Long-term Suppression of Neurodegeneration in Chronic Experimental Optic Neuritis: Antioxidant Gene Therapy

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PURPOSE. To test in mice with experimental autoimmune encephalomyelitis (EAE) a strategy designed to treat patients at risk for axonal degeneration and persistent visual loss from optic neuritis and multiple sclerosis.

METHODS. The authors cloned the human extracellular superoxide dismutase (ECSOD) or catalase (CAT) gene into recombinant adenoassociated virus (AAV). Transgene expression was evaluated by immunohistochemistry of infected RGC-5 cells and after intravitreal injection of AAV-ECSOD or AAV-CAT, or both, into the right eyes of DBA/1J mice. Control cells and left eyes were inoculated with AAV-GFP. Animals were sensitized for EAE, followed by serial contrast-enhanced MRI for 6 months, and then were euthanatized. The effects of ECSOD and CAT modulation on the EAE optic nerve were gauged by computerized analysis of optic nerve volume, myelin fiber area, axonal cell loss, and retinal ganglion cell (RGC) loss.

RESULTS. Western blot analysis of infected RGC-5 cells revealed that expression of ECSOD increased 15-fold and that of CAT increased 3.5-fold. One month after intraocular injections, transgene expression increased 4-fold for AAV-ECSOD and 3.3-fold for AAV-CAT. Six months after intraocular injections and EAE sensitization, combination therapy with ECSOD and catalase decreased RGC loss by 29%, optic nerve demyelination by 36%, axonal loss by 44%, and cellular infiltration by 34% compared with the contralateral control eyes inoculated with AAV-GFP. Compared with the normal optic nerve, it limited RGC loss to 9%.


Experimental autoimmune encephalomyelitis (EAE) is an autoimmune inflammatory disorder of primary central nervous system demyelination that has been frequently used as an animal model for testing treatments against multiple sclerosis (MS).1–10 The optic nerve is a frequent site of involvement in both EAE and MS.1,11–20 Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite are mediators of demyelination and disruption of the blood-brain barrier (BBB) in EAE.21–27 Cellular defenses against ROS include catalase and superoxide dismutase (SOD). SOD dismutates superoxide to hydrogen peroxide (H2O2), and catalase detoxifies the H2O2 to H2O and O2. Three isozymes of SOD exist. Copper/zinc SOD (SOD1) is primarily cytosolic but has also been found in mitochondria. Manganese SOD (SOD2) is expressed exclusively in mitochondria. Extracellular SOD (ECSOD), as its name implies, is primarily extracellular. ECSOD is a tetramer composed of two disulfide-linked dimers; each subunit of approximately 34 kDa binding copper and zinc has been shown to offer central nervous system (CNS) neuroprotection.28–31 In earlier studies, we targeted hydrogen peroxide for detoxification by catalase gene inoculation.32 It reduced demyelination of the optic nerve by one third, a month after sensitization for acute EAE. By detoxifying superoxide and its metabolite, hydrogen peroxide, using intraocular injections of the catalase gene construct into transgenic mice overexpressing ECSOD, we more than doubled the level of protection against demyelination to 78% in acute EAE.33 Although promising, it is unclear whether these effects against demyelination persist beyond the acute episode of EAE. In both earlier reports, the effects on axonal or ganglion cell loss that result in permanent visual disability were not tested. In this study, we used the chronic EAE animal model to test the long-term persistence of the beneficial effects of catalase, ECSOD, or combined gene inoculations on suppressing demyelination, and, to develop a treatment strategy directed against the neurodegeneration of optic neuritis and MS that contributes to disability, we evaluated those effects on neuronal and axonal loss in the optic nerve.

METHODS

Recombinant Adenoassociated Virus

The AAV vector pTRUF22, containing the 381-bp cytomegalovirus (CMV) immediate early gene enhancer/1352-bp chicken β-actin promoter-exon 1-intron 1/woodchuck postregulatory element (WPRE), linked to GFP through a 637-bp poliovirus internal ribosomal entry site (IRES), was used to accept the human catalase cDNA (CAT) or human ECSOD upstream of the IRES-GFP. The resultant pTRUF22-CAT and pTRUF22-ECSOD plasmids were amplified and then purified by cesium chloride gradient centrifugation and packaged into AAV serotype 2 capsids by transfection into human 293 cells using standard procedures. Genome titers of the recombinant AAV (rAAV) were determined using real-time PCR.34 The AAV-packaged viruses contained 1011 to 1012 genome copies per milliliter.

