Ceruloplasmin Upregulation in Retina of Murine and Human Glaucomatous Eyes

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Purpose. Ceruloplasmin (*Cp*) expression is increased locally as a response to many neurodegenerative conditions. The purposes of this study were to confirm findings of Cp upregulation in glaucoma, detect the time course of this upregulation in a glaucoma model, and better localize its expression in the retina.

Methods. mRNA and protein were extracted from the retina and brain of DBA/2 and C57BL/6 mice and were subjected to analysis by RT-PCR and immunoblotting. In addition, eyes from the same mouse strains were subjected to immunohistochemistry using antibodies specific for Cp. Eyes from human subjects with or without glaucoma were also subjected to immunohistochemical analysis for Cp.

Results. Cp mRNA and Cp protein were upregulated in the retinas of glaucomatous DBA/2 mice. Upregulation of Cp occurred at approximately the time of extensive retinal ganglion cell (RGC) death and increased with increasing age to 15 months in the retinas but not in the brains of these animals. No age-related Cp upregulation was detected in the reference normal mouse strain (C57BL/6), which can develop significant nonglaucomatous RGC loss toward the end of the same time frame. Cp upregulation was also detected in most eyes from the patients with glaucoma. Cp upregulation was localized to the Müller cells within the retinas and in the area of the inner limiting membrane.

Conclusions. Cp is upregulated in the retina of a commonly used glaucoma model (the DBA/2 mouse) and in most human glaucomatous eyes. The timing of this upregulation suggests that it may represent a reactive change of the retina in response to a noxious stimulus or to RGC death. Such Cp upregulation may represent a protective mechanism within the retina. (*Invest Ophthalmol Vis Sci.* 2007;48:727–732) DOI:10.1167/iovs.06-0497

Ceruloplasmin (Cp) is an α-2 glycoprotein involved in the transportation of copper in the bloodstream, and it is mainly synthesized in the liver.[1] In addition, Cp is present in the central nervous system (CNS), where it is also synthesized locally.[2–4] In astrocytes, Cp expression can be induced in vitro by interleukin (IL)-1β and tumor necrosis factor (TNF)-α.[5] Although Cp is known as a soluble protein, a membrane-bound form has been recently identified on glial cells that is almost identical to the circulating Cp and that has heme oxidase activity.[6,7]

Although the ferroxidase activity of Cp has been well known for the past 35 years,[8] its precise role in brain iron metabolism is less clear. It appears to be involved in iron efflux (as originally proposed) and uptake in brain cells.[9] The conversion of Fe^{2+} to Fe^{3+} allows iron to bind to apotransferrin, thus forming transferrin, the principal iron transporter across cell membranes. It has been proposed that this dual role of Cp (in both influx and efflux of iron) allows it to function as an iron detoxifier in the CNS that becomes especially important under conditions of acute iron level changes or limited oxygen.[9] When there is a congenital absence of Cp, iron accumulates in the brain and retina, leading to neurologic defects and retinal degeneration.[10,11] Damage to neurons from the accumulated iron is thought o be caused by oxidative stress and free radical formation.[12] The generation of a *Cp* gene–deficient mouse ($Cp^{-/-}$)[13] has allowed further insight in the role of Cp in the CNS. These animals, though healthy at birth, show impaired motor coordination, decreased ability to cope with oxidative stress, and retinal degeneration in advanced age.[13]

Changes in the levels of brain Cp have been detected in patients with Alzheimer disease.[4,14–16] Cp increases in the areas that undergo neuronal degeneration are mainly localized to astrocytic processes and the neuropil rather than to neurons themselves, and it is suggested that the increases are inappropriately low relative to the iron accumulation that occurs in this disease.[15]

In the retina, *Cp* expression has been reported in humans[3] and in mice.[2] It appears to localize in Müller cell bodies in the inner nuclear layer (INL), and in astrocytes in the retinal ganglion layer. Although murine *Cp* has one major RNA transcript in most tissues, a second less abundant transcript is present in the eye.[2] Cp expression has been shown to increase in the RGC and inner nuclear layers after optic nerve crush[17] and pressure-induced retinal ischemia (Levin and Johns, unpublished data[17]) and in Müller cells of streptozotocin-induced diabetes in rats.[18] Here again, a protective role for Cp has been suggested, though some reports indicate that Cp can also have toxic effects by enhancing iron/cysteine-induced DNA damage[19] and LDL oxidation.[20]

