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Gene delivery to cornea

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Abstract

This paper reviews the strategies of *in vivo* gene delivery to the cornea. A number of studies have demonstrated the feasibility of targeted delivery of oligonucleotides, small interfering RNA (siRNA), plasmid DNA, and viral vectors to the corneal cells *in vivo*, specifically stromal keratocytes and corneal epithelial cells, via intrastromal injection, iontophoresis, electroporation, and gene gun. Intrastromal injection of plasmid DNA and adenovirus each can result in efficient transgene expression to stromal keratocytes. The introduction of foreign genes into intact corneal epithelium specifically requires more invasive procedures such as gene gun to disrupt the tight junction barrier and/or cell membranes. The combination of iontophoresis and electroporation was found to be effective in delivering siRNA but not plasmid DNA into the corneal epithelium. Nanocarriers such as polymeric micelles are promising methods of corneal gene delivery.

1. Introduction

The transparent and avascular cornea is an ideal tissue for examining the efficacy of gene therapy of congenital and/or acquired diseases. Since the first attempt of corneal gene therapy reported in 1994 [1], gene therapy has been explored for many corneal diseases such as allograft rejection, herpes simplex keratitis, corneal neovascularization, corneal haze, and corneal dystrophies [2–4]. Current gene delivery methods can be divided into viral and non-viral carrier systems. Despite high expression efficiency of viral vectors, inherent immunogenicity and toxicity limit their uses in human eyes. Non-viral vectors can overcome the immunogenicity and toxicity problems but their expression efficiency is relatively low. Physical and chemical enhancement methods have been investigated to increase gene delivery to corneal cells. However, successful delivery of genes into the target corneal cells and the retention of genes in the cells remain challenging. Readers are encouraged to go to a number of recently published reviews on the topic of corneal gene therapy [2,3]. The focus of the present review is to provide an overview on the strategies of gene delivery to the cornea *in vivo* and particularly recent advances in gene delivery methods (Fig. 1).

2. Anatomy of the cornea

The cornea primarily consists of a stratified epithelium, a thick collagenous stroma, and a monolayer of endothelium [2]. The thickness of the human cornea varies from 0.5 mm at the

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center to 0.7 mm at the periphery. The tear film of 4–9 μm covers the epithelium. The corneal epithelium consists of approximately 6–7 layers of stratified epithelial cells that migrate from the inner layer to the outer surface in about 2 weeks. Tight junctions exist between the epithelial cells, forming the major barrier to topical drug delivery. The stroma is mainly composed of an extracellular matrix and the predominant stromal cells are the keratocytes. The thick stroma (> 90% of the corneal thickness in human) plays an important role in maintaining corneal transparency. The endothelium with a thickness of 5 μm is a non-regenerated monolayer cells, forming a leaky barrier to the aqueous humor due to gap junctions between the endothelial cells. The major function of the endothelium is to regulate the hydration of the stroma.

3. Gene delivery to the corneal epithelium

Although the outermost corneal epithelium is the most accessible, e.g., to the administration of eye drops, the tight junctions in the epithelium create a major barrier to topical gene delivery [5]. For the delivery of hydrophilic molecules, the paracellular route of the epithelial layer is the predominant pathway and the permeability is a function of molecular size. It is generally accepted that macromolecules such as genes cannot be effectively delivered into the corneal epithelium by passive diffusion mechanism using eye drops in the absence of permeation enhancers. Even topical applications of viral vectors failed to deliver and express transgenes in the epithelial layer without the removal of tear film [6] and scarification of the cornea [7]. Various enhancers capable of opening the tight junctions in mucosal membranes such as ethylenediamine tetraacetic acid (EDTA) and cytochalasin B have been tested in corneal epithelial cells [8]. For example, a novel cell-penetrating peptide for ocular delivery was found to enter corneal epithelium after topical application in mice and was suggested to have potential for gene delivery [9]. Excimer laser ablation procedure was used to ablate the basal epithelial rabbit eyes before topical application of viral vectors expressing green fluorescent protein (GFP), which allowed transduction of genes into the basal epithelial cells and the stromal fibroblasts [10]. Nanocarriers such as polymeric micelles [11] and nanoparticles [12] were also investigated to examine the efficacy of corneal gene delivery. This section provides a summary of common injection and physical enhancement methods reported in the literature that allow and/or enhance gene delivery in the epithelial layer.

