

An empty E1⁻, E3⁻, E4⁻ adenovirus vector protects photoreceptors from light-induced degeneration

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Abstract We have previously identified a neuroprotective effect associated with empty (E1⁻, E3⁻, E4⁻) adenovirus vector delivery in a model of light-induced, photoreceptor cell death. In this study, we further characterize this protective effect in light-injured retina and investigate its molecular basis. Dark-adapted BALB/c mice, aged 6–8 weeks, were exposed to standardized, intense fluorescent light for 96 or 144 h. Prior to dark adaptation, all mice received intravitreal injection of 1×10^9 particles of an empty (E1⁻, E3⁻, E4⁻) adenovirus vector in one eye and vehicle in the other. Following light challenge of 96 or 144 h, histopathological analysis and quantitative photoreceptor cell counts were conducted. Semiquantitative assessment of messenger ribonucleic acid (mRNA) for the apoptosis related genes: *p50*, *p65*, *IκBα*, *caspase-1*, *caspase-3*, *Bad*, *c-Jun*, *Bax*, *Bak*, *Bcl-2*, *c-Fos*, and *p53* using quantitative reverse transcriptase polymerase chain reaction was performed on eyes following 12 h of light exposure. Following 96 h of light exposure, the photoreceptor cell density for E1⁻, E3⁻, E4⁻ adenovirus vector and vehicle-

injected eyes were 87.5 ± 9.5 and 79.3 ± 10.1 , respectively, ($p=0.79$). After 144 h of light exposure, the photoreceptor cell density was preserved in vector-injected eyes as compared to vehicle treated eyes, 68.9 ± 10.0 and 49.2 ± 4.6 , respectively ($p=0.016$). Relative mRNA levels of *c-Fos* and *c-Jun* at 12-h light exposure after injection differed significantly between vector- and vehicle-injected eyes ($p=0.036$, 0.016 , respectively). The expression of the other apoptosis-related genes evaluated was not significantly affected. This study investigates the molecular basis of photoreceptor neuroprotective pathway induction associated with E1⁻, E3⁻, E4⁻ adenovirus vectors. The results indicate that empty adenovirus vectors protect photoreceptors from light-induced degeneration by the modulation of apoptotic pathways. Gene expression changes suggest that the suppression of *c-Fos* and *c-Jun* upregulation contributes significantly to the neuroprotective effect. Understanding the molecular basis of the neuroprotective pathway induction in photoreceptors is critical to the development of novel therapies for retinal degenerations.

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Introduction

Adenovirus (AdV), adeno-associated virus, and lentivirus vector platforms continue in active development for ocular gene therapy [1–3]. A number of factors determine the relative advantages and disadvantages of each platform and include but are not limited to vector tropism, transduction efficiency, transgene size, latency, duration of expression, vector-related toxicity, and integration requirements for transgene expression. Associated and potentially therapeutic

tic effects of the empty or null vectors themselves are described but are not well understood [4, 5]. While the magnitude of the reported protective effect is small compared to that resulting from, e.g., overexpression of a specific neuroprotective transgene, such as pigment epithelium-derived factor (PEDF), the effect is significant [5], and to date, there has been no attempt to optimize for therapeutic benefit. Understanding the mechanistic origin of these phenomena may lead to greater understanding of neuroprotection, photoreceptor degeneration, and the requirements for future therapy.

There are clinical settings (e.g., chronic disease) in which prolonged transgene expression may be desired. Currently, the risk of prolonged gene expression is unknown for most proteins and must be evaluated on a protein-by-protein basis. In the eye for example, even increased expression of wild-type rhodopsin or peripherin/rds can result in the degeneration of photoreceptors [6, 7]. Expression changes induced by the vector platform itself are potentially long-term results of vector administration that must be considered. It is conceivable that platform-induced gene expression changes could persist for longer than those induced by transgene expression and should be understood.

