In Vivo Gene Delivery and Visualization of Corneal Stromal Cells Using an Adenoviral Vector and Keratocyte-Specific Promoter

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PURPOSE. This study was conducted to determine whether intrastromal injection of adenoviral construct could be used to transfect corneal stroma cells effectively in vivo and to determine whether a tissue-specific promoter could be used to express exogenous genes in keratocytes.

METHODS. An adenoviral construct with a cytomegalovirus (pCMV)-driven enhanced green fluorescent protein (EGFP) reporter gene was injected into the stroma of murine corneas. In vivo expression was quantitated and samples were analyzed by in vivo stereomicroscopy, and ex vivo expression was determined by confocal three dimensional (3-D) reconstruction. The 3.2-kb keratocan promoter was used to drive tissue-specific reporter gene expression in vivo.

RESULTS. EGFP expression was first detected in vivo 11 hours after injection of adeno-EGFP in the corneal stroma, with a duration of approximately 3 weeks. Ex vivo wholemount corneal confocal analysis with 3-D reconstruction allowed visualization of EGFP expression in corneal stroma cells, to accurately assess cellular architecture and distribution in the corneal stroma. Naked pCMV-EGFP plasmid DNA did not express the reporter gene to the levels of the adeno-EGFP. The 3.2-kb keratocan promoter was capable of driving EGFP tissue-specific expression in the cornea.

CONCLUSIONS. Intrastromal injection of adenovirus packaged DNA constructs is a rapid and efficient way to deliver and express genes in the corneal stroma. Intrastromal injection is also capable of delivering tissue-specific promoter constructs to the corneal stroma for gene expression. Furthermore, 3-D reconstruction provides a powerful tool for enhanced visualization of the corneal stroma environment and cellular biology.

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The cornea provides an ideal environment for in vivo biomicroscopy due to its noninvasive accessibility and clarity. A multitude of recently developed potential gene therapies are applicable to the ocular surface without invasive surgery, making this tissue an excellent candidate for in vivo visualization and quantitation of gene delivery and expression.1-5

Direct gene transfer was initially demonstrated in murine skeletal muscle and later was shown to be effective in cardiac muscle.6-7 This gene-transfer technology has also been used in the ocular surface to deliver VEGF cDNA to the corneal stroma.8 Naked DNA delivery by intrastromal injection is effective, but the efficiency and stability is less than favorable. Introduction of an exogenous gene to the corneal stroma has also been performed using lamellar flap generation.9 This technique was also effective, but reporter gene expression was not detected for 72 hours, and detection methods were terminal; moreover this gene delivery method can be considered moderately invasive.

In an effort to make gene therapy for ocular diseases possible, vectors have been researched to provide a more stable and/or robust expression of the desired gene. The vector types which appear to be the most effective are viral vectors. Adeno-associated, lentivirus, and retroviral vectors have all been shown to deliver and express a gene in ocular surface cells effectively, with various expression levels and duration.10-12 A noteworthy problem that has yet to be addressed is the lack of cell and tissue specificity using these approaches, since the promoters have all been ubiquitous (pCMV).

A potential candidate promoter to drive cell-specific expression of a gene of interest in the corneal stroma is the 3.2-kb 5'-flanking region of the keratocan gene. The stroma is the central and largest layer of the adult cornea and consists of several different cell types with most of the cells being keratocytes.11 Keratocytes express a cornea stroma-specific keratan sulfate proteoglycan (KSPG) in the adult vertebrate cornea called keratocan.12 The 3.2-kb 5'-flanking region of the keratocan gene was identified by Liu et al.13 in 2000 to be able to drive a β-galactosidase reporter specifically in the corneal stroma of adult transgenic mice.15

The effectiveness of intrastromal injection to deliver an adenovirus vector to the corneal stroma, which is minimally invasive, rapidly expressed, and allows nonterminal detection, is reported herein. Moreover, the in vivo visualization and quantification of enhanced green fluorescent protein (EGFP) expression and three-dimensional (3-D) reconstruction of mouse cornea using confocal microscopy for better analysis of gene delivery and expression in the cornea is shown. To date, intrastromal injection coupled with a viral vector to deliver and drive in vivo gene expression in the corneal stroma has not been documented. Furthermore, in the current study the ability to use the 3.2-kb keratocan promoter to drive tissue-specific expression of an EGFP reporter gene in the corneal keratocyte is demonstrated.

