Depletion of taurine and glutamate from damaged photoreceptors in the retinas of dogs with primary glaucoma

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Objective—To determine whether taurine and glutamate contents are reduced in damaged photoreceptors in dogs with primary glaucoma (PG) in a manner consistent with an ischemia-like release of both of these amino acids from damaged cells.

Sample Population—Retinas from 6 dogs with PG and 3 control dogs.

Procedure—Serial, semithin sections of each canine retina were stained with toluidine blue to identify damaged photoreceptors or via immunogold techniques to quantify taurine and glutamate content in retinal cells.

Results—Regions with a thin outer nuclear layer and pathologic nuclear changes in photoreceptors were evident in retinas of dogs with PG. The density of immunostaining for taurine in damaged photoreceptors was significantly reduced to (mean ± SEM) 37.5 ± 2.6% of the density in adjacent undamaged photoreceptors. Photoreceptors with decreased taurine immunostaining also had decreased glutamate immunostaining, consistent with ischemia-like release of both of these amino acids from damaged cells. Immunostaining for glutamate, but not taurine, was increased in presumptive radial glial cells (ie, Müller cells) in damaged regions, consistent with an ischemia-induced redistribution of amino acids in dogs with PG.

Conclusions and Clinical Relevance—Retinal damage in dogs with PG includes ischemia-like losses of taurine and glutamate from photoreceptors and accumulation of glutamate, but not taurine, in nearby Müller cells. These changes are consistent with glutamate release and depletion of intracellular taurine in damaged regions, perhaps contributing to progressive damage in these areas. (*Am J Vet Res* 2005;66:791–799)

Glaucoma is a group of diseases in which there is a progressive loss of retinal ganglion cells. Some types of severe glaucoma may also result in the death of cells of the **inner nuclear layer (INL)** and **outer nuclear layer (ONL)**, in addition to ganglion cells. Death of INL cells and photoreceptors has been reported in quail with hereditary glaucoma,[1] mice with hereditary glaucoma,[2,3] humans with secondary angle-closure glaucoma,[4,5] and perhaps in humans with primary open-angle glaucoma.[6] There is a loss of INL and ONL cells in dogs with **primary glaucoma (PG)**,[7-9] which is a type of narrow- or closed-angle glaucoma.[10]

Several pathologic mechanisms may contribute to glaucomatous damage, including ischemia,[11-14] a lack of neurotrophic factors,[15] and increased production of nitric oxide.[16,17] There may be altered blood flow with several types of glaucoma,[11,12] perhaps leading to ischemia. Altered retinal blood flow has been reported[18] in dogs with hereditary open-angle glaucoma, but to our knowledge, such changes have not been reported in dogs with PG. Ischemia releases glutamate and taurine from retinal cells.[19] Ischemic release of glutamate may contribute to the high vitreal concentrations of glutamate reported for several types of glaucoma, including dogs with PG,[20] quail with hereditary angle-closure glaucoma,[1] and primates with glaucoma.[21] However, such high vitreal concentrations of glutamate are not seen for all types of glaucoma.[22]

Increased extracellular concentrations of glutamate are selectively toxic to cells of the inner retinal layers, including ganglion cells and cells of the INL.[23,24] Photoreceptors of the ONL appear to be less vulnerable to glutamate-induced cell death, compared with vulnerability for cells of the INL and ganglion cells.[24,25] Chronically high concentrations of glutamate in the diet may eventually lead to death of photoreceptors in rats.[26]

Hypoxia or ischemia leads to redistributions of glutamate and taurine that may be detected by use of immunohistochemical techniques.[27-29] Both of these amino acids are reduced in neuronal cell bodies and dendrites during ischemia.[29] Glutamate, but not taurine, may also accumulate in glial cells,[29] including radial glial cells (ie, Müller cells),[28] during ischemia.

In another study[9] conducted by our research group, we found that glutamate is redistributed in dogs with PG in a manner consistent with ischemia, including a loss of glutamate from photoreceptors. We hypothesized that in dogs with PG, ischemia induces the release of taurine and glutamate from photoreceptors. Although photoreceptors may be resistant to high extracellular concentrations of glutamate,[24,25] presumably because of a low number of ionotrophic glutamate receptors,[30] a depletion of taurine from photoreceptors as a result of excessive release may contribute to additional damage and death of these cells. Analysis of evidence suggests that low intracellular concentrations of taurine lead to the death of photoreceptors. Taurine-

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deficient diets in cats,[31] inhibition of taurine uptake in rats,[32] and disruption of the gene coding for the taurine transporter in mice[33] also result in the death of photoreceptors.