Replication-deficient AdV vectors are typically characterized by a large capacity, short latency, transduction of both dividing and nondividing cells, high expression levels, and relative ease of production. AdV vectors, however, induce multigene response including dose-dependent ocular immune responses that are associated with inflammation and shorter periods of expression [8–10]. Among the genes induced by AdV vectors is nuclear factor kappa B (NFkB), a key regulator of apoptosis [11]. We have previously reported that intraocular AdV-mediated gene transfer of PEDF significantly increased retinal cell survival following retinal ischemia–reperfusion injury [12] and light-induced photic injury [5]. The protective effects of PEDF were in part attributed to the modulation of apoptotic pathways [5, 12]. The molecular basis of a separate protective effect noted in the empty (E1⁻, E3⁻, E4⁻) AdV vector is not yet understood and is evaluated in this study.

Materials and methods

Animals

Female BALB/c mice were used at 4 to 8 weeks of age. The animals were housed under a 12-h (7:00 A.M. to 7:00 P.M.) light/dark cycle with 60 lux at the center of the cage prior to the start of experiments. The animals were anesthetized by intramuscular injection of 80 mg/kg of ketamine hydrochloride. All of the animals were treated under deep sedation in accordance with the Association for

Research in Vision and Ophthalmology resolution on the use of animals in research.

Light-induced retinal degeneration alone was induced in six control mice by a predetermined level of fluorescent light exposure sufficient to induce degenerative change. Twenty-four mice received intravitreal injection of adenoviral vectors or vehicle (3% trehalose) followed by light exposure.

Adenoviral vectors and intraocular injection procedures

The vectors are deleted for E1A, E1B, E3, and E4 and lack an inserted transgene. The specific empty vector has been reported in prior publication as AdNull.11 (GenVec, Gaithersburg, MD, USA) [13]. Mice in the experimental group were injected prior to excessive light exposure. Mice received either no injection, vehicle injection, or intravitreal injection of 1×10^9 particles of AdNull.11. Intravitreal injection was performed with a Hamilton syringe fitted with a 33-gauge beveled needle (Hamilton, Reno, NV, USA). The needle was passed through the sclera at the equator into the vitreous cavity. The injection occurred with direct observation of the needle in the center of the vitreous cavity. Eyes with intraocular hemorrhages, lens trauma, or other complication during the viral injection were excluded from this study.

Exposure to light

Immediately after vector delivery, rats were dark adapted for 3 or 72 h. Following dark adaptation, all eyes were carefully examined by slitlamp biomicroscopy and indirect ophthalmoscope, to rule out the presence of ocular injury or toxicity. Eyes with any signs of trauma or inflammation were excluded from further study.

The animals were then housed in an animal cage that was surrounded on all sides by commercially available fluorescent tubes (EFD21EN, Toshiba, Tokyo). Light exposure was continuous at a constant 2,500 lux as measured at the center of the cage. No area of the cage allowed avoidance of the diffused 2,500-lux light. The temperature in the center of the cage during the period of light exposure was maintained at room temperature. All experiments were conducted in a well-ventilated space. The animals had access to water at all times. Animals in the control and treatment groups were exposed to identical environmental conditions throughout the experimental period.

Morphometric analysis

Eyes with or without viral vector administration prior to light exposure were enucleated after 96 or 144 h of light

exposure. All eyes were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min. After rinsing with PBS, the eyes were oriented in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN, USA) with the cornea facing forward and with 12 o'clock positioned superiorly and then snap frozen in liquid nitrogen after which they were stored at -80°C until sectioning. At cryosectioning, five serial sections ($10\ \mu\text{m}$), beginning at the superior edge of the optic nerve, were obtained at $100\text{-}\mu\text{m}$ intervals. In sections including the optic nerve, the optic nerve tissue was excluded from cell counts. All specimens were processed using Hematoxylin staining (Contrast-blue, KPL Laboratory, Gaithersburg, MD, USA).