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In vivo stereomicroscopic images of mouse eyes 24 hours after intrastromal injection of adeno-EGFP. Adeno-EGFP was delivered to mouse corneas by intrastromal injection and visualized 24 hours later by in vivo stereomicroscopy. The intensity of EGFP expression was demonstrated by excitation with blue epifluorescence in the presence of bright light (A). Stereomicroscopic image during excitation of adeno-EGFP with blue epifluorescence (B).

Materials and Methods

Plasmid Construct

Adeno-EGFP. The adenovirus construct with an EGFP reporter gene driven by the CMV (cytomegalovirus) promoter used in this study was described by He et al. in 1998. Briefly, the gene encoding EGFP from pEGFP-C1 (BD Biosciences, San Jose, CA) was cloned into the pAdTrack-CMV vector generated by He et al. for production of EGFP-traceable viruses.

pKera3.2-EGFP. The full-length EGFP cDNA fragment was first amplified by PCR using the pGFP-N1 vector (Clontech/BD Biosciences, Bedford, MA) as a template, with the EcoRI 5'-EGFP primer (5'-GATGGAATTCACCCGTCGACCATGTTG-3') and the SalI 3'-EGFP primer (5'-GTATGGATCTTACGTCAGGCCTGATG-3'). A proofreading enzyme mix (Advantage2; Clontech/BD Biosciences) was used in the following profile: 94°C, 1 minute; 62°C, 1 minute; and 72°C, 1 minute, for 50 cycles. A PCR product (750 bp) was then cut with EcoRI and SalI and subcloned into the Kerapr3.2-int/RTA vector (Liu C-Y, unpublished results, 2004) predigested with EcoRI and SalI. The resultant DNA clone was designated as Kerapr3.2-int/EGFP. DNA sequencing by the DNA core facility at the University of Miami School of Medicine confirmed the fidelity of PCR-derived EGFP.

Animals

C57/BL6 and C57BL/6TgN(ACTbEGFP)1Osb (The Jackson Laboratory, Bar Harbor, ME) were used in these studies which were 8 to 10 weeks of age after preoperative examination for exclusion criteria such as ocular disease, wound, or infection. Animal care and use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The Cleveland Clinic Foundation.

Intrastromal and Subconjunctival Injections

Intrastromal injection was performed by first creating a small tunnel from the corneal epithelium to the anterior stroma with a 33-gauge needle (Hamilton Co., Reno, NV). Another 33-gauge needle attached to a 10-μL syringe (Hamilton Co.) was passed through the tunnel into the corneal stroma, and 2 μg of plasmid DNA or 1.3 × 106 pfu of adenoviral vector in a volume of 2 μL was injected. Subconjunctival injection was performed by first creating a corneoscleral tunnel beginning 1 mm peripheral from the limbus into the sclera. Similar to the intrastromal injection, another 33-gauge needle attached to a 10-μL syringe (Hamilton) was passed into the conjunctiva, and 2 μL plasmid DNA was injected. EGFP expression was analyzed using a corresponding fluorescence filter with a stereomicroscope (Leica Microsystems Inc., Bannockburn, IL) at various time points and captured with a digital camera (SpotCam RT KE; Diagnostic Instruments, Inc., Sterling Heights, MI). The actual surface area of the cornea where EGFP expression could be detected was approximately 70% of the entire cornea. EGFP expression was quantitated on computer (Image Pro Plus; Media Cybernetics Inc., Carlsbad, CA). Green intensity per pixel of corneas at specific time points was calculated in a consistent region of interest (ROI).

Immunohistochemistry

Eyes were enucleated 24 hours after intrastromal injection, snap frozen, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Scientific, Naperville, IL). Frozen sections 5 μm thick were blocked and probed for EGFP. EGFP immunostaining was performed using an AlexaFluor 594 directly conjugated anti-green fluorescent protein rabbit IgG fraction (Molecular Probes, Eugene, OR). The specimens were mounted with antifade medium with 4′,6′-diamidino-2-phenyindole (DAPI, Vectorshield; Vector Laboratories, Burlingame, CA) and visualized with fluorescence microscopy.

