Adenoviral Gene Therapy With Catalase Suppresses Experimental Optic Neuritis

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Objective: To determine if adenoviral-mediated transfer of the gene for catalase (CAT), the reactive oxygen species scavenger, suppresses experimental optic neuritis.

Clinical Relevance: Gene therapy with CAT delivered by an adeno-associated viral vector was previously shown to suppress experimental optic neuritis. Because the transduction of protein expression with recombinant adeno-associated viral vector is relatively slow, taking weeks to reach full levels, we studied the effects of replication-deficient adenovirus containing CAT in suppressing experimental optic neuritis. Transduction with adenovirus occurs within days of inoculation; thus, it may be more applicable for the treatment of patients with acute optic neuritis.

Materials and Methods: Replication-deficient adenovirus containing CAT was injected above the right optic nerve heads of SJL/J mice that were simultaneously sensitized for experimental allergic encephalomyelitis. For controls, the left eyes were injected with the replication-deficient adenovirus without CAT or no virus. The histological effects of CAT on the lesions of experimental allergic encephalomyelitis were measured by computerized analysis of the myelin sheath area (for demyelination), optic disc area (for optic nerve head swelling), the extent of the cellular infiltrate, extravasated serum albumin labeled with immunogold (for disruption of the blood-brain barrier), and the in vivo hydrogen peroxide reaction product.

Results: After 1 month, cell-specific catalase activity, evaluated by the quantitation of catalase immunogold, was increased about 2-fold each in endothelia, oligodendroglia, astrocytes, and axons of the CAT-inoculated right optic nerves compared with the control left optic nerves. The increased cellular levels of catalase reduced demyelination by 30%, optic nerve head swelling by 25%, cellular infiltration by 26%, disruption of the blood-brain barrier by 61%, and in vivo levels of hydrogen peroxide by 81%.

Conclusions: Adenoviral-mediated gene transfer increased catalase levels in all optic nerve cell types, and it persisted for 1 month after inoculation. The increased cellular levels of catalase suppressed demyelination and blood-brain barrier disruption at the foci in the optic nerve where prior magnetic resonance imaging and histopathologic studies have demonstrated the demyelinating inflammation of experimental and human optic neuritis. Together, they suggest that gene therapy with CAT may be helpful in the treatment of patients with optic neuritis.


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REACTIVE oxygen species (ROS) are mediators of demyelination and disruption of the blood-brain barrier (BBB). Reactive oxygen species include superoxide and nitric oxide, released by infiltrating inflammatory cells, and their metabolites hydrogen peroxide (H$_2$O$_2$), peroxynitrite, and hydroxyl radical. The role these ROS play in altering the permeability of the BBB and demyelination has been inferred from the beneficial effect of ROS scavengers on the clinical deficits and histopathologic lesions associated with experimental allergic encephalomyelitis (EAE), a frequently used animal model for multiple sclerosis (MS). Scavengers of ROS include catalase and superoxide dismutase. The latter dismutates superoxide to H$_2$O$_2$, and catalase detoxifies the H$_2$O$_2$ to nontoxic water and molecular oxygen.

Endogenous levels of these ROS scavengers in the optic nerve and brain are inadequate to protect these central nervous system tissues against ROS-induced injury in EAE. Increasing catalase levels by the exogenous administration of this enzyme reduces disruption of the BBB and the demyelination of experimental optic neuritis. Catalase, however, is a protein that must be administered by daily injections, even with the conjugation of polyethylene glycol to prolong the half-life of
MATERIALS AND METHODS

RECOMBINANT ADENOVIRUS

The replication-deficient adenovirus (Ad) containing the human gene for catalase (CAT) and the vector without the catalase gene were provided by Ron Crystal, MD (New York Hospital–Cornell Medical Center, New York), and constructed as previously described. The CAT complementary DNA was under the control of the adenovirus major late promoter. This construct was used to transfect human 293 cells (human embryonic kidney cells), and the resulting Ad-CAT virus was harvested, purified, and concentrated to a titer of $1 \times 10^{10}$ infectious plaque-forming units per milliliter using standard procedures.