The number of nuclear cells in the outer nuclear layer (ONL) was counted in two sample areas, in each of ten standard sections, per eye. The areas to be counted were assigned in a standard fashion such that retina located approximately $200\ \mu\text{m}$ from the optic nerve, lacking artifacts such as retinal detachment, tissue distortion, and staining artifact, were used. The mean ONL cell count was then calculated for each eye and analyzed statistically.

Apoptosis-related gene expression analysis

Eyes treated with no injection, vehicle injection, or injection with AdV vector were enucleated after 12 h of continuous light exposure. Naïve eyes, without injection or light exposure, were also enucleated following 15 h of dark adaptation for gene expression analysis. The retina was removed from experimental eyes and immediately frozen in liquid nitrogen. Retinal tissue was stored at -80°C until ribonucleic acid (RNA) preparation.

Total retinal RNA was isolated by the acid guanidine thiocyanate–phenol–chloroform extraction method using TRIzol® (Invitrogen, Carlsbad, CA, USA). We utilized DNaseI (RNase-free; TAKARA BIO) to remove genomic deoxyribonucleic acid (DNA) contamination. Two hundred nanograms of total RNA was applied to reverse transcription with 25 U of SuperScript II reverses transcriptase (Invitrogen) in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) to generate complementary DNA (cDNA). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out with 10 ng of cDNA using Assay-on-Demand™ Gene Expression for 12 apoptosis-related genes (*p53*, *c-Fos*, *c-Jun*, *Bad*, *Bak*, *Bax*, *Bcl-2*, *Caspase-1*, *Caspase-3*, *IkB*, *p50NFkB*, and *p65NFkB*). We used *acidic ribosomal phosphoprotein P0* (Applied Biosystems) as an endogenous control gene [14]. qRT-PCR was performed in triplicate for each sample with a commercial system (ABI PRISM® 7900HT Sequence Detection System, Applied Biosystems). The expression level of each gene was assigned arbitrary units (relative to

baseline samples) using described comparative Ct methods [15, 16].

Statistical analysis

Statistical analysis of all eyes was performed using paired or unpaired *t* test. *p* values less than 0.05 were prospectively assigned as the value required for the reporting of significance.

Results

Effect of intravitreal injection of empty AdV in light-induced photoreceptor cell death

Three days prior to initiation of light exposure, female BALB/c mice received no injection or intravitreal injection of 1×10^9 particles of empty AdV. Typical histologic changes are shown in Fig. 1. In uninjected eyes, the photoreceptor cell density and the thickness of the ONL were predictably reduced corresponding to the time course of light injury (Fig. 1a–c). In contrast, photoreceptor cell density was relatively preserved in eyes following intravitreal injection of empty AdV as compared to corresponding uninjected eyes (Fig. 1d,e).

Morphometric analysis was performed to quantitatively compare photoreceptor cell counts in each group. The photoreceptor cell counts of uninjected eyes at baseline, 96 h, and 144 h were 108.8 ± 7.3 , 79.3 ± 10.1 , and 49.2 ± 4.6 (mean \pm SD), respectively. Following intravitreal injection with empty AdV, the photoreceptor cell counts at 96 and 144 h were 87.5 ± 9.5 and 68.9 ± 10.0 , respectively. These results represent significant preservation of the photoreceptor layer in eyes treated with empty AdV at 144 ($p=0.016$) but not at 96 h ($p=0.79$; Fig. 2).

Apoptosis-related gene expression analysis

The apoptosis-related genes tested in this study were *p53*, *c-Fos*, *c-Jun*, *Bad*, *Bak*, *Bax*, *Bcl-2*, *Caspase-1*, *Caspase-3*, *IkB*, *p50NFkB*, and *p65NFkB*. Retinal mRNAs of the four anti-apoptosis genes were *Bcl-2*, *IkB*, *p50NFkB* and *p65NFkB*. The eight pro-apoptosis genes were *p53*, *c-Fos*, *c-Jun*, *Bad*, *Bak*, *Bax*, *Caspase-1* and *Caspase-3*. Relative messenger RNA (mRNA) expression levels of *c-Fos* increased in the retinas with light exposure alone ($p=2.37 \times 10^{-10}$) and light exposure and vehicle injection ($p=0.017$) when compared to untreated baseline eyes. Relative mRNA levels of *c-Fos* in the retinas treated with light exposure and empty AdV injection were preserved and less than those in the retinas with light exposure alone