Confocal Microscopy and Wholemount 3-D Reconstruction

Corneas were excised and fixed for 1 hour in 4% paraformaldehyde. After a brief wash in PBS, four small peripheral incisions were made in the cornea to flatten it on a slide. Corneas were mounted with antifade medium containing DAPI (VectorShield; Vector Laboratories) and placed at 4°C for 48 hours to allow the DAPI to penetrate. Corneas were then analyzed using a spectral laser scanning confocal microscope (model TCSSP; Leica, Heidelberg, Germany) with the corresponding lasers for DAPI and EGFP. Zstacks were generated in 0.48-μm increments and 3-D reconstructions were performed on computer (Volocity software; ImproVision Inc., Lexington, MA).

Results

To determine whether EGFP expression could be expressed and visualized in vivo 24 hours after intrastromal injection of adeno-EGFP, mice were anesthetized and placed under a stereomicroscope. Figure 1A shows simultaneous illumination with bright and epifluorescent light to highlight the intensity and efficiency of the adeno-EGFP vector. Figure 1B shows the eye with just the epifluorescent light. At 24 hours after injection, a significant amount of gene expression, as visualized by EGFP intensity and distribution, was detected. The transected area of the cornea was 70% of the total surface area of the cornea, as measured at 24 hours. To document the earliest time point of expression, a 1-week time course was established, images were captured at 11 hours, 24 hours, and 1 week after injection (Fig. 2), and green intensity per pixel was calculated. The earliest time point for EGFP visualization in vivo was 11 hours after injection. Expression could still be detected in vivo 1 week after injection and decreased significantly after 10 days. In some experiments, EGFP expression could be detected to a minimal degree at 21 days (data not shown).

To investigate the localization of EGFP-positive cells, immunohistochemistry of 5-μm sections using a directly conjugated anti-EGFP antibody and DAPI (Molecular Probes; Fig. 3A–D) was initially performed. Control noninjected mouse cornea...
5-μm sections stained with DAPI (Fig. 3A) and anti-EGFP (Fig. 3B) showed no positive EGFP staining. Adeno-EGFP–injected corneal sections showed positive EGFP staining throughout the entire corneal stroma 24 hours after injection (Figs. 3C, 3D). Based on an in vivo high-magnification (100×) stereomicroscope image of adeno-EGFP–injected corneas (Fig. 3E), it was realized that 5-μm corneal sections provide limited information about the cornea cell population and presence of EGFP-positive cells.

The interactions of various whole cells in an x–y plane are impossible to capture in one section and difficult to analyze in serial sections. For these reasons, a novel 3-D reconstruction analysis of images acquired from full-thickness fluorescence confocal micrographs of wholemount adeno-EGFP–injected corneas was performed. This technology provides a unique better understanding of the EGFP-positive cells throughout the cornea. Using image analysis software (Volocity software; ImproVision, Inc.), 3-D images from stacks of confocal images were obtained with a 63× objective and a step-size of 0.48 μm and showed similar patterns of EGFP expression in the corneal stroma (Fig. 4) at the limbal (Fig. 4A), paracentral (Fig. 4B), and central (Fig. 4C) regions. No EGFP was detected in naïve control corneas (Fig. 4E). A better understanding of the number and distribution of the cells in the corneal stroma was also gained compared with 5-μm tissue sections. A 5-μm confocal section (Fig. 4D) generated in the same fashion recreates the similarity to traditional 5-μm tissue sections and demonstrates the limitations to proper visualization of gene expression using tissue sections. Moreover, it demonstrates our ability to detect and localize a specific gene of interest in corneal stroma cells. The 3-D images generated from confocal stacks using the image-analysis software could be spun on a 360° axis, allowing for a spatial and perceptual visualization of corneal regions (Fig. 5). Images taken after 3-D reconstruction of an adeno-EGFP–transfected cornea representing the ability to visualize at different angles and planes are shown in Figure 5 from the top (Fig. 5A), bottom (Fig. 5B), and at a tilt (Fig. 5C). Using this capability, it was determined that EGFP expression was localized to the cells of corneal stroma with no epithelial or endothelial cells transfected.

FIGURE 2. Time course of in vivo EGFP expression and quantitation. Intrastromal injection of adeno-EGFP was visualized at 11 hours, 24 hours, and 1 week after injection and quantitated. Expression decreased moderately at 1 week, but expression was still detectable in vivo. Micrographs obtained at the commensurate time points are shown.

