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Adenovirus-Mediated Delivery of Catalase to Retinal Pigment Epithelial Cells Protects Neighboring Photoreceptors from Photo-Oxidative Stress

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Abstract

Oxidative stress is involved in the pathogenesis of many diseases. Overexpression of antioxidant enzymes by gene therapy may protect tissues from oxidative damage. Because the reactive oxygen species hydrogen peroxide can diffuse across cell membranes, we hypothesized that overexpression of the antioxidant catalase within certain cells might protect neighboring cells. To test this hypothesis, we transduced retinal pigment epithelial (RPE) cells *in vitro* and *in vivo* with adenovirus carrying the catalase gene (Ad.CMV.catalase). After transduction of only a subset of RPE cells *in vitro*, all cells in the culture were protected from exogenous hydrogen peroxide. Similarly, *in vivo*, eyes injected with Ad.CMV.catalase had high catalase levels in the RPE, which protected the adjacent photoreceptors from light damage and reduced photoreceptor oxidative stress as measured by the markers 4-hydroxynonenal and nitrotyrosine. Both *in vitro* and *in vivo*, gene therapy with Ad.CMV.catalase protected neighboring cells from oxidative stress-induced terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) positivity. The data provide a paradigm for antioxidant gene therapy with catalase, designed to protect not only transduced cells, but also neighboring cells.

Introduction

Oxidative Stress is thought to result in cell death in a number of diseases. Gene therapy using adenovirus to deliver the antioxidant enzyme catalase has been shown to protect a variety of cell types from oxidative stress-mediated injury. Protected cells included those in the optic nerve (Guy et al., 1999), the cochlea (Kawamoto et al., 2004), the myocardium (Zhu et al., 2000), and the lung parenchyma (Danel et al., 1998). In the rodent retina, photo-oxidative stress-induced cell death can be ameliorated by administration of antioxidant compounds including *N*-acetylcysteine, dimethylthiourea, and ascorbate (Li et al., 1985;

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Lam et al., 1990; Tanito et al., 2002), but it is unknown whether overexpression of the antioxidant catalase would protect against photo-oxidative stress.

The retinal cells most damaged by photo-oxidative stress are photoreceptors, the sensory neurons for vision. The combination of high oxygen tension, focused light, and a high concentration of easily oxidized lipids causes these cells to be particularly susceptible to light damage. Similarly, in age-related macular degeneration (AMD), photoreceptor cell death may occur, at least partially, through a photo-oxidative stress-mediated pathway. This is supported by the finding that dietary antioxidants reduce the risk of developing advanced AMD (Age-Related Eye Disease Study Research Group, 2001). Also, long-term bright light exposure (BLE) increases oxidative stress and has been shown to change macular pigmentation and decrease visual acuity in humans (Marlor et al., 1973; Young, 1988).

One potent reactive oxygen species is hydrogen peroxide (H_2O_2), which can react with iron in the Fenton reaction to produce the highly reactive and damaging hydroxyl radical. After BLE, as photoreceptor damage progresses, H_2O_2 levels increase in photoreceptors (Yamashita et al., 1992), suggesting that this reactive oxygen species may contribute to BLE pathogenesis. Levels of glutathione peroxidase, an enzyme that can degrade H_2O_2, also increase in photoreceptors after BLE (Ohira et al., 2003).

The enzyme catalase catalyzes the breakdown of H_2O_2. It is localized to intracellular peroxisomes and is found at high concentrations in the liver (Bai et al., 1999). In the retina, it is located in the inner segments of rat photoreceptors and retinal pigment epithelial (RPE) cells (Atalla et al., 1987). To determine whether increased catalase levels might protect photoreceptors from BLE-induced damage, catalase was overexpressed in the retinas of BLE-exposed animals through somatic gene transfer via adenovirus.

Because H_2O_2 can readily diffuse across cell membranes (Ohno and Gallin, 1985), we hypothesized that overexpression of catalase in the RPE might protect the retina by increasing degradation of H_2O_2 produced by BLE in the nearby photoreceptors. The laminar retina provides an excellent opportunity to test this hypothesis. Our results demonstrate that overexpression of catalase in the RPE protects photoreceptors from BLE.

Materials and Methods

Adenovirus amplification and purification

The Ad.CMV.catalase virus was created and produced as described (Woo et al., 1998). Briefly, 293 cells were infected with 10^{10} particles of Ad.CMV.catalase and incubated for 40 hr at 37°C, collected, and centrifuged. The pellet was resuspended in 10 m*M* Tris-HCl (pH 8.1), freeze-thawed three times, and centrifuged. The supernatant was added to 5 plates and the process was repeated until Ad.CMV.catalase had been harvested from fifty 150-mm plates. After Ad.CMV.catalase was purified by CsCl gradient centrifugation, the concentration was determined by spectrophotometry at 260 nm. Ad.CMV.GFP (Zolotukhin et al., 1996) and Ad.CMV.lacZ (Lemarchand et al., 1992) were amplified as described above.

