Review

Corneal gene therapy

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Abstract

Gene therapy to the cornea can potentially correct inherited and acquired diseases of the cornea. Factors that facilitate corneal gene delivery are the accessibility and transparency of the cornea, its stability ex vivo and the immune privilege of the eye. Initial corneal gene delivery studies characterized the relationship between intraocular modes of administration and location of reporter gene expression. The challenge of achieving effective topical gene transfer, presumably due to tear flow, blinking and low penetration of the vector through epithelial tight junctions left no alternative but invasive administration to the anterior chamber and corneal stroma. DNA vaccination, RNA interference and gene transfer of cytokines, growth factors and enzymes modulated the corneal microenvironment. Positive results were obtained in preclinical studies for prevention and treatment of corneal graft rejection, neovascularization, haze and herpetic stromal keratitis. These studies, corneal gene delivery systems and modes of administration, and considerations regarding the choice of animal species used are the focus of this review. Opportunities in the field of corneal gene therapy lie in expanding the array of corneal diseases investigated and in the implementation of recent designs of safer vectors with reduced immunogenicity and longer duration of gene expression.

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Keywords: Cornea; Eye; Gene therapy; Corneal graft rejection; Herpetic stromal keratitis; Corneal neovascularization

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This article is dedicated to the memory of Milo Gibaldi, Ph.D.
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1. Introduction

Corneal gene therapy initially emerged in 1994 when its potential in correcting acquired corneal inflammatory diseases was noted following successful transduction of corneal tissues using replication-deficient adenovirus [1]. Inherited corneal diseases such as corneal endothelial dystrophies [2] are natural candidates for corneal gene therapy [3–5]. However, most of the research has focused on modulation, including immunomodulation, of acquired medical conditions. This is feasible since control of the corneal microenvironment can be attained by induction or knock-down of proteins using corneal gene therapy.

Local corneal gene delivery has the potential to achieve low and continuous concentrations of biologically active proteins thereby improving the efficacy and safety of the treatment [6]. This methodology can be applied to the delivery of various cytokines and growth factors to the cornea thus affecting immune response, inflammation, angiogenesis and proliferation and cell differentiation. Corneal gene therapy also holds promise in obtaining local protein expression at concentrations unattainable by systemic administration of the recombinant protein [7].

While corneal gene therapy has developed as a part of the natural growth in the field of gene therapy, several advantages are apparent in making the cornea particularly attractive for gene transfer. The cornea has a well-defined anatomy [8] and is easily accessible [8–11] during ambulatory visits, as well as surgical procedures [12,13]. The cornea can be treated non-invasively [13] as the anatomic location of corneal epithelium permits direct topical instillation of the gene delivery system [14]. The perfect transparency of the cornea allows rapid and non-invasive visual observation [8,9,15] using standard ophthalmologic methods e.g., slit-lamp biomicroscopy, with high magnification thus estimating the effectiveness and safety of the treatment [16].

Additional factors facilitate gene therapy to both anterior and posterior parts of the eye and are therefore relevant to corneal gene transfer. For instance, the eye is a partially immune-privileged site [17,18] thereby enabling the use of otherwise immunogenic or proinflammatory vectors [18]. In terms of experimental design, the fact that the eye is a paired organ enables the contralateral eye to serve as an internal control [19,20]. Also, the ocular tissues are easily anesthetized [16], and are treatable during trabeculectomy, cataract surgery and corneal transplantation [21]. In the same spirit, ex vivo gene transfer to a donor cornea prior to corneal grafting entails several advantages when compared to ex vivo manipulations of other grafts: The cornea can be maintained in culture for up to 1 month [8,9]; its small size enables modification of the complete tissue [18]; and the major target cell (the corneal endothelial cell) forms a monolayer on the corneal surface and is therefore perfectly accessible to gene vectors [7].

Due to the importance of corneal gene therapy and its inherent advantages, studies have been conducted throughout recent years on various types of animals to investigate new treatments for major corneal disorders such as allograft rejection following corneal transplantation, herpetic stromal keratitis (HSK), corneal neovascularization and corneal haze. The potential of corneal gene therapy in decreasing the incidence of blindness is vast, as it can enhance survival of corneal grafts as well as ameliorate corneal disorders that currently lead to the need for corneal transplantation thus obviating the need for an allograft [22]. A particular challenge in corneal gene delivery
stems from the sensitivity of the organ in that even minimal inflammation might impair vision [21]. This calls for extra caution in designing the corneal gene therapy in terms of the delivery system, mode of administration and dosing regimens.

2. Background

2.1. Anatomy, physiology and immunology relevant for corneal gene therapy

2.1.1. Anatomy and physiology

The cornea is the avascular tissue on the surface of the eye that is directly exposed to the external milieu, see Fig. 1A. The cornea refracts light (with the lens), provides protection from microscopic pathogens (with the conjunctiva and tear film) and confers mechanical strength (with the sclera) thereby shielding the eye from physical injuries. Regulated hydration and the precise architecture of the cornea contribute to its unique transparency [23] that is essential for transmittance of incident visible light through the lens to the retina thereby enabling vision [24].

The adult cornea is approximately 11–12 mm wide and has a height of about 9–11 mm. Its thickness varies from 0.5 mm at the center to 0.7 mm at the periphery. The central 4 mm of the corneal diameter is considered the optical area [25].

From anterior to posterior the cornea is composed of 5 layers (see Fig. 1B): epithelium, Bowman’s membrane, stroma, Descemet’s membrane and endothelium. The epithelium, which is considered “tight” [24], has a thickness of about 50 μm and is self regenerated with a complete turnover time of 5–7 days [26]. It protects the eye from microbes and toxins [23], prevents fluid loss [26] and with the tear film, assists in maintaining the optical smoothness of the corneal surface [23]. The tear film, which has a thickness of 4–9 μm [27], lubricates the cornea and provides nutrients and regulatory factors necessary for epithelial maintenance. Bowman’s membrane is an acellular layer, 12 μm thick, having a random arrangement of collagen fibers and proteoglycans. Its physiological role is still unclear [23].

The stroma makes up more than 90% of the corneal thickness and is primarily composed of an extracellular matrix that is composed of collagen and proteoglycans. The predominant stromal cells are the keratocytes, which are corneal fibroblasts [23] that synthesize, maintain and repair the extracellular matrix [24] and occupy 3%–5% of the stromal volume [28]. The unique regular arrangement of collagen fibers is a key factor in corneal transparency [25].

Descemet’s membrane, the basement membrane of the corneal endothelium, has a thickness of 8–10 μm in adults and is comprised of collagen and laminin, a glycoprotein. The endothelium is a monolayer, 5 μm thick, whose major role is the regulation of stromal hydration. On one hand the endothelium is a “leaky” barrier to the aqueous humor due to gap junctions between cells that allow transfer of nutrients and electrolytes [23]. On the other hand it pumps water from the stroma [25]. Thus, increased corneal thickness usually indicates a pathologic condition of the endothelium [28]. The endothelium does not replicate in humans after birth, therefore cell loss is compensated by enlargement of the remaining cells [25].

Other relevant ocular compartments are the anterior chamber, the vitreous body and the conjunctiva. The anterior chamber is a compartment between the cornea and the iris that is filled with approximately 0.2 ml of aqueous humor, which satisfies the metabolic needs of the lens and the cornea [29]. The vitreous body accommodates 80% of the eye’s volume. It supports retinal metabolism and serves as a diffusion barrier between the anterior and posterior part of the eye [30]. The conjunctiva is a thin mucous membrane that lines the inner surface of the lids [26] and along with the cornea, forms the ocular surface [31]. In addition to its roles in facilitating the movement of the eye and as a reservoir for tears [32], the conjunctiva fulfills an important role in immune responses of the ocular surface, playing host to T and B lymphocytes, mast cells, neutrophils and dendritic cells [33].

2.1.2. Immunology

The immune response of the eye entails interactions between local and systemic immune cells, which include macrophages, lymphocytes, eosinophils and antigen-presenting cells; and soluble mediators of the immune system, which encompass complement, cytokines and immunoglobulins [34].
Despite the availability of various components for an immune response, the ability of the eye to tolerate inflammation is very limited, since a conventional immune response, as observed for instance after the penetration of an antigen or a pathogen through the skin, might destroy the microanatomy of the visual axis needed for accurate vision. This explains the need for a special type of immunity in the eye, termed immune privilege [33].

Ocular immune privilege is a result of various elements. Among these are passive elements such as the blood-ocular barrier that physically prevents cellular infiltration [34], the low expression of major histocompatibility complex class I and II molecules and the lack of lymphatic drainage. Active aspects of ocular immune privilege include the constitutive expression of inhibitors of complement activation by corneal endothelial cells and of Fas ligand (which induces apoptosis of activated T cells) by corneal cells. The aqueous humor also contributes to immune privilege as it contains immunosuppressive factors.

Another aspect of immune privilege is the eye’s ability to regulate the systemic immune response to eye-derived antigens through a mechanism termed anterior chamber-associated immune deviation. In this case the immune response induced by penetrating antigens is deficient in B cells that secrete complement-fixing antibodies and in T cells that mediate delayed hypersensitivity.

The result of ocular immune privilege is that immune defense takes place with no inflammation. To emphasize this concept, when privilege is successful corneal allografts are accepted and ocular infections do not inflame the eye. Failure of immune privilege leads to the opposite and blindness is likely to occur [33].

The cornea itself takes an active role in immune protection of the structure and function of the eye surface in that corneal epithelial cells and keratocytes secrete cytokines. Together with the lacrimal gland, tear film and the conjunctiva, the cornea constitutes the ocular mucosal-associated lymphoid tissue [34].

