Periocular Triamcinolone Enhances Intraocular Gene Expression after Delivery by Adenovirus

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PURPOSE. A noninvasive imaging technique was used for serial assessment of gene expression after intraocular gene transfer. Bioluminescence after intravitreal administration of an adenovirus vector containing the firefly luciferase gene was measured serially and noninvasively. The optical signal was then used as a bioassay to determine whether periocular immune modulation affects intraocular transgene expression.

METHODS. Sixty-two, 8-week-old, male BALB/c mice were used. The correlation of optical signal intensity was determined by tissue luciferase level after injecting 30 mice with one of three intravitreal doses of Ad-Luc-GFP (108, 5 × 108, or 109 particles in 1 µL). Ocular bioluminescence was measured at days 2, 5, 8, and 14. The bioluminescence was then directly compared with measured tissue luciferase levels. The remaining 32 mice were divided into two groups. One group (n = 16), was injected with periocular corticosteroid (400 µg in 10 µL). Two days later, Ad-Luc-GFP was administered by intravitreal injection (109 particles in 1 µL). The remaining mice (n = 16) were injected with the same dose of intravitreal Ad-Luc-GFP without corticosteroid pretreatment. Ocular bioluminescence was then assessed longitudinally on days 2, 4, 6, 8, 11, 14, 22, and 30 after intravitreal injection in n = 10 mice per group. The optical signal intensity over the entire study period. The remaining 12 mice (n = 6, each group) were used to assess histologic differences between the two groups.

RESULTS. In vivo measurement of ocular bioluminescence was well correlated with tissue luciferase levels (Spearman’s correlation, r = 0.969, P < 0.001). Periocular TA injection markedly decreased the acute inflammatory reaction associated with intravitreal Ad-Luc-GFP and was associated with a significant increase in the duration of peak luciferase expression as well as the total period of luciferase expression.


Gene therapy approaches to ocular disease are being actively explored.1–3 Intraocular gene delivery can be performed by using viral or nonviral methods.4,5 Viral vectors are currently the most efficient tools for intraocular and periocular gene delivery.6,7 Adenovirus has been a useful tool in intraocular gene transfer experiments both in vitro and in vivo. An adenovirus vector containing the gene for pigment epithelium-derived factor has recently completed phase 1 clinical evaluation in the setting of “wet” age-related macular degeneration.2,6–8,11

Adenovirus vectors use the coxsackie and adenovirus receptor (CAR) as the primary receptor for attachment.12 CAR is expressed on many ocular cell types that line the anterior and posterior segments of the ocular cavity.13 In the posterior segment, photoreceptors, ganglion cells, Müller cells, and retinal pigment epithelial cells can be transduced at variable levels, in part dependent on the method and location of delivery.14 Although broad tropism for intraocular cells is an advantage of adenovirus vectors, a principal disadvantage is that they evoke a host immune response that contributes to rapid loss of transgene expression in the target tissues.14–17 Attempts to prolong transgene expression and enhance vector utility, have led to the evaluation of other potentially less immunogenic viral vectors such as adeno-associated virus (AAV) and lentivirus, as well as progressive gutting of the adenovirus to diminish the amount of immunogenic viral protein produced.18–20 Each of these approaches is associated with unsolved disadvantages.21–23

Corticosteroids are widely known to blunt immune response and have been shown to diminish virus-associated immune response in nonocular therapy, as well as to increase the efficiency of gene transfer and expression.24–27 The eye however, is an immune-privileged organ and the degree to which ocular immune modulation affects adenovirus-mediated gene transfer, gene expression, and expression patterns is not yet fully understood.14,28 The corticosteroid, triamcinolone acetonide (TA), is commonly used in the clinical treatment of ocular diseases including but not limited to ocular surface and intraocular inflammatory processes as well as macular edema and in the treatment of ocular neovascularization.29–31

