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Prospects for gene therapy in corneal disease

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Abstract

Transfer of cDNA to corneal cells has been accomplished using viral and nonviral vectors. Studies examining the feasibility and optimal methods for vector-mediated gene transfer to the cornea have, as in other tissues, been performed using histochemical or fluorescent marker genes. These have used corneal cells or cell lines *in vitro*, and whole corneas maintained in *ex vivo* culture. Gene-based interventions have been examined in specific corneal disorders such as allograft rejection, postexcimer laser scarring, and herpes simplex keratitis using experimental models.

As the feasibility of genetic modification of corneal cells has been successfully demonstrated, there is great potential for gene therapy vectors in the treatment of human corneal disease. Continued improvements in vectors for gene transfer will improve the efficacy and safety of gene therapy. In addition to use of cDNA transfer as an alternative to drug or protein treatments in acquired corneal disorders, our expanding knowledge of the genetic basis of inherited corneal disorders will ultimately lead to the development of specific and effective gene therapies in this category of diseases.
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Introduction

A number of characteristics of the cornea compared with other tissues confer significant potential for gene treatment of corneal diseases. These include the relatively simple histological structure of the cornea, its accessibility for examination and manipulation, its ability to be maintained in *ex vivo* culture for several weeks, and the relative immune privilege of the anterior chamber.[1] Ongoing advances in our understanding of the biological basis of corneal diseases such as the inherited epithelial, stromal, and endothelial dystrophies,[2,3] allograft rejection,[4] herpes simplex keratitis,[5] and others provide a growing number of possible targets for the development of genetic therapies.

Corneal diseases tend to affect primarily a single layer of tissue, and gene therapy approaches for specific diseases will most likely require modification of the primary site of pathology. For example, the corneal epithelium is rapidly dividing and is readily accessible to topical agents. The stroma and keratocytes are physically sequestered by the overlying epithelium/Bowman's layer and the lamellar structure of the extracellular matrix. The endothelium is a nonreplicative monolayer, directly accessible to interventions *in vivo* via the anterior chamber and *ex vivo* in whole corneal storage. Numerous *in vitro* and nonhuman *in vivo* studies reported to date have established the feasibility of delivery modalities for each layer of the cornea. Furthermore, studies in animal models have shown that the exogenous administration of specific genes can inhibit pathological processes affecting different corneal layers. These results collectively emphasize the significant potential of these approaches.

Techniques for gene transfer to the cornea

Epithelium

The accessibility of the corneal epithelium is a clear practical advantage for potential gene therapy applications. Furthermore, the central role of epithelial cells in the well-defined keratoepithelin dystrophies makes this layer an appealing target for genetic modification.[6] Earliest attempts to genetically modify corneal epithelium used the physical method of bombardment with gold microparticles coated with DNA encoding green fluorescent protein (GFP) marker. This approach successfully delivered the marker gene to the target cells without corneal damage or ocular irritation.[7]

There has been greater interest in use of recombinant viral vectors for gene transfer, principally on account of the ability of viruses to generate high levels of transgene expression. A replication-deficient adenovirus type 5 (Ad5) bearing *lacZ* cDNA encoding β-galactosidase was used by Tsubota *et al*[8] to infect corneal epithelial cell lines as well as *ex vivo* corneal epithelial cells with 100% transduction efficiency, meaning that all target epithelial cells were transduced. Application of the same Ad5 construct failed to yield transgene expression *in vivo* in rat corneal epithelium[8] and *ex vivo* to rabbit corneal epithelium.[9] Upregulation of the pro-inflammatory cytokines interleukin-6, -8, and ICAM-1 was observed in response to Ad5 infection.[8] This upregulation was inhibited with coadministration of topical betamethasone, suggesting a possible approach to improve the usefulness of adenovirus-mediated gene transfer to the ocular surface epithelium.

Stroma

In an alternative approach, the commonly used surgical technique of stromal hydration was used to inject a saline solution containing naked plasmid DNA directly into mouse corneal stroma.[10] This method demonstrated expression of *lacZ* in epithelial cells and keratocytes at the remarkably early interval of 1 h postinjection, peak expression levels at 24 h, and minimal inflammatory reaction. Use of this technique with a plasmid encoding vascular endothelial growth factor (VEGF) resulted in marked corneal vascularization. Similar experiments with a plasmid encoding the VEGF inhibitor Flt-1 showed significant inhibition of the vascularization induced by controlled-release VEGF pellets implanted into mouse corneas.[10]