2.2. Vectors and methods of corneal gene therapy

Many of the prominent gene vectors, knock-down methods and physical techniques for gene therapy have been used in corneal gene delivery. We briefly describe these vectors and methods.

2.2.1. Viral vectors

2.2.1.1. Adenovirus. Adenoviral vectors, commonly used in gene therapy, have a broad range of host cells [35,36] and can infect both dividing and non-dividing cells. Since they do not integrate into the genome, their expression is transient [36,37].

Major hurdle in using adenoviruses for gene therapy is the immunogenicity of their capsid and the proteins they express. Most adenoviral vectors are derived from adenovirus serotype 5 [38], having a genome of 35 kb. The first generation adenoviral vectors are E1-gene deleted, with partial or complete deletion of the E3 gene [39], allowing a transgene capacity of 8.5 kb [36]. Removal of the E1 and E3 genes renders the virus mostly replication-deficient and increases the vector capacity, respectively [39]. Second generation adenoviral vectors have additional deletions of part of the E2 and/or most of the E4 genes, thereby decreasing the immune response to viral proteins [40]. Third generation adenoviral vectors, also termed gutless adenoviral vectors, contain only the sequences needed for the initiation of viral DNA packaging and replication. Therefore they are less immunogenic [39,41] and have substantial capacity of transgene containment, >30 kb [40].

2.2.1.2. Adeno-associated virus. Adeno-associated viruses have a small (4.7 kb) genome, are non-pathogenic [39] and can infect both dividing and non-dividing cells [40]. Despite the fact that adenov-associated viral vectors do not encode for viral proteins, a humoral immune response is elicited following their administration due to the viral capsid [38]. In contrast to wild-type adenov-associated viruses that demonstrate site specific genome integration, adeno-associated viral vectors integrate at sites of double-stranded DNA breaks [39].

2.2.1.3. Retroviruses. Retroviral vectors, commonly used in gene therapy [40], integrate into the host genome and therefore offer long term transgene expression [37]. Due to their random integration they might cause insertional mutagenesis i.e., insert the transgene into a coding region [40]. For instance, as a part of a clinical trial for the treatment of X-linked severe combined immunodeficiency, vector integration up-regulated a proto-oncogene, LMO-2, leading to T-cell leukemia in 3 patients [42], one of whom has died. Retroviral vectors can infect dividing cells but not non-dividing cells [39].

2.2.1.4. Lentiviruses. Lentiviruses are a subtype of retroviruses that have the ability to infect both dividing and non-dividing cells [37], which was one advantage of constructing gene vectors from them [36]. The most extensively used lentiviral vector is based on human immunodeficiency virus type 1 [38]. A recent design of lentiviral vectors circumvented the risk of insertional mutagenesis by integration deficiency [43].

2.2.1.5. Herpes simplex virus-1 (HSV-1). HSV-1 is strongly neuro-tropic [40] and can replicate in epithelial cells [36]. The genome size of HSV-1 is 152 kb [37] and this enables HSV-1 vectors to accommodate relatively large and/or multiple transgenes. Several types of vectors were developed based on HSV-1: amplicon, replication defective and replication competent attenuated vectors [44]. The latter group was studied for gene therapy for the peripheral nervous system, against cancer and as live attenuated HSV-1 vaccines [45].

2.2.2. Non-viral vectors

2.2.2.1. Naked DNA. Naked plasmid DNA is a negatively-charged, large macromolecule with a molecular weight of ≥2000 kDa [46]. Naked DNA loses its stability once it enters...
the body due to susceptibility to extracellular and intracellular nucleases [46,47]. The characteristics of naked DNA facilitate usage of physical techniques to improve cell entry [36], and compounding with cationic lipids or cationic polymers to enhance in vivo stability and cell penetration [36,46]. It is important to note that intramuscular injection of naked DNA used for vaccination in preclinical studies, elicited an immune response that protected animals against infections [36]. In corneal gene therapy, naked DNA was mostly studied for treatment of HSK, see Table 3.

2.2.2.2. Minimalistic immunologically defined gene expression (MIDGE) vectors. MIDGE vectors are double-stranded linear, covalently closed expression cassettes that contain the cytomegalovirus (CMV) promoter, the gene of interest and a polyadenylation site [48]. They are less immunogenic than standard bacterial plasmids. MIDGE vectors are not commonly used in gene therapy, although they were studied for their utility in corneal gene therapy [48–50].

2.2.2.3. Cationic liposomes. Cationic lipids spontaneously create complexes with negatively-charged DNA [36]. To improve the stability of the complex, which is termed lipoplex, the cationic lipid is often combined with a neutral lipid and/or a surfactant [47]. Creation of lipoplex protects the DNA from degradation [51] and cationic lipoplex facilitates penetration via the negatively-charged cell membrane [36,51]. Cationic lipoplexes do not elicit cellular immune response, are less efficient than viral vectors, and have limited use in vivo due to their inherent toxicity [47].

2.2.2.4. Polyethylenimines. Polyethylenimines, which appear as linear or branched isomers, are the most extensively used cationic polymers for gene delivery [52]. Polyethylenimines spontaneously form nanoparticles with DNA [53] and have exhibited relatively high transfection efficiency in various organs [54] although they are still less efficient than viral vectors [36]. Since polyethylenimines are considered toxic in vivo [54], studies are ongoing to expand their in vivo potential by chemical modifications [55].

2.2.2.5. Polyamidoamine dendrimers. Dendrimers are branched polymers with a well-defined spherical shape [56] and very low polydispersity [57]. When using polyamidoamine dendrimers as gene carrier a higher number of layers (‘generations’) around the core is associated with both increased transfection efficiency and toxicity [56]; and the in vivo transfection efficiency is comparable to polyethylenimines [58].

2.2.3. Physical methods

2.2.3.1. Electroporation. Electroporation is the enhancement of DNA penetration through the cell membrane by creating transient and localized membrane pores using electric fields. In vivo electroporation occurs by injecting plasmid DNA with subsequent induction of short electrical pulses from electrodes located in the target tissues [36]. As an advantage, electroporation usually increases gene expression by 100- to 1000-fold in comparison to naked DNA administration. The disadvantage is that it is potentially harmful to the cell membrane. Electroporation is currently being studied for gene therapy in a wide variety of tissues including skin, skeletal muscle, retina and cornea [59].

2.2.3.2. Gene gun. The term, gene gun, refers to the ballistic delivery of DNA particles laden with heavy metal, usually gold, to cells using pressure and speed [36,60]. While this approach was most commonly used for genetic vaccination and immunomodulation of the skin, it was also studied in other tissues e.g., brain, liver [60], and cornea [48,61,62]. A major benefit of the gene gun approach is the potential to obtain locally-defined gene delivery in particular intracorneal tissues [61].

2.2.3.3. Sonoporation. Sonoporation is the enhancement of DNA penetration through the cell membrane by creating transient and localized membrane pores using ultrasound [60]. Sonoporation increases gene expression by 10- to 15-fold in comparison to naked DNA administration [59] and is safe [13,63]. It has been studied for gene delivery to solid tumors, muscle [59] and the cornea [13]. Ultrasound contrast agents that enhance cavitation e.g., Albunex, promote transfection [60].

2.2.4. Knock-down oligonucleotides and expression cassettes, and RNA aptamers

2.2.4.1. Antisense oligonucleotides. Antisense oligonucleotides are typically 15–20-nucleotide [64] single-stranded DNA sequences that hybridize in the cell to sense mRNA sequences by reverse complementarity. The product inhibits gene expression via several mechanisms [65]. The first antisense oligonucleotide drug that was available in the clinical setting, Vitravene® [64], is an intravitreal injection against CMV-induced retinitis [65].

2.2.4.2. Small interfering RNA (siRNA). Synthetic siRNA are short, typically <30-nucleotide double-stranded RNA sequences [66] that act in the cytoplasm of cells where they can knock-down the expression of subsequent genes [67] more efficiently than antisense oligonucleotides [68]. Such siRNA sequences may be formulated for administration by various delivery systems, and are being tested in intravenous, intraarterial and intravitreal administrations [69] in addition to other routes.

2.2.4.3. Short hairpin RNA (shRNA). shRNA vectors are DNA-directed expression cassettes encoding a single RNA palindromic sequence of 50–70 oligonucleotides that can hybridize with itself to form a stem-loop “hairpin” structure [70,71]. Once in the cytoplasm the structure can be cleaved to 21-nucleotide siRNA duplexes. In comparison to the latter, shRNA viral vectors potentially offer prolonged gene suppression [70,72] and shRNA plasmid DNA vectors are easily amplified to obtain large quantities.
2.2.4.4. RNA aptamers. Following synthesis by in vitro selection and amplification from a sizeable pool of RNA sequences, RNA aptamers (ligands) bind with high affinity to small molecules and proteins, thereby potentially inhibiting the activity of the latter [73,74].

3. Previous studies and relevant diseases

3.1. Corneal diseases and manifestations of metabolic diseases treatable by gene therapy

Corneal gene therapy has the potential to treat and cure inherited and acquired corneal disorders and metabolic diseases affecting the cornea. Preclinical work in this field has concentrated on treating and preventing several acquired disorders such as corneal graft rejection, HSK, corneal neovascularization and corneal haze. Studies were also conducted to evaluate the effect of systemic gene therapy on the manifestations, including corneal, of mucopolysaccharidosis VII, a metabolic disease. Therefore, these diseases are described below.

3.1.1. Corneal transplantation and graft rejection

Corneal transplantation, the sole treatment for many corneal diseases that often lead to blindness [75,76], is the most commonly transplanted solid tissue [76,77] with approximately 40,000 annual transplants in the United States [75]. While corneal transplantation success rates of 75% survival through 5 years [77] and 60% survival through 10 years are relatively high, there is no tendency of improvement with the passage of time [78], thereby emphasizing the need for additional clinical solutions.

