Enhanced Short-Term Plasmid Transfection of Filtration Surgery Tissues

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Purpose. To quantify and localize plasmid transfection of filtration surgery tissues using two delivery techniques.

Methods. Full-thickness filtering procedures were performed on eyes of New Zealand albino rabbits. In 10 eyes, naked plasmid DNA in saline was either injected beneath Tenon's capsule at the filtration site or absorbed into a collagen shield that was then placed external to the sclerostomy and under the Tenon's capsule. Forty-eight hours after surgery, levels of the reporter gene, chloramphenicol acetyltransferase (CAT) were measured in samples of ocular tissues. In two additional eyes, the β-galactosidase (*β-Gal*) reporter gene expression was localized histologically.

Results. Injection of plasmid DNA in saline vehicle into the filtration bleb produced readily detectable CAT activity in bleb tissue (conjunctiva, Tenon's capsule, and sclera) whereas CAT activity was nearly undetectable in samples of the cornea, iris–ciliary body, and tissues located opposite the bleb site. Delivery of the plasmid DNA into the bleb through a collagen shield increased CAT activity 30-fold over injection of plasmid in saline (2711 ± 567 mU/mg versus 92 ± 38 mU/mg). β-Gal activity was imaged only in the region of the bleb, and microscopic examination showed β-Gal activity localized to Tenon's capsule fibroblasts, with minimal β-Gal activity observed in inflammatory cells or scleral fibroblasts.

Conclusions. Transfection of filtration tissues is enhanced by absorption of naked DNA into a collagen shield. Furthermore, transfection is localized to the fibroblasts and inflammatory cells of the filtration bleb site. Gene therapy using naked plasmid DNA and a simple collagen shield delivery vehicle may be useful for regulating wound healing after glaucoma surgery. (*Invest Ophthalmol Vis Sci.* 2000;41:4158–4162)

Attempts to use gene transfer to correct diseases in humans began in 1991,[1] and recently, the first successful clinical outcome was reported in two young patients who had severe combined immunodeficiency.[2] To date, there are no reports of successful treatments of ocular diseases in patients using gene transfer. However, there are a limited number of reports investigating gene transfer with human cells in culture and animal ocular tissues. Adenovirus vectors were used to transduce genes into intraocular tissues by injecting the vector into the anterior chamber and vitreous cavity of mice and rabbits.[3,4] Corneal graft donor buttons were infected in organ culture by an adenovirus vector expressing the *β-Gal* reporter gene.[5] A cationic lipid preparation was used to assist transfection of a plasmid vector expressing β-gal applied topically and intraocularly in rats,[6] and a gene gun was used to transfect a plasmid expressing green fluorescent protein into corneal epithelial cells of rabbits.[7] A human retinal pigmented epithelial (RPE) cell line transduced by an adenoviral vector containing the human heme oxygenase gene[8] and replication-deficient adenoviral vector to transduce the anterior segments from postmortem human eyes.[9] These experiments demonstrated the general feasibility of using viral vectors and plasmids to deliver genes to ocular tissues.

Complications that have been reported for adenoviral vectors include severe inflammatory reactions and development of high neutralizing antibody titers that limit repeated applications.[10–12] Adenoassociated virus (AAV) vectors do not produce significant inflammation but instead generate a neutralizing antibody response.[13] Retrovirus vectors in general only are effective on dividing cells and have the potential to integrate at oncogenic sites in the genome.

The objective of many gene therapies, such as those to correct metabolic disorders including cystic fibrosis, hemophilia, or sickle cell disease, is the long-term expression of the transgene by stable integration into the genome. However, reduction of scarring during wound healing presumably requires only short-term expression of a transgene. Transfection of cells with naked plasmid vectors generally results in short-term expression of the transgene and has the additional advantage of not inducing inflammation, which can increase scarring. The success rate of glaucoma filtration surgery has been improved by the use of antimetabolites that reduce scarring at the filtration site and subsequent failure of the filter.[14] However, antimetabolite therapy is a nonspecific manipulation of the wound-healing process that is reported to increase the risk

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of bleb leaks and hypotony with a possible increased risk of infection.[15–18] The complex process of wound healing involves the gene-regulated expression of multiple growth factors, angiogenic factors, enzymes, and inhibitory substances. Therefore, the use of plasmid transfection to inhibit or increase selected gene expression could provide an opportunity to regulate the process in a targeted fashion.