INDUCTION OF EAE AND INTRAOCULAR INJECTIONS

Experimental allergic encephalomyelitis was induced in 20 SJL/J mice, after they were sedated with methoxyflurane (Metofane; Pitman-Moore Inc, Terre Haute, Ind), by sensitization with homologous spinal cord emulsion in Freund complete adjuvant (Difco Laboratories, Detroit, Mich), which was injected subdermally into the nuchal area. While the animals were under sedation, a 32-gauge needle attached to a Hamilton syringe was inserted through the pars plana. The needle tip was visualized in the vitreous with the use of the indirect ophthalmoscope, and it was positioned directly over the optic nerve head. Then 5 µL of Ad-CAT was injected into the vitreous of the right eyes of SJL/J mice, after they were sedated with methoxyflurane.

RESULTS

CELLULAR LEVELS OF CATALASE

A prerequisite for demonstrating CAT-mediated suppression of EAE is the presence of increased levels of intra-cellular catalase in transduced tissues. No differences in catalase activity were seen between the control left eyes that received either the adenovirus injection without CAT or the left eyes that received no viral injection. One month after a single ocular injection of recombinant adenovirus, the levels of catalase immunogold in transduced right optic nerves from animals with EAE were significantly increased compared with the contralateral control nerves; $P < .01$. These cell types had the highest levels of CAT transcription. Catalase immunogold labeling was also significantly increased in axons by 1.95-fold (115 ± 29 vs 70 ± 10 for the contralateral control nerves; $P < .01$) and in astrocytes (2.32-fold, 149 ± 6 vs 64 ± 5; $P < .01$). These cell types had the highest levels of CAT expression within days of inoculation of the recombinant virus.

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bulbar nerve were stained with toluidine blue for light-microscopic examination. Ultrathin sections (90 nm) were placed on nickel grids for immunohistochemical analysis.

Non-specific binding of antibodies was blocked by floating the grids on either 5% normal goat serum in triethanolamime-buffered sodium (pH 7.2), 0.01 mol/L, with polysorbat 20 for 30 minutes for catalase immunostaining, or 2% teleost gelatin and 2% nonfat dry milk in triethanolamime-buffered sodium (pH 7.2), 0.01 mol/L, with polysorbat 20 for 30 minutes for albumin immunostaining. They were then reacted with rabbit anti-CAT antibodies or with rabbit anti-albumin antibodies, respectively, in the same buffer for 2 hours at room temperature. After washes in phosphate-buffered sodium, 0.1 mol/L, the grids were reacted with the secondary goat antirabbit IgG antibodies conjugated to 10 nm of gold for 1 hour at room temperature. After washes in buffer, grids were rinsed in deionized water. For examination by low-magnification transmission electron microscopy, the immunogold particles were enlarged by silver enhancement using a kit (Ted Pella), according to the manufacturer’s specifications. To check for non-specific binding of the secondary antibody, control grids were incubated in the buffer, followed by the gold-labeled antibody. Immunolabeled and control specimens were photographed by transmission electron microscopy without poststaining.

MORPHOMETRIC ANALYSIS

Morphometric analysis was performed in a masked manner, as previously described. Briefly, 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 were manually traced using the National Institutes of Health (Bethesda, Md) image software and a computer (Macintosh; Apple Computer, Inc, Cupertino, Calif). The number of glial cells and inflammatory cells in the retrolbulbar optic nerve were also quantitated by thresholding of the darker staining cell nuclei. Cell-specific catalase activity and extravasated serum albumin immunogold were similarly quantitated. The immunolabeled sections were examined without poststaining using a transmission electron microscope (H-7000; Hitachi Ltd, Tokyo, Japan) operating at 75 kV. Photographs were made at a magnification of ×2500. Ten micrographs of each cell type were taken of each optic nerve. The negatives were digitized into computer memory using a scanner (Umax; Umax Data Systems, Fremont, Calif). Silver-enhanced immunogold particles and H2O2 reaction products were enlarged to a final magnification of ×7500, thresholded, and counted with the software and computer system. Cell-specific catalase activity was quantitated by counting the number of silver-enhanced immunogold particles in endothelial cells, astroglial cells, oligodendroglial cells, axons, and microglial cells. Values were expressed as the mean ± SEM for each cell type. Mean particle counts for each nerve were obtained by taking the mean value of the 10 micrographs. Each mean value was expressed as the number of particles per unit area. The extent of demyelination was quantitated by threshold measurements of the myelin sheaths that were derived from the axonal micrographs for each optic nerve. Increases in the myelin sheath area (less demyelination) thereby indicated a beneficial treatment effect. Grouped t tests were used to assess differences in the myelin areas, optic nerve head areas, optic nerve cell counts, and immunogold and H2O2 particle counts between the CAT-transduced right eyes and the control left eyes and between the left eyes injected with the empty adenovirus and the left eyes that received no ocular injection.

