Optic Neuropathy Induced by Reductions in Mitochondrial Superoxide Dismutase

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PURPOSE. Reactive oxygen species (ROS) are suspected to play a pivotal role in the pathogenesis of Leber hereditary optic neuropathy (LHON), caused by mutated complex I subunit genes. It seems surprising that optic neuropathy has not been described in animals with a knockout of genes encoding critical anti-ROS defenses. If ROS have a role in the optic nerve injury of LHON, then increasing mitochondrial levels of ROS should induce optic neuropathy.

METHODS. To develop an animal model system for study of oxidative injury to the optic nerve, mitochondrial defenses were decreased against ROS by designing hammerhead ribozymes to degrade SOD2 mRNA. Several potential ribozymes were analyzed in vitro. The one with the best kinetic characteristics was cloned into a recombinant adenovirus-associated virus (rAAV) vector for delivery and testing in cells and animals. The effects of the AAV-expressing ribozyme on murine cell growth, SOD2 mRNA and protein, cellular ROS levels, and apoptosis were evaluated by RNase protection assay, immunoblot analysis, and ROS- and apoptosis-activated fluorescent probes. The rAAV-ribozyme was then injected into the eyes of DBA/1J mice, and the effect on the optic nerve was evaluated by ocular histopathologic examination.

RESULTS. The AAV-expressing ribozyme decreased SOD2 mRNA and protein levels by as much as 85%, increased cellular superoxide, reduced mitochondrial membrane potential, and culminated in the death of infected cell lines by apoptosis without significantly altering complex I and III activity, somewhat spared in the most common LHON mutation (G11778A), although adenosine triphosphate (ATP) synthesis is markedly reduced. When inoculated into the eyes of mice, the AAV-expressing ribozyme led to loss of axons and myelin in the optic nerve and ganglion cells in the retina, the hallmark of optic nerves examined at autopsy of patients with LHON.

CONCLUSIONS. The striking similarity of the optic neuropathy to the histopathology of LHON is powerful evidence supporting ROS as a key factor in the pathogenesis of LHON. (Invest Ophthalmol Vis Sci. 2003;44:1088–1096) DOI:10.1167/iovs.02-0864

Oxidative stress has been implicated in many disorders associated with mutated mitochondrial DNA. The most common of these is Leber hereditary optic neuropathy (LHON). In this disorder a multitude of mutations in mitochondrial (mt)DNA encoding the reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase (complex I of the respiratory chain) share the same phenotype,1–3 thus suggesting a common pathway for optic nerve injury.5,6 How these mutations in complex I induce degeneration in the optic nerve is unclear. However, deficiencies in respiratory chain function and reactive oxygen species (ROS) are believed to play pivotal roles in the pathogenesis of LHON.1,7,8

Under normal physiologic conditions, cells mitigate oxygen toxicity predominantly through antioxidant enzymes. They include superoxide dismutase (SOD), catalase, and glutathione peroxidase.9 Mitochondria contain their own version of SOD, the manganese (Mn)SOD that is encoded by the SOD2 gene located in the nucleus. SOD catalyzes the dismutation of two superoxide radicals, producing H2O2 and molecular oxygen. The essential role of SOD2 in mitigating ROS is illustrated by the loss of viability of SOD2 nullizygous (sod2/−/−) mice.10 Without any mitochondrial SOD they die shortly after birth. These animals do not exhibit any retinal or optic nerve abnormality, unless the lifespan is extended briefly by treatment with antioxidant compounds. Still, only central retinal thinning and a minor reduction of optic nerve cross-sectional area was described.11 The absence of a phenotype in heterozygous SOD2+/− makes transgenic MnSOD knockout animals unsuitable for evaluating the role ROS may play in optic nerve degeneration. Another animal model system is needed to test this hypothesis.

In this report, we describe the use of a ribozyme targeted against SOD2 mRNA to reduce expression of MnSOD and increase mitochondrial levels of ROS. Using this technology, we tested whether the optic nerve degeneration of LHON could be recapitulated by delivery of the ribozyme to the mouse visual system using an adeno-associated viral (AAV) vector.