Before such new therapeutic approaches can be used, it is necessary to develop practical methods of gene delivery that minimally alter current surgical techniques. In this study, we used a rabbit model of filtration surgery to compare the efficiency of two delivery techniques for plasmid vectors, to measure transfection in different locations of the eye, and to identify the specific tissues and cells that were transfected.

METHODS

Plasmid Vectors and Purification

The pMP6-CAT plasmid expressing the chloramphenicol acetyltransferase (*CAT*) gene was kindly provided by Mohan Philip (RPR-Gen Cell, Santa Clara, CA) and Lyle Moldawer (Department of Surgery, University of Florida, Gainesville).[19] The plasmid contains a mammalian expression cassette consisting of the cytomegalovirus (CMV) immediate early promoter and enhancer followed by a hybrid intron consisting of an adenovirus major late intervening sequence, a mouse immunoglobulin intervening sequence, and a simian virus (SV)40 polyadenylation sequence. The 5′ heterologous intron and SV40 polyadenylation signal sequence are designed to increase the stability of the transgene mRNA and to direct the mRNA out of the nucleus into the cytoplasm where it can be effectively translated. The expression cassette containing the *CAT* reporter gene is flanked by AAV left and right terminal repeats. The AAV terminal repeats are intended to enhance transgene expression in primary and slowly dividing cells and to serve as origins of replication. The pCMVβ plasmid expressing the *β-Gal* reporter gene was kindly provided by Barry Byrne (Department of Pediatrics, University of Florida, Gainesville). The *β-Gal* insert was excised from the pCMVβ plasmid (Clontech, Palo Alto, CA) and cloned into the *Sna*BI and *Sph*I sites of pTR-UF5 under the transcriptional control of the CMV promoter with a human growth hormone polyadenylation sequence. Plasmid DNA was isolated from bacterial cultures using alkaline lysis followed by anion-exchange column chromatography (Qiagen, Valencia, CA). Endotoxin levels were measured to be below 0.05 endotoxin units (EU)/μg DNA, by using a kit (E-Toxate; Sigma, St. Louis, MO).

Filtration Surgery Procedure

This study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Animal Care and Use Committee of the University of Florida College of Medicine. Six New Zealand albino rabbits were anesthetized with intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), and topical anesthesia was achieved with proparacaine eye drops. Five animals were used for the *CAT* experiment, and one rabbit was used for the *β-Gal* experiment. Viscoelastic (Amvisc; Iolab, Claremont, CA) was injected into the anterior chamber through a paracentesis before entering through the filtration site. No attempt was made to remove the viscoelastic at the end of the procedure. This was intended to maintain the chamber, repress bleeding, and prevent flow of the plasmid into the anterior chamber and away from the desired site of transfection in the sclerostomy and bleb tissues. Filtration surgeries were performed using a limbus-based approach, and a full-thickness sclerostomy was made using a Kelly Decemet punch (Stortz Instrument Co., St. Louis, MO) inserted through a limbal stab incision.

In the right eye of five rabbits, the pMP6-CAT plasmid was applied by injection after closure of the conjunctiva and Tenon's capsule with an 8.0 Vicryl suture. A 30-gauge needle was used to inject 100 μg of plasmid dissolved in 100 μl of phosphate-buffered saline (PBS) beneath the conjunctiva and Tenon's capsule. Care was taken to approach the bleb site from as far away as possible and to inject with the needle tip centered just posterior to the sclerostomy site. Antibiotic ointment was placed on the eye at the end of surgery.

In the left eye of the same five rabbits, the pMP6-CAT plasmid was applied using a collagen shield placed under the conjunctiva and Tenon's layers before closure of the wound. A collagen shield (Bio-Cor II 24-Hour Collagen Corneal Shield; Bausch & Lomb, Tampa, FL) measuring 16 mm in diameter was cut into four equal pie-shaped pieces. One of the pieces was soaked for 10 minutes in 20 μl of PBS containing 100 μg of plasmid. The shield piece was then placed under the conjunctiva and Tenon's capsule on top of and posterior to the sclerostomy site. The wound was closed with an 8.0 Vicryl suture, and antibiotic ointment was placed in the eye. No corticosteroid was administered.