Cell Culture and Western Blot Analysis of ECSOD and Catalase

Rat retinal ganglion cells (RGC-5) were grown in Dulbecco modified Eagle medium (DMEM; Fisher Scientific, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin streptomycin (Sigma, St. Louis, MO) at 37°C with 5% CO2. Cells grown in 15-cm dishes were infected with AAV-CAT or AAV-ECSOD at multiplic-
ities of infection (MOIs) of 5000. Two days later, cells were trypsinized and harvested. Cells were manually homogenized in cold lysis buffer and stored at -80°C for later analysis.

For Western blot immunodetection, 15 μg protein was separated on a 10% SDS polyacrylamide gel and electrotransferred to a nitrocellulose membrane. We incubated the membrane with polyclonal anti–ECSOD antibodies (Stressgen Bioreagents, Victoria, BC, Canada) or with a monoclonal anti–catalase antibody (Sigma-Aldrich, St. Louis, MO). Anti–mouse β-actin antibody was used as an internal control for protein loading. We used goat anti–rabbit (or anti–mouse) IgG horse-radish peroxidase (HRP)-conjugated secondary antibodies (Sigma), followed by detection of complexes using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ). Specific signals were quantified by densitometry using NIH Image (available by ftp from zippy.nimh.nih.gov or from http://rsb.info.nih.gov/nih-image). Each ECSOD or catalase signal was normalized to the β-actin signal from the same sample, and the normalized values were expressed as a percentage of the signal from the control cells infected with AAV-GFP.

**Intraocular Injections**

For the intraocular injection of AAV, 4-week-old DBA/1J mice were anesthetized by inhalation with 2% isoflurane. A local anesthetic (proparacaine HCl) was applied topically to the cornea, and then a 32-gauge needle attached to a syringe (Hamilton, Reno, NV) was inserted through the pars plana. Two microliters of AAV was injected into the vitreous cavity. The right eyes of 20 mice received AAV-ECSOD. Another 15 mice received AAV-CAT. Another group of 10 mice received intravitreal injections of AAV-ECSOD, followed 2 days later by AAV-CAT into the right eyes. As internal controls, the left eyes were injected with AAV-GFP. Thus, mice that received both antioxidant genes were injected twice with AAV-GFP into the left eyes, first when they received AAV-ECSOD and again when they received AAV-CAT. To test any potential toxicity of GFP on RGCs and optic nerve axons, the left eyes of 10 normal mice were injected with AAV-GFP.

**Induction of EAE**

Experimental allergic encephalomyelitis (EAE) was induced in 30 mice by sensitization with 0.2 mL sonicated homologous spinal cord emulsion in complete Freund adjuvant (Difco, Detroit, MI) injected subdermally into the nuchal area on the same day as the intraocular injections. All mice in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the approval of the University of Florida Institutional Care and Use Committee.

**MRI Analysis**

High-resolution three-dimensional (3D) MRI of mouse optic nerve was performed using a 4.7-Tesla magnet (Oxford Instruments, Abingdon, Oxfordshire, UK) 1, 4, and 6 months after EAE sensitization and viral gene inoculations. The animals were anesthetized with isoflurane (1.5%–2%) while they were in a prone position, their heads firmly fixed in a purpose-built surface coil. T1-weighted 3D image acquisitions were performed immediately after intraperitoneal administration of gadolinium (Gd)-DTPA (Berlex Laboratories, Hopkinton, MA) at a dose of 0.2 mmol/kg body weight. With a visual workstation (O2; Silicon Graphics, Sunnyvale, CA), a camera (2.212; ParaVision, Højbjerg, Denmark), and software developed by the University of Florida Brain Institute (Gainesville, FL) MRI core, the volume of the optic nerve was quantified. For statistical analysis, AAV-GFP–inoculated left eyes were compared with right eyes, which received the anti-ROS genes. Statistical analysis was performed with the use of Student’s t-test for unpaired data.

**Light and Electron Microscopy**

Six months after AAV and EAE inoculations, mice were overdosed with sodium pentobarbital. They were then immediately perfused by cardiac puncture with fixative, 4% paraformaldehyde, and 2% glutaraldehyde in 0.1 M PBS buffer (pH 7.4). Eyes with attached optic nerves were dissected, further processed by immersion in 2.5% glutaraldehyde, and postfixed in 1% osmium tetroxide and 0.1 M sodium cacodylate-HCl buffer (pH 7.4). Tissue was then dehydrated through an ethanol series to propylene oxide, infiltrated, and embedded in epoxy resin that was polymerized at 60°C overnight. Semithin longitudinal sections (0.5–1.0 μm) of the optic nerve head and the retrobulbar nerve were stained with toluidine blue for light microscopic examination. Ultrathin sections (90 nm) were cut, examined, and photographed by transmission electron microscopy (H7600; Hitachi, Tokyo, Japan) at 80 kV.