Assuming that ischemia-induced depletion of taurine contributes substantially to the death of photoreceptors during glaucoma, then ischemia-induced redistributions of taurine and glutamate would be expected in damaged regions of retinas in dogs with PG. We used immunogold-staining techniques to determine whether taurine and glutamate concentrations were decreased in damaged photoreceptors and whether glutamate was increased in nearby Müller cells of dogs with PG.

Materials and Methods

Sample population—Six retinas from dogs with PG and 3 retinas from control dogs were used in the study. The 6 retinas from dogs with PG were used in another study[9] conducted by our research group in which we evaluated retinal damage in 25 retinas of dogs with PG and found evidence of glutamate redistribution. That study was conducted by use of several immunohistochemical methods. We selected 6 of those retinas that had signs of focal damage to the ONL for postembedment immunogold staining to detect colocalization of glutamate and taurine in adjacent serial sections of the ONL.

Collection of retinas—Retinas from enucleated eyes of dogs with naturally developing glaucoma were obtained from several veterinary ophthalmologists in private clinical practice and ophthalmologists at the Colorado State University Veterinary Teaching Hospital. Retinas of control dogs were obtained from dogs euthanatized for unrelated studies and used in adherence with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. For control dogs, an ophthalmic examination (slit-lamp biomicroscopy, binocular indirect ophthalmoscopy, and applanation tonometry) was performed; all control eyes were found to be normal on the basis of this examination, and the dogs were then euthanatized.

Tissue preparation—All eyes were rapidly enucleated, and the dorsotemporal quadrant of each globe, including the area centralis, was harvested.[9] Several samples from this quadrant of each eye were immediately placed in a solution of 0.3% glutaraldehyde-4% paraformaldehyde in PBS solution (0.05M phosphate; pH, 7.4); samples remained in this solution until they were processed. Samples were embedded in epoxy resin by use of a procedure described elsewhere.[34]

Staining of sections—Serial, semithin sections (0.5 µm in thickness) were obtained from each control and glaucomatous retina. Sections were mounted on glass slides. To detect pathologic signs of damage, including thin layers and nuclear changes, some sections were stained with toluidine blue for 5 minutes at 40°C.

Postembedment immunogold quantifications of glutamate and taurine contents were performed in adjacent sections of damaged retinal cells. Adjacent sections were immunohistochemically stained for glutamate or taurine by use of silver-intensified immunogold techniques (essentially the methods described to localize amino acids in ischemic retinas of rats in another study[28]). Sections were etched by incubation for 7.5 minutes in sodium ethoxide diluted 1:5 in ethanol, rinsed in ethanol, and then rinsed in water. Sections were blocked by incubation with 5% goat serum in PBS solution (pH, 7.4) for 15 minutes, followed by a rinse in PBS solution. Sections were then incubated in primary antibodies for 16 hours at 23°C, followed by a rinse in PBS solution. Monoclonal antibody Tau-1 (culture supernatants in dilutions [1:10 to 1:50] of PBS solution) was used to localize taurine.[35] Antisera to glutamate[a] diluted 1:2,000 were used to localize glutamate. Similar results were also obtained by use of another specific antisera to glutamate.[9,b] Sections were then rinsed in PBS solution and incubated for 3 hours at 23°C in ultrasmall, gold-labeled secondary antibodies[c] in dilutions (1:5 to 1:20) of PBS solution containing 1% bovine serum albumin. Sections were then rinsed in PBS solution for 45 minutes, followed by final, brief rinses in distilled water. Silver intensification was performed by incubation for 6 minutes in a freshly prepared solution (8.4mM silver nitrate, 0.05mM hydroquinone, and 0.15M citrate buffer [pH, 4.85]). Absorption control specimens in which the antibodies were previously incubated with glutamate or taurine conjugated to bovine serum albumin revealed almost no staining of sections (data not shown).

Image analysis—Digital images were captured by use of a microscope[d] with special software.[e] For quantification of staining density, images were analyzed by use of an image-analysis program.[f] For measurements of staining density, the brightness of images was adjusted on the basis of undamaged ONL cells. To allow for quantitative comparison of immunostaining densities among images obtained at various times and possibly differing light intensities, staining densities of