3.1. Injection

Intrastromal injection has been used to deliver naked DNA plasmids into the cornea for gene expression in the corneal epithelium of mice [13,14]. The expression of *LacZ* reporter gene driven by CMV-promoter (cytomegarovirus) for the protein β -galactosidase (β -gal) occurred as early as 1 hr after intrastromal injection and lasted for 10 days [13]. It is believed that the rapid gene expression was related to the ability of the injected DNA plasmids to enter the epithelial cells by both endocytosis and intrastromal pressure effect due to the volume change from the injection of isotonic buffer. When plasmids that express small interfering RNA (siRNA) against vascular endothelial growth factor (VEGF) were injected into the corneal stroma of mouse at 8 hr before alkaline-induced corneal trauma or 1 week after the injury, expression of VEGF was effectively suppressed compared to the control group [15]. Intrastromal injection was suggested to be suitable mainly for treating acute corneal diseases [13] because the durations of gene retention and expression in the cornea after intrastromal injection were relatively short irrespective of the rapid expression.

3.2. Electroporation

Electroporation uses electric pulses to create transient pores in a cell membrane [16]. The permeant is transported through these pores mainly by diffusion with some contributions from electrophoresis and electroosmosis. The pores would then reseal over time after the termination of the electric field [17]. Electroporation has been widely used in the transfection of cells *in*

vitro, but *in vivo* electroporation studies on gene delivery to the cornea are limited. A previous study has investigated the delivery of cyanine 3 (Cy3) labeled glyceraldehydes-3-phosphate dehydrogenase (GAPDH) siRNA (MW ~ 13 kDa) to the epithelium of mice with electroporation of the siRNA solution on the eye using a custom-made applicator [18]. However, the use of electroporation alone to deliver siRNA into the cornea epithelium was considered not very effective. For the delivery of macromolecules greater than 1 kilobase pairs such as plasmid DNA into the cornea, plasmid delivery can be significantly enhanced when the DNA plasmid is first injected into eyes and electric pulses are subsequently applied to the eyes. Application of electric pulses to mouse eyes to deliver plasmids expressing luciferase or GFP after intrastromal or subconjunctival injection of the plasmids resulted in gene expression over the entire surface of the cornea in both epithelial and stromal layers [19]. The amount of protein expressed was about 1000 fold higher than that with the injection of DNA alone. At optimal field strength of 200 V/cm, no trauma, corneal edema, or inflammation was observed. Electroporation parameters such as electric field, pulse number, pulse length, and pulse interval are critical for effective gene delivery and not causing damages to the cornea.

3.3. Iontophoresis

Iontophoresis uses an electric field to enhance the delivery of compounds across the plasma membrane of cells. The mechanisms of iontophoretic transport across a membrane include the direct interactions of the electric field with the charge of an ionic compound (electrophoresis or electromigration), convective solvent flow affecting the transport of both neutral and ionic compounds (electroosmosis), and electric field-induced pore formation in the membrane (electroporation or electro-permeabilization) [20]. Among these three mechanisms, electromigration is expected to be the dominant driving force for charged macromolecules such as RNA, siRNA, and plasmid DNA. siRNA and oligonucleotide (ODN), which are highly negatively charged and have high charge to molecular weight ratios, are good candidates for iontophoretic delivery. When performed correctly under low to moderate electric current conditions and with short duration, transcorneal iontophoresis is considered to be safe [21]. This method has been examined in human for the delivery of small molecules [22]. In a previous iontophoresis study, Cy3-labeled GAPDH siRNA was successfully delivered into the corneal epithelium of mouse eyes by 1 min application of 0.5 mA iontophoresis [18]. In another study, single-stranded cyanine 5 (Cy5) labeled antisense ODN targeting VEGF-R2 receptor was reported to be delivered to all corneal layers of rat eyes using 0.3 mA iontophoresis for 5 min [23]. Plasmid DNA, possibly due to its large molecular size, could not be iontophoretically delivered into the epithelial layer. Therefore, the effectiveness of corneal iontophoretic delivery is highly dependent on the size of the macromolecule.

3.4. Combination of iontophoresis and electroporation

Although both iontophoresis and electroporation use electric fields to assist the transport of compounds across a membrane, they work by different mechanisms. Whereas iontophoresis primarily assists the transport of charged compounds across a membrane barrier through the mechanism of electromigration, electroporation facilitates the transport by the electropermeabilization of the membrane as the dominant mechanism. The combination of iontophoresis and electroporation have been found to be more effective in delivering and retaining siRNA in the cornea compared to iontophoresis or electroporation alone [18]. In this study, the siRNA was mainly localized in the epithelium of the cornea.