Allograft rejection occurs when a cell-mediated immune response that involves T lymphocytes stimulates a cascade of events that lead to graft destruction [34]. Damage to the endothelium is crucial during this process [25]. Risk factors for immunologic allograft rejection, which is the most common reason for graft failure [15,76,77,79,80], include neovascularization [81,82], inflammation, glaucoma and a history of previous graft rejection [33]. Factors that facilitate successful corneal transplantation include the immune privilege of the tissue as well as the site, which obviates the necessity for systemic immunosuppression or human leukocyte antigen matching, and the fact that the donor cornea can be stored ex vivo for up to a month before transplantation [75]. The latter is particularly appealing for gene therapy approaches as it enables modulation of the donor cornea prior to transplantation.

It is important to note that the majority of corneal transplantations involve full-thickness tissue replacement (penetrating keratoplasty), since this procedure produces favorable visual acuity results [75]. Transplantation of the anterior cornea leaving the Descemet’s membrane and endothelium intact (anterior lamellar keratoplasty), is associated with a decreased risk of graft rejection because the endothelium is the predominant rejection site [83].

3.1.2. Herpetic stromal keratitis

There are 50,000 new cases [84] and approximately 500,000 people suffering repeated episodes of ocular HSV-1 infections in the United States every year [85]. After the virus penetrates, often causing a corneal epithelial infection [86], it progresses in a retrograde manner to ganglia where it resides for the remaining life of the host [87]. In 20% of the cases of ocular HSV-1 infections [88] the reactivation of the virus from latency, mostly in the trigeminal ganglion [87], leads to a viral attack on the eye that is accompanied by an immune response orchestrated by virus specific CD4+ T lymphocytes producing T-helper 1 (Th1) cytokines. This leads to a variable degree of HSK [88]. Chronic corneal inflammation associated with HSK that involves infiltration of cells, edema, neovascularization and scarring is potentially threatening to one’s vision. Thus, HSK is the leading infectious cause of blindness in the developed world and is also the reason for 5–10% of penetrating keratoplasty operations performed [89].

The current pharmacotherapeutic regimens for HSK mainly include antiviral drugs and corticosteroids [87]. It is unequivocal that additional therapeutic approaches for treating HSK are required [89]. To this end, efforts are underway to develop a vaccine that will prevent a primary infection and/or recurrent attacks of ocular HSV-1 [90]. Encephalitis as a complication of ocular HSV-1 infection is potentially lethal in the murine model [91,92] as well as in humans [93].

3.1.3. Corneal neovascularization

The cornea is normally avascular and corneal neovascularization, which is always pathologic [94], can occur in any layer of the cornea [87] causing visual impairment. The sprouting of new vessels in the cornea changes the ocular surface microenvironment [34] and might lead to corneal opacity [94].

Corneal neovascularization plays a pivotal role in inflammation [87], which might explain the fact that neovascularization in the host cornea is a risk factor for corneal transplant rejection [95]. Depending on the etiology of corneal neovascularization, traditional treatments involve the use of anti-inflammatory, antimicrobial or immunosuppressant drugs. Clearly, there is a need for additional therapeutic approaches [94].

3.1.4. Corneal haze

Haze is the term used to depict corneal opacity following excimer laser procedures [96]. Following photorefractive keratectomy most patients develop a mild haze that in the majority of cases resolves over time [97]. Corneal haze occurs during the wound healing process when stromal keratocytes transform to activated fibroblasts and subsequently proliferate and migrate to the anterior stromal compartment where they synthesize new collagen and extracellular matrix, thus causing opacity [98]. The current treatment for corneal haze is topical mitomycin C, which presumably induces activated keratocyte apoptosis. Clearly, there is a need for additional therapies [96]. It should be noted that corneal opacity (or opacification) is the term used to describe cloudiness due to other reasons than excimer laser procedures, for instance corneal cauterization [99].

3.1.5. Mucopolysaccharidosis VII

The mucopolysaccharidoses are a family of inherited disorders of a deficiency in lysosomal enzymes, involved in the degradation of glycosaminoglycans, resulting in a broad spectrum
of manifestations [100]. The normal corneal stroma contains approximately 4% glycosaminoglycans and pathologic deposition of keratan sulfate and dermatan sulfate causes corneal opacification. Mucopolysaccharidosis VII, also known as Sly disease, is a very rare disease in which the deficiency of β-glucuronidase causes, in addition to systemic effects, progressive corneal clouding [101] due to the storage of glycosaminoglycans in stromal lysosomal vacuoles [102]. This pathology might necessitate penetrating keratoplasty [100]. No pharmacotherapy is currently available to treat the corneal manifestations of mucopolysaccharidosis VII [101].

3.1.6. Additional proposed corneal diseases

It was suggested in the literature that corneal gene therapy is a promising approach for the correction of additional corneal diseases such as corneal dystrophies [23] and in particular corneal endothelial dystrophies, which are primary diseases of the corneal endothelium that have an inherited component [2]. These dystrophies encompass congenital hereditary endothelial dystrophy, Fuch’s endothelial dystrophy [3–5,103] and posterior polymorphous dystrophy [3,4]. Other diseases that were mentioned as candidates for corneal gene therapy include keratoconus, inherited stromal disorders [103], iridocorneal endothelial syndrome, [2–4], recurrent anterior segment inflammation of Behcet’s disease [21,104] and infectious diseases of the cornea [5,10]. Rosenblatt and Azar further proposed that dry eye syndrome, recurrent erosions, neurotrophic keratopathy, persistent epithelial defects and chronic epithelial inflammatory disease can all potentially be treated by corneal gene therapy [105].

3.2. In vivo corneal gene delivery studies

The seminal works of gene delivery to the cornea appeared in 1994–1995 and concentrated on the transfer of reporter genes [1,3]. George et al. were the first to study the prevention of graft rejection by ex vivo genetic manipulations of the cornea prior to transplantation, in 1996 [8]. Additional seminal works involving corneal gene therapy were conducted by Rouse et al. to treat HSK (1997 [88]) and Rakoczy et al. [106] to correct corneal neovascularization.

In reviewing previous studies involving corneal gene delivery, this article highlights the in vivo studies that use ocular gene transfer to correct corneal diseases, as opposed to works lacking in vivo experiments or studies using only extra-ocular gene transfer to manage corneal diseases. Information regarding previous research in the area of in vivo corneal gene delivery that includes vectors, genes, animal species, doses, modes of administration, results of studies and references is summarized in Tables 1–5. Each table summarizes an application of corneal gene delivery to treat a specific corneal malady with the exception of Table 1, which describes the transfer of reporter genes. Several corneal gene delivery studies that involve ocular modes of administration [107–111] and extra-ocular modes of administration [99,112–116], are not covered in Tables 1–5. The latter studies, however, are mentioned in Section 4.

3.2.1. Transfer of reporter genes

The initial works that exhibited transfer of genes to the cornea were directed at studying the ability of gene delivery methods to transfer genes to either the eye [1] or the retina [117]. When characterizing the ocular distribution of gene transfer, expression was noted in the cornea, among other ocular tissues. In 1995, Budenz et al. published the first study that aimed mainly to examine gene delivery to the cornea and the trabecular meshwork [3].

The use of reporter genes offers the advantages that (a) the analytical method for the measurement of their expression is usually sensitive and easy; (b) they lack bioactivity and are considered generally safe, although it has been documented that at least for several transgenes, very high expression is potentially toxic to cells; and (c) studies involving reporter genes do not involve induction of disease that is potentially time and effort consuming. These are some of the reasons that more studies in corneal gene delivery were conducted by utilizing reporter genes than using bioactive proteins for the correction of any single corneal disease (see Tables 1–5).

Moreover, corneal gene delivery studies that use reporter genes involve a wider array of gene vectors and methods than corneal gene therapy studies to treat any single corneal disease. For instance, viral vectors that were used in studies of reporter genes that characterized gene transfer to (among other ocular tissues) the cornea include adenovirus, adeno-associated virus, retroviruses, lentiviruses, HSV-1 and baculovirus. The physical methods that were used to deliver reporter genes to the cornea are electroporation, gene gun and sonoporation.

Reporter gene delivery studies in the cornea have used many of the most common reporter genes e.g., chloramphenicol acetyl transferase, green fluorescent protein, enhanced green fluorescent protein, luciferase and lacZ, which is the most common reporter gene in corneal gene delivery (see Table 1). The major aspects that were studied were the ability to transfer genes to the cornea using various vectors, physical techniques and modes of administration, ocular distribution of gene expression, safety of gene transfer vectors and methods in the cornea, and relationship between the dose and magnitude of corneal gene expression. Thus, corneal gene delivery studies that used reporter genes laid the foundation for corneal gene therapy studies of corneal diseases.

3.2.2. Corneal transplantation and prevention of graft rejection

A summary of corneal gene therapy studies for prevention of graft rejection appears in Table 2. Corneal gene therapy for prevention of graft rejection is challenging since corneal transplantation is at least partially successful. In order to justify the effort, cost and risk of advancing gene therapy research related to the prevention of graft rejection into clinical trials, it is desirable to demonstrate prolonged life span of the transplant and reduced treatment refractoriness leading to rapid graft rejection in preclinical studies.