For immunostaining, tissue specimens were postfixed in 5.0% acrolein, 0.1 M sodium cacodylate-HCl buffer (pH 7.4), and 7% sucrose and then were dehydrated through an ethanol series and embedded (LR-White; Ted Pella, Redding, CA) polymerized at 50°C overnight. Ultrathin sections (90 nm) were placed on nickel grids for immunocytochemistry. Nonspecific binding of antibodies was blocked by 5% normal goat serum in 0.01 M Tris-buffered saline (pH 7.2). They were then reacted with CAT or ECSOD antibody in the same buffer. After washes in 0.1 M PBS, the specimens were reacted with the secondary antibodies conjugated to 6 nm gold. After washes in buffer, grids were rinsed in deionized water, and the immunogold particles were enlarged by silver enhancement using a kit (Ted Pella, Redding, CA) according to the manufacturer’s specifications. The specimens were photographed by transmission electron microscopy without poststaining.

**Morphometric Analysis**

Morphometric analysis was performed in a masked fashion. Images of toluidine blue–stained sections of the optic nerve were captured with a video camera mounted on a light microscope, and the digital image was entered into computer memory. After initial calibration with a stage micrometer, the optic nerve head areas and the diameter of retrobulbar optic nerve (photographed at a magnification of 40×) were manually traced using the NIH IMAGE software and a computer (Macintosh; Apple, Cupertino, CA). Numbers of glial cells/inflammatory cells in the retrobulbar optic nerve were quantitated by thresholding of the darker staining cell nuclei at a magnification of 400×. Quantification of RGCs, axons, and myelin was made from the electron micrographs at a magnification of 2500×. RGCs were quantitated by thresholding of the darker electron-dense cell nuclei. The extent of demyelination was quantitated by threshold measurements of the myelin sheaths. Increases in myelin sheath area (less demyelination) thereby indicated a beneficial treatment effect. We inverted photographs to increase the contrast of the axons and manually erased the cells in the area counted. Then we used the threshold feature to highlight the axons and count the numbers of axons in each 1000-μm² area for each micrograph. Statistical analysis was performed by Student’s t-test for unpaired data (two-tailed). Values were expressed as the mean ± SEM. Differences were compared between the right eyes treated with ECSOD, CAT, or ECSOD+CAT to the control left eyes injected with GFP. In addition, experimental EAE eyes were compared with eyes of normal unsensitized mice inoculated with AAV-GFP or normal unsensitized mice that received no intraocular injections. P < 0.05 was considered significant.

**RESULTS**

**Expression of ECSOD and CAT**

Western blot analysis revealed increased expression of the human ECSOD and CAT 1 day after AAV infection of RGC-5 cell cultures (Fig. 1A) or the mouse optic nerve (Fig. 1B). For RGC-5 cells infected with AAV-ECSOD, monoclonal antibody against ECSOD revealed 35- and 31-kDa bands. In addition, 70-kDa and 62-kDa dimers were observed. However, ECSOD was barely detectable in control AAV-GFP–infected cells. In the
mouse optic nerve, we detected an increase in ECSOD 1 month after intravitreal injection of AAV-ECSOD (31-kDa and 35-kDa bands). Quantitative analysis showed a 15-fold increase in ECSOD in cultured RGC-5 cells (Fig. 1A) and a 4-fold increase in the murine optic nerve (Fig. 1D) compared with controls inoculated with AAV-GFP. After infection with AAV-CAT, we found an increase in catalase expression (60 kDa) in RGC-5 cell cultures (Fig. 1A) and in the murine optic nerves (Fig. 1B) compared with controls infected with AAV-GFP that had a faint band indicating the presence of endogenous murine catalase. Quantitation revealed that the AAV-CAT–infected cells and nerves had approximately 3.5-fold more catalase expression than the AAV-GFP controls (Figs. 1C, 1D).