3.5. Gene gun

Gene gun is a particle-mediated delivery system. It uses an adjustable low-pressure helium pulse to sweep gene-coated microcarriers from the inner wall of a small plastic cartridge directly into the target *in vivo*, thus providing rapid and direct gene transfer to the target. Gene

gun has been used to transfer various genes into the corneal epithelium [24–29]. The gene-coated gold particles are delivered into the epithelial cells where the gene is translocated into the nucleus via a mechanism that is not well defined. In a previous study, β -gal expression was detected in the epithelium on days 2 and 4 after gene gun treatment. The gold particles disappeared from the epithelium by day 14 after the treatment at pressure of 300 psi probably due to the turnover of the epithelial cells [28]. The transfection was restricted to the epithelium, irrespective of the size of microcarriers and pressure used. While gene gun is a useful method for cornea epithelial cell gene delivery, mild corneal inflammation was reported in the treatment [28].

3.6. Nanocarriers

Nanocarriers have attracted great attention in gene delivery to the cornea due to the size of these carriers, which are in the same nanometer range as the paracellular pore diameter of the corneal epithelium [5]. Among these carriers, eye drops were developed using poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles loaded with *LacZ* gene plasmids encoding for β -gal whose expression was driven by different promoters, i.e., cytomegalovirus early gene (pCMV-*LacZ*), keratin 12 gene (pK12-*LacZ*), and keratocan gene (pKera 3.2-*LacZ*) [8,11]. PEO-PPO-PEO copolymers are Food and Drug Administration (FDA) approved and have been widely used in ophthalmic products. After topical applications of the polymeric micelle eye drops containing epithelium-specific pK12-*LacZ* to mice and rabbits, β -gal activity was observed to increase in both mouse and rabbit corneas [11]. With the eye drops of polymeric micelles containing pKera 3.2-*LacZ* to the animals, stroma-specific *LacZ* expression was found in the stroma only after the pretreatment of 5 mM EDTA before the topical application [11]. This suggests that the epithelial barrier accounted for the lack of gene delivery into the stroma; the gene was only delivered to the stroma after the perturbation of the tight junctions in the epithelial layer by EDTA. The delivery mechanisms of this nanocarrier were believed to be paracellular transport and endocytosis evidenced by enhanced delivery after the pretreatment of EDTA, a tight junction opener, and retarded by the pretreatment of RGD peptide (arginine-glycine-aspartic acid), an endocytotic inhibitor with a critical integrin-binding motif.

Among the polymers explored for the construction of corneal gene nanoparticles, hyaluronan and chitosan are most attractive owing to their desirable toxicity profiles and physicochemical properties [30–32]. Hyaluronan (HA) and chitosan (CS) or chitosan oligomer (CSO) nanoparticles containing plasmid DNA models (e.g., GFP) were tested in rabbit eyes [12]. Following topical administrations of the nanoparticle (HA:CSO and HA:CS at a mass ratio 1:2) suspensions to the rabbit eyes, gene expression was detected at a dose of 25 μ g/eye for HA:CSO nanoparticles and 50 μ g/eye for HA:CS nanoparticles. The expression was significantly increased with increasing the dose to 50 μ g/eye for HA:CSO nanoparticles and remained apparently the same when the dose was further increased to 100 μ g/eye. The expression lasted as long as 7 days for HA:CSO nanoparticles. The enhanced transfection and extended expression of these nanoparticle systems compared to naked plasmid DNA suspensions were attributed to the incorporation of HA in the nanoparticles and nanoparticle interactions. The nanoparticles prepared from fluoresceinamine-labeled HA (HA-fl) and CS or CSO without the plasmids were found to penetrate through the corneal epithelium of rabbit eyes after 2 h post-instillation of nanoparticles. The nanoparticles were deposited inside the cells rather than in the intercellular spaces, indicating a transcellular transport mechanism. These nanoparticles retained in the epithelial cells longer than the control HA-fl solution, suggesting a stronger interaction of HA with the cornea when HA-fl was in a particulate form, and thus a slower clearance of the nanoparticles from the eye. These results indicate that HA, along with CS, is suitable for the fabrication of nanoparticles for corneal gene delivery due to not only its biocompatibility, biodegradability, and mucoadhesion but also its interactions with

various HA receptors on the corneal epithelial cells, which likely promote the HA cell uptake [33,34].