The fact that immunologic graft rejection is the major reason for transplant failure [15,76,77,79,80] may explain the high prevalence of corneal gene therapy studies, which aim to inhibit the cascade of immunological events that lead to graft rejection.
<table>
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<th>Vector</th>
<th>Gene</th>
<th>Animal</th>
<th>Dosing, per eye</th>
<th>Mode of administration</th>
<th>Results/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Mice</td>
<td>$3 \times 10^7$–$10^8$ pfu, 10 $\mu$l</td>
<td>Intracameral injection</td>
<td>Expression in 20–30% of endothelial cells for 50 days [1]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Mice</td>
<td>$10^3$–$10^4$ pfu/ml, 0.3–0.5 $\mu$l</td>
<td>Intravitreal injection</td>
<td>Expression in endothelium 1 week post-administration [117]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Mice</td>
<td>$5 \times 10^5$ pfu</td>
<td>Intravitreal injection</td>
<td>Expression in endothelium 1 week post-administration [162]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Mice</td>
<td>$10^5$–$10^6$ particles/ml, 2–3 $\mu$l</td>
<td>Intracameral or intravitreal injection</td>
<td>Expression in endothelium for 14 days following both modes of administration [3]</td>
</tr>
<tr>
<td>Naked DNA, cationic lipoplexes</td>
<td>lacZ</td>
<td>Rats</td>
<td>Lipoplexes: 0.8 $\mu$g plasmid DNA, 10 $\mu$l</td>
<td>Topical administration</td>
<td>Naked DNA: no expression; lipoplexes: expression in epithelium was stable for 7 days and was decreased 1 month post-administration [163]</td>
</tr>
<tr>
<td>Cationic lipoplexes</td>
<td>lacZ</td>
<td>Rats</td>
<td>0.8 $\mu$g plasmid DNA, 10 $\mu$l</td>
<td>Topical, intracameral, intravitreal or subretinal administration</td>
<td>Expression in epithelium; intracameral, intravitreal, subretinal: expression in epithelium and endothelium; expression was stable for 7 days and was decreased 1 month post-administration [164]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Rabbits</td>
<td>$8 \times 10^5$ pfu, 20 $\mu$l</td>
<td>Intracameral injection</td>
<td>Expression in endothelium 48 h post-administration [12]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>lacZ</td>
<td>Mice</td>
<td>4–100 $\mu$g, 4 $\mu$l</td>
<td>Topical application to scarified cornea with or without HSK</td>
<td>Expression was stronger than without HSK, 30 h post-administration [88]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>lacZ</td>
<td>Mice</td>
<td>100 $\mu$g, 4–5 $\mu$l</td>
<td>Topical application</td>
<td>No expression               [137]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>GFP</td>
<td>Rabbits</td>
<td>1 $\mu$g</td>
<td>Gene gun</td>
<td>Expression in epithelium for 7 days [61]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>CD-1 mice, immunocompetent or immunocompromised</td>
<td>$4 \times 10^6$ pfu, 1 $\mu$l</td>
<td>Intravitreal injection</td>
<td>Expression in endothelium for 1 week (immunocompetent mice) or 5 weeks (immunocompromised mice) [4]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Balb/C mice, immunocompetent or immunodeficient</td>
<td>$2 \times 10^6$ pfu, 2 $\mu$l</td>
<td>Intracameral injection</td>
<td>Expression in endothelium for 3 weeks (immunocompetent mice) or 15 weeks (immunodeficient mice) [165]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Rats</td>
<td>$1 \times 10^5$–$1 \times 10^6$ pfu/ml, 5 $\mu$l, 3 times for 1 day</td>
<td>Topical administration</td>
<td>No expression [14]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>lacZ</td>
<td>Rats</td>
<td>2.5 $\mu$g, 5 $\mu$l</td>
<td>Intracameral injection with electroporation</td>
<td>Expression in endothelium for 21 days; peak on day 3 [21]</td>
</tr>
<tr>
<td>MoMLV-based retroviral vector</td>
<td>lacZ</td>
<td>Rabbits</td>
<td>100 $\mu$l, 0, 12 and 36 h after lamellar keratectomy</td>
<td>Topical administration</td>
<td>Expression in stroma 4 days post-surgery [159]</td>
</tr>
<tr>
<td>Plasmid DNA having a keratin 12, corneal epithelial specific, promoter</td>
<td>lacZ</td>
<td>Rabbits</td>
<td>2.5 $\mu$g</td>
<td>Gene gun</td>
<td>Expression in epithelium 2 days post-administration [62]</td>
</tr>
<tr>
<td>MoMLV-based retroviral vector</td>
<td>lacZ</td>
<td>Rabbits</td>
<td>Ex vivo keratolimbal graft transduction; orthotopic autograft implantation</td>
<td>Expression in epithelium for 6 months [166]</td>
<td></td>
</tr>
<tr>
<td>HSV-1</td>
<td>lacZ</td>
<td>Mice, rats</td>
<td>$1 \times 10^5$ pfu, 5 $\mu$l</td>
<td>Topical administration with or without corneal scarification, intracameral or intravitreal injection</td>
<td>Mice and rats: expression in epithelium after topical application with, but not without, corneal scarification; rats but not mice: expression in endothelium after intracameral and intravitreal injection; expression declined on day 7 and ended on day 14 [167]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>GFP</td>
<td>Rabbits</td>
<td>0.5, 5 or 50 $\mu$g/ml, 2 ml</td>
<td>Iontophoresis</td>
<td>Expression in all layers of the cornea [168]</td>
</tr>
<tr>
<td>HIV-1-based lentiviral vector</td>
<td>GFP</td>
<td>Mice</td>
<td>$2 \times 10^3$ transducing units/ml, 2 $\mu$l</td>
<td>Intracameral or intravitreal injection</td>
<td>Intracameral: expression in endothelium for 12 weeks; intravitreal: no expression [169]</td>
</tr>
<tr>
<td>Virus Type</td>
<td>Gene</td>
<td>Animals</td>
<td>Dose/Amount</td>
<td>Injection Route</td>
<td>Expression Details</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------</td>
<td>---------</td>
<td>-------------</td>
<td>----------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>GFP</td>
<td>Monkeys</td>
<td>$10^7$ pfu or $10^9$ pfu, 20 μl</td>
<td>Intracameral injection</td>
<td>High dose ($n=2$), strong inflammation; low dose ($n=1$), no expression; low dose ($n=1$), expression in endothelium after repetitive injections</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>GFP</td>
<td>Mice</td>
<td>$10^8$–$10^7$ infection units</td>
<td>Intravitreal injection</td>
<td>Expression in endothelium for 14 days</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>lucZ</td>
<td>Rats</td>
<td>$10^1$ pfu/ml, 2 μl, with or without cauterization</td>
<td>Intracameral injection</td>
<td>With or without cauterization, expression in endothelium for 10 days</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>lucZ</td>
<td>Mice</td>
<td>0.05–3600 ng, 2 μl</td>
<td>Intrastromal injection</td>
<td>Expression in stroma and epithelium from 1 h to 5 days; peak on day 1</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>EGFP</td>
<td>Mice</td>
<td>1 μg, 2 μl</td>
<td>Intrastromal injection</td>
<td>Expression in stroma and epithelium from 4 h to 6 days; peak on day 1</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>GFP</td>
<td>Rats</td>
<td>0.5 μg/μl, 5 μl</td>
<td>Intrastromal injection with electroporation</td>
<td>Expression in stroma during days 1–15; peak on days 2–6</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>lucZ</td>
<td>Rats</td>
<td>$10^7$ pfu, 25 μl, followed by inflammation induction</td>
<td>Intracameral injection</td>
<td>Expression in endothelium was induced by inflammation</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>GFP</td>
<td>Mice</td>
<td>2 μg, 1 μl</td>
<td>Intrastromal injection with electroporation</td>
<td>Increased endothelial expression using a higher dose</td>
</tr>
<tr>
<td>BIV-based lentiviral vector</td>
<td>EGFP</td>
<td>Mice</td>
<td>$5 \times 10^4$ or $5 \times 10^5$ transducing units</td>
<td>Intravitreal injection</td>
<td>Increased endothelial expression using a higher dose</td>
</tr>
<tr>
<td>Cationic lipoplexes, adeno-associated virus</td>
<td>CAT, lucZ</td>
<td>Rabbits</td>
<td>50 μg, 50 μl of plasmid DNA in lipoplexes; CAT: $1 \times 10^{11}$ pfu, 25 μl; lucZ: $5 \times 10^{11}$ pfu, 10 μl</td>
<td>Intrastromal injection via a lamellar flap</td>
<td>Expression in stroma following all modes of administration; adeno-associated virus: up to 21 days</td>
</tr>
<tr>
<td>Naked DNA having a CMV or keratocanstromal specific promoter, adeno-associated virus</td>
<td>EGFP</td>
<td>Mice</td>
<td>2 μg plasmid DNA or $1.3 \times 10^6$ pfu adenovirus, 2 μl</td>
<td>Intrastromal injection of adenovirus or naked DNA having a CMV promoter; or subconjunctival injection of naked DNA having different promoters</td>
<td>Expression in stroma during days 1–15; peak on days 2–6</td>
</tr>
<tr>
<td>Cationic lipoplexes</td>
<td>Luciferase</td>
<td>Rabbits</td>
<td>Naked DNA: 2 μg/μl; lipoplexes: 40–85 μg; 100 μl</td>
<td>Topical instillation or intravitreal injection</td>
<td>Expression in stroma during days 1–15; peak on days 2–6</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>EGFP with or without anti-CD4 antibody fragment</td>
<td>Rats</td>
<td>$5 \times 10^4$–$6 \times 10^7$ pfu, 1–5 μl</td>
<td>Intracameral injection</td>
<td>Expression of EGFP in endothelium on day 3</td>
</tr>
<tr>
<td>Plasmid DNA with FuGENE® 6</td>
<td>EGFP</td>
<td>Rats</td>
<td>$5 \mu$ plasmid DNA, 0.4 ml</td>
<td>Subconjunctival injection</td>
<td>Expression in stroma during days 3–7</td>
</tr>
<tr>
<td>HIV-based lentiviral vector</td>
<td>GFP</td>
<td>Rats</td>
<td>$10^5$, $10^6$, and $10^7$ pfu, 2–10 μl</td>
<td>Intracameral injection</td>
<td>Expression in endothelium 3 weeks post-administration; higher expression was obtained for $10^6$ and $10^5$ pfu</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>GFP</td>
<td>Rats</td>
<td>10 μg, 12 μl</td>
<td>Intracameral injection with ultrasound and microbubbles</td>
<td>Expression in stroma during days 1–8 with gradual decrease thereafter</td>
</tr>
<tr>
<td>ELAV-based lentiviral vectors pseudotyped with VSV-G or rabies-G envelope proteins</td>
<td>EGFP</td>
<td>Mice</td>
<td>$1.5 \times 10^3$–$1.4 \times 10^4$ transducing units/ml, 2 μl</td>
<td>Intracameral or intravitreal injection</td>
<td>Expression in stroma for 10 months post-administration; rabies-G pseudotype; 38% of eyes, expression in stroma and epithelium; intravitreal: no expression</td>
</tr>
<tr>
<td>Plasmid DNA having a CMV or ubiquitin promoter</td>
<td>Luciferase, IL-10</td>
<td>Mice</td>
<td>$5 \mu$, 1 μl</td>
<td>Intrastromal injection with electroporation</td>
<td>Expression mostly in stroma; IL-10: CMV, decline after 1 day; ubiquitin, stable for 4 days; luciferase: ubiquitin, lasting 28 days</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Heme oxygenase-l</td>
<td>Rabbits</td>
<td>$10^8$ pfu/ml, 10–20 μl</td>
<td>Intracameral or intravitreal injection</td>
<td>Intracameral: expression in endothelium and epithelium; intravitreal: expression in endothelium</td>
</tr>
</tbody>
</table>