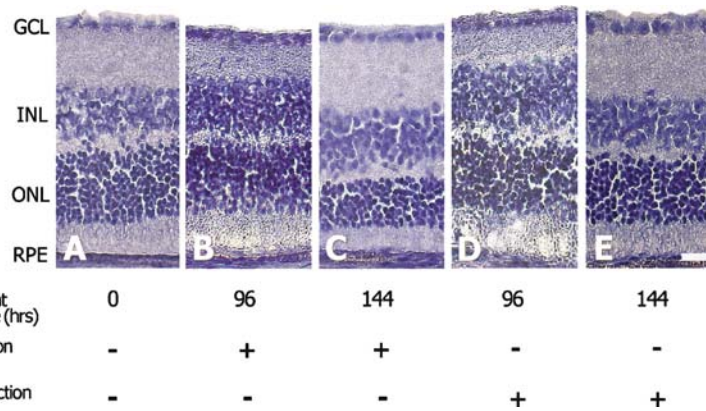


Fig. 1 Light micrographs of the treated and untreated BALB/c retinas following light exposure. Mice received no injection (a), intravitreal injection of vehicle (b, c), or 1×10^9 viral particles of empty AdV vector (d, e). Three days after injection, the mice were exposed to continuous fluorescent light (2,500 lux) for 0 (a), 96 (b, d), and 144 h

(c, e). Eyes injected with empty AdV showed moderate protection of photoreceptor cells (d, e) when compared to vehicle-injected eyes (b, c). *GCL* Ganglion cell layer, *INL* inner nuclear layer, *ONL* outer nuclear layer, *RPE* retinal pigment epithelial layer (Scale: bar=20 μ m)

($p=1.51 \times 10^{-7}$) and light exposure and vehicle injection ($p=0.036$; Fig. 3a).

The results of relative mRNA expression levels of *c-Jun* were similar to *c-Fos* expression. *c-Jun* gene expression levels were induced and significantly higher in the retinas with light exposure alone ($p=3.10 \times 10^{-5}$) and light exposure and vehicle injection ($p=1.16 \times 10^{-3}$) when compared to untreated baseline eyes. The intravitreal injection of empty AdV in eyes with light exposure inhibited the induction of mRNA expression of *c-Jun* in the retinas with light exposure alone ($p=7.69 \times 10^{-4}$) and with light exposure and vehicle injection ($p=0.016$; Fig. 3b). The mRNA expression levels of other genes tested (*p53*, *Bad*, *Bak*, *Bax*, *Bcl-2*, *Caspase-1*, *Caspase-3*, *IkB*, *p50NFkB*, and *p65NFkB*) were not significantly changed between eyes

with light exposure and vehicle injection and eyes with light exposure and empty AdV injection (Table 1).

Discussion

The molecular basis of a retinal neuroprotective effect associated with intravitreal delivery of an empty AdV vector, in the setting of intense retinal light exposure, is

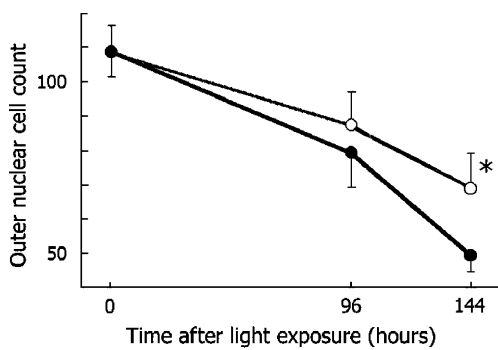


Fig. 2 Morphometric analysis of nuclear cell count in ONL of light-exposed retina. Outer nuclear cell counts of vehicle-injected eyes with 0, 96, or 144 h of continuous light exposure (solid circles) were 108.8 ± 7.3, 79.3 ± 10.1, and 49.2 ± 4.6, respectively. Outer nuclear cell counts of AdV-injected eyes with 96 or 144 h of light exposure (open circles) were 87.5 ± 9.5 and 68.9 ± 10.0, respectively. After 144 h of light exposure, the photoreceptor cell density was preserved in vector-injected eyes as compared to vehicle treated. Asterisk, $p=0.016$ (error bars=1 SD)