4. Gene delivery to the corneal stroma

The corneal epithelial barrier hinders the delivery of macromolecules into the stroma from the surface of the cornea. For example, a previous study has shown that polymeric micelles containing plasmid DNA with a keratocyte-specific promoter did not reach the stroma unless the cornea was pretreated with EDTA to open tight junctions in the corneal epithelium [11]. Other studies have found that gene gun was unable to provide gene delivery across the corneal epithelium and transfect stromal keratocytes [24,29]. It is believed that a more invasive approach such as injection or its combination with physical enhancement such as electroporation or ultrasound is necessary to achieve stromal gene delivery.

4.1. Injection

Direct injection of naked DNA plasmids into the stroma was an effective method for rapid gene delivery to the stroma. Gene expression was detected on the stroma after intrastromal injection of naked plasmid DNA [13]. Intrastromal injection of an adenoviral vector constructed with GFP gene using a keratocyte-specific promoter localized the gene expression to the stroma [35]. The GFP expression was first detected at 11 hr post-injection with duration of approximately 3 weeks and the adenovirus did not transfect the endothelium and epithelium. In another study, the injection of albumin nanoparticles encapsulating plasmids was shown to sustain gene release and prolong the gene expression in the cornea [36]. The albumin nanoparticles entered the corneal keratocytes and retained in the cells more than 4 weeks. The expression of the gene and its effect on corneal neovascularization reduction lasted over 5 weeks.

Subconjunctival injection is an alternative approach to introduce genes into cornea. siRNAs targeting VEGFs significantly inhibited neovascularization induced by cytosine phosphate guanine (CpG) oligodeoxynucleotides and herpes simplex virus infection in mouse eyes at day 4 after subconjunctival injection [37]. Subconjunctival injection of brain-specific angiogenesis inhibitor 1 (BAI1) gene plasmid significantly reduced neovascularized corneal area induced by epithelial debridement [38]. When a plasmid expressing both GFP and BAI1 was administered subconjunctivally 1 week after epithelial debridement, green fluorescence was detected in the corneal stroma with expression retained for 7 days.

4.2. Injection and electroporation

Injection of plasmid DNA into the stroma or subconjunctival space and subsequent electroporation on the eye generally increased gene delivery and expression in the stroma compared with injection alone without electroporation. Electric field strength affects not only the gene expression but also the safety profiles of the treatment. Electroporation was found not to cause cornea damages or inflammation at an optimal electric field of 200 V/cm [19]. In a previous study, plasmid DNA with GFP gene and cytomegalovirus promoter was injected into the corneal stroma of rats, and electric pulses were applied to the cornea with an electric probe [39]. Transgene expression was detected in the stroma on day 1, peaked on days 4 and 5, and then decreased until day 15. Histology study disclosed GFP expression in keratocytes within the targeted area. Gene transfer was most effective using eight electric pulses at 20 V with a pulse length of 50 ms. In another study, it was demonstrated that the type of promoters used in the construction of plasmid affects the duration of gene expression. After intrastromal injection and electroporation, luciferase expression from plasmid with a long-acting human ubiquitin C (UbC) promoter lasted for at least 4 weeks, while the expression from the plasmid with a short-term cytomegalovirus immediate early promoter (CMV_{iep}) lasted for 7 days

[40]. In the same study, CMV_{iep}-driven interleukin 10 (IL-10) expression in the corneas of mice peaked at day 1 whereas UbC-driven IL-10 expression sustained for 4 days after treatments with intrastromal injection and subsequent electroporation [40]. Multi-doses of plasmids and electroporation extended the expression. The method of combining intrastromal injection and electroporation provided gene expression located mostly in the stromal layer. Although the combination of subconjunctival injection and electroporation was shown to deliver gene into the stroma, gene expression was also detected in other eye tissues such as the limbus [19]. Therefore, intrastromal injection and electroporation is believed to be more specific in targeted delivery of genes to the stroma.

4.3. Intrastromal injection and ultrasound

Ultrasound is widely used for clinical imaging and its safety has been established. Ultrasound with microbubbles can increase gene transfer by lowering the acoustic pressure threshold needed to induce the microjets for cellular membrane penetration of macromolecules. Acoustic parameters such as duty cycle, exposure time, ultrasound power, and concentration of microbubbles affect the efficiency of gene delivery. In a previous study, GFP plasmid was injected into the center of the cornea and ultrasound was applied with microbubbles on the corneal surface using a probe [41]. The injection alone could provide gene transfer to a limited degree, but plasmid injection followed by ultrasound with microbubbles strongly increased the gene transfer efficiency without apparent tissue damage. The GFP gene expression was distributed mainly in the keratocytes within the area exposed to ultrasound.