Viral infection of RPE cells *in vitro*

Human adult retinal pigment epithelium (ARPE-19) cells (Dunn et al., 1996) were plated on Nunc (Naperville, IL) 8-well (1 cm^2/well) glass chamber slides at 5×10^4 cells/cm^2. Twenty-four hours later, cells at 90% confluence were infected with adenoviral vector. Infection with 5×10^4 viral particles resulted in transduction of 1% of cells, as determined by green fluorescent protein (GFP) fluorescence or catalase immunocytochemistry. Infection of 10% of cells was achieved by infecting with 5×10^5 particles/well, and 100% of cells with 5×10^6 particles. For the catalase enzymatic assay, 100% of cells were transduced and the catalase assay was performed 24 hr after infection.

Catalase activity

Catalase activity in RPE cells was measured as described (Cohen et al., 1970). Briefly, cells were scraped and sonicated in buffer and centrifuged for 10 min at 10,000 × *g*. Ethanol was added to the supernatant at a 1:100 dilution and incubated for 30 min on ice. Triton X-100 was added at a 1:10 dilution and incubated for 5 min on ice. Duplicate 50-μl aliquots were used for the remainder of the assay. A 500-μl volume of fresh H_2O_2 was added to the aliquots and incubated for 3 min on ice. The reaction was stopped by addition of 100 μl of H_2SO_4. Immediately, 700 μl of $KMnSO_4$ was added and the absorbance at 480 nm was determined by spectrophotometry. Homogenized liver was used as a positive control. Results were normalized to protein concentration, using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL), to obtain the final units of catalase activity (*y* axis of Fig. 2).

Hydrogen peroxide exposure

Cells were incubated for 16 hr in 500 μM H_2O_2 in Hanks' balanced salt solution (HBSS). Cells in control wells were incubated in HBSS. Cells were then fixed in 4% paraformaldehyde for 10 min.

In vivo adenovirus injections

All procedures adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Pennsylvania (Philadelphia, PA) Animal Care and Use Committee. Thirty-six hours before BLE, Ad.CMV.catalase was delivered into the right eye, and the control vector, Ad.CMV.lacZ, into the left eye of each mouse via a transscleral transchoroidal approach as previously described (Bennett et al., 1994). Briefly, after anesthesia, a 30-gauge cannula was inserted into the subretinal space of the temporal peripheral retina, and 1 μl of virus (5×10^9 particles) was injected. For *in vivo* assessment of adenoviral transduction, mice were given a subretinal injection of Ad-GFP and fundus photography was performed 36 hr later with a Kowa (Tokyo, Japan) camera with a blue excitation filter as described (Bennett et al., 1997).

Retina X-Gal staining

Ad.CMV.lacZ-injected eyes were evaluated for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. Enucleated eyes were immediately placed into phosphate-buffered 4% paraformaldehyde, pH 7.4, for 2 hr at 4°C. The eyes were then

rinsed in phosphate-buffered saline (PBS) and incubated in X-Gal solution [10 m*M* $K_3Fe(CN)_6$, 10 m*M* $K_4Fe(CN)_6 \cdot 3H_2O$, 2 m*M* $MgCl_2$, 0.01% $C_{24}H_{39}O_4Na$ (deoxycholate), 0.02% NPO_4 in PBS] overnight at 37°C. Each eye was then rinsed in PBS, embedded in O.C.T. medium, and cryosectioned according to previously published methods (Dorn et al., 1995).

Bright light exposure

Male BALB/c mice (Jackson Laboratory, Bar Harbor, ME), 2–3 months of age, were reared with a 12-hr lightdark cycle. Thirty-six hours before BLE, Ad.CMV.catalase was delivered into the right eye, and the control vector, Ad.CMV.lacZ, into the left eye of each mouse. No-BLE (control) mice were maintained in room light (200 lx) before euthanasia (12 eyes). The experimental groups were exposed to 8 klx of cool white fluorescent light in a well-ventilated, air-conditioned room for 7 hr, from 2 to 9 A.M. The mice were housed in clear Plexiglas cages, were allowed to roam freely, and were given free access to food and water. Outside the cages, arrayed on the top and sides, were 12 fluorescent lights (4 ft long, 40 W) (F4ORES; General Electric, Cleveland, OH). Mice were killed 24 hr after the end of light exposure for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis and immunolabeling.