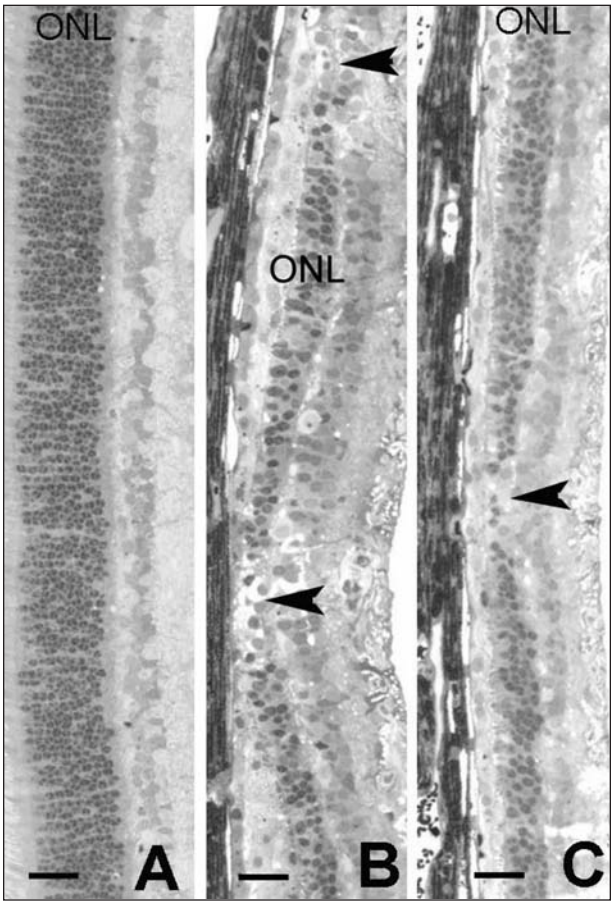

Figure 1—Photomicrographs of retinas from a control dog (A) and 2 dogs with primary glaucoma (PG; B and C). Notice that the outer nuclear layer (ONL) in the control retina is relatively uniform in thickness, whereas thin regions of the ONL (arrowheads) are common in glaucomatous retinas. Toluidine blue stain; bar = 50 µm.

ONL cells were adjusted (gray scale) to a value of 100 and the background staining of regions of blank plastic was set at a value of 0.

Statistical analysis—Data were analyzed to detect significant differences by use of *t* tests and an ANOVA. Statistical software[§] was used for the calculations. Variability was expressed as the SEM unless otherwise specified.

Results

Damage to ONL cells in dogs with PG—Regional thinning of the ONL was commonly seen in glaucomatous retinas, presumably because of the death of photoreceptors (**Figure 1**). Regions of the retina with a thin ONL often also had a thin INL, but the INL appeared relatively unaffected in some thin regions of the ONL. The number of ganglion cells was greatly reduced in all glaucomatous retinas. In many regions, there was an increase in the number of ONL cells that had nuclear changes suggestive of cell damage, especially in or near thin regions of the ONL. These changes included dark, homogeneous staining of nuclei with toluidine blue and a smaller nuclear size (**Figure 2**). Other ONL nuclei had abnormally light staining or were not visible in sections stained by use of toluidine blue.

Immunostaining for taurine in damaged ONL cells of glaucomatous retinas—Photoreceptors of control retinas contained the highest amounts of taurine immunoreactivity (**Figure 3**). High amounts of taurine immunoreactivity were also evident in some cells of the