Delicate and nonregenerative intraocular tissues such as the retina are not amenable to biopsy or direct sampling. Serial assessment of transgene expression would facilitate translational gene therapy efforts. A noninvasive method permitting serial and quantitative assessment of expression, in vivo, would be ideal. The use of optical imaging techniques in combination with vectored luciferase permits longitudinal in vivo assessment of luciferase expression in superficial tissues.32–36 The
utility of this approach in ocular gene therapy has only recently been explored.57 Traditional fluorescent reporter genes such as green fluorescent proteins (GFPs) have also been used in the noninvasive assessment of gene expression in the observable retina and trabecular meshwork.36,58 Optical imaging of bioluminescence resulting from luciferase expression using current-generation technology is now significantly more sensitive than direct observation techniques. The increased sensitivity results from luminescence capture methods over extended periods.34 Other potential advantages of bioluminescence over fluorescence imaging include an emission spectrum that is less affected by tissue autofluorescence and a greater signal intensity than that of GFP.54

In this study, we applied noninvasive optical imaging techniques to the measurement of luminescence, after intravitreal delivery of an adenovirus vector containing luciferase (Ad-Luc-GFP) as the expressed transgene. The kinetics of transgene expression were observed in the same population of animals for up to 30 days, the optical signal was correlated with tissue levels of luciferase and the effect of periocular corticosteroid injection on transgene expression was quantitatively assessed.

Materials and Methods

Adenoviral Vectors Expressing Luciferase and GFP

Adenoviral vectors (Ad-Luc-GFP) were constructed that express firefly luciferase and GFP from a cytomegalovirus (CMV) immediate early promoter expression cassette on a backbone provided by adenovirus serotype 5. The vectors are deleted for E1A, E1B, and E3. Luciferase and GFP expression was verified by transfection experiments in a cultured ARPE-19 cell line.

Animals

All animal procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sixty-two, 8-week-old male BALB/c mice (Harlan, Indianapolis, IN) were used. Thirty mice were used for quantitative assessment of the correlation between optical signal intensity and tissue luciferase assay level. Another 20 were divided into two groups (control versus corticosteroid, \( n = 10 \)) each and were used for analysis of periocular TA's affect on transgene expression. The remaining 12 mice were divided into two groups (control versus corticosteroid, \( n = 6 \) each group) and used for histologic analysis at days 2 and 18.

Periocular Triamcinolone Injection

Two days before intraocular vector delivery, 16 mice assigned to the corticosteroid pretreatment group received periocular injection of TA (400 \( \mu \)g/10 \( \mu \)L Kenalog-40; Bristol-Myers Squibb Co., Princeton, NJ). Briefly, after the mouse was anesthetized with ketamine and xylazine (45 mg/kg and 4.5 mg/kg, respectively) and topical proparacaine was applied, the superior-temporal quadrant of the right eye was exposed. With the aid of a binocular dissecting microscope (Stemi 2000; Zeiss, Thornwood, NY) for visualization, a 30-gauge needle attached to a microvessel micropipette (H9262/L of TA was used to deposit TA gently onto the episcleral surface, beneath the conjunctiva/Tenon's capsule. The needle was held in position for approximately 5 seconds after injection, to allow for distribution of the drug and to minimize injection site leakage. At the end of the procedure, the white material containing TA was confirmed to be in the periocular position in all mice.