Genetic modification of keratocytes has received particular attention in efforts to inhibit haze formation following refractive laser stromal ablation. A G1BgSvNa retroviral vector containing herpes simplex virus (HSV) thymidine kinase (HStk) resulted in 90% growth inhibition of cultured human keratocytes.[11] Following superficial keratectomy of rabbit corneas *in vivo*, the same vector bearing *lacZ* yielded 25–40% keratocyte transduction efficiency; treatment of HStk-transduced keratocytes with ganciclovir resulted in a statistically significant reduction in corneal haze.[11] Inhibition of rabbit keratocyte growth *in vitro* has also been demonstrated using a pG1XSvNA retroviral vector containing an antisense cyclin G1 (aG1) construct by McDonnell and colleagues.[12] Transduction efficiency was 34% and cell proliferation was inhibited by approximately 50%. Transduction with aG1 was associated with increased incidence of apoptosis. This study was extended to inhibit stromal haze after excimer laser photorefractive keratectomy (PRK) in rabbit eyes.[13] Retroviral vector containing an antiproliferative dominant negative mutant cyclin G1 (dnG1) was applied as drops after PRK. The dnG1 construct demonstrated significant inhibition of corneal haze at 2 weeks post-PRK. Later timepoints were not reported.[13]

Long-term genetic modification of keratocytes may be a useful therapy for keratoconus and inherited stromal disorders. Mucopolysaccharidosis VII (MPS VII) is one such disorder that has been investigated, an autosomal recessive disorder arising from systemic deficiency of lysosomal β-glucuronidase (GUSB) and causes ocular abnormalities including corneal clouding, retinal degeneration, and glaucoma. Using a mouse model of MPS VII with corneal clouding, an adenoviral vector encoding GUSB was applied directly to corneal stroma after lamellar keratectomy. This resulted in widespread distribution of GUSB expression in cells in the corneal stroma, epithelium, and endothelium at 5 and 30 days post-treatment.[14] Corneal expression of GUSB was associated with morphologic improvement as well as rapid and almost complete reversal of vacuolar histopathological changes. Even taking into account the shorter lifespan of mouse than man, these results must be considered short-term for an inherited disorder in which lifelong expression of a functionally active therapeutic gene would be necessary. As long-term expression of a therapeutic gene requires integration into the host cell genome, retroviruses are at present the vectors of choice for these applications. Lentivirus is one such vector and has been found to transduce primary keratocyte cultures with high efficiency and sustained expression of GFP up to 60 days.[15] Efficient gene transfer using the same retroviral vector was also reported *in situ* for endothelial and epithelial cells and for keratocytes at the cut edge of the specimen.[15] As with other vectors, marker gene expression was not detected in keratocytes which were not directly exposed to retrovirus-containing media.

Endothelium

Following exposure of whole-thickness cornea *ex vivo* to optimal concentrations of recombinant adenovirus bearing *lacZ*, high proportions of rat (90%) and rabbit (>75%) endothelial cells were transduced. Expression was restricted to the endothelium, peaked at day 3–5 postinfection and was found to diminish to very low levels at 14–21 days.[9,16] Following adenovirus-mediated *lacZ* transfer *ex vivo*, rabbit corneas were then transplanted as allografts into recipient rabbits. Short-term expression of the marker gene in donor corneal endothelial cells was demonstrated *in vivo*, without significantly increased clinical or histopathological evidence of ocular inflammation.

Moreover, stable corneal thickness measurements postgrafting indicated that endothelial function remained satisfactory, excluding significant cytopathic effects of the virus or the tissue manipulation.[9] Further examining the feasibility of such approaches in clinical management, a similar adenovirus vector was shown to transduce human corneal endothelium *ex vivo*. High (90–100%) levels of target cell transduction were found, with expression of the marker gene for a maximum of 7 days post-transduction.[17] A similar vector was used to transfer cDNA encoding CTLA-4 Ig, an immunomodulatory protein which blocks T-lymphocyte activation, into human corneas in *ex vivo* culture: cumulative secretion of functionally active CTLA-4 Ig by the cornea into culture medium was demonstrated up to 28 days post-transduction.[17] The significance of this study is the demonstration that transfer of immunomodulatory genes, or other genes that might influence graft function post-transplantation, to donor human corneas is feasible during the period of *ex vivo* storage using current eye banking methods.

