differences between glaucomatous and healthy dogs and that such differences could serve as the ba-sis for future research and potentially as diagnostic or prognostic tools. The optic nerve was chosen as an an-tigen source for several reasons. Damage to the axons of the optic nerve occurs prior to loss of retinal gan-glion cell bodies in glaucoma. Moreover, neither lowering of IOP nor blockade of apoptotic pathways appears to halt axon loss, although antiapoptotic treatments can protect ganglion cell bodies. Disruption of axonal transport secondary to the increase in IOP occurs in dogs and humans and certainly plays an important role in axonal loss. However, given the inconsistent relationship between IOP and axonal damage, the possibility exists that axonal damage may be initiated prior to an increase in IOP. The purpose of the study reported here was to determine whether glaucomatous optic neuropathy in dogs involves immune-mediated mechanisms.

Materials and Methods

Animals—Sixteen dogs with GDRG and 17 healthy control dogs were enrolled in the study. Owner consent for study inclusion was obtained for all dogs. The study protocol was approved by the Clinical Studies Review Committee of Tufts University Cummings School of Veterinary Medicine. Optic nerves were harvested from euthanized dogs. Sample collection—Three milliliters of blood was obtained from all dogs with GDRG and all healthy control dogs via jugular venipuncture by use of a 0.5-inch, 20-gauge needle. Serum was separated by centrifuga-tion, transferred to nonreactive tubes, and held at –80°C until the time of analysis. Optic nerves were harvested from euthanized dogs within 1 hour after death. Perineural tissues were com-pletely removed, and nerves were trimmed such that no optic nerve head, retinal, or scleral tissue was included. Optic nerve tissues were then frozen at –80°C until use.

Western blot technique—Sample handling and western blotting were performed in a manner similar to that described in previous reports. Optic nerves were used to provide an antigen source. Previously harvested optic nerves were minced by hand and then homogenized in ice-cold cell lysis buffer containing protease and phosphatase inhibitors (20mM Tris [pH, 7.5], 150mM NaCl, 1mM EDTA, 1mM ethylene glycol tetraacetic acid, 1mM Na3VO4, 1mM β-glycerophosphate, nonionic surfactant, 200mM Na2VO4, aproteinin [2 mg/mL], and leupeptin [2 mg/mL]). Pooled extracts were centrifuged and separated from the pellet, then refrozen at –80°C until use. Nerves and extracts were kept chilled at all times during handling to minimize proteolysis. The method of Lowry et al was used to determine protein content in the optic nerve extract.
Pooled optic nerve extracts were subjected to 10% SDS-PAGE at 180 V and 500 mA for 1 hour by use of an electrophoresis cell. Nerve extracts were first mixed with 2X SDS loading buffer with β-mercaptoethanol at a 1:2 ratio and boiled for 5 minutes. Extracts were loaded at 25 µg of protein/lane, with high- and low-weight molecular markers included on each gel. After electrophoresis, the gels were blotted onto nitrocellulose membranes at 30 V overnight by use of a wet blotting method. The blots were then blocked for 6 hours with 5% nonfat dry milk in Tris-buffered saline (0.9% NaCl) solution with Tween.

Blots were cut into strips corresponding to lanes. Strips were incubated overnight with serum from the study dogs (diluted 1:100 in blocking buffer) as primary antibody. Strips were then washed multiple times (3 × 10 minutes) with Tris-buffered saline solution with Tween and finally with Tris-buffered saline solution alone. After the washing stage, strips were incubated with secondary antibody (goat anti-dog IgG diluted 1:10,000 in blocking buffer) for 15 minutes. Bands were made visible by reaction with enhanced chemiluminescence reagents followed by exposure to x-ray film. As a negative control sample, 1 strip/blot was incubated with blocking buffer in the absence of serum, then treated with secondary antibody and enhanced chemiluminescence reagents as described previously. As a positive control sample, 1 strip/blot was incubated with anti-myelin basic protein C-16 IgG in blocking buffer as primary antibody (1:200 dilution), then treated with goat anti-human IgG secondary antibody (1:5,000 dilution) and enhanced chemiluminescence reagents.