METHODS

Hammerhead Ribozyme and Target

We designed a ribozyme against SOD2 (RzSOD2) to cleave the murine SOD2 mRNA at position 432 (GenBank accession number NM_013671; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The ribozymes are synthetically engineered molecules, capable of cleaving target RNA in a highly specific manner by blocking gene expression and therefore preventing production of targeted proteins.12 RzSOD2 coding sequences were generated by the extension of two overlapping DNA oligonucleotides (Life Technologies, Gaithersburg, MD) flanked by PsI and BamHI restriction sites, and were ligated into pT7/T3-19, containing a T7 RNA polymerase promoter. The 13-nt ribonucleotide targets were purchased from Dharmaco (Boulder, CO) and were 5′-end labeled with [γ-32P]-adenosine triphosphate (ATP; ICN, Irvine, CA) using polynucleotide kinase (Promega, Madison, WI).

In Vitro Analysis of Hammerhead Ribozyme

Time course and multiple-turnover kinetic analysis of ribozyme cleavage of RNA oligonucleotides were performed as previously reported.12

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Briefly, pT7/T3-19 containing ribozyme sequences were linearized at downstream restriction sites, and transcripts were synthesized using T7 RNA polymerase and were labeled during transcription to estimate ribozyme levels by specific radioactivity. The ribozyme was incubated with RNA target in 40 mM Tris HCl, 20 mM MgCl₂ (pH 7.5) at 37°C. The cleavage products were separated on 10% denaturing acrylamide gels that were analyzed with a phosphorescence imager (PhosphorImager; Molecular Dynamics, Sunnyvale, CA). Kinetic constants (Kₚ, Kₖₑₛₜ) were determined using Lineweaver-Burke double-reciprocal plots.

**rAAV-Ribozyme Construct**

The rAAV vector, pTR-UF12 (University of Florida Vector Core), was used to accept the ribozyme into the Xpl and HindIII sites. Expression of the ribozyme was regulated by a hybrid cytomegalovirus (CMV) enhancer and a chicken β-actin promoter. The hammerhead ribozyme was preceded by an intron from the actin gene and followed by an internally processing hairpin ribozyme. The ribozyme cassette was followed by an internal ribosome entry site (IRES) derived from poliovirus and a humanized gfp gene. Recombinant AAV was purified through iodixanol step gradients and heparin-agarose affinity columns. Each virus preparation contained 10¹⁵ to 10¹⁶ particles per milliliter and 10⁵ to 10⁶ infectious centers per milliliter. No helper adeno virus was used in this preparation.

**rAAV Delivered Ribozyme in Cell Culture**

Mouse fibroblast (NIH/ST3) cells and human embryonic kidney (293) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Fisher Scientific, Fair Lawn, NJ) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin streptomycin (Sigma, St. Louis, MO) at 37°C with 5% CO₂. Cells were plated in a 15-cm dish. After they were allowed to grow to approximately 70% confluence, they were infected at an increasing multiplicity of infection (MOI) from 100 to 10,000 physical particles per cell. After incubations for various periods, cell morphology and pathology were examined by light microscopy, and the cell number was quantitated with a hemocytometer.

**Detection of ROS, Mitochondrial Membrane Potential, and Apoptosis**

To detect intracellular ROS generation, we used two probes (Molecular Probes, Eugene, OR). The probe 2’-7’ dichlorofluorescein diacetate (DCFDA) was used to detect cytosolic hydrogen peroxide (H₂O₂). DCFDA has no fluorescence until it passively diffuses into cells where the intracellular esterase cleaves the acetates, and the oxidation of DCFDA by H₂O₂ produces a green fluorescent signal. The mitochondria-specific dye dihydroethidium was used to detect intracellular superoxide. Superoxide oxidizes dihydroethidium to a red fluorescent signal. Loss of mitochondrial membrane potential (∆Ψm) was detected by the loss of rhodamine-123 signal. NIH/ST3 cells infected with the AAV-expressing ribozyme and the control were incubated for 15 minutes at 37°C with 10 μM DCFDA, 1 μM dihydroethidium, and 5 μM rhodamine-123. We assessed apoptotic cell death with a kit, according the manufacturer’s specifications (Roche Molecular Biochemicals, Indianapolis, IN). Cells were washed and then observed under a fluorescence microscope and quantitated with flow cytometry (FACScan; BD Biosciences, Lincoln Park, NJ).