FIGURE 3. Localization of EGFP expression in 5-μm tissue sections immunostained with anti-EGFP fluorescence-conjugated antibody after intrastromal injection of adeno-EGFP and counterstained with DAPI (A, C). Naïve (noninjected) mouse cornea sections showed no positive signal for EGFP (B), but adeno-EGFP–injected sections showed extensive positive signals in the corneal stroma (D). High-magnification (100×) in vivo stereomicroscopy revealed a dense population of EGFP-positive cells in the corneal stroma 24 hours after injection (E).
The data generated from the 3-D reconstruction analysis demonstrated an abundant amount of transfected EGFP-positive cells in the corneal stroma. To investigate further the transfection efficiency and pattern of intrastromal injection using adenovirus, a comparison confocal analysis was performed between the adeno-EGFP-injected cornea and the naive control corneas.

Figure 4. Three-dimensional reconstruction analysis of wholemount cornea confocal images. Z-stack images of wholemount corneas were compiled electronically and used to generate a 3-D image of adeno-EGFP-injected and DAPI-stained corneas. The limbal (A), paracentral (B), and central regions (C) of the cornea were reconstructed for 3-D analysis. A 5-μm section (D) was also generated using this technology to show the limitations of tissue sections and the comparison of this technique to traditional methods. No EGFP was detectable in naive control corneas (E).

Figure 5. Rotation of 3-D reconstructed wholemount cornea confocal images. The 3-D reconstructed images of wholemount corneas could be manipulated and viewed in a 360° manner as indicated by the top (A), bottom (B) and tilt (C) images 24 hours after adeno-EGFP was delivered to mouse corneal stromal cells by intrastromal injection.
chicken β-actin promoter–driven EGFP transgenic mouse line C57BL/6-TgN(ACTbEGFP)1Osb (The Jackson Laboratory) (Fig. 6). The EGFP transgenic mouse (tg/0) had EGFP-positive cells in the corneal epithelium and endothelium; however, corneal stroma EGFP-positive cells were more sporadic in the transgenic mouse than in the adeno-EGFP–injected corneas, further showing the high efficiency of this procedure.

One of the caveats of using a ubiquitous promoter such as pCMV to drive the expression of exogenous genes in tissues that contain different populations of cells is that the expression cannot be targeted to a particular cell type of interest. The main goal of gene therapy is to deliver the gene of interest to a particular cell type at an ideal expression level. The use of tissue-specific promoters is ideal for this. To test if a gene of interest could be delivered to a particular cell type using intrastromal injection and expression EGFP in only a certain population of cells, we used the keratocyte-specific 3.2-kb keratocan promoter (pKera). To ensure an equal comparison of promoter constructs and due to the unavailability of an adenoivirus packaged pKera-EGFP construct, CMV-EGFP and pKera-EGFP naked plasmid DNA was used. CMV and pKera3.2 were both able to drive EGFP expression in the mouse cornea (Figs. 7A, 7B). Even though injection of naked DNA with CMV-EGFP did not have the high efficiency or expression level of the adeno-EGFP, delivery was still localized to the corneal stroma, as previously described. The 3-D confocal reconstruction analysis also demonstrated that pKera3.2 drove EGFP expression in the corneal stroma to a lesser degree than did the CMV promoter. This decrease in expression was indicative of our ability to deliver the EGFP reporter gene through intrastromal injection and cause it to be expressed by a specific cell population, the keratocytes. To confirm corneal specificity of the pKera 3.2-EGFP construct, subconjunctival injections of CMV-EGFP and pKera 3.2-EGFP were performed. Figure 7C shows CMV-EGFP was expressed in both the cornea and in the conjunctiva and sclera; whereas, pKera 3.2-EGFP are expressed only in the cornea. To demonstrate the intracellular expression and morphology of the cells expressing EGFP, a 63× confocal

![Figure 6](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932927/) Efficiency of adenovirus-mediated intrastromal injection. Three-dimensional confocal reconstructed images of (A) adeno-EGFP–transfected and (B) chicken β-actin promoter–driven EGFP transgenic mouse corneas. EGFP-positive cells in the adeno-EGFP cornea were more abundant than the transgenic mouse. Furthermore, the stroma-specific delivery is highlighted by the absence of EGFP in the corneal epithelium and endothelium compared with the positive EGFP in the transgenic mouse cornea.