Increases in *Cp* expression and immunoreactivity in the retina in primate experimental glaucoma and in human glaucoma have been recently reported by two independent groups.[21,22] We attempted to confirm these findings of Cp upregulation in glaucoma and to gain further insight into the role of Cp upregulation in the pathophysiology of the disease by studying its time course, detecting the upregulated gene, and better localizing its expression in the retina.

Methods

Animals and Tissues

DBA/2J and C57BL/6 mice ranging from 3 to 18 months of age were used in this study. Mice were kept under a 12-hour light/12-hour dark

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cycle and fed ad libitum. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care Committee. Experimentation on human specimens was approved by the local institutional review board.

Mice were anesthetized with a mixture of ketamine/xylazine/acepromazine (10.8/1.2/54 mg/kg), and blood was obtained transcardially under anesthesia before humane killing. Mice were then perfused with a solution containing heparin (1000 U/mL) in normal saline to remove the remaining blood, and the eyes and brain were immediately removed. The retina was isolated and stored in tissue storage agent (RNAlater; Ambion, Austin, TX) at −20°C, as was the brain. Blood serum collected was also stored at −80°C after centrifugation at 1000 rpm for 5 minutes in the presence of a cocktail of protease inhibitors (Roche, Indianapolis, IN).

Mice used for immunohistochemistry were initially perfused with the solution described to remove blood and then with 4% ice-cold paraformaldehyde in phosphate-buffered saline (PBS). Eyes were enucleated and further treated in the same fixative for 24 to 72 hours before further processing.

Archival material from patients with glaucoma ($N = 6$) who underwent enucleation was also studied. Eyes obtained postmortem from patients without significant ocular history ($N = 6$) served as controls. Specimens were included in the experimental group if the clinical history indicated the presence of glaucoma (any type) or if histologic examination revealed the presence of typical glaucomatous optic neuropathy. Specimens were excluded if the clinical history or histologic analysis indicated the presence of vitreous hemorrhage or active inflammation. Specimens had been fixed by immersion fixation in 10% buffered formalin at the time of enucleation and embedded in paraffin. Clinical data of the human eye samples studied are summarized in Table 1. Because information on the specimens was obtained through pathology records (and not through the patient records), information on treatment and IOP control was incomplete; however, all eyes in this category showed histologic evidence of advanced glaucomatous neuropathy.

Reverse Transcription–Polymerase Chain Reaction

Total RNA and protein were extracted using the TRIzol protocol (Gibco-BRL, Grand Island, NY). Tissue was homogenized in TRIzol, mixed with chloroform, and centrifuged, and the aqueous and the nonaqueous phases were separated. The nonaqueous phase was subjected to dialysis to recover the proteins, whereas the aqueous phase containing the RNA was further purified (RNeasy Micro Kit; Qiagen, Valencia, CA). The amount of RNA isolated was quantitated spectrophotometrically, and the concentration was adjusted for subsequent reverse transcription with random primers (Superscript First-Strand Synthesis System; Invitrogen, Carlsbad, CA).

Real-time PCR was performed using primers designed based on the sequences of mouse Cp and Cp-GPI mRNA (GenBank NM_007752 for Cp and AK086999 and AK043248 for Cp-GPI). Anticipated product sizes were 157 bp and 158 bp for Cp and Cp-GPI, respectively. Primer sequences were TCCCTGGAACATACCAAACC (common forward primer), ATTTATTTCATTCAGCCAGACTTAG (reverse primer for Cp), and CCAGGTCATCCTGTAACTCTGA (reverse primer for Cp-GPI). To accurately quantitate the amount of Cp and Cp-GPI in the various samples and to account for variations that might have been introduced by variable efficiency of reverse transcription, the product of a housekeeping gene was also amplified from the same cDNA samples in separate reactions. The gene *rps11* (*Mus musculus*, similar to 40S ribosomal protein S11; GenBank XM_193290) encoding for a ribosomal protein, was used for this purpose. Primer sequences used for rps11 amplification were CGTGACGAAGATGAAGATGC (forward) and GCACATTGAATCGCACAGTC (reverse). SYBR Green PCR amplification was performed at three cDNA dilutions (1 ng, 10 ng, 100 ng) for each run, with each dilution in triplicate. Reactions were run at least twice for each mouse strain, age group, tissue, and cDNA level (ABI Prism 7900HT Sequence Detection System [SDS]; Applied Biosystems, Foster City, CA), and results were analyzed (SDS 2.1 software; Applied Biosystems). Normalized relative concentrations of Cp mRNA in the various age groups were compared with analysis of variance (ANOVA) and post hoc Fisher LSD testing.