Abbreviations: BIV, bovine immunodeficiency virus; CAT, chloramphenicol acetyl transferase; cfu, colony-forming units; CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; EIAV, equine infectious anaemia virus; GFP, green fluorescent protein; HIV, human immunodeficiency virus; HSK, herpetic stromal keratitis; pfu, plaque-forming units; IL, interleukin; MoMLV, Moloney murine leukemia virus.

* Among various organs and ocular tissues, only expression in the cornea is described.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Gene</th>
<th>Animal</th>
<th>Dosing, per eye</th>
<th>Treatment</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>lacZ</td>
<td>Rabbits</td>
<td></td>
<td><em>Ex vivo</em> graft transduction; orthotopic transplantation</td>
<td>Expression for 4 days with no effect on corneal function</td>
<td>[8]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>TNFR-Ig</td>
<td>Recipients: New Zealand white rabbits; donors: Dutch Belted rabbits</td>
<td></td>
<td><em>Ex vivo</em> graft transduction</td>
<td>Mock adenovirus shortened survival of grafts in comparison to TNFR-Ig adenovirus-treated or untreated grafts</td>
<td>[123]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>CTLA4-Ig</td>
<td>Recipients: Lewis rats; donors: Brown Norway rats</td>
<td>1 × 10^5 pfu/ml, 100 μl</td>
<td><em>Ex vivo</em> graft transduction or intravenous injection</td>
<td><em>Ex vivo</em> and systemic administrations prolonged graft survival from 9 days to 18 days (medians), respectively</td>
<td>[119]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>IL-4</td>
<td>Recipients: Lewis rats; donors: Wistar–Furth rats</td>
<td>1 day post-transplantation</td>
<td><em>Ex vivo</em> graft transduction</td>
<td>No prolongation of graft survival</td>
<td>[9]</td>
</tr>
<tr>
<td>MIDGE</td>
<td>IL-4, IL-10, CTLA4, IL-4 with CTLA4</td>
<td>Recipients: Balb/C mice; donors: C3H mice</td>
<td>2 μg</td>
<td>Glucocorticoid pharmacotherapy with gene gun bombardment to the epithelium 10 days post-transplantation</td>
<td>All transgenes: no prolongation of graft survival over pharmacotherapy positive control</td>
<td>[49]</td>
</tr>
<tr>
<td>MIDGE</td>
<td>IL-4 with CTLA4</td>
<td>Recipients: Balb/C mice; donors: C3H mice</td>
<td>0.1 μg, repeated twice within 1 min</td>
<td>Pharmacotherapy with gene gun bombardment to the epithelium 1 day post-transplantation</td>
<td>Prolongation of graft survival from 27±19 to 64±28 days</td>
<td>[50]</td>
</tr>
<tr>
<td>MIDGE</td>
<td>IL-10</td>
<td>Sheep</td>
<td>10 μg, repeated to a total dose of 300 μg</td>
<td><em>Ex vivo</em> graft transduction</td>
<td>Prolongation of graft survival from 20 days to 55 days (medians)</td>
<td>[18]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>IL-10</td>
<td>Sheep</td>
<td></td>
<td><em>Ex vivo</em> graft transduction</td>
<td>P40 IL-12: prolongation of graft survival from 20 days to 45 days (medians); IL-4: no effect</td>
<td>[118]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Monomeric anti-CD4 antibody fragment</td>
<td>Recipients: Fisher 344 rats; donors: Wistar–Furth rats</td>
<td>Injection of 2–5 × 10^7 pfu</td>
<td><em>Ex vivo</em> graft transduction with or without intracameral injection to donor corneas 3 days pre-transplantation</td>
<td>Eyelid: prolongation of graft survival from 26.6±5.7 to 61.7±25.6 days; leg: no effect</td>
<td>[48]</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Endostatin:K5 IDO</td>
<td>Rabbits</td>
<td></td>
<td><em>Ex vivo</em> graft transduction</td>
<td>Prolongation of graft survival from 14 and 18 days to 39 days (medians)</td>
<td>[82]</td>
</tr>
<tr>
<td>EIAV-based</td>
<td>Endostatin:K5 IDO</td>
<td>Rabbits</td>
<td></td>
<td><em>Ex vivo</em> graft transduction</td>
<td>Prolongation of graft survival from 11 days to 21 days (medians)</td>
<td>[76]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>CTLA4-Ig with or without IL-10</td>
<td>Recipients: Lewis rats; donors: Dark Agouti rats</td>
<td>Injection of 1 × 10^5 or 1 × 10^10 pfu 1 day pre-transplantation</td>
<td><em>Ex vivo</em> graft transduction with or without intraperitoneal injection</td>
<td><em>Ex vivo</em>: prolongation from 13.1±0.3 (control) to 15.8±0.6 (CTLA4-Ig) or 15.3±0.2 (CTLA4-Ig and IL-10) days; systemic, CTLA4-Ig: low dose 22.7±4.3, high dose 40.4±9.2 days (medians)</td>
<td>[15]</td>
</tr>
<tr>
<td>Cationic lipoplexes or adenovirus</td>
<td>Viral IL-10</td>
<td>Recipients: Lewis rats; Donors: Wistar–Furth rats</td>
<td>Injection of 1 × 10^5 or 1 × 10^10 pfu 1 day pre-transplantation</td>
<td><em>Ex vivo</em> graft transduction or intraperitoneal injection</td>
<td><em>Ex vivo</em>: lipoplexes or adenovirus, no effect; systemic: prolongation from 10.56±0.34 (control) to 14.6±1.01 (low dose) days (medians); high dose, no effect</td>
<td>[79]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>NGF, CTLA4-Ig</td>
<td>Recipients: Lewis rats; donors: Dark Agouti rats</td>
<td>Injection of 1 × 10^5 pfu 1 day pre-transplantation</td>
<td><em>Ex vivo</em> graft transduction, intraperitoneal injection, or both</td>
<td>NGF: ex vivo, prolongation from 13.1±0.3 (control) to 16.8±1.4 days (medians); systemic, no effect; NGF ex vivo with CTLA4-Ig: ex vivo, no effect; systemic, no rejection (6/7 animals) for 70 days post-transplantation</td>
<td>[80]</td>
</tr>
</tbody>
</table>

**Abbreviations:** CTLA4-Ig, cytotoxic T lymphocyte antigen 4-immunoglobulin; EIAV, equine infectious anaemia virus; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; K5, kringle 5 of plasminogen; MIDGE, minimalistic immunologically defined gene expression; pfu, plaque-forming units; NGF, nerve growth factor; TNFR-Ig, tumor necrosis factor receptor-immunoglobulin.
Table 3

In vivo corneal gene therapy for prevention and treatment of herpetic stromal keratitis