![Figure 7](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932927/) Tissue-specific gene delivery by intrastromal injection. Naked CMV-EGFP (A) and pKera3.2-EGFP (B) plasmid DNA was delivered to the corneal stroma by intrastromal injection. Tissue-specific expression of EGFP was accomplished with the keratocyte specific promoter. (C) Subconjunctival injections of CMV-EGFP and pKera3.2-EGFP (dotted line delineates the limbus) and demonstrates the ability of pKera3.2-EGFP to drive corneal-specific expression of EGFP, but expression of CMV-EGFP was present in cornea, sclera, and conjunctiva. (D) Illustrates the keratocyte morphology of a corneal stroma cell injected with pKera3.2 EGFP.
micrograph (4× digital zoom) was generated of a cell expressing EGFP under the control of the pKera3.2 promoter. Figure 7D shows the intracellular expression of EGFP by a cell with a keratocyte-like morphology, further supporting the ability of this technique to deliver a gene of interest and express a protein effectively in a cell- and/or tissue-specific manner.

**DISCUSSION**

In the present study, intrastromal injection was used, and we report several novel results. First, the ability to deliver a gene of interest and express a protein (EGFP) into the corneal stroma cells rapidly (11 hours) and efficiently with an adenoviral vector and intrastromal injection was demonstrated. Second, 3-D reconstruction was performed on wholemount mouse corneas using confocal microscopy. Finally, a tissue-specific gene promoter was shown to drive corneal stromal-specific expression of a reporter gene.

Gene delivery to the stroma is not novel, but this is the first report of adenovirus technology coupled with intrastromal injection. The actual mechanism of how intrastromal DNA injection delivers DNA to corneal stroma cells and is expressed is currently unknown. A possible explanation is that the high amount of pressure generated during the injection forces DNA through pores in the plasma membrane. Similar to the work published by Stechschulte et al., we also noticed that the endothelium could not be transfected with the intrastromal injection technique. The most likely explanation for this is that Descemet's membrane acts as a barrier, and either naked DNA or adenoviral particles can get to the endothelium. However, in contrast to naked DNA transfections, in most instances intrastromal injection of adenovirus is limited to the corneal stroma and does not transfet the epithelium. Although this could also be the result of a selective barrier effect of the basement membrane isolating the epithelium from adenovirus transfection, another possible explanation could be a difference in force used during the intrastromal injection. It is possible for the injection to force fluid through a weakened or damaged part of the basement membrane, consequently exposing the basal epithelium for transfection. Adenoviral vectors alleviate the need for high volume and pressure due to their independent ability to introduce DNA in cells at a very high efficiency. Nevertheless, the potential long-term benefits of this technology are in the field of gene therapy to express a therapeutic protein of interest rapidly, efficiently, and transiently.

Another interesting observation from this work is that 3-D reconstruction analysis of wholemount corneas surpasses the limitations of tissue sections through visualization of cellular interactions, tissue architecture, and spatial perception. With a host of recently discovered fluorescent proteins that can be simultaneously visualized because they possess distinct spectral properties and have stable expression, the application of 3-D reconstruction analyses become more valuable in understanding in vivo biology. The method used in the current report provides the ability to gain a better understanding of corneal architecture and cellular biology through 3-D reconstruction, which allows for 360° visualization of an analyzed tissue. This is useful in comparing the quantity, morphology, distribution, and interactions of cells populating the corneal stroma under normal conditions and how these variables change after an introduced stimulus such as disease, inflammation, gene transfer, or stress.

Even though limitations exist in the penetration of antibodies in the process of wholemount immunofluorescence, a host of tissue and/or cell-specific promoters exist and a multitude of gene-delivery techniques are currently available. Tissue-specific promoters are not only effective for 3-D reconstruction, they are invaluable tools for gene therapy. Gene therapy is a very complex process, with the major obstacles being localized delivery, gene expression level, and control of expression. Tissue-specific promoters circumvent at least two of these obstacles, since promoter analyses provide an understanding of expression localization and expression levels.

This study presents novel findings in the field of ocular surface gene delivery and the 3-D reconstruction of whole-mount corneas. Together these findings not only provide further advancements in gene therapy, but they provide for better understanding of corneal cell biology and architecture.

**References**

18. Yu TS, Moctezuma-Anaya M, Kubo A, Keller G, Robertson S. The heart LIM protein gene (Hlp), expressed in the developing and
adult heart, defines a new tissue-specific LIM-only protein family. 


