Loss of glutamine synthetase immunoreactivity from the retina in canine primary glaucoma

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Abstract

Purpose Changes in retinal glutamate distribution occur in primary glaucoma (PG) in dogs. Although the redistribution resembles that induced by ischemia, decreases in glutamine synthetase (GS) activity may also induce a similar glutamate redistribution. We examined the distribution of GS, glutamate, and glial fibrillary acidic protein (GFAP), a marker for reactive glia, in PG retinas by immunohistochemistry to determine whether decreases in GS and formation of reactive glia are associated with glutamate redistribution and neuronal damage.

Animals Sections from 14 control dog eyes and 22 eyes from dogs with PG.

Methods Sections from 14 control and 22 glaucomatous globes were immunohistochemically stained for GS, glial fibrillary acidic protein or glutamate.

Results In semiquantitative immunogold studies, decreases in GS staining density were strongly correlated with glutamate redistribution and neuronal damage. In less quantitative immunoperoxidase staining of acute (≤5 days after clinical signs) and chronic PG retinas, GS immunoreactivity was decreased in focal regions of some acute PG retinas, and there were widespread decreases in chronic PG retinas. GFAP immunoreactivity was increased in Müller cells primarily in severely damaged regions of chronic PG retinas.

Conclusions Decreases in GS immunoreactivity were associated with glutamate redistribution. These decreases in GS occurred even in mildly damaged regions of retina before retinal thinning. Reactive Müller cells were seen primarily in chronic PG in severely damaged regions. Decreases in GS may potentiate ischemia-induced early glutamate redistribution and neuronal damage in canine PG.

Key Words: glaucoma, glial fibrillary acidic protein, gliosis, glutamate, glutamine synthetase, Müller cells

INTRODUCTION

Glaucoma is characterized by a progressive loss of retinal ganglion cells that is often associated with increased intraocular pressure1,2 and altered blood flow.3 PG in dogs is a very severe retinal disease that often damages all layers of the neuroretina.4,5 We have previously found focal regions with redistributions of glutamate in canine PG that are consistent with ischemia.5,6 This redistribution includes increased concentrations of glutamate in Müller cells and decreased concentrations in neurons. We have recently found that microvessel losses are associated with glutamate redistribution and neuronal damage in PG.7 These results are consistent with microvessel losses leading to local ischemia, glutamate release, and neuronal damage. However, glutamate redistribution and neuronal damage were seen near the remaining microvessels in PG, suggesting that microvessel loss was not the only factor leading to glutamate redistribution and neuronal damage.

Another way in which glutamate may redistribute in retinal cells is by altered glutamate metabolism. Glutamine synthetase (GS) is a major enzyme involved in the metabolism of glutamate in glial cells. GS catalyzes the amidation of glutamate to glutamine, which is an essential part of the cycling of the transmitter pool of glutamate between neurons and glia. Decreased GS activity will lead to increased levels of glutamate in glial cells and decreased levels of glutamate in neurons in a manner similar to the redistribution of glutamate seen in PG in dogs. Decreased GS activity has been seen in glaucoma in rats.8 Decreased GS activity has also been reported after hypoxia in the brain9 and some of the
glutamate redistribution in ischemia has been attributed to decreased GS activity.\textsuperscript{10} GS requires ATP to synthesize glutamate from glutamate, and ischemia-induced ATP depletion inhibits GS activity. In retinal detachment with presumptive hypoxia, the redistribution of glutamate and other amino acids is suggestive of decreased GS activity.\textsuperscript{11} In addition to loss of GS enzymatic activity in hypoxia/ischemia, loss of GS immunoreactivity has also been reported after ischemia in the retina.\textsuperscript{12}

Decreased GS activity may contribute to higher extracellular glutamate levels and subsequent neuronal damage. Decreased GS activity may lead to an accumulation of glutamate in glial cells. Models of glutamate transport by GLAST, the main glutamate transporter in the retina,\textsuperscript{13} suggest an increase in the intracellular level of glutamate in glial cells may lead to a proportionate increase in the minimum extracellular level of glutamate.\textsuperscript{14,15}

Reactive Müller cells are induced in different types of retinal diseases, including some types of glaucoma.\textsuperscript{16,17} The reactive glial cells alter their cytoskeleton, thus allowing their detection by immunohistochemical staining for glial fibrillary acidic protein (GFAP) and other cytoskeletal components. Other changes that occur in reactive glia include altered expression of enzymes. Thus one possible mechanism leading to changes in the expression of GS would be reactive changes in the glial cells.