Immunoblotting

The nonaqueous phase from TRIzol extraction was dialyzed through a 3500-Da membrane (Millipore, Bedford, MA) at 4°C for 48 hours in 0.1% SDS, then centrifuged at 10,000*g* for 10 minutes. The supernatant was stored at −80°C until use. After resuspension in a buffer containing 25 mM Tris (pH 1.7), 1.6% SDS, 8% glycerol, and 0.7 M βME, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Nonspecific binding was blocked with buffer in PBS (SuperBlock Blocking Buffer; Pierce, Rockford, IL). Membranes were then incubated with the primary antibodies anti-Cp (A0031 at 1:1000; DAKO, Carpinteria, CA) and anti–α-actin (sc1616 at 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed with a horseradish peroxidase (HRP) chemiluminescence system (ECL; Amersham, Piscataway, NJ). Luminescence was measured with an automated imager (digital imaging station 440CF; Eastman Kodak, Rochester, NY). Relative optical density of each band was measured (Image Tool software; University of Texas

Table 1. Characteristics of Human Specimens Used in the Cp Immunohistochemistry Experiments*

	Glaucomatous Eyes		Control Eyes		
	(+)	(−)	(+)	(−)	Group Comparison
Number	5	1	0	6	—
Age, mean ± SD (y)	69 ± 10		72 ± 17		$P > 0.7$
Sex					
Male	0	0	0	4	$P > 0.06$
Female	5	1	0	2	
Specimens obtained					
Autopsy	1	0	0	6	$P < 0.02$
Enucleation	4	1	0	6	
Type of glaucoma					
Chronic angle closure	3	1	—		—
Chronic secondary	1	0			
Unspecified	1	0			

* Most glaucomatous eyes were obtained following enucleation for end-stage painful glaucoma. In contrast, all of the control eyes were obtained at autopsy.

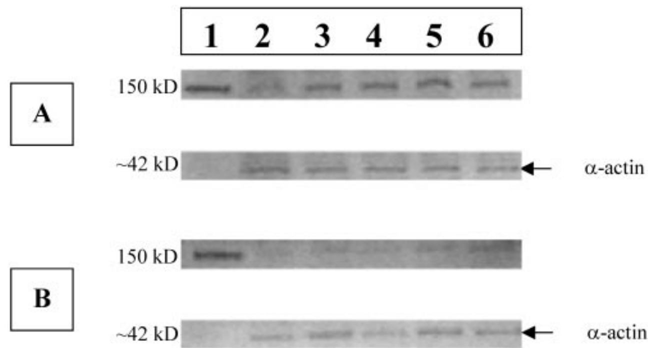

FIGURE 1. Cp and α-actin immunoblotting of DBA/2 retina (**A**) and C57BL/6 (**B**) mice. Tissue pooled from at least five individual eyes (**A**, **B**). Cp immunoreactivity in retina increases with age in DBA/2 animals but remains constant in C57BL/6 mice. *Lane 1*: mouse blood serum (Cp-positive control). *Lane 2*: three-month-old animals. *Lane 3*: six-month-old animals. *Lane 4*: nine-month-old animals. *Lane 5*: twelve-month-old animals. *Lane 6*: fifteen-month-old animals.