Transmission electron microscopy confirmed the increased expression of ECSOD and catalase in the retina and optic nerve after intravitreal injection of antioxidant genes. Compared with eyes inoculated with AAV-GFP that showed endogenous levels of murine ECSOD (Fig. 2A), we found that ECSOD immunogold particles were increased in optic nerve endothelial cells of eyes inoculated with AAV-ECSOD (Fig. 2B) and in ganglion cells of the retina. ECSOD immunogold was highly represented in the optic nerve sheath of control eyes (Fig. 2C) and in AAV-ECSOD–inoculated eyes (Fig. 2D). Compared with ganglion cells of control eyes (Fig. 2E), catalase immunogold was substantially increased in RGCs of AAV-CAT–inoculated eyes (Fig. 2F). Although we found endogenous murine catalase immunogold in axons of the control optic nerve (Fig. 2G), intracocular injections of AAV-CAT increased the number of catalase immunogold particles in optic nerve axons (Fig. 2H).

**MRI Analysis**

The benefits of transgene modulation of antioxidant gene expression on experimental optic neuritis were tested first in live animals using serial volume measurements of the optic nerve. Three-dimensional magnetic resonance images were obtained 1, 4, and 6 months after EAE sensitization and intravitreal injection with AAV-ECSOD (Figs. 3A-D), AAV-CAT (Figs. 3E-H), and both antioxidant enzyme genes (Figs. 3I-L). The ratios of optic nerve volumes between right and left eyes were less than 1 in all three groups 1 month after sensitization for EAE.

This finding is indicative of the protective effect against optic nerve swelling by anti-ROS gene therapy compared with the unprotected control left eyes injected with AAV-GFP.

Four and 6 months after EAE sensitization, the ratios of right eye and left eye optic nerve volumes increased to values greater than 1 for AAV-ECSOD (Fig. 3D), AAV-CAT (Fig. 3I), and both antioxidant genes (Fig. 3L). These findings are indicative of the neuroprotective effect of anti-ROS gene therapy against atrophy of the optic nerve. Our MRI findings were confirmed in the optic nerves excised 6 months after sensitization for EAE. Compared with the optic nerves excised from normal unsensitized mice (Fig. 3M), marked atrophy was present in a control left eye of an EAE animal (Fig. 3N). However, the right optic nerve protected with both AAV-ECSOD and AAV-CAT was not atrophic and appeared similar to the optic nerves of normal animals.

**Optic Nerve Atrophy**

Histopathologic examination confirmed the protective effect of antioxidant gene therapy against atrophy of the EAE optic nerve detected by serial MRI. The retrobulbar optic nerve from a representative control eye inoculated with AAV-GFP had a small caliber indicative of atrophy (Fig. 4A). Compared with controls, the retrobulbar optic nerves of experimental eyes inoculated with AAV-ECSOD (Fig. 4B), AAV-CAT (Fig. 4C), or both antioxidant genes (Fig. 4D) had much larger diameters. These findings are suggestive of the long-term protective effect of antioxidant gene therapy against degeneration of the optic nerve induced by chronic EAE.

**Retinal Histopathology**

Atrophy of the EAE optic nerve was mirrored in the retina. Six months after inducing EAE, light microscopy revealed that RGCs were lost in AAV-GFP–inoculated EAE eyes, with the ganglion cell layer composed predominantly of small, darkly staining glial cells (Fig. 4E). The retinas of eyes treated with AAV-ECSOD showed more ganglion cells (Fig. 4F) than did the retinas of control eyes. They were larger, ovoid, and much lighter staining (arrows) than the smaller, darker glia that were
also seen with AAV-ECOSD treatment (Fig. 4F). In contrast, the large, pale-staining cells with elliptical nuclei characteristic of ganglion cells were increasingly more prevalent, and glial cell prominence was decreased, in experimental eyes inoculated with AAV-ECOSD (Fig. 4G) or AAV-ECOSD/H11001 AAV-CAT (Fig. 4H), respectively.

Next we used transmission electron microscopy to identify cells in the ganglion cell layer. In control eyes inoculated with AAV-GFP, we found mostly small cells with electron-dense nuclei and a thin rim of cytoplasm characteristic of microglia (Fig. 4I). In contrast, eyes treated with AAV-ECOSD had larger cells with pale, elliptical nuclei and cytoplasm characteristic of RGCs, though cells with shrunken nuclei and condensed chromatin, indicative of apoptotic RGCs, were also present (Fig. 4J). Compared with control and AAV-ECOSD-inoculated eyes, fewer microglia and apoptotic RGCs were found in AAV-CAT (Fig. 4K) or AAV-ECOSD+AAV-CAT–treated eyes (Fig. 4L) that had predominantly RGCs with a normal ultrastructural profile. In addition, eyes receiving combined antioxidant gene treatment exhibited the least amount of swelling of the nerve fiber layer overlying the ganglion cells.