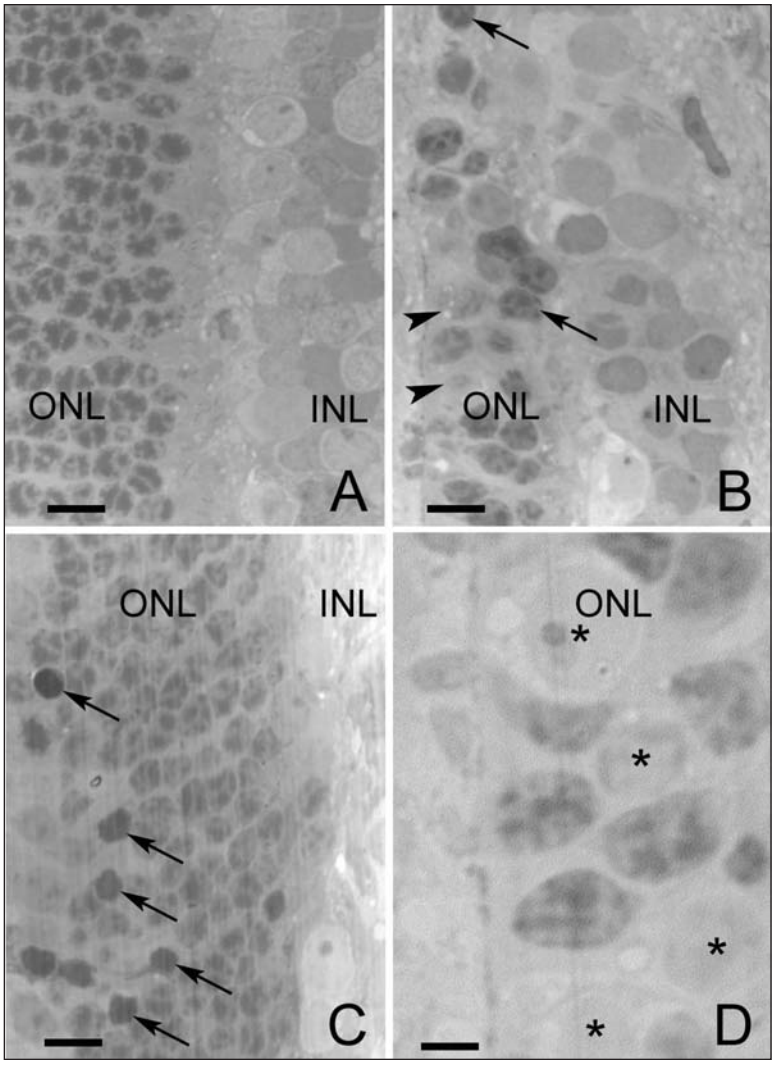

Figure 2—Photomicrographs of retinas from a control dog (A) and dogs with PG (B through D). Notice acute cell damage in the ONL of glaucomatous retinas. The ONL and inner nuclear layer (INL) in the control retina and the density of staining of nuclei in the ONL is fairly homogeneous (A), whereas the morphologic characteristics of ONL cells are often altered in thin regions of a glaucomatous retina (B). In PG retinas, the morphology of ONL cells is often altered in thin regions, with some nuclei stained more lightly than typical (arrowheads), whereas others are stained more heavily (arrows; B and C). In some regions of PG retinas with no obvious thinning of the ONL, cells are found to have signs of acute damage including darkly stained nuclei (arrows). In the higher magnification of thin ONL, abnormally lightly stained nuclei are apparent (asterisks; D). Toluidine blue stain; bar for A, B, and C = 20 μm and for D = 3 μm.

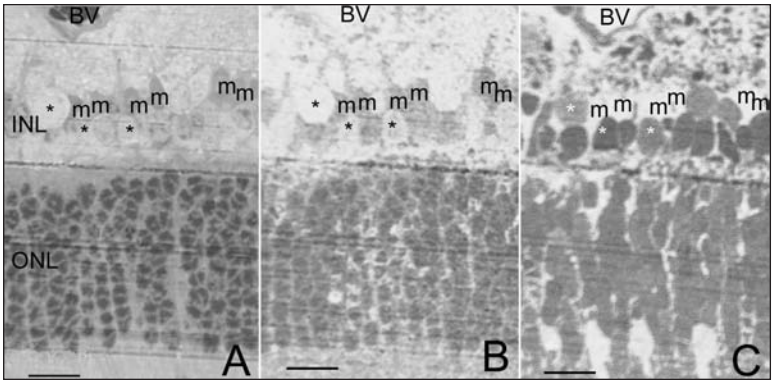

Figure 3—Photomicrographs of retinas from a control dog immunohistochemically stained for taurine and glutamate. A—In the INL, staining with toluidine blue reveals that putative radial glial cells (ie, Müller cells [m]) had more densely stained nuclei than nearby putative neurons. Notice the blood vessel (BV) and horizontal striations created during sectioning that aid in aligning adjacent sections. B—A semithin section of retina obtained adjacent to that for panel A and immunohistochemically stained for taurine reveals that many putative Müller cells had moderate amounts of taurine immunoreactivity, but many other putative neurons had lower amounts of taurine (dark asterisks). Notice that cells of the ONL contain relatively homogeneous high amounts of taurine. C—Another semithin section of retina obtained adjacent to that for panel A and immunohistochemically stained for glutamate reveals that putative Müller cells generally had extremely low amounts of glutamate immunoreactivity but that many putative neurons contain high glutamate immunoreactivity (white asterisks). Bar = 20 μm.