Developed blots were evaluated for overall number of bands as well as presence and density of individual bands. Corresponding molecular weights were assigned through use of prestained molecular weight standards. Bands were deemed present on the basis of direct visual inspection of blots as well as lane plots generated with imaging software. Bands were deemed to be present when a band was visible on the developed blot and a peak of > 30 U greater than background corresponding to the same molecular weight appeared on the generated lane plot. Band density was determined with imaging software and normalized to the entire area under the curve as in previous studies.

Statistical analysis—Statistical analyses were performed with statistical software. The number of blots per band was compared between healthy dogs and dogs with GDRG via the Wilcoxon test. Differences between groups in the presence or absence of individual bands were assessed via the Fisher exact test. Densitometry data were evaluated for molecular weights for which a band was present in > 25% of dogs in both the GDRG and healthy control groups. Data were evaluated for normality of distribution via the Shapiro-Wilks test. Densitometric values were then compared via the unpaired t test and Wilcoxon test as deemed appropriate. Values of P ≤ 0.05 were considered significant.

Results

Animals—The median age of the 16 dogs with GDRG was 7 years (range, 1 to 12 years). Eleven breeds were represented, the most common of which were American Cocker Spaniel (n = 4) and Basset Hound (3). Five dogs were castrated males, 4 were spayed females, 3 were sexually intact females, and 1 was a sexually intact male. On initial examination, the median IOP in the affected eye was 51 mm Hg (range, 35 to 70 mm Hg). Twelve dogs were blind in the affected eye, whereas 4 retained some degree of retinal and optic nerve function (menace response, dazzle reflex, or indirect pupillary light reflex present). Median duration of clinical signs of GDRG, as reported by owners, was 5 days (range, 1 to 14 days). Examination of the contralateral, unaffected eye (when present) revealed no relevant abnormalities, with the exception of gonioscopic findings and minor changes not likely to be of relevance to the present study (eg, nuclear sclerosis, iris atrophy, and trichiasis). Schirmer tear test values were considered unremarkable (> 15 mm/60 s). Results of clinicopathologic tests were unremarkable for each, providing no evidence of potentially confounding diseases. No evidence of major underlying disease processes was noted on physical examination in any dog.

The median age of the healthy control dogs was 5 years (range, 3 to 9 years). Ten breeds were represented, including 2 each of German Shepherd Dog, English Pointer, Labrador Retriever, and pit bull–type dog. Six dogs were castrated males, 6 were spayed females, 3 were sexually intact females, and 1 was a sexually intact male. Laboratory testing and ophthalmic and physical examinations yielded unremarkable results for all of these dogs.

The median age of the euthanized dogs from which optic nerves were extracted was 5 years (range, 2 to 12 years). Seven were mixed-breed dogs, and 3 were Labrador Retrievers. Each had grossly unremarkable eyes, no history of ocular disease, and no reported history.
of potentially confounding disease. The most common reasons for euthanasia or death included elective euthanasia because of behavioral concerns (n = 3), orthopedic disease (3), and vehicular trauma (2).

**Western blots**—Multiple bands representing serum autoantibodies against optic nerve tissues were evident on western blots from all clinically normal and GDRG-affected dogs (Figure 1). Dogs with GDRG had a median of 9 bands/blot (range, 6 to 15 bands/blot). Clinically normal dogs had a median of 7 bands/blot (range, 4 to 13 bands/blot). The difference between groups with regard to the number of bands per blot was not significant (P = 0.33).

Significant (P ≤ 0.05) differences in band presence were evident at 38, 40, and 68 kDa. An apparent difference between groups was also observed at 43 kDa, but the difference was not significant (P = 0.16). Dogs with GDRG were more likely than clinically normal dogs to have bands at the 38-, 40-, and 68-kDa positions (Figure 2).

Significant differences in band density were found at 40, 48, and 53 kDa, and the difference at 57 kDa neared significance (P = 0.06). The Shapiro-Wilk test detected deviation from a normal distribution only for the 48-kDa band. Given that sample sizes were small, parametric and nonparametric tests were performed to compare groups at each molecular weight. Significance was achieved with the unpaired t test and the Wilcoxon test for the 48- and 53-kDa bands, but differences for the 40- and 57-kDa bands were significant or approached significance only when the unpaired t test was used. Dogs with GDRG had greater autoreactivity (ie, greater band density) than did clinically normal dogs for the 40- and 53-kDa bands and lower autoreactivity than in clinically normal dogs for the 48-kDa band (Figure 3).