We examined the immunohistochemical distribution of GS in the retinas of dogs with PG to determine whether decreases in the amount of GS occur in PG and whether the decreases are associated with glutamate redistribution and neuronal damage.

METHODS

Acquisition and processing of eyes

Retinal samples were of two types: (1) plastic-embedded retinas which were acquired for immunohistochemical studies of glutamate distribution, and (2) archived, paraffin-embedded retinas (acquired by RRD), which were used for studies of the effects of chronicity of glaucoma and retinal position on GS and GFAP immunoreactivity. The retinas that were plastic embedded were from dogs with PG undergoing enucleation as part of treatment. Control plastic-embedded retinas were obtained from dogs euthanized at a local Humane Society. A complete ophthalmic examination (slit-lamp biomicroscopy, binocular indirect ophthalmoscopy, and applanation tonometry) was performed prior to euthanasia and found to be normal in each control dog. Eyes were obtained with adherence to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research with approval from the Animal Care and Use Committee of Colorado State University.

The 10 plastic-embedded glaucomatous retinas were acquired from several veterinary ophthalmologists and from the Colorado State University Veterinary Medical Center. In the plastic-embedded eyes, no more than two globes were obtained from any single breed. The 12 paraffin-embedded glaucomatous samples were obtained from eight Cocker Spaniels, three Basset Hounds, and one Dachshund. The duration between presentation of the dogs to ophthalmologists for clinical signs and subsequent enucleation was known for the archived specimens. Retinas acquired ≤ 5 days after onset of clinical signs were considered acute, while retinas acquired > 5 days after onset of signs were considered chronic.

Fixation and sectioning

Plastic-embedded specimens were fixed as previously described.\textsuperscript{5} Eyes were rapidly dissected and the dorso-temporal quadrant of the globe was then placed in 0.3% glutaraldehyde-4% paraformaldehyde in 0.05 M of phosphate buffered saline (PBS). Quarters were then processed and embedded in epon as previously described.\textsuperscript{6} Paraffin-embedded retinas were fixed by immersion in either 4% buffered formalin or Bouin's fixative/ethanol, without penetration of the globe. The samples were embedded in paraffin and sagittally sectioned at 5-µm thickness.

Staining of paraffin sections

Paraffin sections from PG and control dog eyes were stained for GS using an avidin-biotin method. In brief, sections were dewaxed in xylene and graded ethanol. Sections underwent antigen retrieval by autoclaving for 15 min in a 0.1-M citrate buffer, pH 6.0. Afterwards, endogenous peroxidase was inactivated in 3% peroxide for 15 min, and sections were incubated in primary mouse monoclonal antibody to GS (1 : 100 in PBS) (Chemicon International, Temecula, CA, USA) overnight. A Vectastain elite kit for mouse antisera (Vector Laboratories, Burlingame, CA, USA) was then used to visualize the primary antibody using peroxidase with diaminobenzidine as chromogen. All slides were dehydrated through graded ethanol, cleared in xylene, and coverslipped. Controls for specificity included omission of the primary antibody.

Staining of plastic-embedded eyes

Postembedding immunogold and toluidine blue staining were performed on adjacent, 0.5-µm sections using the same methods we have previously employed.\textsuperscript{6,7} Serial semi-thin sections (0.5 µm) of retinas embedded in Epon were made using an ultramicrotome. Some sections were stained for 20 min at 60 °C with 1% toluidine blue in 1% sodium borate buffer. Other, near-adjacent sections were etched and immunogold stained for glutamate or GS using a method for silver-intensified immunogold labeling. Sections mounted on gel-coated slides were etched with sodium ethoxide diluted 1 : 5 in ethanol for 20 min, rinsed in ethanol and water, and blocked with 5% normal goat serum diluted in 0.05 M PBS. Sections were rinsed in PBS and incubated overnight in 1 : 400 dilutions of rabbit antisera to glutamate (Sigma-Aldrich, St Louis, MO, USA) or 1 : 100 dilutions of mouse antibody to GS (Chemicon International) in PBS. After rinsing in
PBS, sections were incubated for 4 h in secondary gold-labeled (ultrasmall particles) anti-rabbit or anti-mouse antisera (Electron Microscopy Sciences, Hatfield, PA, USA) diluted in PBS (1:20). Sections were rinsed for 30 min in PBS and then two rapid changes of water. Staining was silver enhanced for 6 min, rinsed in water, and the slides coverslipped for image capture. Controls for specificity included omission of the primary antibody.