Health Science Center, San Antonio, TX). The amount of Cp in each lane was normalized in relation to the amount of the housekeeping protein (α-actin) in the same lane. Normalized concentration graphs were created (at least five membranes analyzed per point). Normalized concentrations were compared using ANOVA with post hoc Fisher least significant difference (LSD) testing. Immunoblots of individual animal tissues and tissues pooled from a number of animals (minimum pool from five eyes per lane) were performed to account for individual eye variability in the amount of RGC loss in the DBA/2 retina.[23]

Positive controls included mouse blood and rat testis lysate (Transduction Laboratories, Lexington, KY; BD Biosciences, San Jose, CA) for Cp and α-actin antibodies. Specificity of the Cp antibody for the intact protein was confirmed by elimination of staining on omission of primary antibody.

Immunohistochemistry

Tissue sections (4–5 μm) were collected on positively charged slides (ESCO; Superfrost Plus Microscope Slides; Erie Scientific, Portsmouth, NH) and dried in an oven for 1 hour at 60°C. Sections were then deparaffinized in xylene and rehydrated in graded alcohols. Tissues were digested with proteinase K (0.03% in distilled water for 20–40 minutes). Testing of the human specimens under various digestion lengths was necessary because of the variable conditions of time to fixation, amount of fixation, and processing of this archival material. Sections were then rinsed three times for 3 minutes each with PBS and were incubated with the primary antibodies (anti-Cp DAKO 1:200, anti-glutamine syntheses, 1:50; Transduction Laboratories) for 2 hours at room temperature in a solution containing 1% BSA in PBS. They were then rinsed three times with PBS and treated with the appropriate Alexa-conjugated secondary antibody (1:400; Molecular Probes, Eugene, OR) for 20 minutes at room temperature. After rinsing, the sections were treated with DAPI (5 μg/mL) and washed, and the slides were coverslipped and observed with a microscope (Axioscope II; Carl Zeiss, Oberkochen, Germany) using appropriate excitation wavelengths and filters. Antibody specificity was verified by immunoblotting. Negative controls were incubated without primary antibodies. Staining of human specimens was considered positive if it was present in at least part of the retina after digestion for either 20 or 40 minutes.

RESULTS

Immunoblotting of retinas from DBA/2 mice of various ages (protein pooled from at least five eyes per lane) revealed increasing amounts of Cp with age starting at approximately 6 months (Fig. 1A). In contrast, such an increase in Cp expression was not seen in the brains of the DBA/2 animals (data not shown) or in the retinas (Figures 1B) and brains of C57BL/6 mice. Time course of changes in the amounts of Cp in the retina and brain of DBA/2 animals and the control C57BL/6 mice are shown in Figure 2. Cp immunoreactivity was constant in DBA/2 brain and C57BL/6 retina and brain (P = 0.59, P = 0.96, and P = 0.97, respectively; ANOVA), whereas it increased in the DBA/2 retina ($P < 0.049$; ANOVA). This increase started after the 6-month point but reached statistical significance at the 15-month point ($P < 0.05$; Fisher post hoc test).

To confirm that the Cp detected on immunoblotting was locally synthesized in the retina (rather than transported there