**Discussion**

The present study was conducted to investigate the role of immune-mediated mechanisms in GDRG. Identifying at-risk dogs prior to the onset of glaucoma is inherently difficult, and our study population consisted of dogs with overt clinical disease. Thus, our intent was not to determine whether any differences in western blot patterns between healthy dogs and dogs with GDRG suggested a role for autoantibodies in the initial pathogenesis of disease; were the result of early, transient, subclinical increases in IOP that damages ocular tissues, alters ocular immune privilege, and subsequently contributes to the onset of clinically evident disease; or were the consequence of long-standing glaucomatous changes within the eye of longer duration. Longitudinal follow-up studies may provide more information regarding the temporal relationship between autoantibody changes and onset of glaucoma.

The described western blot technique was effectively used to identify significant differences in electrophoretic patterns of serum autoantibodies against optic nerve antigens between dogs with GDRG and clinically normal dogs. A high degree of variability between dogs within groups as well as considerable overlap between groups in band numbers and individual band presence and density suggests that this technique would not likely have promise as a diagnostic tool in the clinical setting. The observed differences provide additional support for consideration of immune-mediated mechanisms in the pathogenesis of GDRG in dogs, however, and should serve as a stimulus for future research. In particular, the relationship between changes in autoantibodies and stage of disease requires further investigation, given that the glaucomatous dogs in the present study all had advanced disease.

Although our results established a connection between GDRG and changes in autoantibodies against op-
tic nerve antigens, these results do not allow any conclusions to be made as to whether immune-mediated mechanisms are involved in the initial pathogenesis of disease or are a consequence of established disease that may in turn accelerate or intensify the disease process. Glaucoma, particularly in veterinary patients, has historically been considered a disease of high IOP. However, so-called normal-tension glaucoma, in which characteristic optic nerve and visual field changes occur in the absence of a documented increase in IOP, is well characterized in people. Conversely, ocular hypertension without glaucoma, in which IOP is high but optic nerve changes and visual field loss are absent, can also develop in people. Existence of these 2 disease subtypes serves as a reminder that glaucoma is not purely an aqueous outflow problem, but rather a disease affecting the optic nerve and retinal ganglion cells. High IOP and aqueous outflow obstruction are risk factors for development of disease in people, not absolute requirements. In dogs, normotensive glaucoma has not been documented and high IOP appears to lead consistently to vision loss. However, dogs with goniodysgenesis typically do not develop high IOP or glaucoma until midlife, and many dogs with goniodysgenesis never develop glaucoma. Therefore, congenital malformations are not likely to be the sole factor in development of hypertensive glaucoma in dogs. This realization has prompted the search for additional pathophysiologic mechanisms underlying onset of the disease.

Inflammatory and autoimmune mechanisms may play a role in the development of glaucoma. Glaucomatous globes are commonly removed in veterinary practice a role in the development of glaucoma. In the glaucomatous dogs of the present study, like the studies in humans, a normal-tension form of glaucoma also exists. The present study is the first to provide a comprehensive description of western blot–derived autoantibody profiles in dogs with GDRG.

The western blot technique used in our study allows for high-throughput screening for autoantibodies, but it is also necessarily a retrospective technique. The glaucomatous dogs of the present study, like the human subjects of previous studies, were evaluated for changes in autoantibody patterns after the onset of clinically evident disease. Therefore, it was not possible to determine whether the autoantibodies identified had been up- or downregulated because they played a role in disease development or because their expression had been altered as a response to glaucomatous damage (ie, whether the changes were cause or effect). Even if not a part of the initial pathogenesis of glaucoma, changes in autoreactivity are still of interest because they may
serve as an amplifying step, leading to worsening of disease and eventual vision loss. Longitudinal studies involving dogs of at-risk breeds or colonies of purpose-bred dogs with an identified predisposition to glaucoma could potentially allow correlation of autoantibody changes with stage of disease. In particular, tracking dogs with goniodysgenesis over time would provide information regarding the relationship of autoantibody changes to stage of disease.