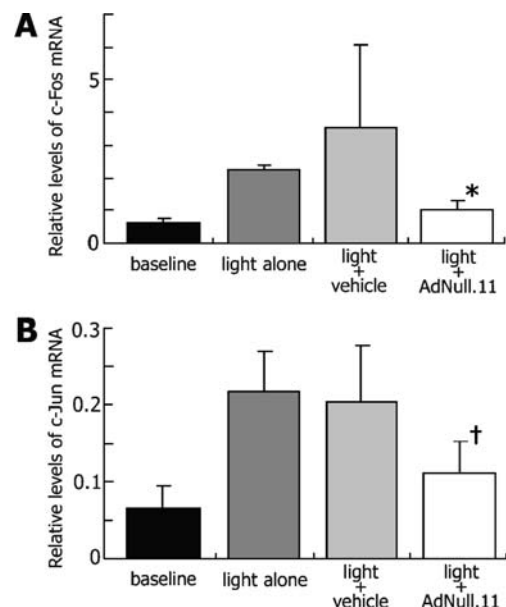


Fig. 3 Analysis of *c-Fos* (a) and *c-Jun* (b) mRNA levels in retinas of BALB/c mice by qRT-PCR. Mice were either kept in darkness (black column) or exposed to 2,500 lux for 12 h with no injection (dark gray column), vehicle injection (light gray column), or AdV injection (open column). *c-Fos* and *c-Jun* mRNA expressions were reduced in retinas of vector-injected eyes when compared to retinas of vehicle-injected eyes (asterisk, $p=0.036$, cross, $p=0.016$)

Table 1 Comparison of relative mRNA levels between eyes with light exposure and vehicle injection and eyes with light exposure and AdV injection

Genes	Light+vehicle ^a	Light+AdV ^b	<i>p</i> value
<i>p53</i>	0.52±0.03	0.16±0.03	0.069
<i>c-Fos</i>	3.53±0.95	1.01±0.11	0.036 ^c
<i>c-Jun</i>	0.20±0.03	0.11±0.02	0.016 ^c
<i>Bad</i>	0.13±0.03	0.09±0.01	0.277
<i>Bak</i>	0.33±0.03	0.34±0.05	0.932
<i>Bax</i>	0.32±0.04	0.28±0.02	0.309
<i>Bcl-2</i>	0.04±0.01	0.05±0.01	0.669
<i>Caspase-1</i>	0.02±0.004	0.02±0.01	0.624
<i>Caspase-3</i>	0.02±0.002	0.02±0.002	0.184
<i>IκB</i>	5.22±1.87	1.01±0.18	0.068
<i>p50NFκB</i>	0.56±0.18	0.17±0.03	0.067
<i>p65NFκB</i>	0.69±0.10	0.53±0.09	0.307

Data are mean±SD.

^aRelative mRNA levels of the retina treated with light exposure and vehicle injection

^bRelative mRNA levels of the retina treated with light exposure and AdV injection

^cSignificant

explored in this work. Mean ONL cell counts were significantly protected (63% preserved) in empty vector-injected eyes as compared to eyes injected with vehicle (45% preserved), following 144 h of intense light exposure. Associated gene expression changes suggest that the protective effect involves the suppression of transient upregulation of *c-Fos* and *c-Jun*, both components of transcription factor AP-1.