**Optic Nerve Histopathology**

Light microscopy revealed foci of inflammatory cells, and loss of toluidine blue staining, indicative of demyelination—the
classical hallmark of optic neuritis and MS—was evident in the control EAE optic nerves (Fig. 5A). These findings were seen to a lesser degree in the retrobulbar optic nerves of animals treated with AAV-ECSOD (Fig. 5B) or AAV-CAT (Fig. 5C). They were seen least with combined antioxidant gene treatment (Fig. 5D), when the optic nerve myelination and cellularity most closely resembled those of normal unsensitized mice.

In control eyes of EAE mice inoculated with AAV-GFP, transmission electron microscopy revealed that loss of toluidine blue staining in the retrobulbar optic nerve was caused not only by loss of myelin lamellae enveloping axons but also by loss of axons themselves (Fig. 5E). Optic nerves treated with AAV-ECSOD had more remaining axons than the eyes of EAE mice inoculated with AAV-GFP (Fig. 5F). Some axons were naked, whereas others were enveloped by thin sheaths of myelin indicative of remyelination. Optic nerves protected with AAV-CAT exhibited less axonal and myelin loss than GFP-inoculated EAE controls or those inoculated with AAV-ECSOD (Fig. 5G). Nerves inoculated with both antioxidant genes showed a near normal complement of fibers with relatively preserved myelin lamellae (Fig. 5H)

The optic nerve head is unmyelinated in mice, as it is in humans. Here inflammatory cells thought to mediate the axonal damage were absent. Cystic spaces were present at foci where axons were missing in eyes of EAE mice inoculated with AAV-GFP (Fig. 5I). Compared with these controls, axonal loss was less prominent in experimental eyes treated with AAV-ECSOD (Fig. 5J), AAV-CAT (Fig. 5K), or both antioxidant genes (Fig. 5L). Hydropic degeneration of axons with disruption of axonal filaments and tubules and aggregation and dissolution of mitochondria cristae were least severe in the nerves protected by inoculation with both antioxidant genes. Just behind the lamina sclerales of EAE controls inoculated with AAV-GFP, many axons with disrupted contents were surrounded by thin myelin lamellae, indicative of remyelination (Fig. 5M). Apoptotic glial cells were evident in control EAE optic nerves and those of eyes treated with AAV-ECSOD (Fig. 5N). Resident axons of AAV-ECSOD-treated nerves were abnormal, exhibiting mitochondrial vacuolization and dissolution of cristae (Fig. 5O). Axons without disruption of mitochondria or cytoplasmic axonal filaments and tubules were most evident in eyes treated with AAV-CAT (Fig. 5P) or with both antioxidant genes (Fig. 5Q). Thus, not only did antioxidant gene therapy suppress axonal loss, it preserved the structural integrity of resident axons.

**FIGURE 3.** Suppression of optic atrophy. Serial MRI of mice inoculated with AAV-ECSOD into the right eyes and AAV-GFP injected into the left eyes showed no difference in the caliber of the optic nerves at 1 month (A) or left optic atrophy at 4 months (B) and no difference at 6 months after EAE sensitization (C). Histogram of optic nerve volume showed a significant loss in the control left eyes compared with the ECSOD-treated right eyes at 4 months and 6 months after EAE sensitization (D). With AAV-CAT inoculation of the right eye, no difference in caliber was seen compared with the control left eye at 1 month (E). Atrophy of the untreated left optic nerve became apparent at 4 months (F) and at 6 months (G), but atrophy was suppressed in the right eye by treatment with AAV-CAT. Quantitative analysis of optic nerve volume showed a significant loss in the left eye compared with the treated right eye at 4 months and 6 months after EAE sensitization (H). With AAV-ECSOD+AAV-CAT inoculation of the right eye, a slight decrease in swelling of the optic nerve compared with the control left eye was apparent at 1 month (I). However, at 4 months (J) and 6 months (K), the left optic nerve atrophied, whereas the protected right optic nerve did not. Quantitative analysis showed that at 1 month, protection against swelling in the right optic nerve compared with the control left eye resulted in a ratio of less than 1 in optic nerve volume (L). Later, protection against atrophy reversed the right eye/left eye optic nerve volume ratio. Excised optic nerves of a normal animal are shown (M) for comparison with those of the EAE animal (N). The right eye treated with both antioxidant genes was of normal caliber, whereas the left optic nerve inoculated with AAV-GFP was markedly atrophic (N, arrow). *P < 0.05. L, left; R, right.
RGC Quantitation