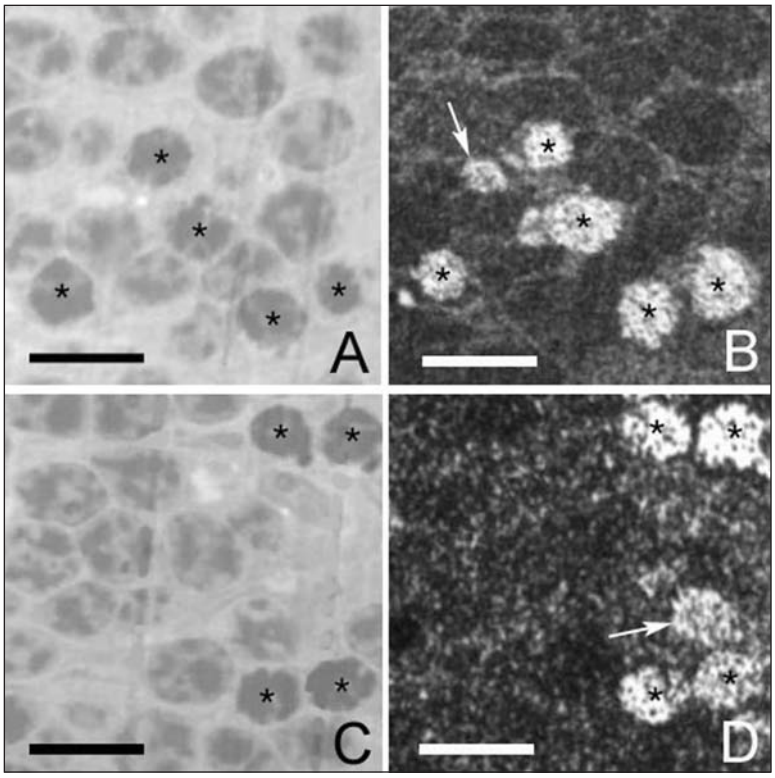

Figure 4—Photomicrographs of regions of damaged cells of the ONL in glaucomatous retinas. Two regions of glaucomatous retina stained with toluidine blue contain damaged ONL cells with darkly stained nuclei (asterisk; A and C). Two sections obtained adjacent to those in panels A and C were stained with immunogold to reveal taurine immunoreactivity in damaged cells (B and D). Notice the cells with a low amount of taurine immunoreactivity that do not have visible nuclei in the corresonding toluidine blue-stained section (white arrows). Bar = 3 μm.

INL, including putative Müller cells. Immunostaining for taurine was relatively homogeneous in control ONL cells (SEM of 3.8 for immunostaining density for taurine). Cells of the ONL that had a large decrease in the amount of taurine immunoreactivity were infrequent (generally < 1% of the cell bodies of the ONL). Cells of

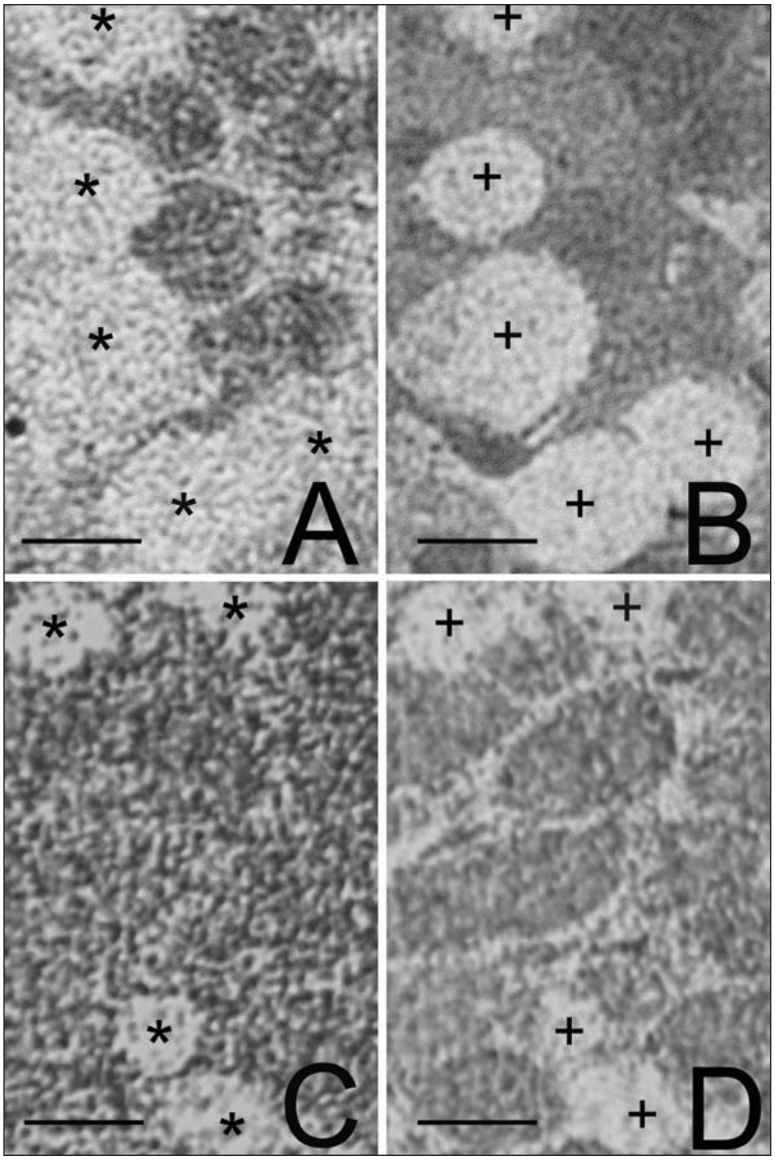

Figure 5—Photomicrographs of glaucomatous retinas stained with immunogold to reveal several ONL cells with a decrease in taurine immunoreactivity (asterisks; A and C) and adjacent sections revealing a decrease in glutamate immunoreactivity (pluses; B and D). Notice that glutamate immunoreactivity was decreased in cells of the ONL that had decreased taurine immunoreactivity. Bar = 3 μm.