<table>
<thead>
<tr>
<th>Vector</th>
<th>Gene †</th>
<th>Animal</th>
<th>Dosing, per eye</th>
<th>Mode of administration</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia virus</td>
<td>gD</td>
<td>Rabbits</td>
<td>10⁷ pfu, 30 and 51 days pre-infection</td>
<td>Intradermal injection</td>
<td>No effect on HSK during 16 days post-infection</td>
<td>[126]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>gB1s</td>
<td>Rabbits</td>
<td>Subconjunctival: 100 μg, 100 μl 14, 35, 56 and 77 days pre-infection; intramuscular: 300 μg, 14 days pre-infection</td>
<td>Subconjunctival or intramuscular injection</td>
<td>Complete survival and reduction of lesions following intramuscular, but not subconjunctival, administration</td>
<td>[180]</td>
</tr>
<tr>
<td>HSV-1</td>
<td>IL-2, IL-4, IFN-γ</td>
<td>Mice</td>
<td>10⁷ pfu, repeated 1 or 3 times, last or only immunization 21 days pre-infection</td>
<td>Intraperitoneal injection</td>
<td>All transgenes: complete survival and prevention of corneal scarring 28 days post-infection</td>
<td>[127]</td>
</tr>
<tr>
<td>HSV-1</td>
<td>IL-12p35, IL-12p40</td>
<td>Mice</td>
<td>10⁷ pfu, 21, 42 and 63 days pre-infection</td>
<td>Intraperitoneal injection</td>
<td>Both transgenes: complete survival and prevention of corneal scarring 28 days post-infection</td>
<td>[128]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>A cocktail of gB, gC, gD, gE and gI</td>
<td>Mice</td>
<td>50 μg, 100 μl, 21, 42 and 63 days pre-infection</td>
<td>Intramuscular injection</td>
<td>Complete survival and prevention of corneal scarring 28 days post-infection</td>
<td>[131]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>gD, gD-IL-2</td>
<td>Mice</td>
<td>45 μg, 21 and 28 days pre-infection</td>
<td>Subconjunctival injection</td>
<td>Complete survival and prevention of stromal, but not epithelial, keratitis</td>
<td>[129]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>gD-IL-2</td>
<td>Mice</td>
<td>90 μg, 10 μl, 21 and 28 days pre-infection</td>
<td>Subconjunctival injection</td>
<td>Complete prevention of stromal, but not epithelial, keratitis 10 days post-infection</td>
<td>[133]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>gD-IL-2</td>
<td>Mice</td>
<td>90 μg, 10 μl, 21 and 28 days pre-infection</td>
<td>Topical conjunctival application</td>
<td>Complete prevention of stromal, but not epithelial, keratitis</td>
<td>[130]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-2, IL-4</td>
<td>Mice</td>
<td>100 μg, 4–5 μl, 3, 2 and 5 days post-infection</td>
<td>Topical application to scarified cornea</td>
<td>IL-2 or IFN-γ: no effect</td>
<td>[137]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-2, IL-4, IFN-γ</td>
<td>Mice</td>
<td>Topical: 100 μg, 4 μl; nasal or intramuscular: 200 μg, 25 μl, 0, 7 and 14 days pre-infection</td>
<td>Topical application to scarified cornea, nasal or intramuscular administration</td>
<td>Topical and nasal, but not intramuscular, administration of IL-4 or IL-10 reduced lesion severity 21 days post-infection; IL-2: no effect</td>
<td>[138]</td>
</tr>
<tr>
<td>Antisense oligonucleotides</td>
<td>TNF-α</td>
<td>Mice</td>
<td>100 nM, 2 μl, −1, 1 and 4 days post-infection</td>
<td>Subepithelial injection</td>
<td>Reduction of HSK symptoms 14 days post-infection</td>
<td>[139]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IFN-α</td>
<td>Mice</td>
<td>5–100 μg; −14, −3, −1 or 1 day post-infection</td>
<td>Topical application to scarified cornea</td>
<td>Increased survival only when treated 1 day pre-infection using DNA doses of 25–100 μg</td>
<td>[143]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IFN-α</td>
<td>Mice</td>
<td>100 μg, 3 μl, topical: −1, 1 or 2 days post-infection</td>
<td>Topical application to scarified cornea, nasal or vaginal administration</td>
<td>Increased survival only when treated 1 day pre-infection using topical application</td>
<td>[144]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IFN-β</td>
<td>Mice</td>
<td>100 μg, 3 μl, −3, −1 or 1 day post-infection</td>
<td>Topical application to scarified cornea</td>
<td>Increased survival only when treated 1 day pre-infection using topical application</td>
<td>[91]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-12, IP-10</td>
<td>Mice</td>
<td>100 μg, 4 μl, 3 and 6 days pre-infection</td>
<td>Topical application to scarified cornea</td>
<td>Balb/C: suppression of lesions using both transgenes; GKO: IP-10, but not IL-12, suppressed lesions</td>
<td>[140]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-18</td>
<td>Mice</td>
<td>100 μg, 4 μl, 2 and 4 days pre-infection</td>
<td>Topical application to scarified cornea</td>
<td>Suppression of lesions 12–21 days post-infection</td>
<td>[141]</td>
</tr>
<tr>
<td>Plasmid DNA encoding shRNA complexed with cationic liposomes</td>
<td>MMP-9</td>
<td>Mice, (Balb/C, GKO)</td>
<td>2 plasmids, each 2 μg, 1 μg/μl, 2 days pre-infection</td>
<td>Intrastomal injection</td>
<td>Suppression of lesions during 21 days post-infection</td>
<td>[142]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-10, IL-2, GM-CSF</td>
<td>Mice</td>
<td>Topical: 4–100 μg, 4 μl; intramuscular: 200 μg; 9 days post-infection to animals with mild HSK</td>
<td>Topical application to scarified cornea (all transgenes) or intramuscular administration (IL-10)</td>
<td>Topical: IL-10, but not IL-2 or GM-CSF, reduced lesion severity 32 days post-infection; intramuscular: no effect</td>
<td>[88]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IFN-α</td>
<td>Mice</td>
<td>Subconjunctival: 10 μg, 10 μl; systemic: 40 μg, 100 μl mixed with TargeTran™; 1 and 3 days post-infection</td>
<td>Subconjunctival or intramuscular injection</td>
<td>Subconjunctival and systemic administrations equally suppressed lesions 10 days post-infection</td>
<td>[146]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IFN-α</td>
<td>Mice</td>
<td>25 μg, 3 μl, 12, 24 or 48 h post-infection; repeated 3 times at 12-hour intervals</td>
<td>Topical application to scarified cornea</td>
<td>Increased survival only when treated 12 h post-infection</td>
<td>[92]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IFN-α</td>
<td>Mice</td>
<td>100 μg, 3 μl, 1 day post-infection</td>
<td>Topical application to scarified cornea</td>
<td>Increased survival</td>
<td>[147]</td>
</tr>
</tbody>
</table>

Abbreviations: gB1s, secreted HSV-1 glycoprotein B; gD, HSV-1 glycoprotein D; GM-CSF, granulocyte-macrophage colony-stimulating factor; HSK, herpetic stromal keratitis; IFN, interferon; IL, interleukin; IP-10, IFN-inducible protein-10; MMP-9, matrix metalloproteinase-9; pfu, plaque-forming units; shRNA, short hairpin RNA; siRNA, small interfering RNA; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

† The gene can be encoded by the vector or knocked-down.
Table 4

**In vivo** corneal gene therapy for control of corneal neovascularization

<table>
<thead>
<tr>
<th>Vector</th>
<th>Gene a</th>
<th>Animal</th>
<th>Dosing, per eye</th>
<th>Mode of administration</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense ODNs</td>
<td>bFGF</td>
<td>Rabbits</td>
<td>120 pmol, in a pellet containing bFGF</td>
<td>Implantation of a hydrogel, pre-incubated with bFGF and oligonucleotides, into a stromal pocket</td>
<td>Reduced NV 6 days post-implantation</td>
<td>[155]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>VEGF, sFlt-1</td>
<td>Mice</td>
<td>12 μg, 2 μl, sFlt-1: 1 day before NV challenge</td>
<td>Intrastromal injection</td>
<td>VEGF induced NV; sFlt-1 reduced NV 7 days post-administration</td>
<td>[16]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-1 RA</td>
<td>Mice</td>
<td>1 μg, 2 μl, with concomitant injury</td>
<td>Subconjunctival injection</td>
<td>Reduced NV 5 days post-administration</td>
<td>[151]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>sFlt-1</td>
<td>Rats</td>
<td>10^11 pfu/ml, 2 μl, 2 or 24 h pre-injury</td>
<td>Intracameral injection</td>
<td>Reduced NV 4 days post-administration</td>
<td>[106]</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>sFlt-1</td>
<td>Rats</td>
<td>4 × 10^11 pfu/ml, 2 μl, 3 weeks pre-injury</td>
<td>Intracameral injection</td>
<td>Reduced NV 4 days post-injury</td>
<td>[149]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>VEGF RNA antisense</td>
<td>Mice</td>
<td>2 × 10^8 pfu/ml, 2 μl, 1 day pre-injury</td>
<td>Subconjunctival injection followed by electroporation</td>
<td>Reduced NV 14 days post-injury</td>
<td>[150]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>K5</td>
<td>Rats</td>
<td>50 μg, 25 μl, with concomitant injury</td>
<td>Subconjunctival injection</td>
<td>Reduced NV during 36 days</td>
<td>[82]</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Endostatin; K5 fusion gene</td>
<td>Rabbits</td>
<td></td>
<td>Ex vivo transduction</td>
<td>Reduced NV during 36 days</td>
<td>[140]</td>
</tr>
<tr>
<td>RNA aptamer</td>
<td>Angiopoietin-2</td>
<td>Rats</td>
<td></td>
<td></td>
<td>Reduced NV 6 days post-implantation</td>
<td>[155]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>Fh23K, Flt24K</td>
<td>Mice</td>
<td>1 μg, 2 μl, 2 days pre-injury</td>
<td>Intrastromal injection</td>
<td>Both transgenes reduced NV 9 days post-administration</td>
<td>[152]</td>
</tr>
<tr>
<td>Plasmid DNA with FuGENE® 6</td>
<td>BAI1-ECR</td>
<td>Rabbits</td>
<td>5 μg, 0.4 ml, 2 or 3 doses with 1-week interval, 1 week post-injury</td>
<td>Subconjunctival injection</td>
<td>Reduced NV 7 and 14 days after the last administration</td>
<td>[81]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-12, IP-10</td>
<td>Mice</td>
<td>100 μg, 4 μl, 3 and 6 days before HSV-1 infection</td>
<td>Topical application to scarified cornea</td>
<td>Both transgenes reduced NV during 20 days post-infection</td>
<td>[141]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>IL-18</td>
<td>Mice</td>
<td>100 μg, 4 μl, 2 and 4 days before HSV-1 infection; 1 μg, 2 μl, 1 day before CpG ODN pellet implantation</td>
<td>HSV-1 challenge: topical DNA application to scarified cornea; CpG challenge: intrastromal DNA injection</td>
<td>Reduced NV 4 and 7 days post-implantation</td>
<td>[141]</td>
</tr>
<tr>
<td>siRNA duplexes with or without TargeTran™</td>
<td>VEGF</td>
<td>Mice</td>
<td>Subconjunctival: 10 μg, 10 μl; systemic: 40 μg, 100 μl mixed with TargeTran™; 1 and 3 days after HSV-1 infection; subconjunctival 1 day or systemic 6 and 24 h after CpG ODN pellet implantation</td>
<td>Subconjunctival or intravenous injection</td>
<td>HSV-1 challenge: both administrations equally reduced NV 10 days post-infection; CpG challenge: both administrations reduced NV 4 and 7 days post-implantation</td>
<td>[146]</td>
</tr>
<tr>
<td>Plasmid DNA encoding shRNA complexed with cationic liposomes</td>
<td>MMP-9</td>
<td>Mice</td>
<td>2 plasmids, each 2 μg, 1 μl, 2 days before HSV-1 infection</td>
<td>Intrastromal injection</td>
<td>Reduced NV during 21 days post-infection</td>
<td>[142]</td>
</tr>
<tr>
<td>Naked DNA, albumin polyplexes</td>
<td>Fh23K</td>
<td>Mice</td>
<td>Polyplexes: 2 μg, 2 μl, 3 weeks pre-injury</td>
<td>Intrastromal injection</td>
<td>Polyplexes, but not naked DNA, reduced NV 14 days post-administration; expression of Fh23K for 8 days (naked DNA) or 5 weeks (polyplexes)</td>
<td>[154]</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>Cytochrome P4504B1</td>
<td>Rabbits</td>
<td>2 μg, 4 μl</td>
<td>Intralimbal injection</td>
<td>Induced NV 3 days post-administration</td>
<td>[157]</td>
</tr>
<tr>
<td>PEI polyplexes, dehydrated</td>
<td>bFGF</td>
<td>Rats</td>
<td>0.1, 1, 10 μg</td>
<td>Corneal pocket implantation</td>
<td>Induced NV; peak on days 15–21 (0.1 μg), 18–24 (1 μg) and 24–30 (10 μg)</td>
<td>[156]</td>
</tr>
</tbody>
</table>