DEMYELINATION

In experimental optic neuritis, loss of the myelin sheaths that envelop axons is a hallmark of the histopathologic features at the ultrastructural level. Transmission electron microscopy of the optic nerve revealed that all animals sensitized for EAE exhibited foci of demyelination, naked axons, and axons enveloped by thin sheaths of myelin that were suggestive of remyelination. Mononuclear inflammatory cells and reactive astroglial cells comprised the optic nerve cellular infiltrate that predominantly involved the retrolbulbar optic nerve. We found no evidence of myelin injury induced by the intravitreal injection. The left eyes that received the empty adenovirus had a mean myelin area of 26.0 ± 1.5 × 10^3 μm² vs 25.0 ± 0.6 × 10^3 μm² for uninjected left eyes (P > 0.5). Indicative of the suppression of demyelination by Ad-CAT delivery, however, CAT-inoculated optic nerves had 30% more myelin (less demyelination), with a mean myelin area of 37.0 ± 2.0 × 10^3 μm² vs 26.0 ± 1.5 × 10^3 μm² (P < 0.01) for the control left eyes that received the empty Ad (Figure 1, B). Figure 2 shows representative transmission electron micrographs of the optic nerve inoculated with Ad-CAT having less demyelination (Figure 2, C) than the controls (Figure 2, D). Therefore, gene transfer of catalase achieved therapeutic protection from EAE-induced demyelination.

OPTIC DISC EDEMA

Optic disc edema, seen in about 40% of patients with acute optic neuritis, was evident in animals with EAE. Lateral displacement of the peripapillary retina and filling of the optic cup indicated optic disc edema at the light-microscopic level. The peripapillary retinas of SJL/J mice that are highly susceptible to the induction of EAE also showed a genetically induced degeneration of photoreceptors, with the outer nuclear layer reduced to a single cell layer that was symmetric between the right and left eyes. Ultrastructurally, intracellular edema of unmyelinated axons contributed to the optic nerve head swelling. These histopathologic features were seen to some degree in both CAT-transduced nerves and contralateral control nerves. In addition, we found no evidence of glaucomatous injury. There was no cupping, smaller optic nerve head areas, induced by the transient rise of intraocular pressure following the intravitreal injection. The left eyes that received the empty adenovirus had a
mean optic nerve head area of 4.2 ± 0.2 × 10⁴ µm² vs 4.2 ± 0.2 × 10⁴ µm² for un.injected left eyes (P > .05). On the other hand, CAT delivery by adenovirus reduced optic disc edema by 25%, with a mean optic head nerve area of 3.2 ± 0.3 × 10⁴ µm² vs 4.2 ± 0.2 × 10⁴ µm² for the control left eyes that received the empty adenovirus (Figure 1, C). These differences were significant (P < .05). Thus, EAE-induced swelling of the optic nerve head was reduced by CAT inoculation.

**OPTIC NERVE CELL COUNT**

For all groups, light-microscopic evaluation of the myelinated segment of the optic nerve, commencing just posterior to the lamina scleralis, revealed foci of inflammatory cells and reactive astroglial cells. Comparisons of the control left eyes that received the adenovirus inoculation without CAT had a mean optic nerve cell count of 218 ± 16 cells × 10⁵ µm² vs 211 ± 22 cells × 10⁵ µm² for the left eyes that received no viral inoculation. This difference was not significant, thereby suggesting that adenovirus did not increase the inflammatory response in the EAE nerve. However, Ad-CAT inoculation reduced the optic nerve cell count by 26% to a mean value of 161 ± 15 cells × 10⁵ µm² vs 218 ± 16 cells × 10⁵ µm² for the control left eyes that received the empty adenovirus (Figure 1, D). These differences were significant (P < .05).