TUNEL and immunohistochemistry analysis

After fixation, TUNEL analysis was performed with an *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN). Immediately after TUNEL labeling, immunohistochemistry was performed as described (Dunaief et al., 2002). Chamber slides were blocked with normal donkey serum (diluted 1:100) in PBS containing 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBT) for 1 hr at room temperature. Cells were then exposed to anti-catalase (diluted 1:200; Athens Research & Technology, Athens, GA) for 2 hr at room temperature followed by a fluorophore-labeled secondary antibody and mounted with 4′,6-diamidino-2-phenylindole (DAPI) to counterstain nuclei blue. Cells were analyzed by fluorescence microscopy with a Nikon TE-300 microscope (Nikon, Tokyo, Japan) and SpotRT Slider camera (Diagnostic Instruments, Sterling Heights, MI) with Image-Pro Plus software, version 4.1 (MediaCybernetics, Silver Spring, MD).

For TUNEL analysis, enucleated eyes were immediately placed into phosphate-buffered 4% paraformaldehyde, pH 7.4, for 2 hr. The eyes were embedded and cryosectioned according to previously published methods (Milam et al., 1993) and frozen sections were labeled with the TUNEL kit described above. TUNEL-positive nuclei were counted in 15 sections per eye, cut in the sagittal plane at 100-μm intervals spanning nasal and temporal sides of the optic nerve, thus including both transduced and nontransduced portions of the eye. The number of TUNEL-positive cells was compared between experimental animals and controls, using the Student *t* test (Williams and Howell, 1983).

For retinal immunohistochemistry, cryosections were blocked in normal donkey serum and then labeled with anti-4-hydroxy-2-nonenal (HNE) (diluted 1:10,000; Alpha Diagnostic International, San Antonio, TX), anti-catalase (diluted 1:200; Athens Research & Technology), or anti-nitrotyrosine (5 μg/ml; Upstate Cell Signaling Solutions, Lake Placid,

NY) in PBT overnight at 4°C. Before incubation with anti-nitrotyrosine, antigen retrieval was performed. Sections were microwaved in 10 m*M* sodium citrate, pH 6.0. Sections were rinsed with PBS, incubated in Cy3-conjugated donkey anti-rabbit IgG (diluted 1:200 in PBT) for 1 hr, rinsed in PBS, and mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). The labeled sections were viewed with a laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Results

We first tested the ability of the adenoviral vector, Ad. CMV.catalase (Woo et al., 1998), to transduce an RPE cell line (ARPE-19). Immunocytochemistry revealed increased anti-catalase label in cells infected with Ad.CMV.catalase compared with uninfected cells (Fig. 1). Consistent with this, cells infected with Ad.CMV.catalase had 10-fold higher catalase activity than controls ($p < 0.001$) (Fig. 2). Infection with lower titer virus resulted in lower catalase activity. The highest levels of RPE catalase activity achieved after high-titer Ad.CMV.catalase infection were similar to the levels of endogenous catalase activity in the positive control extract of liver, a catalase-rich tissue.

To determine whether Ad.CMV.catalase can protect these cultured RPE cells from H_2O_2-induced damage, we first infected ARPE-19 cells with Ad.CMV.catalase and then assessed their ability to survive H_2O_2 exposure. In contrast to uninfected (Fig. 3A) or Ad.CMV.GFP-infected cells (Fig. 3B), cells infected with Ad.CMV.catalase (Fig. 3C) were completely negative for TUNEL, an indicator of DNA fragmentation associated with cell death. TUNEL-positive cells in the uninfected and Ad.CMV.GFP-infected wells had condensed nuclei, consistent with the nuclear condensation that occurs during apoptosis. There were no visible changes in Ad.CMV.catalase-infected cells after H_2O_2 exposure. GFP fluorescence decreased in Ad.CMV.GFP-infected cells after H_2O_2 exposure.

To determine whether transduction of only a portion of the RPE cells would result in a bystander effect, ARPE-19 cells were infected with an Ad.CMV.catalase titer sufficient to transduce approximately 10% of the cells, as determined by counting cells labeled by anti-catalase immunocytochemistry (Fig. 3C and F). Similar to wells in which 100% of RPE cells were transduced (not shown), these wells showed no TUNEL-positive cells after H_2O_2 exposure. This result indicates that, *in vitro*, catalase-overexpressing cells can protect their neighbors from oxidative stress. The ability of transduced cells to protect their neighbors had limits, as infection with higher titers of Ad. CMV.catalase was required to protect RPE cells from higher concentrations of H_2O_2 (not shown).