Intraocular Injection of Vectors

To correlate optical signal intensity and tissue luciferase activity, 30 mice received intravitreal injection of \( 10^9 (n = 10) \), \( 5 \times 10^9 (n = 10) \), or \( 10^8 (n = 10) \) viral particles of Ad-Luc-GFP in the right eye. The left eye served as the noninjected control. The remaining 58 mice were used to assess the effect of periocular corticosteroid on transgene expression. Each of these mice received 10\(^9\) particles of Ad-Luc-GFP in the right eye with the left eye serving as a noninjected control. Under anesthesia with ketamine/xylazine (45 mg/kg and 4.5 mg/kg, respectively) and topical proparacaine, intravitreal injection was performed with a pump microinjection apparatus (Harvard Apparatus, Holliston, MA) and pulled glass micropipettes, as previously described.59 Each micropipette was calibrated to deliver 1 \( \mu \)L of vehicle containing \( 10^9 \), \( 5 \times 10^9 \), or \( 10^9 \) particles of Ad-Luc-GFP on depression of a foot switch. With the guidance of the dissecting microscope, the sharpened tip of the micropipette was carefully inserted through the sclera just behind the limbus of the right eye into the vitreous cavity and the foot switch was depressed. The pipette tip was held in situ briefly to allow for distribution of vector and to minimize the potential for efflux at the site of injection. The injection volume was verified by assessment of the meniscus and remaining volume in the micropipette.

Optical Imaging with Luciferin

Eyes injected with vectors containing the luciferase gene were imaged later with an optical imaging system (IVIS 200; Xenogen, Alameda, CA). In brief, before imaging, intraperitoneal (IP) luciferin solution (0.2 mL of 15 mg/mL, 150 mg/kg, \( \beta \)-luciferin potassium salt; Promega, Madison, WI) was injected. The animals were then placed in the imaging device where they were exposed to a maintenance dose of inhalation anesthetic (continuous flow of 2.5% isoflurane via nose cone). The images were acquired with the following settings: high-resolution bin, F1 stop, 120 seconds of exposure time, 24 \( \times \)4 cm field of view, and height 0.8 cm. The intraocular luciferase kinetic study was performed using 10 mice at day 1 after vector injection. The settings were, continuous picturing mode starting at 8 minutes after luciferin injection for up to 25.5 minutes yielding eight pictures. After determining the intraocular luciferase kinetic curve, all animals were imaged in single-image mode with the same settings, at a standard time (10 minutes after luciferin injection).

Ocular Tissue Luciferase Assay

Thirty mice were used to establish the correlative relationship between optical signal intensity and tissue luciferase. In this study, mice receiving one of three doses of Ad-Luc-GFP in an injection were euthanized at days 2 (\( n = 2 \), each dose), 5 (\( n = 3 \), each dose), 8 (\( n = 2 \), each dose), and 14 (\( n = 3 \), each dose) immediately after optical imaging. The whole eyes were harvested and immediately frozen in liquid nitrogen and stored at –80°C until assay. The luciferase assay was performed with a commercial kit (Luciferase Assay System with Reporter Lysis Buffer; Promega) in accordance with the manufacturer's protocol. The resulting signal luminescence was measured with a microplate reader (Synergy HT; BioTek, Winooski, VT).

Optical Imaging of the Control and Corticosteroid Groups

Twenty mice in the control (\( n = 10 \)) and corticosteroid (\( n = 10 \)) groups were imaged on day 2, 4, 6, 8, 10, 14, 18, 22, and 30, after vector injection. The optical signal intensity was measured at each time point. The signal intensity curve was plotted for control and corticosteroid-treated groups.

Signal Analysis of Optical Imaging

The photon count was calculated using the manufacturer's software (Living Image 2.5; Xenogen). The circle corresponding to the ocular region of interest (ROI) was assigned a diameter of 10 mm and applied to the mouse image. The average photon count in this region of interest (ROI) was calculated automatically by the software. The untreated left eye of the animal was used to determine the background photon count.
Histologic Examination

Twelve eyes, six receiving vector injection only and six pretreated with TA and then injected with vector, were submitted to histologic examination on days 2 and 18 after vector injection. The eyes were then fixed in 10% formalin, embedded in paraffin blocs, and sectioned. Under the fluorescence microscope, GFP expression was examined after the deparaffination process with xylene. Hematoxylin and eosin staining was performed to evaluate the degree of inflammation associated with vector administration, with and without corticosteroid treatment.