In contrast to short-term applications in such circumstances as transplantation, long-term genetic modification of corneal endothelial cells would be a significant therapeutic advance for endothelial disorders such as Fuchs' dystrophy and inherited diseases. Integration of a putative therapeutic gene into the host cell genome would be required, for which candidate vectors are recombinant adeno-associated virus (rAAV) or retrovirus. rAAV has been used *in vivo* to transduce rabbit corneal endothelium after injection into the anterior chamber, although it is noteworthy that highest level expression required induction of intraocular inflammation by intravitreal lipopolysaccharide injection.[18] Nevertheless, evidence of *lacZ* expression in endothelial cells up to 60 days postinjection suggests the feasibility of longer term genetic modification. Moreover, recombinant HIV-based lentivirus vectors delivered by a similar intracameral injection approach have yielded gene expression in mouse endothelium for up to 12 weeks duration and without any requirement for induction of intraocular inflammation.[19] The clear safety limitations for clinical gene therapy applications in corneal disease of HIV-based retrovirus vectors has prompted investigation of non-HIV retroviruses. We have used an equine infectious anaemia virus (EIAV) to transduce *ex vivo* cultured human corneal endothelium (Figure 1). Transduction efficiency of this vector of approximately 50% suggests significant potential for nonhuman retroviral vectors, which have the advantages of decreased pathogenicity and immune responses.

Nonviral gene transfer vectors have several potential advantages over viral vectors. These avoid the potential for viral cytopathogenicity and induced immunogenicity, and are comparatively easy to produce. Such vectors include cationic liposomes and cationic dendrimers.[20–22] All of these studies showed that less than 10% of target cells were transfected with the marker gene, suggesting limited clinical potential of these agents alone. Receptor-targeting peptides include integrin-targeting[23] and transferring receptor-targeting[24] vectors, nonviral systems that improve cell transfection efficiency rates in corneal endothelium to ~25%.

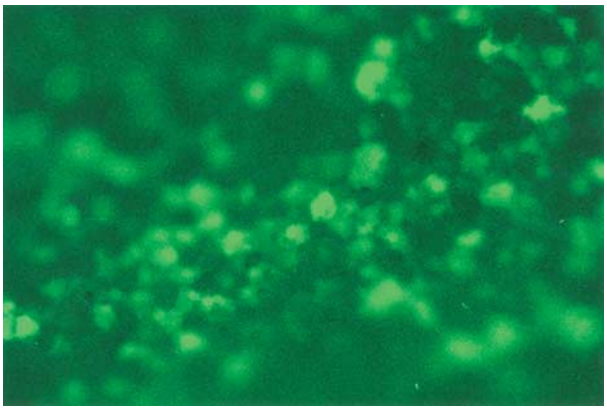

Figure 1 Fluorescence photomicrograph of human corneal endothelial cells transduced *ex vivo* with an equine infectious anaemia virus vector encoding enhanced green fluorescent protein.

Gene therapies for corneal disorders

Corneal graft rejection

Cornea is the most commonly transplanted tissue, with approximately 45 000 cases performed annually in the United States and the United Kingdom. The fact that almost all donor corneas used as allografts in these procedures are stored *ex vivo* for a period of up to 4 weeks prior to transplantation indicates the potential for genetic modification approaches which side-step the possible safety issues of *in vivo* gene therapy in patients. As graft rejection is the leading cause of corneal transplant failure, modulation of the host allogeneic response to the cornea or protection of the donor corneal endothelium by some strategy has significant appeal.

As in other transplanted tissues, corneal allograft rejection is dependent on alloreactive T-cell activation.[25] T-cell activation requires the initial interaction of alloantigen/major histocompatibility complex (MHC) present on the surface of antigen-presenting cells (APC) with the T-cell receptor/CD3 complex present on the T-cell surface.[25] T-cell activation requires additional costimulation interactions between molecules expressed on the T-cell and APC surfaces. Binding of CD28 on the T-cell surface to ligands CD80 and 86 on the APC surface

is the most potent such costimulatory interaction described thus far. A strategy to prolong corneal graft survival by inhibiting the host T-cell costimulatory signal through CD28 has been examined in a rat corneal allograft model using the protein CTLA4-Ig. The protein was expressed from cDNA in an adenoviral vector AdCTLA. These experiments demonstrated that *ex vivo* administration of AdCTLA4 prior to transplantation minimally prolonged graft survival, and protein would in this circumstance be assumed to be secreted into the anterior chamber by donor endothelial cells postgrafting; of interest, a single systemic injection of AdCTLA at the time of transplantation significantly prolonged graft survival.[26]

An alternate immunomodulatory approach to prolong corneal allograft survival is inhibition of the activity of tumor necrosis factor (TNF), a pro-inflammatory cytokine which is present in aqueous humour prior to and at observed onset of endothelial graft rejection.[27] Using a rabbit corneal transplant model, an adenoviral vector AdTNFR containing cDNA encoding a soluble TNF receptor protein was used to transfect donor corneas *ex vivo* prior to transplantation. Transduced corneas showed moderately increased graft survival times compared with control donor corneas incubated in virus-free medium. However, additional control corneas transduced with an adenovirus construct lacking the TNF receptor (Ad0) showed significantly reduced graft survival times compared with AdTNFR-infected and mock-infected corneas. In tandem with the effects of AdCTLA, this suggests harmful immunogenic effects of adenovirus infection which counteract the anti-inflammatory effect of the TNFR or CTLA-Ig constructs.[28]