When the effects of gene therapy on preserving RGCs were quantitated, we found that although treatment with AAV-ECSOD suppressed RGC loss by 9% with a mean of 1274 ± 230 cells/mm² compared with 1156 ± 254 cells/mm² for control EAE eyes injected with AAV-GFP, this difference was not significant (P = 0.44; Fig. 6A). Although AAV-CAT–treated eyes had 13% more RGCs with a mean of 1332 ± 254 cells/mm² compared with 1163 ± 87 cells/mm² for EAE control eyes inoculated with AAV-GFP, this difference was not significant (P = 0.08; Fig. 6A). However, for eyes that received both antioxidant genes, we found ganglion cell loss was suppressed by 29% with a mean RGC count of 1613 ± 248 cells/mm² compared with 1142 ± 139 cells/mm² for GFP-inoculated EAE controls. This difference was highly significant (P = 0.009; Fig. 6A). Compared with the normal optic nerves of mice that were not sensitized for EAE, AAV-ECSOD–treated eyes had a 27% loss of RGCs (P = 0.006). In eyes that received AAV-CAT, ganglion cell loss was 24% (P = 0.004). With combined antioxidant gene treatment, RGC loss was limited to 9% compared with the normal optic nerve (P = 0.22); this difference was not statistically significant. Therefore, combined ROS scavenging preserved the normal complement of RGCs 6 months after sensitization for EAE, whereas neither ECSOD nor catalase did.

Myelin Quantitation

Although AAV-ECSOD–treated nerves had 8% more myelin (less demyelination), with a mean myelin area of 169 ± 59 μm² per 1000 μm², than control nerves of EAE mice, with a mean of 156 ± 37 per 1000 μm² (Fig. 6B), this difference was not statistically significant (P = 0.24). In contrast, AAV-CAT–inoculated nerves had 25% more myelin, with a mean myelin area of 193 ± 23 μm², than the control nerves of EAE mice, with a mean of 143 ± 15 μm² per 1000 μm² (Fig. 6B). This difference was highly significant (P = 0.004). Still, scavenging of superoxide and H₂O₂ with both antioxidant genes offered the greatest protection (36%) against myelin loss, with a mean of 279 ± 29 μm² per 1000 μm², than unprotected controls, with a mean of 178 ± 33 μm² per 1000 μm² (P = 0.0003; Fig. 6B). Compared with the normal optic nerves of unsensitized mice, AAV-ECSOD–treated eyes had a 55% loss of myelin (P = 0.005). AAV-CAT myelin loss was 38% (P = 0.004). However, the 10% loss of myelin in eyes that received dual treatment with...
AAV-ECSOD+CAT\ was statistically indistinguishable from the normal optic nerve myelination of unsensitized mice (\( P = 0.25 \)).

**Axon Quantitation**

Given that loss of myelin staining detected by toluidine blue light microscopy may be caused by myelin or axonal loss and that axonal loss rather than demyelination is associated with permanent visual loss, we also quantified resident axons in the 6-month EAE optic nerves using transmission electron microscopy. Although we found 13% more axons with scavenging of superoxide by AAV-ECSOD with a mean axon count of 104 ± 16.2 per 1000 \( \mu \text{m}^2 \) compared with a mean of 91 ± 17 per 1000 \( \mu \text{m}^2 \) for control EAE nerves that were inoculated with AAV-GFP, this difference was not statistically significant (\( P = 0.14 \); Fig. 6C). Treatment with AAV-CAT reduced axonal loss by 24%, with a mean axon count of 116 ± 6 per 1000 \( \mu \text{m}^2 \) compared with 86 ± 11 per 1000 \( \mu \text{m}^2 \) for control EAE eyes inoculated with AAV-GFP (\( P = 0.04 \)). Neuroprotection was greatest with combined scavenging of superoxide and \( \text{H}_2\text{O}_2 \) by AAV-
**Quantitation of Optic Nerve Cellularity**