Abbreviations: BAI1-ECR, brain-specific angiogenesis inhibitor 1 – extracellular region; bFGF, basic fibroblast growth factor; HSK, herpetic stromal keratitis; HSV, herpes simplex virus; IL, interleukin; IL-1 RA, interleukin-1 receptor antagonist; IP-10, IFN-inducible protein-10; K5, kringle 5 of plasminogen; MMP-9, matrix metalloprotease-9; NV, neovascularization; ODN, oligodeoxynucleotide; PEI, polyethylenimine; pfu, plaque-forming units; sFlt-1, soluble Flt-1; shRNA, short hairpin RNA; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

a The gene can be encoded by the vector or knocked-down.
Since elevated levels of Th1 cytokines were found in rejected corneal grafts and Th1 and Th-helper 2 (Th2) cells are mutually inhibitory [9], various corneal gene therapy studies were conducted to support the Th2 and/or inhibit the Th1 pathway. For example, studies investigated the corneal gene transfer of IL-4 [9,48–50,118] and IL-10 [15,18,49], which are Th2 cytokines, and of cytotoxic T lymphocyte antigen 4-immunoglobulin (CTLA4-Ig) [15,49,119] and p40 IL-12 [118], which are antagonists of Th1 response.

Additional immunomodulatory molecules transferred to corneal grafts as genes include a monomeric anti-CD4 antibody fragment [120]; CTLA4 [48,50], a glycoprotein on the surface of T cells that down-regulates their activation [121]; viral IL-10 [79], a homolog to the human IL-10 that exhibits the immunosuppressive properties but lacks the immunostimulatory effects of human IL-10 [79,122]; indoleamine 2,3-dioxygenase, an intracellular enzyme that arrests activated T cells in the G1 phase [76]; and tumor necrosis factor receptor-Ig, a proinflammatory cytokine and inhibitor of tumor necrosis factor [123].

A different approach to prolonging corneal graft survival is to directly inhibit the activation of endothelial cells and neovascularization processes that are related to graft rejection. Stout et al. studied the corneal gene delivery of endostatin:kringle 5 of plasminogen, a fusion protein that prevents endothelial cell proliferation and migration and also has anti-angiogenic properties. Lentiviral-vector transduction of endostatin:kringle 5 in rabbits prolonged graft survival from 14 and 18 days in the negative control group to at least 39 days [82]. A comprehensive review of graft failure processes that are potentially susceptible to gene therapy can be found elsewhere [124].

The transgenes used to prevent corneal graft rejection often encode for proteins previously shown to prolong corneal graft survival upon administration. Using such transgenes improves the chances of favorable outcomes in the extension of graft survival. For example, tumor necrosis factor receptor-Ig [123], CTLA4-Ig [119] and anti-CD4 antibodies [120] prolonged graft survival when administered as proteins in preclinical studies. The rationale for conducting subsequent gene delivery studies was the anticipation that gene therapy would extend graft survival for longer time spans than protein therapy. The advantages of gene therapy over protein therapy are well known and include benefits, such as continuous and stable protein levels, that should improve the efficacy of the treatment [10,125] and result in fewer side effects [6]. However, unless the needs for optimization of the delivery system, mode of administration and dose are met, it is possible that protein delivery will yield prolonged corneal graft survival in comparison to gene transfer. Such a result was obtained when Larkin et al. incubated de-epithelized donor rat corneas ex vivo with CTLA4-Ig or adenovirus encoding CTLA4-Ig prior to transplantation [119].

The outcomes of corneal gene therapy studies for prevention of graft rejection emphasize the challenges in achieving this goal. Several studies were unable to prolong graft survival [9,120]. Williams et al. were able to extend median graft survival time in sheep from 20 days to 45 [118] or 55 [18] days. However, in both studies the authors expressed their concerns that several
animals were refractory to the treatment and rejected the graft in similar time periods as untreated control group [18,118]. Hoffmann et al. demonstrated extension of graft survival using gene gun from 22.5±1.7 (mean±standard deviation) days in the negative control group to 42±21.5 days in the combined treatment of glucocorticoid with IL-4 and CTLA4 gene transfer. However, the extension was not significantly longer than the positive control group, which received only glucocorticoid treatment that resulted in graft rejection after 34.6±7.8 days [49]. This result highlights the importance of using a positive control in such studies.

A subsequent study by Hoffmann et al. combined pharma-cotherapy of dexamethasone eye-drops with gene gun IL-4 and CTLA4 transfer, using a different DNA dose and administration time compared with the previous study, thereby extending graft survival from 27±19 days in dexamethasone positive control group to 64±28 days in the treated group. An additional outcome of the study was that more aggressive treatments do not necessarily improve, and might be detrimental to graft survival. For example, gene gun treatment of recipient mice on day (−1) prolonged graft survival, while a double treatment of recipient mice on days (−1) and 2-post-transplantation, or treatment of both recipient and donor mice on day (−1), did not [50].

When conducting corneal gene therapy studies for prevention of graft rejection, one should be aware that the use of vectors and physical methods of corneal gene therapy might have a detri-

tmental effect on corneal graft survival. This was demonstrated when mock adenovirus, which did not encode for therapeutic transgenes, significantly shortened graft survival in comparison to the negative control group or the group treated with adenovirus encoding for tumor necrosis factor receptor-Ig [123].

3.2.3. Prevention and treatment of herpetic stromal keratitis

A summary of corneal gene therapy studies for prevention and treatment of HSK appears in Table 3.

In this section, in addition to the description of gene therapy studies for the control of stromal disease resulting from HSV-1, we also briefly review works describing corneal gene transfer, where the major therapeutic index for treatment success was the ability to protect mice from ocular HSV-1-induced encephalitis. These studies, by Carr et al., are relevant for HSK and corneal gene therapy.

There are several potential strategies to control HSK that corneal gene therapy can address: (a) vaccination against a primary ocular HSV-1 infection; (b) prevention of recurrent episodes of viral attacks on the cornea and/or the accompanying immune response due to an existing HSV-1 infection; and (c) treatment of existing HSK symptoms.

Corneal gene therapy studies were conducted for prevention of a primary ocular HSV-1 infection and HSK using gene-based vaccination against viral envelope glycoproteins. Recombinant vaccinia virus was studied by Nesburn et al. [126]. Recombinant HSV-1 type viruses that encode for cytokine adjuvants were studied by Ghiasi et al. [127,128]. Naked DNA vaccination and the effect of DNA-encoded cytokine vaccine adjuvants on the prevention of HSK were studied by Inoue et al. [129,130]. Ghiasi et al. also studied multigenic naked DNA vaccination [131]. In all of these studies the experimental animals were challenged with ocular HSV-1 at least 21 days after the last treatment of an immunological boost.

HSV-1 glycoprotein D (gD) is a viral glycoprotein that was used either as a single glycoprotein or as a part of a cocktail of glycoproteins, in most of the gene-based vaccination studies against infection of ocular HSV-1 and for prevention of corneal scarring and HSK [126,129–133]. This glycoprotein facilitates the binding and entry of the virus into the cell, is extensively expressed by the virus [85], and was identified in previous vaccination studies as more effective than other glycoproteins in protecting animals from HSV [129].