The photoreceptor response to light injury is complex and the result of a multigenic gene response. Current understanding in this area is reviewed by Wenzel et al. [17]. Acute bright light exposure is reported to induce changes in the mitochondrial membrane potential that may be associated with the induction of photoreceptor apoptosis [18]. Bcl-2 family members are known to regulate mitochondrial membrane permeability and integrity [19, 20] However, the ameliorative effect of Bcl-2 overexpression in a transgenic model following excessive light exposure is incompletely understood with points of controversy remaining [21, 22]. Reports indicating that the ablation of the proapoptotic Bcl-2 family members Bax and Bak protect the retina against light damage support a neuroprotective effect for BCL-2 [23]. In general, photo-oxidative stress is believed to downregulate NFκB via involvement of caspase-1, resulting in apoptosis of photoreceptor cells [24, 25].

In this study, gene expression changes show significant suppression of *c-Fos* and *c-Jun* upregulation, both constituents of transcription factor AP-1. Intensive visible light exposure induces apoptotic photoreceptor cell death by activation of the transcription factor AP-1 and AP-1 activation is believed to be essential for light-induced

photoreceptor apoptosis [17]. AP-1 is a complex that consists either of heterodimers of members of the Fos and Jun family or homodimers of members of the Jun family [26, 27]. Light exposure induces complexes of c-Fos, c-Jun, and JunD proteins [28], but JunD is not essential for retinal light damage [29]. The absence of c-Fos is reported to completely prevent light-induced apoptotic photoreceptor cell death [30]. Grimm et al. have evaluated activation of several apoptosis-related genes during light-induced photoreceptor degeneration in wild-type mice (strain 129SV/B16 or BALB/c) and found that intensive light exposure induced *c-Fos* and *c-Jun* gene upregulation [31]. Although the setting (duration and intensity) of light exposure were different than those in this study, these findings are consistent with those reported here. Lastly, while the Grimm study observed upregulation of the *caspase-1* gene [31], we did not detect significant expression change in *caspase-1* gene expression following empty AdV injection.

Reichel et al. have reported that an AdV vector expressing the β-galactosidase reporter gene had a protective effect in the *rd* mouse model of retinal degeneration [4]. While it was not determined whether the protective effect was related to the β-galactosidase protein or the vector, it was negated by immune suppression with depletion of both CD4+ and CD8+ T cells. They therefore hypothesized that the immune response to vector and/or transgene products was protective. The vector used in the current study is a human adenoviral vector, serotype 5, similar to that used in the study by Reichel et al. [4]. We may therefore speculate that the downregulation of *c-Fos* and *c-Jun* could result, at least in part, from immune responses initiated by intraocular injection of the AdV vector. AdV vectors have been tested in human subjects [32, 33], and safety data are available from a phase I clinical trial [33]. An inflammatory response has been considered a disadvantage of this vector platform, but induced immune responses may also have beneficial effects in the setting of retinal degeneration.

We have previously demonstrated that the intravitreal injection of the AdV vector with the a similar genetic backbone to the vector used in this study resulted in the transduction of cells predominantly in the iris, cornea, and ciliary body but not in photoreceptors [34]. It is interesting to note that several studies have demonstrated that the AdV vector induces modification of endogenous multigene expression [34–37]. We thus hypothesize that the intravitreal injection of the AdV vector modifies the endogenous gene expression in the transduced cells of the eye, which might secrete the neuroprotective protein. In future experiments, we will test this hypothesis and others regarding the mechanism of the effect of the AdV vector on neuroprotection, which could provide additional opportunities for the development of new treatments.

In summary, our data indicate that intravitreal injection of an E1⁻, E3⁻, E4⁻ AdV vector increases photoreceptor cell survival resulting from intense light exposure. Associated gene expression changes suggest that the protective effect involves suppression of transient upregulation of *c-Fos* and *c-Jun* genes, both constituents of transcription factor AP-1. The findings provide insight into AdV vector-induced neuroprotective pathways associated with photoreceptor rescue and may have eventual therapeutic implications for retinal degenerations.

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