**RiNase Protection Assay**

To quantify SOD2 mRNA levels, total RNA of NIH/ST3 cells from either control or experimental dishes at each time point (1, 2, 3, and 4 days after infection) was prepared with a kit (RNasy Mini Kit; Qiagen, Valencia, CA), according to the manufacturer’s specifications. Each experimental reaction was conducted using an equal amount of RNA. For analysis of SOD2 RNA, an 80-nt antisense RNA probe corresponding to a fragment of the SOD2 gene was purchased (Dharmacon) and was 5’-end labeled with γ³²P-ATP (ICN, Irvine, CA) using polynucleotide kinase (Promega). The RNAse protection assay (RPA) was performed with a kit (RPAIII; Ambion, Austin, TX). Briefly, gel-purified riboprobes were hybridized with 30 μg total cellular RNA at 42°C for 18 hours followed by RNAse A/T1 digestion at 37°C for 30 minutes. Protected fragments were heat denatured and separated on 6% denaturing polyacrylamide gels. A 304-nt mouse β-actin antisense probe was used as an internal control. Radioactive signals were recorded and quantitated by using a phosphorescence imager (PhosphorImager; Molecular Dynamics). Each SOD2 signal was normalized to the β-actin signal from the same sample, and the normalized values were expressed as a percentage of the signal in the control.

**Immunodetection of SOD2**

For Western blot analysis, we infected NIH/ST3 cells as described earlier. The proteins from total cellular lysates were separated on a 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The membrane was then immunostained with the rabbit polyclonal anti-SOD2 antibodies and then goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies. We used the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ) to detect the labeled proteins. The immunostained fragments were quantified by densitometry, using NIH Image (available by ftp from zippy.nimh.nih.gov or from http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Anti-mouse β-actin antibody was used as an internal control for protein loading. Each SOD2 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. Anti-green fluorescence protein (GFP) antibodies counterstained by IgG conjugated to FITC were used to evaluate AAV transfection in cell culture.

**Oxidative Phosphorylation Assays**

Assays of complex I and III activity and rotenone-sensitive complex I activity were performed on Rz-SOD2 and mock-infected cells by the reduction of cytochrome c with reduced nicotinamide adenine dinucleotide (NADH) and also in the presence of the inhibitor rotenone. Mitochondrial complex I activity was calculated by subtraction from the total value obtained for complexes I and III after the addition of rotenone.

**Experimental Animals**

For the intracocular injection of rAAV, 8-week-old DBA/1J mice were sedated by an intramuscular injection of a mixture of ketamine and xylazine. 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. The needle tip was visualized in the vitreous with the use of an indirect ophthalmoscope and positioned directly over the optic nerve head. Two microliters rAAV ribozyme were injected into the vitreous of the right eye of each mouse. As a control, the left eyes received RAAV without the ribozyme but with the IRES-gfp gene. Mice were maintained in veterinarian-supervised animal care facilities and humanely cared for. The animal protocol was in compliance with the Statement for the Use of Animals in Ophthalmic and Visual Research.

**Light and Electron Microscopy**

Mice were received an overdose of sodium pentobarbital 6 weeks, 4 months, and 6 months after viral inoculation. They were then immediately perfused intracardially with fixative consisting of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS buffer (pH 7.4). The eyes with attached optic nerves were dissected and further processed by immersion in 2.5% glutaraldehyde and then postfixed in 1% osmium tetroxide, 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 μm) of the optic nerve head and retrobulbar nerve were made and stained with toluidine blue for light microscopic examination. Ultrathin sections (80 nm) were cut and examined by using a transmission electron microscope (model; H-7000; Hitachi, Tokyo, Japan) operating at 75 kV.