**Image capture and analysis**

Digital images were captured using a Zeiss Axioplan 2 microscope with Axiovision 3.1 software (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). For measurements of staining density, images were analyzed using ImageJ 1.34 (National Institutes of Health, Bethesda, MD, USA). After inversion, background densities of immunostaining of blank plastic or paraffin were subtracted from each image.

For measurements of glutamate immunogold densities in inner nuclear layer (INL) regions, the average densities in 15-×15-μm INL regions in plastic sections were quantified. Three INL regions were measured in each image. For measurements of glutamate immunostaining densities in Müller cells, the densities in three putative Müller cells per region were measured. In a similar manner measurements of GS immunogold densities in plastic sections were made in three putative Müller cell bodies in each region.

For measurement of GS-staining densities in paraffin sections, average densities were measured in 60-×60-μm boxes in the inner retina. For measurements of GFAP-staining densities, densities were measured in 80-μm lengths of retina from the outer limiting membrane through the INL. Three regions from central and peripheral areas of each eye were measured in each captured image for both GS and GFAP.

**Assessment of damage**

Regions of INL in glaucomatous retinas were classified as mildly damaged or severely damaged by the estimated percentage of damaged cells and the thickness of the INL in the region in plastic sections. Plastic sections were stained with toluidine blue to evaluate damage. Mildly damaged regions contained ≤10% damaged INL cells and an INL thickness of ≥2 cells. Severely damaged regions showed thinning of the INL to < 2 cells in thickness. Damaged INL cells were defined as those with a dark, condensed nucleus and/or swollen, pale cytoplasm. Regions with highly variable thickness of INL or variable percentages of damaged cells were not included in the study unless at least 70% of the region fitted one of the categories.

**Statistical analysis**

Immunohistochemical staining densities obtained from sections were analyzed for statistical significance using either t-tests or ANOVA followed by Tukey’s multiple comparison tests using GraphPad Instat 3 software (GraphPad Software, San Diego, CA, USA).

**RESULTS**

**GS immunoreactivity was decreased in regions with neuronal damage and glutamate redistribution**

We have previously found that a redistribution of glutamate occurs in PG, characterized by a loss of glutamate from neurons and an accumulation of glutamate in glial cells.5-7 The average level of glutamate immunostaining in the INL decreases as the severity of damage increases,7 because of loss of glutamate from many of the INL neurons.

Regions of PG retinas with different degrees of damage and glutamate redistribution were evaluated for GS immunostaining in plastic sections. Damage to INL cells in PG may progress from mild damage — where a normal number of cells are still present but some (≤10%) have clumping of chromatin and pale, swollen cytoplasm — to severe damage, where the loss of cells results in thinning of the INL. To determine whether the severity of damage affected the levels of GS immunoreactivity, damage to INL regions was classified as mild or severe. Cells were classified as damaged if they had dark nuclei with condensed chromatin, or pale, swollen cytoplasm.

Immunogold staining for GS and glutamate was performed on adjacent 0.5-μm plastic sections of control and PG retinas. Control retinas had high levels of GS immunoreactivity in Müller cells and their processes, with low levels of glutamate in the same Müller cells and processes (Fig. 1a,e). Mildly damaged regions that maintained high levels of glutamate had slightly reduced levels of GS (Fig. 1b,f), while mildly damaged regions showing glutamate redistribution had low levels of GS immunoreactivity (Fig. 1c,g). In regions with severe damage, little GS immunoreactivity remained in Müller cells while almost all INL neurons had low levels of glutamate (Fig. 1d,h).

The density of GS immunostaining was measured in Müller cell bodies in regions with different degrees of damage (Fig. 2). In mildly damaged regions of retina where glutamate immunoreactivity was not reduced in 15-×15-μm regions of INL (mild, high glutamate), the immunostaining for GS was significantly reduced. In neighboring areas where INL neurons contained significantly lower levels of glutamate immunoreactivity (mild, low glutamate), GS immunoreactivity showed a further reduction. The density of GS immunostaining decreased as the severity of damage increased and glutamate decreased in INL neurons (Fig. 2).

To further examine the relationship between glutamate and GS immunoreactivity, we plotted the average densities of glutamate immunostaining in the INL (predominantly neurons) against the densities of GS immunostaining in Müller cells. There was a loss of glutamate immunoreactivity in the neurons of the INL as GS immunoreactivity decreased in damaged regions. As shown in Fig. 3(a), the average density of glutamate immunostaining was strongly correlated with the density of GS immunostaining ($R^2 = 0.88$, linear fit).