the INL had a more heterogenous pattern of taurine immunoreactivity. Compared with the amount of taurine immunoreactivity in cells of the ONL, some cells of the INL had extremely low amounts of taurine immunoreactivity (mean ± SEM gray scale, 28.7 ± 2.0), whereas others had high amounts of taurine immunoreactivity (mean gray scale, 64.3 ± 2.7). These included putative Müller cells that also contained extremely low amounts of glutamate.

Taurine immunoreactivity was greatly reduced in damaged ONL cells of glaucomatous retinas (**Figure 4**). Of 30 ONL cells with abnormally dark nuclei in regions with multiple dark nuclei, only 1 did not have an obvious reduction in the density of taurine immunostaining in adjacent semithin sections. A decrease in taurine immunostaining was also seen in ONL cells that had abnormally light staining of nuclei and cell bodies with nuclei that were not visible in adjacent sections stained with toluidine blue. Mean density of immunostaining for taurine in ONL cells that had damaged nuclei was 37.5 ± 2.6, which differed significantly ($P < 0.001$; t test) in comparison to values for adjacent ONL cells with no nuclear changes (100 ± 3.6; n = 50 cells). In most regions of glaucomatous retinas without obvious ONL damage, density of taurine immunostaining remained homogenously high, which was similar to the density of taurine immunostaining for control retinas (data not shown).

Glutamate immunoreactivity in ONL cells with decreased taurine immunoreactivity—In glaucomatous retinas, glutamate immunoreactivity was decreased in ONL cells that had a decrease in taurine immunoreactivity (**Figure 5**). Amounts of glutamate