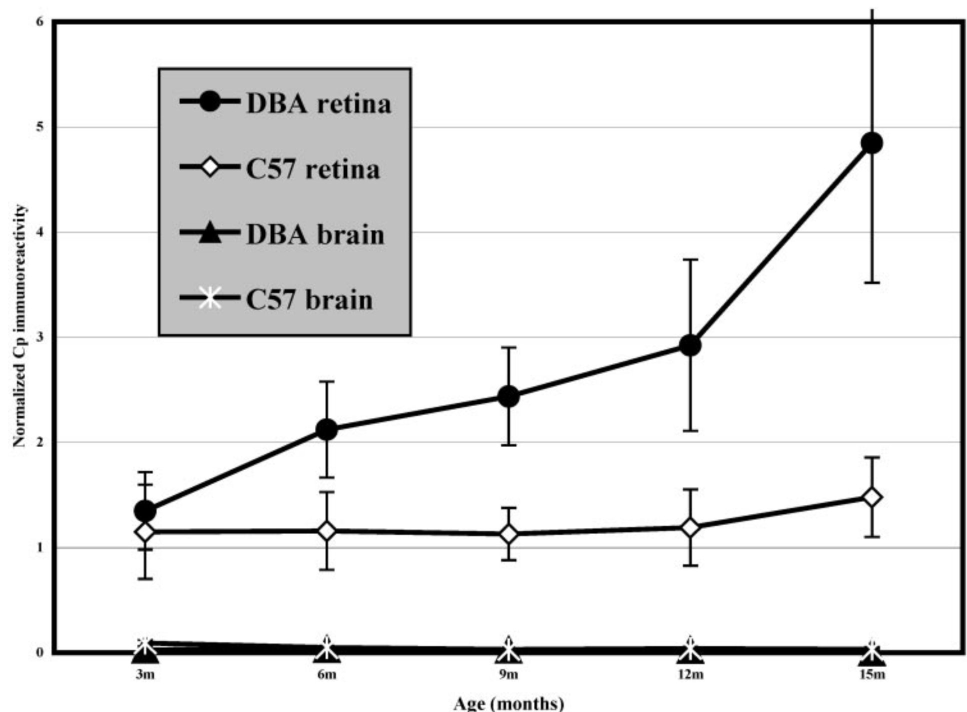

FIGURE 2. Cp immunoreactivity in retina and brains of 3-, 6-, 9-, 12-, and 15-month-old DBA/2 and C57BL/6 mice. Cp staining has been normalized to the staining of α-actin to account for potential differences in the amount of protein loaded in each lane. Notice that Cp staining increases in the retina of the DBA/2 mice after 6 months of age but becomes statistically significant only at 15 months of age. Error bars represent SEM from five different Western blot membranes. Error bars for the brain samples are very small. Each sample contains protein from a pool of at least five eyes from the corresponding age group.

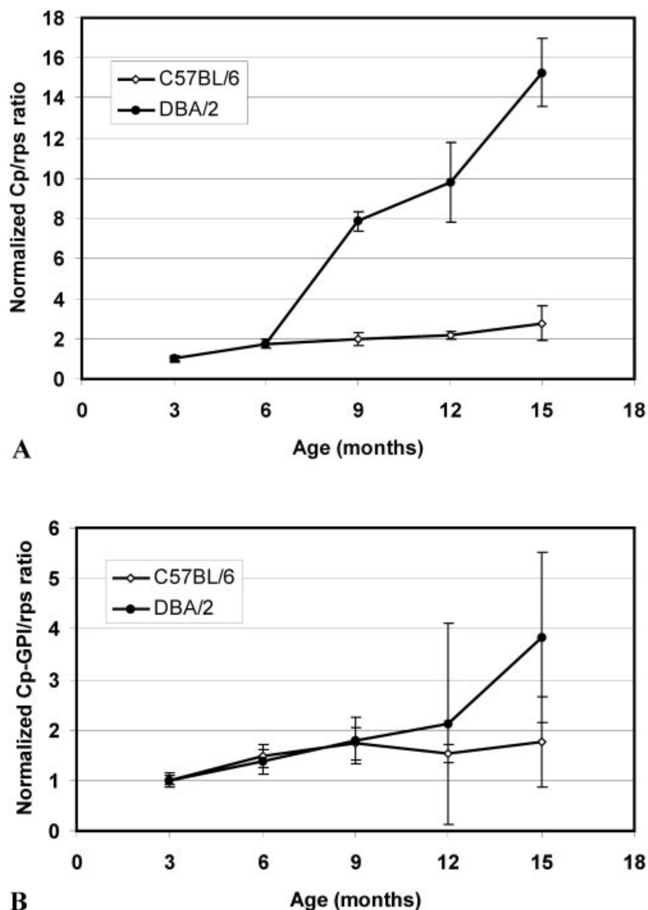

FIGURE 3. Cp (**A**) and Cp-GPI (**B**) mRNA in retina of 3-, 6-, 9-, 12-, and 15-month old DBA/2 and C57BL/6 mice. Cp and Cp-GPI mRNA has been normalized to the amount of the housekeeping ribosomal gene *rps11* and the relative amount of mRNA at 3 months has been set to a value of 1. Notice that Cp mRNA significantly increases in the retina of the DBA/2 mice starting at 9 months of age, whereas Cp-GPI mRNA significantly increases in the retina of the DBA/2 mice starting at 12 months of age. Error bars represent SEM from three different RT-PCR experiments. Each sample contains RNA from a pool of at least five individual eyes from the corresponding age group.