Morphometric Analysis

Morphometric analysis was performed as previously described.5 Briefly, images of toluidine blue-stained sections of the retina and optic nerve were captured with a video camera mounted on a light microscope, and then the data were entered into the computer. Myelin and ganglion cell loss were quantified using the NIH Image software (Macintosh computer; Apple, Cupertino, CA). For statistical analysis, rAAV-ribozyme inoculated eyes were compared with the control eyes that received rAAV-gfp without the ribozyme. Statistical analysis was performed by Student’s t-test for unpaired data.

RESULTS

In Vitro Analysis

The ribozyme recognized a 13-nt sequence in SOD2 mRNA that was determined to be accessible to ribozyme-attack (Fig. 1a). We found the 13-nt target was rapidly cleaved by the ribozyme. It broke the targeted phosphodiester bond producing two fragments. After incubation of target and ribozyme, the labeled target decreased and the γ32P labeled 5’ degradation product increased (Figs. 1b, 1c). The 3’ product fragment was not seen, because it was not labeled after cleavage from the target. Multiple turnover kinetic analysis revealed a Km of 15.4 μM and a Kcat of 0.3 min−1 for RzSOD2 (Fig. 1c). The kinetic constants exhibited here are similar to others we have successfully used in gene therapy experiments15,16; thus, this ribozyme seemed promising for the suppression of SOD2 gene expression.

Effects of the AAV Expressing SOD2 Ribozyme in Cell Culture

Before animal testing, we evaluated the cytotoxicity of the AAV expressing ribozyme in cultured cells. For delivery and prolonged expression in animal tissues, the ribozyme gene was inserted into an AAV-2 vector, and expression was driven by the hybrid CMV enhancer-chick β-actin promoter.13 The ribozyme was inserted upstream of the gfp gene under the control of a poliovirus-derived IRES. Using this bicistronic AAV vector, infected cells expressed both the ribozyme and GFP. Thus, cellular transfection with the ribozyme could be monitored by the fluorescence of GFP.

The SOD2 ribozyme induced apoptotic cell death, reduced mitochondrial membrane potential, and increased superoxide production. One day after infection with the SOD2 ribozyme, murine cells were labeled with the red fluorescence signal indicative of apoptosis. Cells mock-infected with AAV-gfp did not label (Fig. 2a, 2b). Control cells infected with AAV without the ribozyme gene revealed a mitochondrial pattern of green rhodamine-123 fluorescence, indicative of a normal mitochondrial membrane potential (Fig. 2c). However, in cells infected with rAAV-RzSOD the diminished green fluorescence of rhodamine-123 indicated loss of mitochondrial membrane potential (Fig. 2d). Quantitation by flow cytometry (FACScan; BD Biosciences) revealed a 64% reduction in mitochondrial membrane potential. As expected, suppression of SOD2 by the AAV-expressing ribozyme increased mitochondrial levels of superoxide. The red signal of the oxidized mitochondria-specific dye dihydroethidium generated by increased mitochondrial levels of superoxide was present only in cell cultures infected with the RzSOD2 (Fig. 2d). Quantitation by flow cytometry revealed 45% more superoxide in RzSOD2-infected cells compared with the control. Although no green DCFDA fluorescence induced by H2O2 was visually detectable by fluorescence microscopy in mock-infected (Fig. 2e) or RzSOD2-infected cells (Fig. 2f), quantitation by flow cytometry revealed 36% more H2O2 in RzSOD2-infected cells than in the control.