Because *in vitro* data suggested that catalase-overexpressing cells can protect neighboring cells, we tested whether this bystander effect would occur *in vivo*. We first examined the pattern of transduction after subretinal injection of adenovirus vectors. At 36 hr after Ad.CMV.GFP injection, *in vivo* retinal photographs revealed transduction of nearly half of the retina on the temporal side, where the Ad.CMV.GFP was injected subretinally (Fig. 4A). After Ad.CMV.catalase or Ad.CMV.lacZ injection, serial sections immunolabeled with anti-catalase or stained with X-Gal revealed a similar spatial pattern of catalase and LacZ overexpression. At the level of retinal microarchitecture, the catalase overexpression was

limited to the RPE (Fig. 4B). In the Ad.CMV.lacZ-injected (Fig. 4C) and uninjected eyes there was little catalase label. When the gain was increased in these photomicrographs (higher than shown), there was a low level of catalase label in the RPE, as previously reported (Atalla et al., 1987). Our immunohistochemistry was not sensitive enough to detect the previously reported photoreceptor catalase (Atalla et al., 1987), but was able to detect strong signal in the RPE transduced by Ad.CMV.catalase (Fig. 4B). X-Gal staining demonstrated the expression of LacZ in the RPE of Ad.CMV.lacZ-injected eyes (Fig. 4D).

To examine the protective, bystander effect of Ad.CMV.catalase in BLE-exposed mouse retinas, mice were first treated with a subretinal virus injection, followed by BLE. Twenty-four hours after the end of BLE, TUNEL-positive photoreceptor nuclei were detected in uninjected eyes. A similar number of TUNEL-positive photoreceptor nuclei was present in Ad.CMV.lacZ-injected retinas (Figs. 4C and 5). However, the number of TUNEL-positive photoreceptor cells decreased 5-fold when Ad.CMV. catalase was injected 36 hr before the onset of BLE (Figs. 4B and 5). This difference was significant ($p < 0.001$) for two distinct experiments studying a total of 26 retinas. Eyes injected with Ad.CMV.catalase or Ad.CMV.lacZ but not exposed to BLE had no TUNEL-positive photoreceptors.

The mechanism of Ad.CMV.catalase-mediated photoreceptor protection from BLE is most likely reduction of oxidative stress. To determine whether Ad.CMV.catalase reduced oxidative stress, we labeled retinas for the oxidative stress markers 4-hydroxy-2-nonenal (HNE) and nitrotyrosine. HNE is formed as a result of hydroxyl radical-induced lipid peroxidation. The newly formed HNE then forms covalent cross-links with proteins (Esterbauer et al., 1991). Nitrotyrosine also results from free radical-mediated protein modification. In the normal retina there was minimal anti-HNE or nitrotyrosine labeling (Fig. 6A and D). In BLE, Ad.CMV.lacZ-injected retinas there was anti-HNE and anti-nitrotyrosine labeling of the photoreceptor inner and outer segments (Fig. 6B and E). Anti-HNE also labeled projections in the outer plexiform and inner nuclear layers (Fig. 6B). Labeling of BLE, Ad.CMV.catalase-injected retina was markedly reduced (Fig. 6C and F), consistent with a reduction of oxidative stress by catalase overexpression.

Discussion

This study demonstrates that Ad.CMV.catalase reduces BLE-induced photoreceptor oxidative stress and DNA nicking. Eyes injected subretinally with Ad.CMV.catalase had reduced levels of the oxidative stress markers HNE and nitrotyrosine and fewer TUNEL-positive photoreceptors than those injected with Ad.CMV.lacZ. Because recombinant adenovirus targets RPE primarily after subretinal injection (Bennett et al., 1994; Li et al., 1994), the reduced HNE and nitrotyrosine labeling in the Ad.CMV.catalase-injected eyes demonstrates that overexpression of catalase in the RPE was sufficient to block at least a portion of the BLE-induced lipid peroxidation and protein modification in photoreceptors. Because H_2O_2 is the catalase substrate, these results implicate H_2O_2 as a mediator in BLE-induced photoreceptor cell death.

Catalase overexpression in this study was directed to RPE cells, which indicates that catalase need not be overexpressed in photoreceptors in order to protect them. Because

catalase is not secreted and H_2O_2 can diffuse across membranes, it is possible that H_2O_2 produced by BLE in photoreceptors diffuses down a concentration gradient to the RPE cells overexpressing catalase. Evidence of this mechanism is provided by our *in vitro* experiments. RPE cells were infected with Ad.CMV.catalase, causing catalase overexpression in 10% of the cells. Yet, all the cells in the well were protected from H_2O_2-mediated death. It is also possible that catalase directly protects the RPE and that photoreceptor rescue results indirectly from preservation of RPE function. The former possibility seems more likely given that overexpression of catalase in the RPE reduces photore-ceptor HNE and nitrotyrosine levels.