Glaucoma in dogs is often considered as a single disease process in the clinical setting. When enrolling dogs for the study, we attempted to make our inclusion criteria fairly strict to avoid confounding effects from medications or other disease processes. Only dogs with gonioscopic or histopathologic evidence of goniodysgenesis in the contralateral eye were included. Dogs with glaucoma evaluated during the enrollment period that had unremarkable gonioscopic findings (open angles with unremarkable pectinate ligament morphology) in the contralateral eye were deliberately excluded. However, greater stratification of the study group may yield more significant results, as it has in humans. Comparison of glaucomatous dogs with unaffected dogs within single breeds or, ideally, within single lineages may further our understanding of glaucoma as a diverse group of diseases with shared clinical signs. Future identification of genomic risk factors for canine glaucoma may also allow for greater subtyping of disease and could provide another means for identifying at-risk dogs to follow over time with the western blot technique.

In analogous human studies, \textsuperscript{8,12} electrophoretic bands identified through western blotting have subsequently been analyzed via mass spectrometry, permitting identification of specific autoantigens. Identification of autoantigens was outside the scope of the present study but may provide a basis for future research. Some of the electrophoretic bands identified in our study did potentially align with autoantigens identified in human studies. The 68-kDa band that was more prevalent in the glaucomatous dogs may correspond with heat shock protein-70, autoantibodies against which are greater in glaucomatous dogs than in clinically normal dogs.\textsuperscript{15} The 40-kDa band, which was more likely to be present in dogs with glaucoma than in clinically normal dogs and which, when present, had significantly greater autoreactivity in dogs with glaucoma, may likewise represent annexin V or one of the proteoglycan mieties, which have similar molecular weights and reportedly autoreactivity in dogs with SARDS and control dogs. In that study,\textsuperscript{61} negative control strips, treated similarly to those in the Gilmour et al\textsuperscript{60} study and the present study, also had bands at 50 kDa. This band was found to represent the IgG heavy chain present in the retinal extract. Both canine serum and the purified anti-dog IgG secondary antibody were reactive against this IgG fragment, thereby explaining the appearance of the band on the negative control strips.\textsuperscript{61} Finally, Braus et al\textsuperscript{62} identified a band at 47 kDa that was present in serum from dogs with SARDS but not in serum from clinically normal dogs or on negative control strips. This band was subsequently identified as neuron-specific enolase. Other investigators have also found evidence of autoreactivity at or around this molecular weight in clinically normal dogs.\textsuperscript{60}

Although optic nerve tissue rather than retinal tissue was used as the antigen source in the present study and our study population differed, the range of findings at or near this molecular weight in past SARDS studies suggests that some additional caution is required in interpreting our results. Like Gilmour et al\textsuperscript{60} and Braus et al,\textsuperscript{62} we did not detect any bands on our negative control strips, which were also incubated with goat anti-dog IgG. Therefore, this band is unlikely to represent IgG present in our antigen source. Moreover, although we also found the 48-kDa band to be present in serum from all dogs with glaucoma and all clinically normal dogs, a significant difference in band density was evident between the 2 groups, with glaucomatous dogs having less autoreactivity at this molecular weight. The identity of the autoantigen represented by the 48-kDa band is uncertain. Nevertheless, we believe that the changes identified in autoreactivity are a relevant finding.

Overall, we believe that our results provide a basis for further investigations into the role of autoimmunity in the development or progression of canine GDRG. Future work may include following at-risk dogs over time, determining changes within more specific subpopulations of glaucomatous dogs, and evaluating autoreactivity against retinal or trabecular meshwork proteins. Such work may help establish a timeline of events in the pathogenesis of glaucoma and may provide a basis for future screening tools and therapeutic interventions.

\begin{itemize}
\item a. Triton X-100, Sigma, St Louis, Mo.
\item b. Criterion Cell, Bio-Rad, Hercules, Calif.
\item c. Bio-Rad, Hercules, Calif.
\item d. Criterion Blotter, Bio-Rad, Hercules, Calif.
\item e. Goat anti-dog IgG, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
\item f. Anti-myelin basic protein C-16 IgG, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
\item g. Goat anti-human IgG, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
\end{itemize}

References

chamber angle of normal eyes and patients with primary open angle glaucoma and exfoliation glaucoma. Mol Vis 2007;13:408–417.