**BBB DISRUPTION**

Disruption of the BBB, a hallmark of both experimental and human optic neuritis, was seen in all animals sensitized for EAE. In vivo evaluation of the BBB by contrast-enhanced magnetic resonance imaging reveals enhancement of the optic nerve in most patients with acute optic neuritis and in all animals with acute EAE. A standard marker of BBB disruption is the extravasation of serum albumin, which is detected by immunolabeling. Transmission electron microscopy of the optic nerves revealed albumin immunogold labeling in all animals with EAE. Extravasated albumin immunogold in the perivascular compartment located the foci of BBB disruption in EAE. Albumin immunogold confined to the intravascular compartment indicated normal integrity of the BBB. Comparisons of the control left eyes that received the adenovirus inoculation without CAT showed a mean of 656 ± 121 extravasated immunogold particles per 2.6 × 10⁵ µm² compared with 540 ± 93 particles per 2.6 × 10⁵ µm² for the left eyes that received no viral inoculation. Although this difference was not significant (P > .05), it showed a trend suggesting that adenovirus itself may increase BBB disruption in the EAE optic nerve. On the other hand, adenovirally delivered CAT reduced disruption of the
BBB by 61% to a mean value of 256 ± 39 extravasated immunogold particles per 2.6 × 10⁶ µm² compared with 656 ± 121 particles per 2.6 × 10⁶ µm² for the control left nerves that received the empty adenovirus (Figure 1, E). These differences were significant (P < 0.05). Representative transmission electron micrographs of the optic nerve inoculated with Ad-CAT show less extravasated serum albumin (Figure 2, E) than the control left optic nerves, where a marked accumulation of extravasated albumin immunogold in the perivascular space is evident (Figure 2, F). Therefore, CAT inoculation markedly improved BBB integrity.

H₂O₂ REACTION PRODUCT

The perfusion of animals with cerium chloride forms an electron-dense precipitate, cerium perhydroxide, in the presence of endogenously generated H₂O₂.9,10 This reaction product was seen predominantly in a perivascular distribution in animals with EAE. It was also seen along the apical processes of endothelial cells in normal, unsensitized animals. In the interstitial optic nerve of animals with EAE, the reaction product also surrounds infiltrating inflammatory cells. Decreased in vivo levels of H₂O₂ were seen with Ad-CAT inoculation. Mean particle counts in the optic nerve head were reduced by...
81% in CAT-inoculated nerves to a mean of 11 ± 6 particles per 2.6 × 10^6 µm² vs 59 ± 21 per 2.6 × 10^6 µm² for the control nerves that received the empty adenovirus (P < .05) (Figure 1, F). In the retrolubar optic nerve, reaction product counts were reduced by 65% to a value of 81 ± 35 with CAT inoculation vs 231 ± 70 for control nerves that received the empty adenovirus (P > .05). In the optic nerve sheath, particle counts were reduced 52% to a mean of 433 ± 96 with catalase inoculation vs 900 ± 141 in the control nerves that received the empty adenovirus (P < .05). Figure 2 shows representative transmission electron micrographs of the optic nerve head inoculated with Ad-CAT exhibiting less H₂O₂-derived reaction product (Figure 2, G) than the control nerves (Figure 2, H).

**COMMENT**

Gene delivery and expression have been demonstrated in many mammalian tissues, including retina, neural tissues, and endothelial cells, but to our knowledge, only 2 other reports describe gene transfer to the optic nerve. Structural injury to oligodendroglial cells and dysfunction of endothelial cell permeability lead to demyelination and disruption of the BBB, which are the predominant pathogenic tissue alterations of optic neuritis, EAE, and MS. We found that the viral promoters (adenovirus or cytomegalovirus) drove the transgene expression and the suppressive effects of CAT expression needed for optic nerve protection by catalase may also promote remyelination by oligodendroglia. Thus, the restoration of BBB integrity is an important first step in limiting the pathologic effects of EAE. The adeno viral-mediated doubling of catalase levels in each of these important optic nerve cell types. Although the peripapillary retinas of EAE-susceptible SJL/J mice also showed a genetically induced degeneration of photoreceptors, the retinal structure was symmetric between the right and left eyes, and the ultrastructure of the optic nerves appeared normal. Consequently, the photoreceptor abnormality played no role in the differences in optic nerve morphometric measurements obtained between CAT-injected right eyes and control left eyes. The increased cellular levels of catalase protected against ROS-induced optic nerve injury in the EAE animal model of MS.