It is possible that catalase overexpression could have deleterious effects in the retina. Several pieces of evidence suggest that this is unlikely. First, subretinal injection of Ad.CMV.catalase did not result in any TUNEL-positive retinal cells. Second, retinal morphology was normal 7 days after subretinal injection of Ad.CMV.catalase. Third, electroretinograms from Ad.CMV. catalase-injected mice were not significantly different from those of uninjected or Ad.CMV.lacZ-injected mice (not shown).

To our knowledge this is the first demonstration of retinal protection against light-induced retinal degeneration achieved by gene therapy. This study also demonstrates a protective bystander effect. This result provides a proof of principle for gene therapy designed to reduce oxidative stress in retinal degenerations and has important implications for retinal degenerations that are accelerated by light (Marlor et al., 1973; Young, 1988). Current clinical trials using antioxidant therapy have shown a decreased rate of retinal disease progression (Age-Related Eye Disease Study Research Group, 2001). The current study, unlike the Age-Related Eye Disease Study, which involved systemic administration of antioxidant molecules, involves a onetime delivery of high levels of the therapeutic reagent directly to the target tissue. Such an approach is likely to enhance the therapeutic effect and to minimize toxicity to other organ systems. The concept of H_2O_2 diffusing across cells to areas of high catalase levels provides a novel application of gene therapy, potentially applicable to a variety of organs.

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Overview Summary

This study tests the hypothesis that gene therapy with the antioxidant enzyme catalase protects not only the transduced cells, but also their neighbors. Because the reactive oxygen species, hydrogen peroxide, can diffuse across cell membranes, it is possible that it can diffuse down a concentration gradient to cells overexpressing catalase. To test this possibility, 10% of retinal pigment epithelial (RPE) cells *in vitro* were transduced with Ad.CMV.catalase, and cell death caused by hydrogen peroxide exposure was assessed by TUNEL labeling. The transduced cells protected their neighbors. Similarly, *in vivo*, Ad.CMV.catalase transduction of RPE cells protected the neighboring photoreceptors from photo-oxidative stress. These data provide evidence of a “bystander effect” resulting from gene therapy with Ad.CMV.catalase.

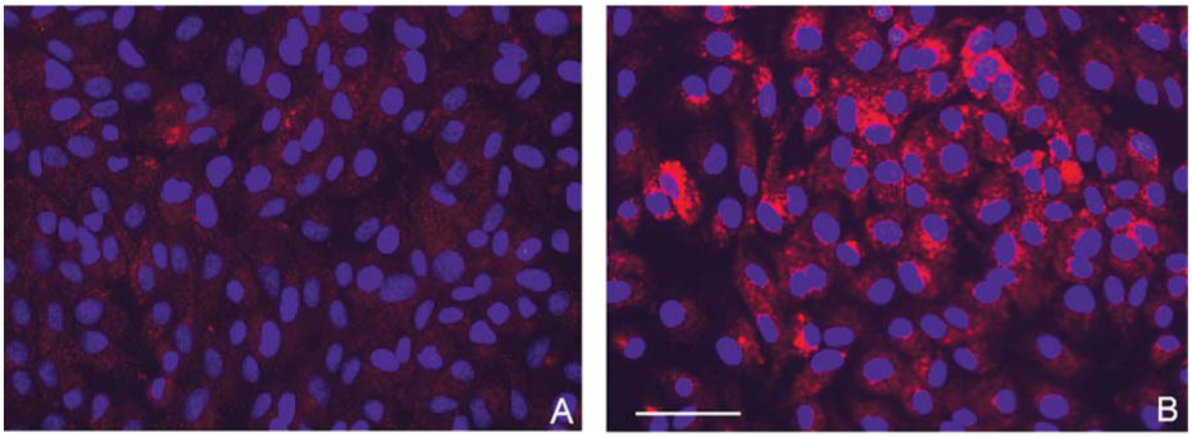

FIG. 1.

Ad.CMV.catalase infection of ARPE-19 cells results in catalase overexpression. Shown is fluorescence microscopy of cells infected with Ad.CMV.catalase and labeled with anti-catalase (red) and DAPI (blue). (**A**) Uninfected cells contain endogenous catalase. (**B**) Immunofluorescence is increased in Ad.CMV.catalase-infected cells. Scale bar: 50 μm.