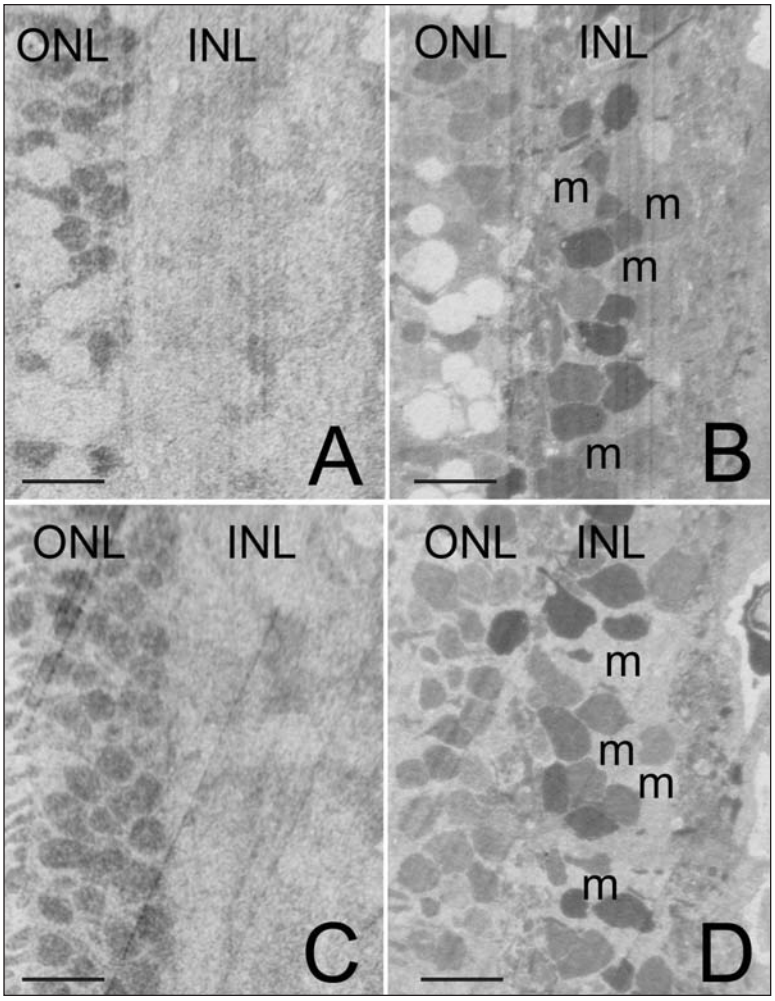

Figure 6—Photomicrographs of retinas obtained from dogs with PG that were immunohistochemically stained to reveal changes in glutamate and taurine immunoreactivity in Müller cells (m). Regions of glaucomatous retinas were subjected to immunogold staining for taurine (A and C) or glutamate (B and D). In panel A, notice that decreased taurine immunoreactivity in cells of the ONL did not have decreased taurine immunoreactivity in Müller cells, compared with staining for control retinas, whereas in panel B, putative Müller cells had an increase in glutamate concentration, compared with concentrations in control retinas. Panel C is a less-damaged region of glaucomatous retina that reveals taurine immunoreactivity was not depleted in cells of the ONL or Müller cells. Panel D is a section of retina adjacent to that of panel C that reveals putative Müller cells had a significant ($P < 0.05$) increase in glutamate immunoreactivity, compared with that for control retinas. Bar = 20 μm.

immunoreactivity were compared among ONL cells that had varying taurine immunoreactivity. Glutamate content in these cells was highly correlated to the amount of taurine immunoreactivity ($r = 0.89$; $P = 0.01$ [n = 60 cells]), which suggested that mechanisms of depletion of these 2 amino acids may be related.

Glutamate and taurine immunoreactivity in Müller cells of damaged regions—Putative Müller cells were examined for evidence of redistribution of amino acids in damaged regions of glaucomatous retinas. In damaged regions of the INL, glutamate immunoreactivity, but not taurine immunoreactivity, increased in cells that had morphologic characteristics consistent with Müller cells (**Figure 6**). Glutamate immunoreactivity in Müller cells increased significantly ($P = 0.01$; *t* test) in regions of glaucomatous retinas that had a loss of amino acids from ONL cells, compared with immunoreactivity for control retinas (gray scale for control retinas, 21 ± 0.8; gray scale for damaged regions, 77.9 ± 6.6 [n = 12 cells from 2 regions]). Müller cells accumulating glutamate in these severely damaged regions did not have significantly increased amounts of taurine immunoreactivity (72.4 ± 1.8 [17 cells from 2 regions]), compared with taurine immunoreactivity for control retinas (70.5 ± 2.5). Some regions that did not have an obvious loss of amino acids from ONL cells still had accumulations of glutamate in Müller cells that differed significantly, compared with glutamate immunoreactivity for control retinas.

Discussion

Studies have documented that ganglion cells, INL cells, and photoreceptors die in dogs with PG. Those studies found regions with thin layers,[7-9] increased **terminal deoxynucleotidyl transferase–mediated uridine triphosphate nick-end labeling (TUNEL)** staining,[8,9] and morphologic changes suggestive of necrosis and apoptosis.[8,9] In the study reported here, a thin ONL and ONL cells with signs of damaged nuclei were used as signs of photoreceptor damage in glaucomatous retinas to determine whether there was ischemia-like redistribution of taurine or glutamate (or both) in damaged regions (Figures 1 and 2).

Multiple mechanisms have been proposed to contribute to the pathogenesis of glaucoma, including ischemia[11-14] and a lack of transport of neurotrophic factors from postsynaptic cells of the brain via axons of the optic nerve.[15] Many of the changes seen in the retina of dogs with PG are consistent with an ischemic mechanism of damage. Ischemia causes necrosis and apoptosis in retinal cells as indicated by TUNEL-positive staining,[36] abnormal staining of nuclei,[37,38] and a delayed loss of photoreceptors.[39,40] Those reported ischemic changes are similar to changes seen in the study of dogs with PG reported here and are consistent with other reports[8,9] of necrosis and TUNEL staining of several types of retinal cells, including ONL cells in dogs with PG.

Decreased amounts of taurine and glutamate in damaged photoreceptors in dogs with PG are consistent with the concept that ischemia releases glutamate and taurine from cells.[19,41,42] In the study reported here, almost all photoreceptors with nuclear damage had a decrease in immunostaining for taurine and glutamate, which is consistent with ischemia-induced changes in these cells (Figures 4 and 5). There is probably variation in the susceptibility of retinal regions to damage during glaucoma, as suggested by focal regions of thin ONL and the apparent clustering of damaged ONL cells in affected retinas of dogs with PG (Figures 1 and 2).