Endothelial cells comprising the BBB are the first line of defense against mediators of EAE injury to myelin and oligodendroglia. Thus, the restoration of BBB integrity is an important first step in limiting the pathologic effects of EAE. The adeno viral-mediated doubling of catalase levels in endothelial cells suppressed the disruption of the BBB by 61%. This restoration of BBB integrity might also have a suppressive effect on EAE by restricting not only H₂O₂ but also other ROS mediators of damage from access to the optic nerve. Hydrogen peroxide is a strong oxidant that can diffuse from the sites of generation in the perivascular space and induce peroxidation of myelin and oligodendroglia at remote sites in the interstitial optic nerve. Oligodendroglia are particularly vulnerable to the effects of H₂O₂. This cell type suffers the greatest injury in both EAE and MS, culminating in the classic demyelinating response. Reductions in perivascular ROS, coupled with the viral transduction of 2-fold increases in cellular levels of catalase in oligodendroglia, partially protected these important cells from the adverse effects of H₂O₂ released into the microenvironment by the inflammatory process, thereby reducing demyelination by 30%.

It was somewhat surprising to find that transgene expression and the suppressive effects of CAT gene transfer on experimental optic neuritis with adenovirus were comparable to those seen with AAV-meditated gene transfer when studied 1 month after inoculation. One factor contributing to this result was that the adenoviral titer was 10³ times higher than that reported in a study using recombinant AAV. We are now able to routinely obtain comparably high viral titers with AAV. Whereas adenovirus has the theoretical advantage of faster cellular transduction, it has the disadvantage of inciting an inflammatory response that contributes to short-lived cellular transduction, often lasting 2 weeks. Comparisons of the optic nerve cell counts between the control left eyes that received the adenovirus inoculation without CAT and the control left eyes that received no viral inoculation were comparable, and they showed no significant differences, thus suggesting that adenovirus did not substantially increase the inflammatory response in the EAE-induced optic nerve. Nevertheless, transgene expression with adenoviral vectors incites inflammation in normal tissues, and it is undetectable 2 months after inoculation. Adenovirus vectors, however, will persist longer in animals that do not mount an effective inflammatory response. Persistent adenoviral transduction is impaired by immune mediators such as nitric oxide that are generated by the inflammatory response induced by adenovirus. Reductions in inflammation induced by ROS scavenging with catalase may prolong the duration of expression of this transgene product in EAE-affected optic nerves 1 month after adenoviral inoculation.

Unlike adenovirus, AAV does not incite an inflammatory response; thus, it has provided long-term transgene expression for as long as 1½ years. For this reason, AAV may be the vector best suited for long-term transgene expression needed for optic nerve protection against future ROS injury by the recurrence of optic neuritis. The comparatively small size (21 nm) of the AAV particle, however, limits the size of packaged genes for transfer with AAV to about 4.5 kilobases (kb). Although this presented no problem for insertion of the 2-kb CAT, the insertion of larger gene(s), such as the myelin basic protein (MBP), its promoter, or both, is too long for incorporation into AAV. Transfer of the MBP gene has the potential to promote remyelination by oligodendroglia that persist in chronically demyelinated nerves, such as those of patients left with poor visual acuity 6 months or more after an attack of optic neuritis. The larger capacity of recombinant adenovirus may accommodate this relatively larger gene, whose transduction in patients blinded by optic neuritis may improve their level of visual function. This newly formed myelin should persist in these chronically demyelinated optic nerves because the inflammatory response has long since subsided. In demyelinated optic nerves with active inflammation, however, ROS scavenging by catalase may also promote remyelination by limiting the damage of myelin basic protein in impaired but not destroyed oligodendroglia. Our work proves that either viral vector—adenovirus or AAV—may be used to transfer small genes such as CAT to suppress...
demetylination and perhaps promote remyelination. Because many advances in therapy for MS were first tested in the EAE animal model,31,42-45 our findings of the suppression of experimental optic neuritis with CAT gene transfer suggests that this form of therapy may be useful in patients with acute optic neuritis.

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