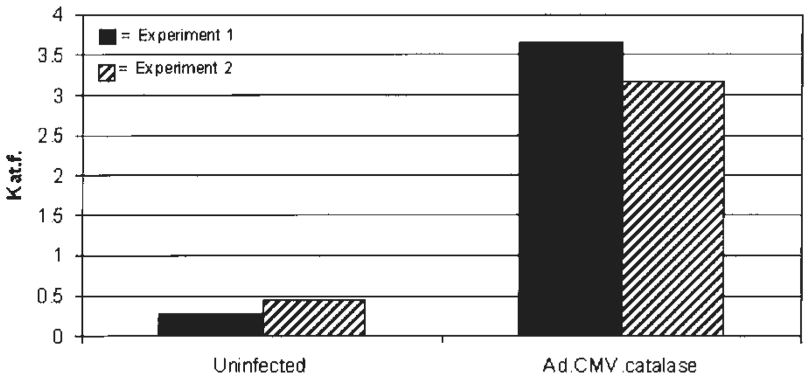

FIG. 2.

Quantitation of catalase activity in infected and uninfected ARPE-19 cells. Kat.f, ratio of the velocity constant of catalase at 0 min to the protein content in grams per milliliter. Each column represents an independent experiment.

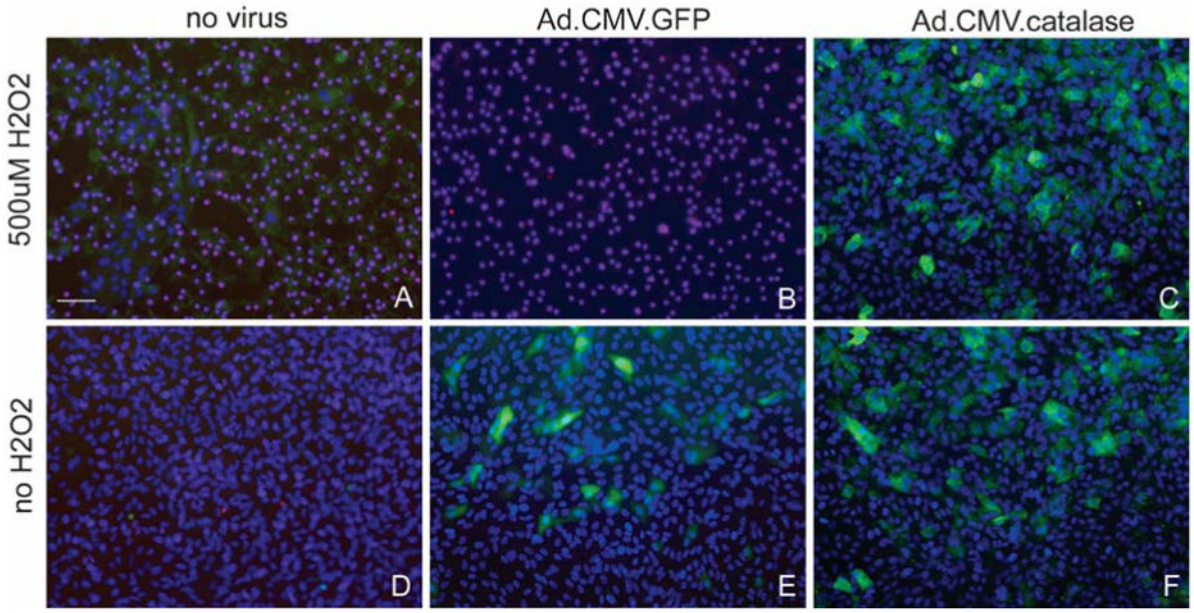

FIG. 3.

Protection of ARPE-19 cells from hydrogen peroxide-induced apoptosis by infection with Ad.CMV.catalase. Cells were exposed to no virus (**A** and **D**), Ad.CMV.GFP (**B** and **E**), or Ad.CMV.catalase (**C** and **F**). They were then incubated with 500 *μM* hydrogen peroxide (**A**–**C**) or HBSS (**D**-**F**). After hydrogen peroxide exposure, cells were analyzed by TUNEL (red), and nuclei were counterstained with DAPI (blue). Uninfected and Ad.CMV.catalase-infected cells were labeled with anti-catalase (green). Ad.CMV.GFP-infected cells were not labeled with anti-catalase; their green color results from GFP fluorescence. After hydrogen peroxide exposure Ad.CMV.GFP-infected cells lose their green fluorescence (**B**). Scale bar: 100 *μ*m.