In contrast to neurons, the levels of glutamate immunoreactivity increased in glial cells as GS decreased in damaged
regions (Fig. 3b; \( R^2 = -0.82 \), linear fit). In severely damaged regions, Müller cells had very low levels of immunoreactivity for glutamate and GS (Fig. 1d,h) and were difficult to distinguish from neurons, so average values for the region were used as estimates of staining density in Müller cells in these regions.

**Effects of chronicity of glaucoma and location within the retina on GS immunoreactivity**

Sagittal sections of archived, paraffin-embedded eyes were immunohistochemically stained for GS using a less quantitative avidin-biotin immunoperoxidase method to allow evaluation of the effects of position within the retina and chronicity of glaucoma on GS immunoreactivity. Control retinas had substantial GS immunoreactivity in Müller cells and astrocytes (Fig. 4). No control retinas had regions with markedly decreased immunostaining for GS except in areas with obvious sectioning or staining artifacts. Two of six acute (≤ 5 days since clinical detection) PG retinas showed distinct patches of retina with decreased GS immunoreactivity (Fig. 4b). Five of six chronic PG retinas (> 5 days since clinical detection) appeared to have relatively homogeneous lighter immunostaining for GS than control retinas (Fig. 4c).

Although the avidin-biotin peroxidase method of immunohistochemical staining is generally considered less quantitative than the immunogold method, measurements of GS...
staining density were performed to confirm our visual impressions. Central and peripheral regions of control retinas did not have significant differences in GS staining density. Mean staining density in peripheral regions was 76.2 ± 8.4 (gray scale) and in central regions the mean staining density was 78.8 ± 7.8 (gray scale) (n = 18; P > 0.4 by t-test). The lack of variation in GS staining intensity in these different locations in control retinas suggests that the focal decreases in immunoreactivity seen in acute PG retinas were unlikely to be due to normal regional differences in GS levels within the retina.

Chronic PG retinas had significantly less GS immunoreactivity than control retinas in central regions (Fig. 5a; P < 0.01). Acute retinas did not have significantly less GS immunoreactivity in central regions than control retinas (P > 0.05), but distinct patches of central retina containing low levels of GS immunoreactivity were evident in 2/6 acute PG retinas (Fig. 4b). Regions of acute PG retina that appeared to be less intensely stained on visual inspection did indeed have significantly lower levels of GS immunoreactivity (Fig. 5b), while adjacent regions were found to have control levels of GS immunoreactivity.

Figure 2. GS and neuronal glutamate immunostaining densities were dependent on the severity of damage. GS staining densities in Müller cells and average glutamate staining densities in the INL (primarily neurons) were measured in regions with different severities of damage. In mildly damaged regions, staining densities were measured in areas with high levels of glutamate immunostaining (Mild, High Glu) and areas with low levels of glutamate immunostaining (Mild, Low Glu). Significant decreases in GS immunostaining densities compared to controls were seen in mildly damaged regions and in severely damaged regions (P < 0.05 by Tukey’s multiple comparison test). Bars = SEM; n ≥ 18 for all PG values and n = 6 for control values.

Figure 3. GS immunostaining density was strongly correlated with glutamate immunostaining densities in neurons and Müller cells. (a) The average immunostaining densities for glutamate in the INL were plotted against GS immunostaining densities (data from Fig. 2). There was a strong correlation (R² = 0.88) between decreases in GS immunoreactivity and decreases in glutamate immunoreactivity in INL regions. The decreases in the average INL levels of glutamate reflect primarily the glutamate loss that occurs in neurons in PG. (b) Glutamate immunostaining in Müller cells increased as GS immunostaining decreased. There was a strong negative correlation (R² = −0.82) between decreases in GS immunoreactivity and increases in glutamate immunoreactivity in Müller cell bodies. Bars = SEM; n ≥ 18 for all PG values and n = 6 for control values.