Virus expressing only GFP had no cytotoxic effect (Fig. 2g), but the AAV-expressing SOD2 ribozyme rapidly diminished the cell populations. Although apoptosis was detectable 1 day after RzSOD2 infection, massive cell death occurred 3 days after infection (Fig. 2h). When the AAV-expressing ribozyme was used to infect human (HEK293) cells, they grew normally, indicating no toxic effects on human cells from this mouse-specific ribozyme (Fig. 2i, 2j).
Effects of Ribozyme on SOD2 mRNA and Protein Expression

Because of the lethal effects of the ribozyme, in repeated attempts we were unable to select mouse cell lines that stably express Rz-SOD2 using the G418 resistance gene in the AAV vector, even though the parent virus consistently led to stable transduction. Therefore, cellular assays of ribozyme-infected cells had to be performed before cell death. We started 1 day after the infection. Compared with the control (AAV-gfp infected) cells, RPAs indicated that levels of SOD2 mRNA initially decreased dramatically (81%) and continued to decrease until 72 hours, when the transcript level appeared to increase (Fig. 3a). Because the remaining cells did not produce GFP, they were apparently not infected with the ribozyme. Therefore, the growth of uninfected cells accounted for the restoration of SOD2 mRNA levels.

**FIGURE 2.** Effects of rAAV-ribozyme in cell culture. One day after infection of NIH/3T3 cells with the SOD2 ribozyme, the murine cells were labeled with the red fluorescence signal indicative of apoptosis. Cells mock infected with AAV-gfp were unlabeled. (a,b) Cells double-stained with rhodamine-123 (pseudocolored green) and dihydroethidium (pseudocolored red; c). In the control cells that are infected with AAV expressing GFP without the ribozyme, rhodamine-123 accumulated in mitochondria. However, in cells infected with rAAV-Rz-SOD2, the green rhodamine-123 fluorescence was subsequently lost and was accompanied by the red fluorescent superoxide signal (d). In contrast, the generation of H_2O_2 (green fluorescence) was not visualized in control (e) or Rz/SOD2—infected cells (f). Cells mock infected by AAV-gfp without a ribozyme gene (g) showed no cytopathic effects, but the cells infected with Rz/SOD2 decreased significantly after 72 hours (h). However, the infection of human 293 cells with the AAV-expressing SOD2 ribozyme revealed no nonspecific effects from this mouse-specific ribozyme (i, j).

**FIGURE 3.** Levels of target mRNA and protein expression. RNase protection assays of total cellular RNA revealed dramatically decreased levels of SOD2 mRNA within 24 hours of the infection and declined further 48 hours after the AAV-Rz-SOD2 infection, but then appeared to increase because of the death of infected cells expressing Rz/SOD2 and overgrowth of the plate by uninfected cells (a). Compared with Western blots of the control cells, blots of the infected cells revealed a substantially reduced SOD2 protein level 24 to 48 hours after infection with AAV-RzSOD2 (b).
We also observed a substantial decline in the targeted mitochondrial protein. Compared with the cells mock-transfected with AAV-gfp, cells infected with Rz-SOD2 had a marked reduction in mitochondrial SOD2 by 64% and 85%, 1 and 2 days after infection, respectively (Fig. 3b).

**Oxidative Phosphorylation Assays**

Compared with the control, at the time of maximal reduction of SOD2 expression, 2 days after infection, no loss of complex I and III or rotenone sensitive complex I activity was detected (8.5 nm cytochrome c reduction per minute per milligram protein for Rz-SOD2 versus 8.8 nm cytochrome c reduction per minute per milligram in the control). Therefore, it appears that cell death resulted from the direct toxicity of ROS induced by the Rz-SOD2; but would the ribozyme have any effect on the optic nerve?

**Effects of Loss of Mitochondrial Gene Expression in the Eye**

Indeed, the SOD2 ribozyme infection had definite deleterious effects on the optic nerve and retina. Compared with the control (Fig. 4a), optic nerve head swelling occurred 6 weeks after inoculation with Rz-SOD2 (Fig. 4b). Ultrastructurally, intracellular edema and mitochondrial accumulation in the un-
loss of retinal ganglion cells, 4 and 6 months after RzSOD2 inoculation, respectively \((P < 0.01; \text{Fig. 5b})\). Compared with mock infection, RzSOD2 decreased the diameter of the optic nerve by a third \((259.3 \mu \text{m} \text{ in RzSOD2-treated retinas versus } 378.6 \mu \text{m in the control}, P < 0.0001)\) at 6 months.