Statistical Analysis

Statistical analysis was performed with commercial software (SPSS ver. 11.0 for Windows; SPSS, Chicago, IL). Spearman’s test was used to analyze the correlation between optical imaging signal intensity and the tissue luciferase assay level. The Mann-Whitney test was used to analyze the correlation between optical signal intensity and tissue luciferase activity. The optical signal intensity (y-axis) is correlated in this two-dimension plot. Thirty mice received intravitreous injection of Ad-Luc-GFP in the right eye. Eyes were harvested at days 2 (n = 2, each dose), 5 (n = 3, each dose), 8 (n = 2, each dose), and 14 (n = 5, each dose) immediately after optical imaging. Bioluminescence was measured just before death. Data from 27 eyes were used in this plot due to the loss of eye tissue in three eyes during preparation. Each dot represents one eye, each with a measured bioluminescence value and a luciferase activity value. Spearman’s test was used to analyze the correlation.

RESULTS

Luciferin Kinetics

To assess the bioactivity of luciferase, the substrate for the reaction, luciferin, must be provided. In the absence of luciferin, there is no bioluminescent signal. The pharmacokinetics of luciferin is in part dependent on the tissue being examined, the route of administration, and the dose used.35–36 After intraperitoneal injection of luciferin, the whole eye signal intensity exhibited approximately linear decay kinetics in the interval between 8 and 25.5 minutes; however, the small decline observed in this interval was not statistically significant (Fig. 1). All animals were therefore imaged at a standard point of examination (10 minutes) after luciferin injection. The 10-minute time point allowed for peak bioluminescence to be achieved and occurred during a period of predictable signal stability.

Correlation between Optical Signal and Tissue Luciferase Level

The ocular signal intensities obtained by the optical imaging system showed substantial correlation with tissue luciferase levels. (Spearman’s correlation test, r = 0.969, P < 0.001; Fig. 2) Any eye showing evidence of rupture (during harvest or removal of attached tissues) before homogenization was not subject to tissue analysis to standardize the amount of tissue submitted for each eye (n = 3).

GFP Expression after Ad-Luc-GFP Intravitreous Injection

As expected, Ad-Luc-GFP intravitreous injection resulted in GFP expression from the cells lining anterior segment structures (e.g., corneal endothelium, lens epithelium, iris epithelium and ciliary body epithelium). Retinal cells did not demonstrate significant expression of GFP over a low level of background autofluorescence (Fig. 3).

Time-Intensity Curve of Intraocular Luciferase Expression

A time-luminescence intensity curve was obtained with the optical imaging system. In mice receiving only an intraocular injection of Ad-Luc-GFP, peak luminescence occurred at day 2 after injection and was followed by a rapid decline in optical signal intensity by day 6. The residual signal decayed slowly to day 14, after which it was negligible when compared with noninjected control eyes (left eyes; Figs. 4, 5A).

Periocular Corticosteroid Effect on Intraocular Transgene Expression

Twenty mice in the corticosteroid and control group were examined under the microscope on days 0, 14, and 30. White TA residue was readily visible at the site of periocular injection.
on days 0 and 14 in all eyes of the TA-treated group, but was not visible by day 30 in any animal. No cataract or ocular discharge was observed in any mouse during the study period. Noninfectious corneal opacity was observed in one eye, in each group at day 30. Periocular TA injection significantly increased the mean duration of transgene expression at peak levels between days 2 and 11. Between days 11 and 18 luciferase expression declined from peak levels but was still significantly elevated until day 30, compared with control animals (Figs. 4, 5B). Because the optical imaging technology is noninvasive, all animals could be imaged longitudinally. There was no need to kill animals at each time point or to biopsy and perform a direct assay on the tissue of interest. Individual differences in transgene expression were easily assessed with this technique.