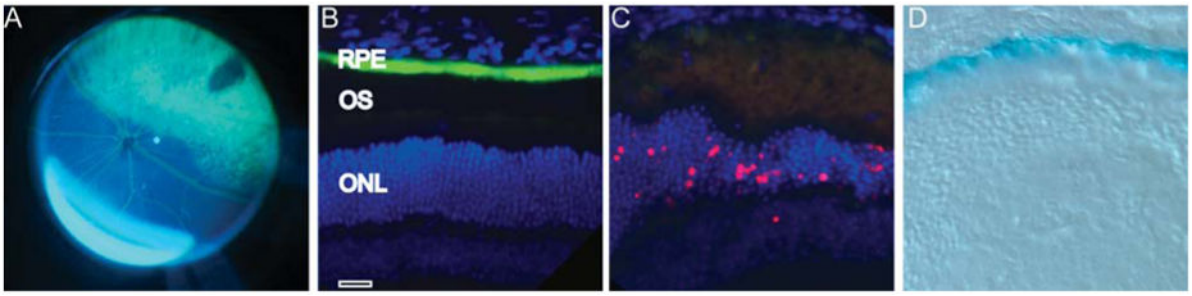

FIG. 4.

Ad.CMV.catalase injection results in catalase overexpression and protection from BLE-induced photoreceptor cell death. (**A**) Fluorescence *in vivo* retinal photograph shows temporal area *(top right)* of retina transduced by Ad.CMV.GFP. (**B** and **C**) Fluorescence microscopy and (**D**) light microscopy of retinas from eyes injected with either (**B**) Ad.CMV.catalase or (**C** and **D**) Ad.CMV.lacZ. BLE retinas injected with (**B**) Ad.CMV.catalase or (**C**) Ad.CMV.lacZ were labeled both with anti-catalase (green) and for TUNEL analysis (red). (**D**) BLE retina injected with Ad.CMV.lacZ was stained with X-Gal. Scale bar: 25 μm. RPE, retina pigment epithelium; OS, outer segments; ONL, outer nuclear layer.

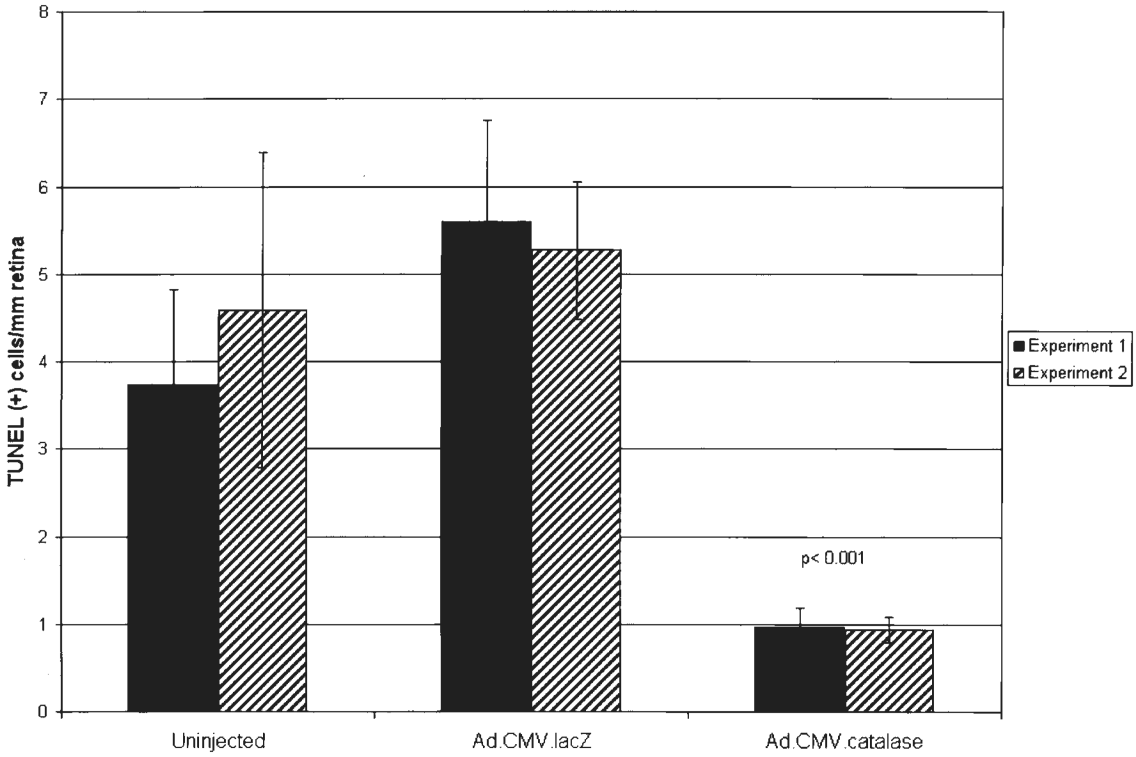

FIG. 5.