Ultrastructural analysis of mock-infected nerves produced normal findings at each time point (Fig. 4a). Four months after inoculation with Rz-SOD2 the retrobulbar nerves revealed foci of hydropic degeneration with dissolution of microtubules and including swollen mitochondria, cytoplasm with low electron density, and white halos around astroglial and oligodendroglial nuclei from swelling of perinuclear cisternae. Axons appeared naked or enveloped by thin sheaths of myelin suggestive of remyelination (Fig. 6b). At 6 months, the injury progressed. These eyes exhibited more severe axonal loss and demyelination. Some optic nerves were almost completely demyelinated (Fig. 6c). Mitochondria appeared morphologically normal in mock-infected nerves (Fig. 6d). Four months (Fig. 6e) and 6 months after inoculation with Rz-SOD2, mitochondria were distended, with dissolution of cristae. Although not apparent earlier, a mild mononuclear inflammatory cell infiltrate and reactive astroglial cells were seen at 6 months (Fig. 6f). Expression of GFP persisted in RGCs at 6 months (Fig. 7) in control animals.

**DISCUSSION**

In the current study, ganglion cells in the retina and axons and myelin in the optic nerve were extremely vulnerable to ROS induced injury. Loss of these cell types is the hallmark of the histopathology of eyes examined at autopsy of patients with LHON.\(^{17}\) The striking similarity of the optic neuropathy shown herein to that of LHON is powerful evidence supporting ROS as a key factor in the pathogenesis of mitochondrial diseases with mutated complex I subunit genes. The marked reductions in ATP synthesis that have been detected in LHON cytoplasmic hybrid (cybrid) cells lines suggest that a deficiency in oxidative phosphorylation also plays a major role in the pathogenesis of LHON.\(^{18}\)

Similar to human disorders associated with mutated complex I subunit genes, manifestation of our disease model took time to evolve.\(^{19}\) Because in vivo gene expression with the AAV vector may take several weeks, it was not surprising that we detected no morphologic changes until 6 weeks after injection, when marked swelling of the optic nerve head developed. At this early time, the retinal ganglion cell layer and axons and myelin of the retrobulbar nerve appeared morphologically normal, degenerating later, 4 and 6 months after injection of the ribozyme. This sequence of initial optic nerve head edema followed by optic atrophy also occurs in LHON with mutated complex I subunit genes. These observations suggest that axonal insult first occurs at the optic nerve head.

Although of patients with LHON also exhibit ophthalmoscopically detectable disc edema during the initial loss of vision,\(^{20}\) often, little is known of the morphologic changes that result until decades after this event, when some eyes are subjected to histopathologic examinations. At this end stage, the optic nerves are found to be severely degenerated, revealing demyelination and axonal loss in the retrobulbar nerve.\(^{17}\)

The similarity to LHON of the evolution of the neuropathy in our animal model system suggests that events in the chain leading to degeneration of the optic nerve may be initiated in the nerve head. Coincidentally, this site has the highest levels of mitochondrial oxidative phosphorylation enzymes in the body.\(^{21,22}\) Still, how is this experimental optic neuropathy, induced by loss of an antioxidant gene, related to LHON that is caused by mutated complex I genes?
It appears that an inverse relationship exists between the activity of complex I and ROS. In vitro data clearly show that the cytotoxicity induced by loss of complex I activity is not from reductions in oxidative phosphorylation but is due to increased production of ROS. We have demonstrated in the current study that increasing ROS levels directly, by attacking expression of a gene that causes the dismutation of superoxide, induces an optic neuropathy with features similar to the

**Figure 6.** Ultrastructural changes in the mouse eye. Compared with mock-infected eyes that received rAAV-gfp (a), optic nerves examined 4 months after inoculation with AAV-Rz-SOD2 revealed axonal degeneration, naked axons (A) denuded of myelin, and axons enveloped by thin sheaths of myelin (arrows), suggestive of remyelination (b). Even more loss of axons resulted in vacuolar changes and spaces between the nerve fibers (arrows) 6 months after inoculation with AAV-Rz-SOD2 (c). Although mitochondria were morphologically normal in mock-infected nerves (d), in ribozyme-inoculated nerves (arrows), they were swollen and devoid of cristae (e). Reactive astroglia (arrows) and phagocytes (arrowhead) were also observed (f). Original magnification: (a–c) $\times 2,500$; (d–f) $\times 10,000$.