through the bloodstream), real time-PCR was performed on retinal tissue from pooled eyes. Soluble and GPI-anchored *Cp* expression was differentiated with specific primers. As can be seen in Figure 3, significant upregulation of soluble *Cp* expression started after 6 months of age and progressively increased to 15 months of age ($P < 0.0001$; ANOVA, Fisher post hoc test), whereas *Cp* expression did not change in C57BL/6 retinas ($P = 0.16$; ANOVA). *Cp*-GPI expression increased significantly after the DBA/2 animals were 9 months of age ($P < 0.0002$; ANOVA, Fisher post hoc test), but no significant increases in its expression were found in C57BL/6 animals at that time point (Fig. 3).

Retinal immunohistochemistry revealed the presence of Cp in the inner retinas of aged DBA/2 mice, specifically on Müller cells and in the area of the inner limiting membrane (Fig. 4). Cp immunoreactivity mostly colocalized with that of glutamine synthase, a marker for Müller cells (Fig. 4A,D). Young, prepathologic, 3-month-old DBA/2 mice showed no Cp staining of Müller cells or of the inner limiting membrane (Fig. 4B), whereas aged DBA/2 mice showed an increase of staining in these areas (Fig. 4E).

Clinical data on the human specimens stained are presented in Table 1. Staining for Cp was more variable in the glaucomatous human eyes, but most of the glaucomatous eyes (five of six specimens studied) showed some staining of the Müller cells. An example of such staining is seen in Figure 5E. Müller cells were identified by GS immunostaining (Fig. 5D). None of the control (nonglaucomatous) human eyes showed any Cp immunostaining of the Müller cells ($P < 0.02$; χ^2 test) (Fig. 5B).

DISCUSSION

Ceruloplasmin is a protein with multiple roles. Its main site of production in the body is the liver,[24] though many other tissues express it.[25,26] Ceruloplasmin manufactured in the liver enters the bloodstream and carries copper. Although this is an important biological function of this protein, it is by no means the only one. Ceruloplasmin cannot cross the blood-brain and blood-retinal barriers, so it is synthesized locally in the central nervous system and the retina.[2,3] In the central nervous system, Cp is believed to act as a ferro-oxidase converting ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). This conversion is important for the delivery of iron to neurons because ferrous iron is toxic. In contrast, ferric iron binds to transferrin, which associates with specific receptors on the cell surface to deliver iron intracellularly. Similarly, Cp probably converts the ferrous iron presented at the neuron surface to ferric iron for excretion, though the exact mechanisms involved are unclear.[9] In addition, Cp bound to glial cells by a GPI link may participate in the processing of other molecules, such as glypican-1.[27]

The expression of Cp within the retina seems to be principally localized in astrocytes and Müller cells.[2] Glial cells are known to express both the soluble and the GPI-anchored forms of Cp.[7] Our results confirm that both forms are also expressed in the retina.

Cp upregulation, which appears to be part of an injury response, has been reported in retinal detachment,[28] light damage,[28] and experimental glaucoma in rats.[29] Cp expression is reported by microarray and immunohistochemical analyses to be upregulated in primate[21] and human[22] glaucoma, but these findings have not been previously confirmed quantitatively at the protein expression level. Our results also indicate an increase in the amounts of Cp in the retina of aged glaucomatous DBA/2 animals compared with young animals of the same strain.

The increase in *Cp* gene expression in the retina of DBA/2 mice starts after the 6th month of age for the soluble form and after the 9th month for the GPI-linked form of the molecule. Because a significant amount of the Cp in the retina is in the GPI-linked form, the actual amount of protein detected by immunoblotting (both soluble and GPI-linked Cp) does not dramatically increase until after the 12th month of age. A smaller increase in the amounts of Cp detected by immunoblotting is obvious from around the 6th month of age (Fig. 2) but, because of some variability between the DBA/2 animals (indicated by the large SE bars in Fig. 2), does not reach statistical significance. Thus, significant increases in the amounts of Cp occur when large numbers of RGCs have already been lost in the DBA/2J animals. The simultaneous absence of such upregulation in the brains of the same animals indicates that it is specific for the retina. In addition, the increase in *Cp* does not appear to be directly related to aging because C57BL/6 mice (a reference nonpathologic strain) do not show any changes in immunostaining, even at advanced ages.