Bilateral, nonblinding surgery was performed on the rabbits to permit fewer numbers of animals to be used. Postoperative care of the animals included daily eye examinations, observation of eating and drinking behavior, and monitoring for signs of distress and pain. No animals displayed signs of infection, distress, or dehydration during the course of the experiment.

CAT Assay

Eyes were harvested 48 hours after the surgeries were performed. For each of the 10 eyes used in the CAT assay, samples were analyzed from four locations. The filtration site specimen consisted of a rectangular block of conjunctiva, Tenon's capsule, and sclera measuring 8 mm concentric with the limbus and 6 mm radially posterior to the limbus. An identical specimen 180° opposite the sclerostomy site was also harvested. The third specimen consisted of the entire clear cornea, and the fourth specimen was the entire iris and ciliary body complex. Tissue samples were stored at −80°C until measured for CAT activity using the radioactive enzymatic protocol described by the manufacturer's technical bulletin (No. 84; Promega, Madison, WI). Briefly, tissue samples were weighed, homogenized in 1 ml of lysis buffer (250 mM Tris [pH 8.0], 5 mM EDTA, 0.02% sodium azide, 1 mM paraoxon, and 10 μg/ml aprotinin), and centrifuged at 17,000*g* for 10 minutes at 4°C. Aliquots of the supernatant solutions (100 μl) were added to 1.5-ml centrifuge tubes containing 25 μl of substrate solution creating final concentrations of 24 μM ^{14}C-chloramphenicol (50 mCi/mmol; duPont NEN Research Products, Beverly, MA) and 214 μM *n*-butyryl coenzyme A. After incubation overnight at 37°C, 1.25 ml xylene was added to the tube, and the reaction was vortexed for 1 minute. The xylene phase containing the butyrylated ^{14}C-chloramphenicol reaction product was transferred to a new 1.5-ml centrifuge tube and extracted three

times with 200 μl of 250 mM Tris (pH 8.0). Levels of radioactivity were measured in 500 μl of the xylene phase using liquid scintillation counting, and the amount of CAT activity present in the sample was calculated using the best-fit equation generated from a standard curve of CAT activity, which ranged from 0.01 mU to 100 mU. Samples were assayed in triplicate and the average CAT units were expressed as milliunits per milligram tissue. Levels of CAT activity in the tissue samples tissues were compared using analysis of variance (ANOVA) and Tukey's post hoc test with $P = 0.05$ considered statistically significant.

Tissue and Cellular Localization of Transfection

Plasmid transfection at tissue and cellular levels was localized by histochemical detection of β-gal activity using a β-gal staining kit (Boehringer Mannheim; Indianapolis, IN). Briefly, 100 μg of the plasmid expressing *β-Gal* was applied to the eyes of a rabbit using the previously described injection method in one eye and the collagen shield method in the other eye. Two days after surgery, the rabbit was killed, and the eyes were fixed in situ for 5 minutes with ice cold 2% formaldehyde and 0.02% glutaraldehyde in PBS. The eyes were then removed and placed in the fixative solution for 2 hours at 4°C. The fixed eyes were permeabilized using 0.02% nonidet P-40 for 15 minutes at room temperature, then rinsed and incubated in the X-Gal substrate solution (Boehringer Mannheim) overnight at 37°C. Tissues were photographed to document gross anatomic localization of *β-Gal* expression and then were embedded in paraffin. Sections were counterstained with nuclear fast red and photographed to document cellular localization of *β-Gal* expression.