**Figure 7.** GFP expression. Six months after an AAV-expressing GFP injection, GFP expression persisted in the retinal ganglion cells.
complex I mutations of LHON. However, the normal complex I activity in our Rz-SOD2-infected cells suggests that the cytotoxicity of our ribozyme was not due to a loss of oxidative phosphorylation, but to the direct effects of oxygen toxicity. Because complex I activity is only mildly reduced in the most common mutation associated with LHON (G11778A),

5,26 many years may pass before mildly elevated levels of ROS induce an injury to the optic nerve severe enough to result in loss of vision. External factors, such as smoking and ethanol intake, that increase oxidative stress are believed to hasten the events leading to visual loss.27 Our model system of disease is further evidence linking oxidative stress to optic nerve degeneration.

The next question is how increased levels of ROS are generated by the mutated complex I gene associated with LHON. Within the mitochondria, production of ROS occurs at complex I and complex III (ubiquinone-cytochrome c oxidoreductase), with complex I being the major source under normal physiologic conditions.2,28 In LHON, the flow of electrons that normally pass along the electron transport chain may be disrupted by the mutated complex I gene. The freed electrons are now available to react with molecular oxygen, generating superoxide to levels beyond the capability of dismutation by the endogenous levels of MnSOD normally present within mitochondria. This oxidative stress may then damage proteins, lipids, and DNA, ultimately culminating in cell death and optic neuropathy.31,32

Clearly, our experimental model system of disease has no human counterpart. With the exception of the A16V mutation associated with idiopathic cardiomyopathy,\textsuperscript{33} no SOD2 polymorphisms have yet been linked to LHON or for that matter with any other diseases in which oxygen toxicity is suspected (amyotrophic lateral sclerosis [ALS], juvenile Parkinson's disease, familial dependent diabetes mellitus [IDDM]). However, at least two polymorphisms in the nuclear-encoded SOD2 may affect mitochondrial SOD activity.\textsuperscript{34–36} Polymorphisms in the targeting sequence that directs the import of the MnSOD into mitochondria from the cytoplasm may diminish SOD levels in mitochondria. The lowered levels of mitochondrial SOD may reduce protection against endogenous levels of superoxide, and cellular injury inducing optic neuropathy may ensue. This scenario may be somewhat analogous to our mouse model of ROS injury.

The selective vulnerability of the optic nerve to mutated complex I genes in LHON remains a mystery.\textsuperscript{26,37} It is likely to continue to be so until an animal model with a mutated complex I gene, encoded by mtDNA, is developed for study. We must emphasize that our mouse model of optic neuropathy was not created by manipulating mtDNA, but rather by reducing expression of a nuclear-encoded mitochondrial gene, SOD2. Currently, the technology to generate a mitochondrial disease model of optic neuropathy by the direct introduction of an artificial gene (or a ribozyme gene) into mitochondria does not exist.\textsuperscript{37,38} However, if ROS play a pivotal role in the pathogenesis of LHON, then perhaps one factor contributing to the vulnerability of the optic nerve to ROS injury may be lower levels of SOD2 expressed in the optic nerve compared with other body tissues and organs. This makes the optic nerve the bodily tissue most highly susceptible to ROS injury. However, the sparing of the retina compared with other organs in homozygous SOD knockout mice with no MnSOD in any bodily tissue may seem somewhat at odds with this hypothesis.\textsuperscript{11,12}

Clearly, without detailed knowledge of the mechanisms inducing visual failure in LHON, it is difficult to devise a strategy for treatment. If ROS play a pivotal role in development of optic neuropathy, then treatments to reduce oxygen toxicity may be beneficial in preventing or restoring visual function. At the present time, there is no treatment for LHON or, for that matter, any other mitochondrial disease.

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