5. Gene delivery to the corneal endothelium

Gene delivery to the corneal endothelium has the potential to modulate corneal graft rejection [42], an immune-mediated reaction targeting the endothelium and causing failure of corneal transplantation. Corneal endothelial cell loss during allograft failure is mediated by apoptosis. Suppressing apoptosis in the graft endothelium by the delivery of an anti-apoptotic gene *bcl-xL* with lentivirus to the corneal endothelium of the donor cornea significantly improved the survival of allografts *ex vivo* [43]. However, a previous study showed that adenovirus encoding GFP can selectively infect the endothelium but increase graft failure [44]. Many other viral and non-viral vectors were explored to deliver genes to the corneal endothelium but with limited success *in vivo* [42]. Successful *in vivo* studies of endothelial gene delivery without using the injection procedure have not been reported in literature. Unlike epithelial and stromal delivery, there is no corneal endothelial cell-specific promoter available at this time for *in vivo* gene delivery to corneal endothelial cells.

Owing to inaccessibility of the innermost endothelial layer and direct contact of this layer to the aqueous humor in the anterior chamber, injection of genes into the anterior chamber could be an administration route for gene delivery into the endothelium. Likewise, gene expression in the endothelium may also provide an approach to treat anterior chamber diseases. The expression of connexin43 (Cx43) in the rat corneal endothelium was knocked down by direct injection of Cx43 antisense oligodeoxynucleotide (AS-ODN) or siRNA into the anterior chamber [45]. Cx43 knockdown accelerated wound healing with complete wound closure 3 days after the treatments. Electric pulses applied after the injection could facilitate the transport of the genes into localized areas of the endothelium. In one study, plasmid DNA with the *LacZ* gene coding for β -gal was injected into the anterior chamber of rats and 8 pulses of 5 to 40 V were applied to the cornea using a circular electrode immediately after the injection [46]. It was found that the β -gal expression in the endothelium were confined to the area where the electrode was applied, indicating localized delivery of the gene by the electric field. No damage to the cornea was observed when the applied voltage was below 20 V but cornea opacity occurred at 40 V despite the higher transfection efficiency at the higher voltage. With this delivery method, transfection efficiency reached its maximum on day 3 and decreased over

21 days post-treatment under the electroporation condition of 8 pulses of 20 V and 50 ms. In another study, a plasmid encoding tissue plasminogen activator (tPA) was injected into the anterior chamber at the rat limbus [47]. With the assistance of electric pulses given subsequently on the surface of the cornea, the plasmid DNA was transferred to a highly selected area of corneal endothelium without inflammation. The biologically active tPA was present in the cornea for 4 days post-treatment. Intracameral fibrin formation and corneal cloudiness were significantly inhibited in treated eyes compared to control eyes.

6. Conclusion

Recent advances in the knowledge of gene therapy can benefit the treatment of corneal diseases. The cornea which is transparent and avascular is also an ideal tissue for developing a strategy to treat congenital and/or acquired diseases. However, it is important to understand that efficient gene therapy relies on the effectiveness of the delivery method, and the advances in gene delivery technologies depends on our understanding of the mechanisms and principles of macromolecule transport across and into the cells in cornea. In recent years, we have observed progress in these technologies. To date, effective gene delivery can be achieved by physical and chemical methods such as direct injection into the corneal stroma, combination of injection with electroporation and ultrasound, direct application using a gene gun, and the use of nanocarriers with chemical enhancers. Despite this, a noninvasive method of gene transfer in the cornea and corneal gene therapy in human is not currently available. For gene therapy of the corneal epithelium, iontophoresis and the combination of enhancers and nanocarriers are the most promising noninvasive methods. For the corneal stroma and endothelium, these two methods are not effective without the deposition of the macromolecules in the stroma and endothelium via injections. The process of putting basic science into practice can be long and difficult, but the continuing efforts in improving corneal gene delivery through fundamental research can lead to successful development in this area.

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Fig. 1. Methods of gene delivery targeting the epithelia, stroma, and endothelia of the cornea reviewed in this paper.