**Histologic Examination of the Eye**

Intravitreous Ad-Luc-GFP injection without TA pretreatment was accompanied with significant inflammatory reaction in the anterior chamber and in the vitreous cavity. The inflammation decreased over the course of the experiment (Figs. 6, 7).
that may currently be less immunogenic have other associated disadvantages, among which are small payload capacity and low transduction efficiency, in the case of recombinant AAV, and, in the case of lentivirus, the risks associated with random integration.\textsuperscript{22,23} As vector technology evolves, the combination of ease of production, low immunogenicity, and a large capacity for genetic material is desirable.

Ad-Luc-GFP in this experiment resulted in effective transduction of cells lining anterior segment structures such as iris pigment epithelium, lens epithelium, trabecular meshwork, and corneal endothelium, consistent with previous reports.\textsuperscript{41,42} Low transduction of retinal elements after intravitreous injection of adenovirus is also consistent with prior work that shows transduction of Müller cells and some ganglion cells after intravitreous injection and of photoreceptors and retinal pigment epithelium after subretinal injection.\textsuperscript{41} Expression of genes for secretable proteins such as neuroprotective growth factors or antiangiogenic proteins from cells lining the anterior portion of the eye certainly has the potential to be therapeutic for retinal and subretinal disease.\textsuperscript{30,43–45} The potential is evidenced by the therapeutic effects after intravitreous injection of diffusible agents (e.g., TA, bevacizumab, pegaptanib, and ranibizumab) as well as therapeutic effects shown in earlier studies in animal models of retinal and choroidal disease after intravitreous injection of adenovirus vectors.\textsuperscript{6,9,41,45–45}

In this study, peak expression levels in animals not receiving TA occurred at days 2 to 5 after intravitreous injection with levels generally declining to undetectable by 2 weeks.\textsuperscript{5} Expression levels were confirmed by both bioluminescence imaging and biochemical assay of luciferase. This relatively short period of expression, while potentially disadvantageous for chronic or lengthy disease states, is a potential advantage when considering acute, brief or transient indications for which long-term expression is neither necessary nor desirable. By extending periods of peak expression and the total period of expression, via periocular treatment with TA, the utility of this vector platform may be significantly increased with expression being measurable at the 30-day time point. The maximum period of intraocular adenovirus expression could be further extended by optimization of immune modulation, but this possibility has not yet been fully investigated.

Adenovirus-initiated immune response has been considered a significant mechanism for viral clearance from host tissues.\textsuperscript{14–17} Intraocular delivery of adenovirus leads to significant and dose-dependent recruitment of inflammatory cells. Inflammation after intravitreous injection may be greater than that induced by inflammation by a similar subretinal dose and may contribute to shorter periods of transgene expression.\textsuperscript{15,46} Both cellular and humoral immune responses are induced by adenovirus.\textsuperscript{14,15,38} Induced cytotoxic T cells may eliminate transduced cells or influence transgene expression, and neutralizing antibodies against viral proteins may affect tolerance for readministration of vector.\textsuperscript{11,15} The eye, however, manifests relative immune privilege and the ocular response to viral particle administration is reported to differ from those of other immune competent tissues.\textsuperscript{28,46–47} By way of example, fewer neutralizing antibodies follow intravitreous injection of adenovirus or recombinant adenoassociated virus than are induced in immune-competent tissues.\textsuperscript{14,15,38} Subretinal injection of adenovirus elicits a predominantly type 2, helper T cell response.\textsuperscript{48} These observations may have important implications for subsequent doses of intraocular vector.\textsuperscript{14,39,40} Tolerance of up to five doses of adenovirus vector is evident in the ongoing gene therapy retinoblastoma treatment trial.\textsuperscript{1}

Corticosteroid effects on the immune system are well described.\textsuperscript{2} Systemic administration of glucocorticoids broadly affects immune responses. Periocular steroid administration
results in a greater localization of immune suppression in the orbit and interior of the eye and relatively little systemic effect and is therapeutic in some settings.49,50 Direct effects on immune pathways and indirect effects such as decreased inflammation and greater competence of the blood–retinal barrier all contribute to the biological effects in the eye.24,51 The ocular