In a related approach using adenovirus and the TNF pathway, lipoadenofection was used to introduce a marker gene under a TNF-inducible promoter into rabbit corneal endothelium.[29] This method utilizes adenovirus to enhance liposome-mediated DNA transfer into cells and may have the potential advantages of decreased immunogenicity and no need for cloned adenoviral constructs. This study demonstrated a 9- to 10-fold upregulation of marker gene expression after TNF stimulation, suggesting the possibility that endogenous TNF levels can be used to control the expression of additional immunomodulatory genes to prolong graft survival.[29] The significance is that it may be possible to commence expression of counter-inflammatory transgenes only at onset of a rejection episode.

An additional gene target for immunomodulation after corneal grafting is interleukin (IL)-10. This cytokine downregulates MHC class II and costimulatory molecule expression on monocytes, macrophages, and dendritic cells, and inhibits the synthesis of pro-inflammatory cytokines. Using a sheep corneal transplant model, *ex vivo* transfection of corneal endothelial cells with an adenoviral construct encoding IL-10 demonstrated very significant prolongation of graft survival. IL-10 expression was detected at 21 days, and no evidence of immunologic or inflammatory responses was present after transplantation.[30] This study involved a similar *ex vivo* donor cornea gene modification strategy and an adenovirus vector, but the more beneficial effect on graft survival indicates the importance of cDNA selection in such studies. There is clear potential for improvement in effects of such gene modification strategies and this is likely to follow improved less immunogenic viral or more effective nonviral vectors. Looking to possible application of these methods in the future, there is no reason why donor cornea modification could not incorporate immunomodulatory with other cytoprotective constructs, and this would be likely to be combined with postoperative immunosuppression of the recipient.

Herpes simplex virus keratitis

It is known that after primary infection, HSV-1 assumes a latent state from which it can reactivate and cause corneal inflammation, vascularization, and scarring. Detailed knowledge of the HSV genome and the immunological response following infection in host tissue is the foundation for recent studies on gene-based interventions in HSV keratitis models.

The host cytokine interferon-α1 (IFN-α1) antagonizes HSV-1 transcription, translation, and assembly. Using a murine model of lethal encephalitis after corneal infection by HSV-1, topical application to the cornea of plasmid DNA encoding IFN-α1 provided a protective effect when administered either before or shortly after infection.[31] Immunization confers protection against viral infection. In an alternative approach to attenuating virus-induced injury, subconjunctival and topical administration of plasmid DNA encoding HSV-1 glycoprotein D linked to interleukin-2 (gD-IL-2) prevented the development of keratitis in mice.[32,33]

These studies used murine models of primary HSV-1 infection and provide significant insights into the pathobiology of HSV-1. They represent DNA vaccine approaches rather than gene therapy as discussed in other contexts. Human application of these approaches appears unlikely as widespread pre- or peri-infection immunization with these vectors would face significant practical obstacles.

Conclusions

Genetic modification has been achieved in cultured corneal cells, *ex vivo* corneas, and *in vivo* animal models.

Recombinant adenovirus vectors remain the most effective studied to date in the cornea, largely on account of the high proportion of target cells transfected by these viruses and the resulting high levels of recombinant protein produced. Nonviral gene transfer systems have not as yet proved effective, although toxicity and immunogenicity of viral vectors are likely to limit the safety of these in clinical applications. However, later-generation adenovirus vectors, in which deletion of more viral genes is engineered, will be of experimental interest especially in immunological applications, in which any corneal cell expression of viral proteins can significantly attenuate the immunomodulatory effect of the therapeutic gene. An additional major challenge in development of gene therapies in corneal disorders is the requirement of long-term, even lifelong, gene expression for inherited disorders. However, this is a category of disease for which there is at present only the nonspecific and partially successful treatment of corneal transplantation. Retroviral vectors are likely to ultimately enable this approach to be feasible.

The cornea remains an ideal model tissue for genetic modification with great potential to assume a vanguard role in the development of successful new gene therapies. Much progress has been made in the genetic modification of the epithelium, endothelium, and keratocytes using viral and nonviral vectors in a variety of model systems. Despite these advances, continued improvements in existing or new gene therapy techniques will be required before these approaches can be applied to human clinical trials.

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