Quantitation of cell death after BLE in Ad.CMV.lacZ- and Ad.CMV.catalase-injected eyes. Each column represents mean counts of TUNEL-positive photoreceptor nuclei from 15 sections per eye of multiple eyes (see below). Solid columns represent experiment 1 (two uninjected, BLE eyes; five Ad.CMV.lacZ, BLE eyes; five Ad.CMV.catalase, BLE eyes). Hatched columns represent experiment 2 (two uninjected, BLE eyes; six Ad.CMV.lacZ, BLE eyes; six Ad.CMV.catalase, BLE eyes). Error bars represent the SEM. The *p* value compares Ad.CMV.lacZ, BLE retinas with Ad.CMV.catalase, BLE retinas.

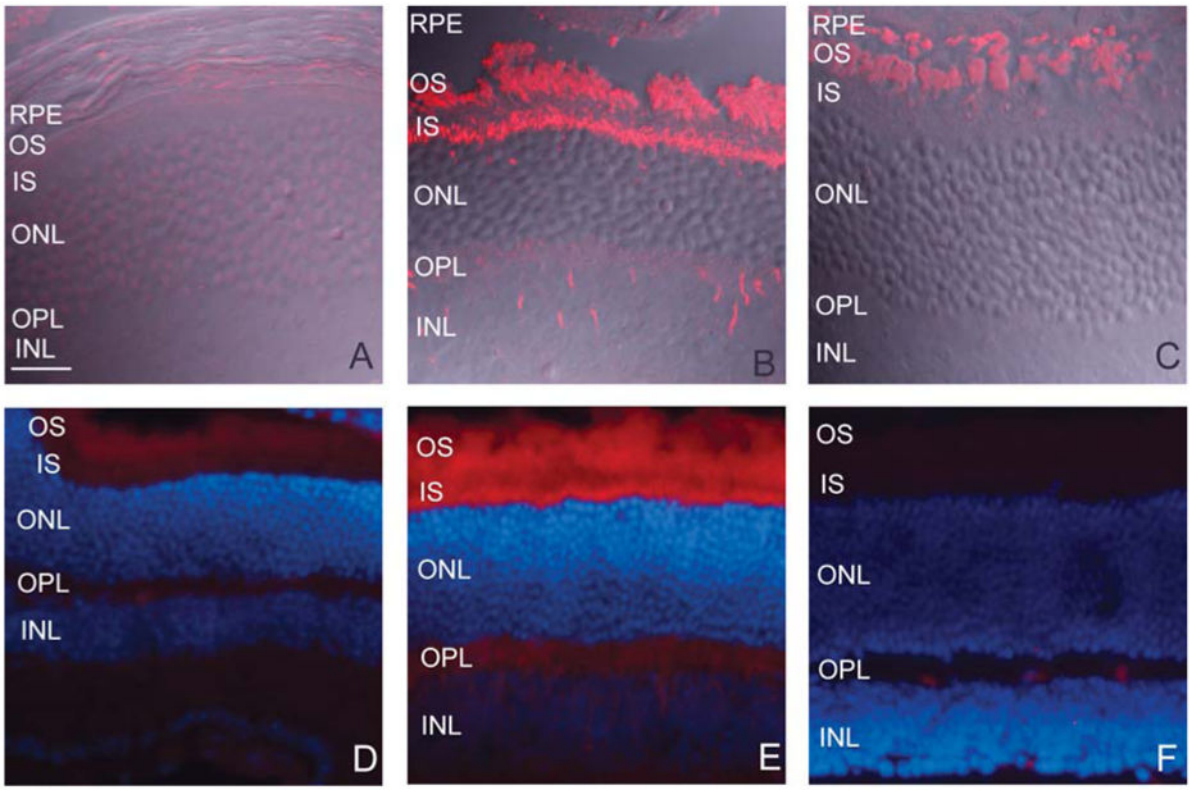

FIG. 6.

BLE causes oxidative stress, which is prevented by treatment with Ad.CMV.catalase. Shown are confocal micrographs of retinas immunolabeled (red fluorescence) with oxidative stress markers anti-HNE (**A**–**C**) or anti-nitrotyrosine (**D**–**F**). Eyes were treated as follows: uninjected, no BLE (**A** and **D**); Ad.CMV.lacZ injection, BLE (**B** and **E**); and Ad.CMV.catalase, BLE (**C** and **F**). RPE, retina pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar: 20 μm.