**Figure 6.** Histologic examination of mouse eyeballs: day 2 after injection of Ad-Luc-GFP (10⁹ particles in 1 µL). Eyes were stained with H&E. (A, C) Representative images from an eye injected with vector without corticosteroid pretreatment. (B, D) Images from a representative eye pretreated with corticosteroid 2 days before vector injection. (C) High magnification of (A, B). *Greater inflammatory reaction seen in eyes not receiving pretreatment with corticosteroid. Inset: optical signal intensities measured on day 2. Arrow: lesser number of inflammatory cells found in TA-pretreatment eyes. VC, vitreous cavity; CB, ciliary body.

**Figure 7.** Histologic examination of eyes 18 days after injection of Ad-Luc-GFP vector (10⁹ particles in 1 µL). Eyes were stained with H&E. (A, C) Representative images from a vector-injected eye without corticosteroid pretreatment. (B, D) Images from a representative eye injected with vector after pretreatment with periocular corticosteroid. (C, D) *Greater inflammatory reaction in the vector-only–treated eye. Inset: optical signal intensities measured on days 2 and 18. VC, vitreous cavity; CB, ciliary body.
immune environment is sufficiently complex, however, that the effects of local steroid administration on immune competence and immune tolerance are not fully understood. The potential for ocular toxicity related to periocular steroid use in clinical application is well described and includes elevated intraocular pressure, secondary cataract, and increased susceptibility to infection.59–61 Although intraocular pressure was not assessed, no pattern of toxicity was observed in this single-dose study.

Transfection of nondividing cells, in the immune-privileged subretinal space and, in the absence of apparent cellular infiltrate, still results in only transient expression.62–5 This deficiency has been in part, explained by regulatory effects on promoter activity.58 Our data indicate that suppression of immune response, as evidenced by diminished intraocular inflammatory response to vector, improves the duration of intraocular gene expression but does not eliminate the eventual loss of transgene activity. The relative importance of local immune response in regulating the expression of vectored gene expression remains to be determined but appears to be both significant and modifiable by using standard clinical approaches such as periocular injection of TA (Fig. 7). The effects of other corticosteroids would be of interest for future studies. Hydrocortisone pretreatment has been associated with stimulation of the cytomegalovirus promoter.55 Hydrocortisone has also been shown to increase cell membrane fluidity and to facilitate the entry of adenoviruses into treated cells, whereas dexamethasone pretreatment has been associated with reduced adenovirus transduction in cultured cancer cell lines, via downregulation of the adenovirus receptor CAR.27,56

In this study we have shown that the optical signal measured by a bioluminescence imaging system correlates with and is quantitatively reflective of in vivo luciferase measurements as determined by classic biochemical assays performed directly on ocular tissues. This finding is consistent with similar correlative assessments in other tissues.32,57 The simple, fast, and noninvasive nature of this technique allows for accurate and high-throughput applications in the eye. By virtue of its superficial location and the clarity of the optical axis, the eye may be an ideal organ for noninvasive optical imaging studies of a translational nature. In our study, we used serial assessment of transgene expression in response to immunomodulatory intervention. Several other applications in animal models have been described.58,59 Before bioluminescence studies are undertaken in humans, it would be necessary to acquire a full understanding of the pharmacokinetics and potential toxicities of luciferin in humans and to adapt the scale of existing imaging devices.

In summary, we used noninvasive, in vivo imaging techniques to assess the effect of periocular delivery of TA on gene expression after intravitreous delivery by adenovirus. In this setting, periocular TA serves to enhance vectored gene expression, as evidenced by significant prolongation of the peak transgene expression period and the total period of transgene expression. These findings may have practical and therapeutic relevance in application in the setting of ocular gene therapy. Finally, optical imaging to detect bioluminescence resulting from expression of adenovirus vectored luciferase in the eye is useful for monitoring the time course and levels of gene expression after ocular gene therapy in vivo.

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