Figure 4. Immunostaining for GS in paraffin sections of archived eyes was similar to plastic sections. (a) Avidin-biotin peroxidase immunostaining of control sections revealed strong GS immunoreactivity in Müller cells and astrocytes homogeneously distributed throughout the length of the retinas. (b) Some acute (≤ 5 days from clinical detection) PG retinas had focal regions with reduced immunostaining for GS. The edge of a region of reduced staining is indicated by arrowheads. (c) Chronic PG retinas had reduced immunostaining for GS distributed in a more homogeneous manner. All images are in the same orientation and at the same magnification. ONL, Scale bar = 30 µm.
Reactive Müller cells may occur in PG

GFAP was expressed in Müller cells of PG retinas. Müller cells did not normally have detectable GFAP immunoreactivity in control sections (Fig. 6a). GFAP immunoreactivity was normally seen in presumptive astrocytes of dog retina, found primarily in the inner layers of control retinas. The expression of GFAP by Müller cells has been used as an indicator of reactivity. GFAP was detectable in Müller cell bodies and processes in the outer nuclear layer primarily in chronic PG (≥ 5 days from clinical detection). Six of six chronic PG retinas from paraffin-embedded eyes had substantial GFAP immunoreactivity in a radial pattern consistent with Müller cells in a large portion of the retinas. In general the GFAP immunoreactivity was seen in most regions of the chronic

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PG retinas, but varied somewhat in intensity and seemed more intense in thinned regions of retina. In contrast, 2/6 acute PG retinas (≤ 5 days from clinical detection) had substantial GFAP in Müller cells, but only in a limited number of regions. High levels of GFAP were usually limited to regions where the inner nuclear layer (INL) was thinned (< 2 cells thick). Measurement of GFAP staining density in 80-µm lengths of retina from INL through the outer limiting membrane confirmed that average GFAP immunoreactivity was increased in the central regions of chronic PG retinas (P < 0.001) but not in the central regions of acute PG retinas (Fig. 6d). No significant differences were seen between GFAP densities in central and peripheral regions of control retinas (P > 0.3; data not shown), confirming that there were no substantial regional differences in GFAP in normal retinas.

DISCUSSION

GS may be decreased in glial cells beginning in early PG

GS immunoreactivity was decreased in PG retinas (Figs. 2, 3). We speculate that the decrease in immunoreactivity may reflect a decreased quantity of functional GS in these regions, resulting in decreased GS enzymatic activity. The decrease in GS immunoreactivity may be due to decreased synthesis or increased degradation/modification. The rate of GS synthesis may be altered by exposure to numerous substances or conditions such as high extracellular glutamate, ischemia, pigment epithelium-derived growth factor, and glucorticoids. GS is also very vulnerable to modification by oxidation and nitrination. Modification of GS by these processes may lead to decreased enzymatic activity and perhaps to decreased immunoreactivity. Oxidative stress and nitrination by peroxynitrite derived from NO may be expected in PG, as these processes are increased in retinal ischemia and in glaucoma.

The decreases in GS immunoreactivity may begin in the early stages of PG. Decreased levels of GS immunoreactivity were seen in some regions of acute PG retinas in paraffin sections (Fig. 5b). In more quantitative immunogold studies, significant decreases in GS immunoreactivity began in mildly damaged regions of INL and further decreases occurred in the more severely damaged regions (Fig. 2).

Reductions in GS may contribute to glutamate redistribution

We have seen a decrease in GS immunoreactivity in PG that is strongly correlated with a redistribution of glutamate in the same regions (Fig. 3). We speculate that this decrease in immunoreactivity may reflect a decrease in functional GS in these regions.

GS is a major enzyme involved in the metabolism of glutamate in glial cells. GS catalyzes the amidation of glutamate to glutamine, which is an essential part of the cycling of the transmitter pool of glutamate between neurons and glia. Decreased GS activity may then account for at least some of the increased levels of glutamate in glial cells and decreased levels of glutamate in neurons seen in PG in dogs.

Loss of GS and ischemia may act in a cooperative manner to increase extracellular glutamate levels and neuronal damage

In the current studies we observed decreased GS immunoreactivity in PG retinas that may reflect decreased GS function.

We have also reported microvessel losses and vascular damage that may lead to ischemia in canine PG. These two processes may act cooperatively to allow glutamate to accumulate in the extracellular fluid. Decreases in functional GS protein may result in glutamate accumulation in glial cells. These increases in glial glutamate will raise the minimum extracellular level of glutamate reached by transporters. Ischemia reduces cellular ATP levels. This reduction in ATP will have several effects on glial cells, including a further reduction in GS activity, and also decreases in the ion gradients that drive glutamate transporters. If severe enough, the ATP depletion-induced changes in ion gradients will then reverse the glutamate transporters and release glutamate into the extracellular fluid, leading to excitotoxic damage to neurons.

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