Because transection by inflammatory cells is thought to be the mechanism of axonal loss, we performed quantitation of optic nerve cellularity (Fig. 5D). AAV-ECSSOD gene inoculation, with a mean of 1226 ± 250 cells/mm² compared with a mean of 1342 ± 230 cells/mm² for EAE nerves injected with AAV-GFP, did not significantly suppress cellular infiltration of the optic nerve (P = 0.5). In contrast, eyes inoculated with AAV-CAT exhibited 29% less cellular infiltration (mean, 1108 ± 140 cells/mm²) compared with a mean of 1560 ± 280 cells/mm² for EAE AAV-GFP-injected nerves (P = 0.01). Dual gene inoculated optic nerves had 34% less cellularity (mean cell count, 1024 ± 241/mm²) than AAV-GFP-inoculated controls (mean, 1554 ± 336/mm²; P = 0.018). Compared with the normal optic nerves of unsensitized mice, AAV-ECSSOD-treated eyes had a 20% increase in optic nerve cell counts (P = 0.08). Although AAV-CAT and AAV-ECSSOD+CAT cell counts increased by 11% and 4%, respectively, they were not significantly different from the normal optic nerves of unsensitized mice.

**Effect of GFP**

RGC, axon, and myelin cell counts of normal eyes of unsensitized mice that received AAV-GFP were similar to those of uninoculated eyes of normal mice (Figs. 5A–D). Thus, AAV-GFP gene inoculation had no deleterious effects on the retina or optic nerve.

**FIGURE 5.** Microscopy of myelin and axonal loss. (A) Representative light micrograph of the control retrobulbar optic nerve from an EAE mouse showed loss of toluidine blue staining, typical of the classical demyelination of EAE, and cellular infiltration (arrows). Eyes treated with AAV-ECSSOD (B), AAV-CAT (C), or both anti-ROS genes (D) had fewer foci of demyelination and inflammation than the control shown in (A). (E) Transmission electron microscopy shows an inflammatory cell, acellular spaces where axons were likely lost, and few remaining axons in the retrobulbar optic nerve of a control eye inoculated with AAV-GFP. Some axons were naked; others were enveloped by thin sheaths of myelin (arrows) indicative of remyelination. The retrobulbar optic nerve of an eye treated with AAV-ECSSOD (E) had a column of axons with many more remaining axons than the control shown in (E). Astrocytic cells were also a prominent finding. (G) Retrobulbar optic nerves protected with AAV-CAT exhibited many axons with thin myelin lamellae (arrows), though a naked axon is evident. (H) The retrobulbar nerve of an eye inoculated with both antioxidant genes showed a near normal complement of axons with preservation of myelin lamellae. (I) In the unmyelinated optic nerve head of a control eye inoculated with AAV-GFP, acellular spaces were present at foci where axons were lost. (J) Compared with the control shown in (I), more axons were evident in an experimental eye treated with AAV-ECSSOD; however, these axons exhibited hydropic degeneration. (K) In the optic nerve head of an eye inoculated with AAV-CAT; many axons were preserved, though some axons were swollen with dissolution of axonal contents. (L) In contrast, less hydropic degeneration was evident in axons of the optic nerve head of an eye treated with both antioxidant genes that had preserved axon filaments and tubules. (M) Just behind the lamina sclerales, axons with aggregation of mitochondria (arrowheads) and dissolution of cristae were most severe in the unprotected control eyes. This axon was surrounded by thin myelin lamellae. (N) An apoptotic glial cell was evident in an optic nerve treated with AAV-ECSSOD, though compared with the control shown in (M), AAV-ECSSOD-treated nerves had fewer axons exhibiting mitochondrial vacuolization and dissolution of cristae. Axons without disruption of axonal filaments and tubules or mitochondria (arrowheads) were most evident in eyes treated with AAV-CAT (P) or by both antioxidant genes (Q). a, axon; ic, inflammatory cell; n, naked axon; ac, astrocyte. *Acellular space.
DISCUSSION

The protective effect of antioxidant gene therapy against demyelination, the classical parameter of disease activity, diminished with increasing exposure to EAE. When studied 1 month after sensitization for acute EAE, overexpression of catalase suppressed optic nerve demyelination by 38%. Here, when studied 6 months after sensitization for chronic EAE, it achieved only a 25% long-term protective effect against demyelination, even with a 1000-fold higher titer virus than that used in our earlier work. By adding the dismutation of superoxide to the detoxification of its metabolite, hydrogen peroxide, we increased the suppressive effect on demyelination up to 36% in chronic EAE. Our earlier results with combined germine ECSOD and catalase gene therapy for acute EAE were more impressive, with a 78% reduction in demyelination. A recent autopsy study of an MS patient with optic neuritis who was treated with corticosteroids and recovered from 20/400 to 20/20 even with extensive myelin loss suggests that demyelination may not be the determinant of persistent visual loss despite the traditional view of optic neuritis and MS that emphasizes demyelination as the primary event in the disease process.