Soluble Cp upregulation appears to occur at approximately the same time that IOP is increased in the DBA/2 animals and before any significant RGC loss. Simultaneously, other molecules, such as C1q, also become upregulated in the retina,[30] which may mean that the upregulation of soluble *Cp* is a stress

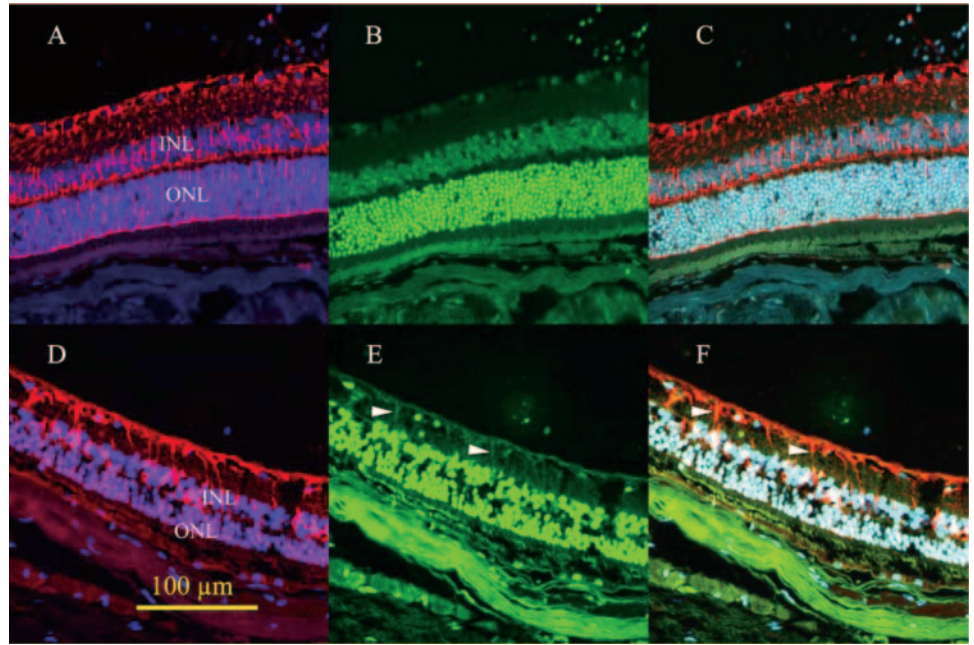

FIGURE 4. Cp (*green*, **B**, **E**) and glutamine synthase (*red*, **A**, **D**) immunostaining in sections from 3-month-old (**A–C**) and 20-month-old (**D–F**) DBA/2 mice. (**C**, **F**) Merged images. Nuclei are stained *blue* with DAPI. Note the labeling of the inner limiting membrane and the Müller end-feet. Müller cells processes are also visible (*arrows*). ONL, outer nuclear layer; INL, inner nuclear layer.

response of this tissue. This is not unexpected because circulating Cp is a well-known stress response protein.[31]

In contrast, *Cp*-GPI upregulation appears to occur later (after the 9th month of age), when significant RGC loss starts in the DBA/2J mice and continues to increase up to at least the 15th month of age. This delayed increase in *Cp*-GPI suggests a connection with the increased oxidative stress imposed on the retina by dying RGCs. The upregulation of the soluble and the GPI-linked form of *Cp* does not result from a generalized upregulation in other tissues of the DBA/2 mice because other CNS tissues do not show this upregulation by immunoblotting.