Several potential mechanisms may cause the release of taurine and glutamate from damaged ONL cells. Assuming that there was ischemia during PG, the induced depletion of ATP may decrease ion gradients, especially sodium gradients, and lead to the release of glutamate[27] and taurine by sodium-dependent cotransporters.[41] Another possible mechanism that may contribute to the release of amino acids is the opening of volume-regulated anion channels that allow the movement of ions and amino acids across the membranes of cells to prevent swelling.[43,44] It is unlikely that the decreased intracellular amounts of glutamate and taurine are attributable to a complete loss of membrane integrity in most of the damaged cells. Many of the damaged cells with decreased immunostaining still retained intracellular amounts of glutamate and taurine in the range of several millimolars, which is several fold higher than the vitreal concentrations reported for taurine and glutamate in dogs.[20]

The accumulation of glutamate, but not taurine, in Müller cells in damaged regions of the retina of dogs with PG is also consistent with ischemia in damaged regions. Glutamate accumulates in glial cells during ischemia in the brain[29] and retina,[28] whereas there is little change in taurine concentrations in glial cells during ischemic insults.[29] In another study[9] conducted by our research group, we reported that glutamate may increase approximately 4-fold in putative Müller cells in some damaged regions of PG retinas. In the study reported here, we quantified taurine immunoreactivity in putative Müller cells with increased glutamate concentrations in damaged regions. Similar to reports[45,46] in other species, taurine was typically found at highest concentrations in photoreceptors of dogs with PG, with low concentrations in some INL neurons and intermediate concentrations in Müller cells. Significant increases in taurine immunoreactivity were not detected in those putative Müller cells that had accumulated glutamate, which is consistent with ischemic-induced changes in glutamate and taurine distribution (Figure 6).

A decrease in taurine concentrations in damaged photoreceptors may contribute to additional damage in these cells. The amount of taurine immunoreactivity in damaged photoreceptors was reduced to approximately 38% of that for adjacent cells that had normal morphologic characteristics. Staining density depends on the logarithm of the antigen concentration,[47] suggesting that the concentration of taurine may have been reduced in these photoreceptors to approximately 23% of that for control retinas. Several lines of evidence suggest that low intracellular concentrations of taurine lead to the death of photoreceptors. Taurine-deficient diets in animals unable to synthesize sufficient amounts of taurine lead to photoreceptor damage and death in cats[31] and possibly rhesus monkeys.[48] Inhibition of taurine uptake may also lead to damage and death of photoreceptors. Guanidinoethyl sulfonate, an inhibitor of taurine uptake, results in a decrease in retinal concentrations of taurine and death of photoreceptors in rats.[32] Mice with a disrupted gene that codes for the taurine transporter also have a severe loss of photoreceptors.[33] In mice lacking the taurine transporter[33] and in dogs with PG,[8,9] TUNEL-positive cells are seen in the ONL, which is consistent with an apoptotic mechanism of cell death in both conditions.

A growing body of evidence suggests that there is a decrease in blood flow during several types of glaucoma.[11-14] We examined whether there were ischemia-like changes in glutamate and taurine distribution in damaged photoreceptors in dogs with PG. Primary glaucoma leads to extremely rapid, severe damage in retinas of affected dogs that involves essentially all layers of the neuroretina.[8,9] Severe damage may be evident within a few days of the time at which signs are first noticed in affected dogs.[8] Mechanisms of pathogenicity that contribute to this severe type of glaucoma may differ from mechanisms that contribute to other types of glaucoma that have a less rapid onset. It remains to be determined whether similar ischemia-like changes in glutamate and taurine distribution are also evident in other types of glaucoma, especially those types with a less rapid onset.

Photoreceptors may be lost in dogs with PG,[7-9] quail with hereditary angle-closure glaucoma,[1] mice

with hereditary glaucoma,[2,3] and possibly in humans with certain types of glaucoma.[4,6] In the study reported here, we found that photoreceptor damage in dogs with PG was associated with a loss of taurine and glutamate from damaged photoreceptors. Assuming that taurine depletion contributes substantially to photoreceptor loss in some types of glaucoma, therapeutic approaches to reduce this depletion may improve the efficacy of treatment. The loss of glutamate from damaged photoreceptors may also lead to high extracellular concentrations of glutamate that would contribute to additional damage and death of retinal cells, especially in INL cells and ganglion cells that are more vulnerable to glutamate-induced cell death.[24,25]

a. Rabbit anti-glutamate, Sigma Chemical Co, St Louis, Mo.
b. AB5018, Chemicon International, Temecula, Calif.
c. Aurion, Electron Microscopy Sciences, Washington, Pa.
d. Zeis Axioplan 2, Carl Zeiss Microimaging Inc, Thornwood, NY.
e. Axiovision 3.1, Carl Zeiss Microimaging Inc, Thornwood, NY.
f. ImageJ 1.28, National Institutes of Health, Bethesda, Md.
g. StatWorks, Heyden & Son, Philadelphia, Pa.

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