Axonal and neuronal loss are increasingly recognized as the cellular events responsible for persistent deficits and disability in patients with optic neuritis and MS. Recent OCT studies showing loss of macular volume in these patients suggest that lack of visual recovery results primarily from loss of retinal ganglion cells. Axonal and neuronal loss also play prominent roles in the pathogenesis of acute EAE, relapsing remitting EAE, and chronic EAE. In our previous reports of acute EAE, we did not examine the effects of catalase or ECSOD on these key parameters of visual disability. In this study of chronic EAE, we did. ECSOD alone had no significant effect against neuronal or axonal loss. This result is in contrast to other studies showing that the extracellular isoform of SOD was highly effective against CNS oxygen toxicity, brain edema, but worsened ischemia. Our previous work showing the suppressive effect of the mitochondrial SOD isoform (MnSOD) suggests that detoxification of mitochondrial rather than cytoplasmic superoxide activity may be key to protecting RGCs against oxidative injury and mitigating neurodegeneration in EAE and perhaps MS.

Unlike superoxide that does not likely diffuse far from the sites of generation in the mitochondria or after discharge into the extracellular space by inflammatory cells, its metabolite, hydrogen peroxide, can diffuse. Thus, by detoxification of hydrogen peroxide using cytoplasmic expression of catalase, we were able to suppress axonal loss by 24% in the chronic EAE optic nerve. Although ECSOD by itself had no significant effect against axonal loss when combined with catalase, it ameliorated axonal loss by almost half compared with unprotected nerves of mice with chronic EAE. When measured against the normal optic nerve, axonal loss was indistinguishable from the optic nerves treated with both antioxidant genes. In addition, the resident axonal population, in dual-treated eyes of chronic EAE mice, had many axons with preserved intraretinal connections compared with unprotected EAE eyes that exhibited disruption of axonal filaments, tubules, and mitochondria. Disruption of axonal tubules has been shown to occur before demyelination and axonal loss in EAE, suggesting it is an early event in the disease process. These findings may have important implications in preventing the permanent disability of optic neuritis and MS resulting from neurodegeneration. To date no treatment has been shown to protect against optic nerve or RGC loss in patients with optic neuritis or MS. The neurodegeneration of MS is thought to be caused by inflammatory cell-mediated transection of axons. Still, this sequence is refractory to immunomodulatory drugs. Addressing potential mechanisms and treatments for axonal injury in MS is the subject of a recent request for applications by the National Institutes of Heath (PAS-07-193). In this study we found that antioxidant gene therapy with catalase or combined therapy, but not monotherapy with ECSOD, suppressed cellular infiltration of the optic nerve in chronic EAE. Suppression of inflammation by corticosteroids is the mainstay of treatment for patients with optic neuritis and MS. Still a recent review found no ultimate beneficial effect of corticosteroids on visual function. In the EAE animal model, others have shown that corticosteroid administration may be harmful by increasing apoptosis of ganglion cells in the retina, despite the suppressive effect on inflammation in the optic nerve. The clinical data suggest that corticosteroids do not have an adverse impact on visual function. Fortunately most patients recover, treated or untreated. For those who do not, the options are limited.

Available treatments for MS, such as glatiramer acetate but not IFN-β, do suppress RGC loss in EAE. Neuroprotection by glatiramer acetate was not dependent on suppression of inflammatory cells. Additional experimental treatments such as gene delivery of the antiapoptotic agent BclXL has been shown to attenuate neurodegeneration of the optic nerve in EAE. Bcl2 increases found in MS lesions may be an endogenous response to prevent apoptosis. Studies showing the antioxidant erythropoietin combined with corticosteroid administration ameliorates RGC loss, together with our work here, further support the suppressive effect on neurodegeneration that can be achieved by transfer of two key antioxidant genes to the retina and optic nerve in the chronic EAE animal model. Taken together, these studies are promising in the quest to prevent axonal and neuronal demise in patients with optic neuritis and MS. Although treatments developed in the EAE animal model have been successfully applied to MS, whether similar strategies applied to our patients will help avert this dreadful sequelae that results in permanent disability remains to be determined.

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