Taken together our results indicate that upregulation of the soluble form of Cp may be triggered by the IOP stress that leads to RGC loss in the retina of DBA/2 mice, whereas upregulation of the Cp-GPI form may be the result of the RGC loss itself. It is conceivable that the cells responsible for the production of the two forms of the protein are not the same and thus follow a different time course in their response to events occurring within the glaucomatous retina. Unfortunately, we did not have access to an antibody specific for Cp-GPI for the immunohistochemical studies described and thus can only speculate on its location.

The presence of increased immunostaining in the human glaucomatous retina further supports the notion that Cp upregulation detected in murine glaucoma is relevant to glaucoma in general and may play a role in the human disease. Human specimens from patients with longstanding severe glaucoma confirm the involvement of Cp in the pathophysiology of the disease; however, the results from experiments on archival material must be interpreted cautiously and further confirmed by experiments performed with more standardized processing. In addition, the specimens used for this study may not be

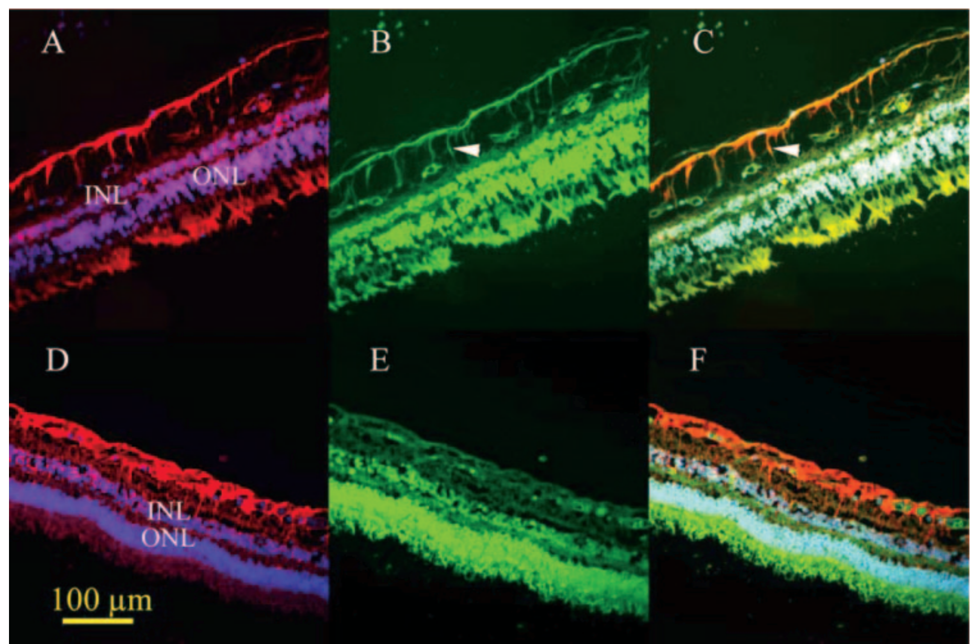

FIGURE 5. Cp (*green*, **B**, **E**) and glutamine synthase (*red*, **A**, **D**) immunostaining in sections from one of the glaucomatous eyes of a patient with longstanding glaucoma (**A–C**) and from a nonglaucomatous control eye (**D–F**). (**C**, **F**) Merged images. Nuclei are stained *blue* with DAPI. Note the labeling of the inner limiting membrane and the Müller end-feet. Müller cells processes are also visible (*arrow*). ONL, outer nuclear layer; INL, inner nuclear layer.

representative of the full range of the disease. Most of the glaucomatous specimens came from females with end-stage glaucoma and were obtained at enucleation, whereas control specimens came mostly from males and were obtained at autopsy.

Localization of Cp staining in the Müller cells by immunohistochemistry in mouse and primate eyes with glaucoma suggests that it is an adaptive mechanism to protect RGCs from excess iron or oxidative stress, as has been previously proposed. Such an explanation would also account for Cp upregulation in other conditions (such as light damage and retinal detachment). Cp is known to be induced in rat astrocytes by IL-1β.[32,33] IL-1β is secreted by microglia in response to injury.[34] An alternative explanation could be that the retinal glial cell stress response caused by high IOP or other unknown factors leads to Cp upregulation. Finally, a third alternative could be that Cp upregulation is a reactive change within the retina in response to the RGC death that occurs with glaucoma.

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