Results

CAT Activity in Ocular Tissues

Figure 1 shows the average levels of CAT activity measured in different ocular tissues 2 days after injection of plasmid into the filtration bleb or absorption of the plasmid into the collagen shield. Two major results were apparent. First, the average level of CAT activity in the filtration bleb was approximately 30 times greater when the plasmid was delivered by a collagen shield compared with injection of the plasmid (mean ± SE of CAT activities, 2711 ± 567 mU/mg versus 92 ± 38 mU/mg, $P = 0.0001$). Second, the average levels of CAT activity in the other ocular tissue samples (cornea, iris–cilliary body, and conjunctiva–Tenon's–sclera opposite the bleb site) was nearly undetectable for both delivery methods (mean ± SE of CAT activity, 1.6 ± 0.1 mU/mg, $P = 0.0001$ compared with bleb tissue transfected by the shield method). Thus, both plasmid delivery systems produced transfection that was highly localized to the filtration site, and the use of a collagen shield to deliver the plasmid greatly enhanced the level of transfection of the filtration bleb without increasing transfection of other ocular tissues.

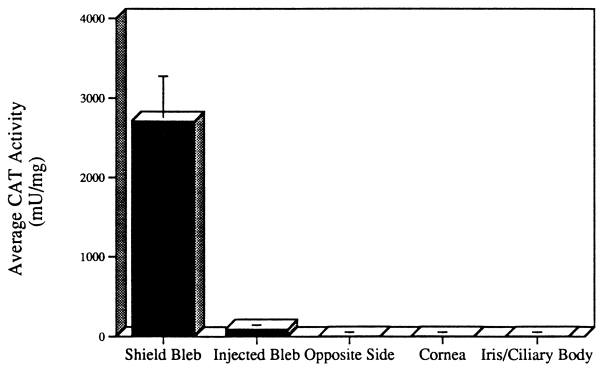

Figure 1. CAT activity in rabbit ocular tissues. Columns show the average ± SE of CAT activity measured in different tissue specimens (Opposite Side, Cornea, Iris/Cilliary Body; $n = 10$ samples of each tissue) 2 days after injection of 100 μg of plasmid into the filtration bleb (Injected Bleb, $n = 5$ samples) or absorption of 100 μg of plasmid into a collagen shield placed into the bleb (Shield Bleb, $n = 5$ samples). Average CAT activity in shield bleb samples was significantly higher than average CAT activity in injected bleb samples ($P = 0.0001$) and was significantly higher than the samples for other sites ($P = 0.0001$).

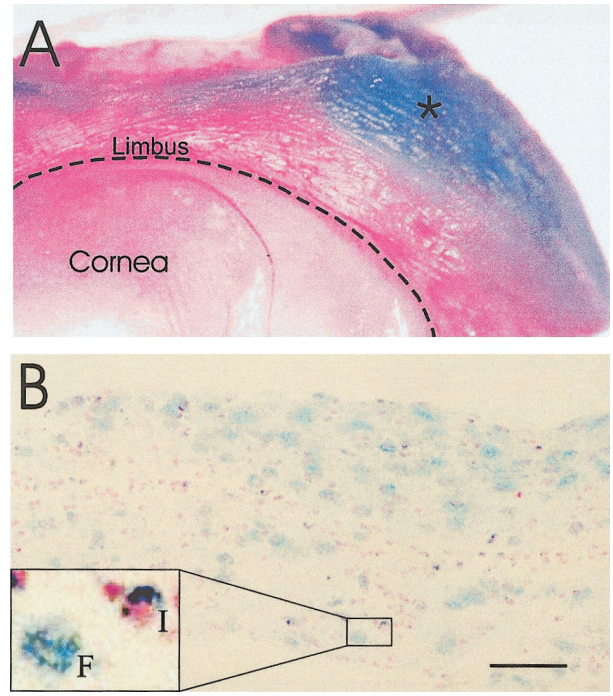

Figure 2. Tissue and cellular localization of transfection. (**A**) The quadrant of a rabbit eye where filtration surgery was performed. Two days after transfection with a collagen shield containing 100 μg of plasmid expressing *β-Gal*, *blue β-Gal* reaction product was localized in the bleb filtration tissues (*). The cornea, limbus (*dotted line*), and surrounding conjunctiva did not generate *β-Gal* staining. (**B**) Pattern of *β-Gal* staining at the cellular level. *Inset*: prominent staining of *β-Gal* is present in fibroblast-like cells (F) of the Tenon's capsule surrounding the bleb; however, minimal *β-Gal* staining was present in inflammatory cells (I). Bar, 50 μm.

Tissue and Cellular Localization of Transfection

As seen in Figure 2A, *β-Gal* staining was localized in the bleb filtration tissues after treatment with the *β-Gal* plasmid delivered by the collagen shield method. The cornea, iris and surrounding conjunctiva did not generate *β-Gal* staining. The observed pattern of localized staining of *β-Gal* reporter gene in the filtration bleb is consistent with the quantitative measurements of CAT activity found in homogenates of the ocular tissues transfected with the *CAT* reporter plasmid. Figure 2B shows the pattern of *β-Gal* staining at the cellular level. This field shows an area of prominent staining of *β-Gal* in fibroblast-

like cells of the Tenon's capsule surrounding the bleb, although other areas of Tenon's capsule within the bleb showed fewer cells staining for *β-Gal*. *β-Gal* staining was not found in cells of the sclera or conjunctival epithelium. Inflammatory cells were observed in the bleb tissues but minimal *β-Gal* staining was present in these cells (Fig. 2B, inset). Identification of fibroblasts and inflammatory cells was based on morphologic features that are characteristic of these two cell types. Specifically, cells were identified as fibroblasts based on the presence of multiple, irregular, cytoplasmic extensions and an oval-shaped nucleus. Inflammatory cells, specifically polymorphonuclear leukocytes, were identified based on the presence of a segmented nucleus and multiple cytoplasmic inclusion bodies. Scleral fibroblasts and conjunctival epithelium were identified by their location in the tissue sections. Similar patterns of *β-Gal* staining were observed in the companion eye transfected by injecting the *β-Gal* plasmid into the bleb.

Discussion

Conjunctival wound healing after glaucoma filtration surgery is a major determinant of the long-term clinical success of the procedure. Failure of glaucoma filtration surgery is most often due to scarring in the conjunctiva level at the bleb and sclerostomy sites. Information from previous investigations in animal models and in patients have helped to formulate a general model of conjunctival scarring.[20] Similar to skin wound healing, conjunctival scarring progresses through a sequence of phases. Initially, after tissue damage and blood vessel rupture, the clotting cascade is activated, which leads to degranulation of platelets and release of multiple growth factors including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, epidermal growth factor (EGF), insulin-like growth factors (IGFs), and fibroblast growth factors (FGFs). The growth factors, together with arachidonic acid metabolites, promote chemotaxis of inflammatory cells to the injury site neutrophil are the first inflammatory cells of the immune system to enter the wound area, accumulating within 6 hours and disappearing by the third day after wounding.[21] Macrophages are the next inflammatory cells to migrate into the wound, becoming the predominant inflammatory cell type at approximately 12 hours after injury, reaching peak numbers on approximately the third day and decreasing by the fifth day. Lymphocytes comprise approximately 70% of the inflammatory cells in the wound area by the 10th day after injury. Newly synthesized collagen fibers and elastin fibers are detected in the subconjunctival wound area as early as 3 days after surgery in animal models, reach a maximum approximately 7 days after surgery, and then slowly decrease in subsequent weeks as the scar tissue remodels.

Intuitively, a major goal of glaucoma filtration surgery is to identify the molecule(s) that play key roles in regulating conjunctival scarring and to develop agents that selectively and controllably inhibit excess scarring and bleb failure. Chemotherapeutic drugs such as 5-fluorouracil and mitomycin C have powerful, although nonspecific effects, on cells by inducing cell death or apoptosis. Both drugs are used clinically to reduce conjunctival scarring in certain high-risk patients, but these drugs have potential side effects. A unique aspect of glaucoma filtration surgery healing is the bathing of wound tissues by aqueous humor. The presence of multiple growth factors in normal aqueous humor and tears, especially latent TGF-β2, suggests that components in aqueous humor can influence conjunctival scarring.[22,23] Recently, repeated injections of a recombinant monoclonal neutralizing antibody to human TGF-β2 significantly inhibited conjunctival scarring in a rabbit model of filtration surgery.[24] Transfection of conjunctival cells with plasmids expressing antisense oligonucleotides, ribozymes, or other genes that block scarring or promote antiscarring activities may provide an additional approach to preventing excessive scarring and failure of glaucoma filtration surgery.

Plasmids generally are not considered optimal vectors for gene therapy, in part because of their presumed inefficiency in cellular transfection. These experiments demonstrated that substantial levels of reporter gene expression could be achieved in ocular filtration surgery tissues by simple injection of naked plasmid DNA into the bleb, and furthermore, absorption of the plasmid into a collagen shield increased the level of transfection by many times. Perhaps even more important for clinical applications, the transfection was almost exclusively localized to the bleb site without significant uptake in other ocular tissues. This suggests that potential unwanted side effects that could result from transfection of other tissues may be avoidable. In contrast, postoperative injections of 5-fluorouracil can produce well-known side effects in tissues away from the surgical site, most notably toxicity to the ocular surface.[25]

The localized gene transfection was achieved using a rabbit filtration surgery model that mimics many of the key parameters of human filtration surgery. However, certain anatomic aspects of the rabbit eye require alterations in technique. The rabbit sclera is very thin and soft, which prevents the construction of a reliable partial-thickness scleral flap and necessitates the use of a full-thickness sclerostomy. In addition, the anterior chamber is very shallow in the rabbit, which, when combined with the absence of a scleral flap, results in a prolonged period of postoperative flat chamber. This model, therefore, necessitates the use of viscoelastic in the anterior chamber to prevent a flat chamber, which would probably promote retrograde flow of plasmid from the bleb into the anterior chamber and away from the desired location of transfection. In human surgery where the sclerostomy is guarded by a scleral flap, the use of a viscoelastic may not be necessary. However, many surgeons routinely leave viscoelastic in the anterior chamber in human surgery,[26–28] which together with a guarded sclerostomy, should minimize retrograde flow of material into the anterior chamber.

The reason that increased transfection was achieved with the collagen shield is not known. It could be because a slow release of the plasmid from the dissolving collagen shield resulted in longer exposure time of the target tissues to the vector. Plasmid injected into the bleb probably diffused away from the site much more rapidly. If this explanation is correct, it suggests that other slow-release vehicles may also enhance transfection.

It is also known that plasmid vectors generally produce relatively short-term gene expression. The level of gene transfection at various time points after intraoperative plasmid application must be delineated in further experiments. However, gene expression that decreases over a period of days or weeks may be desirable in this setting where the primary goal is to regulate wound healing in the short term until a stable fistula for aqueous egress is established. Furthermore, short-term ex-

pression could be amenable to clinical scenarios in which postoperative injections are titrated according to the monitored clinical response. Retroviral and AAV vectors have longer term expression due to integration into the genome, which limits the ability to adjust gene expression. To overcome this potential problem, efforts are under way to develop promoters that can be incorporated into vectors and can be regulated by small molecules, such as tetracycline. Another advantage of naked plasmid vectors is the minimal inflammatory cell and neutralizing antibody humoral response produced compared to adenovirus vectors or lipid–plasmid complexes.

The cellular localization of gene expression predominantly in fibroblasts is an important finding. This seems to indicate that cells that were present in the tissues at the time of exposure to the plasmid were transfected, but that inflammatory cells that appeared later in the healing process were not transfected. Therefore, if gene expression in the inflammatory cells is desired in an eventual therapeutic model, this may require repeated injections of plasmids into the filtration bleb.

Short-term gene transfection may provide advantages over current anti-scarring treatments for glaucoma surgery such as chemotherapeutic drugs (5-fluorouracil and mitomycin C) because of the theoretical ability to selectively target genes that play key roles in regulating conjunctival scarring. In addition, because gene transfection should be effective for days or weeks, it is possible that only a single application to tissues is needed. In contrast, treatment with neutralizing antibodies or other proteins typically require repeated applications to be effective because the therapeutic proteins diffuse away from the site of injection or are inactivated. Transfected cells should continue to synthesize the transgene protein (or an antisense oligo or a ribozyme) for an extended period. Before plasmid transfection can be evaluated for effectiveness with therapeutic transgenes, basic parameters for transfection of conjunctival tissues must be established. The data reported here have helped to delineate many of the issues of surgical technique, vector choice, transfection quantitation, and transfection localization that are unique to glaucoma filtration surgery. The results justify further investigations of gene transfection as a therapeutic adjuvant to manipulate wound healing in glaucoma filtration surgery.

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