The development of a multigenic vaccine encoding 5 glycoproteins by Ghiasi et al. was based on previous studies, which showed that vaccines against only a single HSV-1 glycoprotein were not as successful in protecting the host from eye disease as vaccines against a cocktail of ≥5 glycoproteins [85]. A suggested explanation was that a cocktail of glycoproteins enhances the efficiency of the immunization against a range of variants and strains of HSV-1. This vaccine was injected intramuscularly as naked DNA and protected mice from corneal scarring and ocular HSV-1-induced encephalitis [131].

The use of naked DNA vaccination in corneal gene therapy studies protected experimental animals from HSK [129,130,132,133], corneal scarring [131] and ocular HSV-1-induced encephalitis [129,131]. Naked DNA vaccination is potentially advantageous in comparison to a different contemporary bio-
technological approach, of recombinant protein-based vaccina-
tion. The pharmaceutical production of naked DNA vaccines is considered easier in comparison to recombinant proteins [125]. While recombinant protein vaccination elicits mostly humoral immune response, naked DNA vaccination also induces cell-mediated immune responses, which are often instrumental for effective vaccination [134]. A study on the effect of administra-
tion of recombinant protein or DNA encoding gD-IL-2 (a fusion protein of gD and IL-2) for vaccination against ocular HSV-1 using subconjunctival injection in mice, showed that both treat-
ments significantly reduced HSK symptoms 10 days following HSV-1 infection [133].

Live recombinant viruses [127,128] and naked DNA vaccines [129,130,132,133] against ocular HSV-1 and HSK manifesta-
tions, used DNA cytokine adjuvants such as IL-2 [127,129,130,132,133], IL-4, IFN-γ [127], IL-12-p35, and IL-12-p40 [128]. Cytokine adjuvants are used to improve the cytokine environment for expansion of CD4+ and CD8+ T lymphocytes during vaccination [135]. An advantage of utilizing cytokine DNA over cytokine protein is that the latter has a very short half-life, a limitation that can be circumvented by gene therapy [136].

In corneal gene delivery studies, the use of cytokine DNAs did not affect therapeutic indices such as protection from HSK symptoms, corneal scarring and HSV-1-induced encephalitis, as the vaccinations yielded optimal results even without the adjuvants [127–129]. However, Inoue et al. reported that immunization with gD-IL-2 enhanced cytotoxic effector cell activity and delayed type hypersensitivity response significantly more than gD immunization without IL-2 [129]. These results were the basis for the use of gD-IL-2 in their subsequent studies [130,132,133].
Designing a therapeutic vaccine for the prevention of recurrent HSK episodes is desirable [85,90]. Corneal gene therapy studies to achieve this goal have not yet been conducted. Vaccination during the latent phase of ocular HSV-1 infection requires modulation of the immune response and is considered even more challenging than prophylactic vaccination against a primary infection of ocular HSV-1 [90].

Attempts to attenuate HSK and corneal symptoms in a relatively early stage of the disease progression, were conducted by Rouse et al. [137,138] and Heiligenhaus et al. [139] utilizing gene-based immunomodulation, and by Rouse et al. [140–142] using anti-angiogenesis. In all of these studies at least part of the treatment occurred ≤3 days prior to ocular HSV-1 infection. This approach does not involve immunological memory and is potentially effective only for the short term. Therefore such treatments cannot be applied in the clinic for prevention of a primary infection and are more relevant to the prophylactic treatment of recurrent disease. Corneal gene transfer studies that involve treatment shortly before HSV-1 challenge were also conducted by Carr et al. [91,143,144].

The pathology and tissue destruction of HSK is related to the host immune response to HSV-1 infection that is CD4+ T cell orchestrated and typically involves cytokines of the Th1 type [88], while later appearance of Th2 cytokines is probably associated with lesion reduction [92,145]. This explains the immunomodulation approach underlying gene transfer studies to treat HSK. For example, genes for IL-10, which partially involves cytokines of the Th1 type pattern and was observed in corneas during lesion resolution [88], and IL-4, which modulates the immune response toward the Th2 pattern [137,138], were administered as naked DNA. Tumor necrosis factor-α, a Th1 cytokine [89] that is also a key HSK mediator, was knocked-down using administration of antisense oligonucleotides [139].

Neovascularization is an essential process in HSK that facilitates the infiltration of CD4+ T cells to the stroma. The discovery, during a gene transfer study related to immunomodulation of HSK, that IL-12 can suppress corneal lesions via an anti-angiogenic mechanism [140] led to the evaluation of additional anti-angiogenic approaches for the treatment of HSK. These include administration of naked DNA encoding IL-18, an anti-angiogenic factor [141], and the use of RNAi to knockdown the expression of vascular endothelial growth factor (VEGF) [146] or matrix metalloproteinase-9 (gelatinase B) [142], which are known angiogenic factors.

The importance of the timing of corneal gene transfer for the protection from ocular HSV-1-induced encephalitis, in relation to HSV-1 infection was highlighted in the studies of Carr et al. that utilized gene delivery of IFNs type 1 (e.g., IFN-α1 and IFN-β), which are potent antiviral cytokines [91]. A treatment of the experimental animals that used a single dose of 100 μg naked DNA 1 day prior to HSV-1 infection increased survival, while treatments 14 or 3 days pre-infection and 1 or 2 days post-infection did not [91,143,144]. A different treatment of 3 doses of 25 μg naked DNA administered in 12-hour intervals was effective when it was administered 12 h, but not 24 or 48 h, post-infection. It is interesting to note that while all of these studies used an HSV-1 challenge of 450 plaque-forming units, a separate study showed that by using a decreased HSV-1 challenge of 150 plaque-forming units with a similar single dose of 100 μg naked DNA, even a treatment 1 day post-infection can increase survival of animals [147].

A study by Rouse et al. stands out in that the treatment took place 9 days post-infection using experimental animals, which were pre-selected as presenting a mild [88], although not peak [140], HSK manifestations [88]. This study, which utilized DNA immunomodulation, better mimics a clinical situation where the treatment aims to suppress existing HSK lesions. Additional studies that are relevant to post-infection treatment were conducted by Rouse et al. using an anti-angiogenic approach [146] and by Carr et al. [92,147].

3.2.4. Control of corneal neovascularization

A summary of corneal gene therapy studies for control of corneal neovascularization appears in Table 4.

Corneal neovascularization is usually caused by infectious, inflammatory or traumatic conditions [148]. In corneal gene delivery studies aiming to suppress neovascularization, animal subjects were pre-treated to induce corneal neovascularization by various methods such as chemical injury [106,149–151], mechanical injury [81,152] or both [153,154].

Since corneal neovascularization is a central component in the development of several corneal diseases, other corneal gene therapy studies investigated the potential of treating the disease by reducing neovascularization that is induced as a part of disease progression. For example, following corneal transplantation the delivery of an anti-angiogenic fusion gene, endostatin:K5, inhibited neovascularization and graft rejection [82]. Rouse et al. studied the ability of anti-angiogenic corneal gene delivery to inhibit HSK and the progression of neovascularization progression following HSV-1 challenge [140–142,146]. Most of these studies also included experiments evaluating similar methods in a different corneal model where neovascularization was induced by implantation of a pellet that contains VEGF [140] or CpG-containing oligonucleotides [141,146], which are inducers of VEGF expression [146].

Levels of VEGF, the primary mediator of neovascularization in the eye [149], were found to be elevated in inflamed and vascularized corneas of rats and humans [150]. This may explain the fact that reduction of VEGF levels is the most common approach for inhibiting corneal neovascularization in corneal gene therapy studies. For example, the gene delivery of a secreted form of Flt1, a VEGF receptor [106], was studied using naked DNA [16], adenovirus [106] and adenov-associated virus [149]. Naked DNA encoding for Flt23K or Flt24K, VEGF-binding domains of Flt1 fused with KDEL (an endoplasmic reticulum retention peptide), or albumin polyplexes as vehicles for Flt23K DNA [154], were used for intracellular inhibition of VEGF [152]. RNAi [146] or adenovirus encoding VEGF RNA antisense [150] were used for VEGF knock-down studies. Gene transfer of IL-18, a known anti-angiogenic factor, suppressed corneal neovascularization and reduced VEGF expression in vitro and in vivo [141]. An RNA aptamer that inhibits activity of angiopoietin-2, an enhancer of VEGF angiogenic activity, suppressed neovascularization when implanted, after compounding with a basic
fibroblast growth factor-containing pellet, in a corneal micro-

pocket [155]. Naked DNA administration of IL-1 receptor

antagonist that was shown in a model of penetrating keratoplasty
to reduce VEGF expression was unable to affect VEGF

expression in a model of corneal neovascularization [153].

Other corneal gene therapy studies have used transgenes

encoding for known anti-angiogenic factors from other disease

models, biological systems and target organs [81,82,140,151].

It is important to note that while the majority of corneal gene

therapy studies for control of neovascularization aim to reduce

neovascularization, a few of these studies concentrate on in-

duction of the condition. For instance, in order to study the

potential of gene therapy by the administration of naked DNA
to improve the treatment of corneal diseases, Adams et al.
examined the induction of neovascularization using naked DNA encoding for VEGF [16]. Kuo et al. studied the ability of a
dehydrated form of a polyethylenimine–DNA complex to induce
corneal neovascularization using DNA encoding for basic
fibroblast growth factor. The cornea was used as a model due
to factors facilitating the use of biomicroscopy in following
neovascularization processes, such as accessibility and visibility
[156]. In order to study the importance of the cytochrome
P4504B1 mediated pathway for future anti-angiogenic treat-
ments of corneal neovascularization, Laniado-Schwartzman et al.
investigated the ability of naked DNA encoding for cytochrome
P4504B1 to cause corneal neovascularization [157].

3.2.5. Correction of corneal haze, opacity and manifestations of
mucopolysaccharidosis VII, and facilitation of wound healing

A summary of corneal gene therapy studies to treat corneal
haze, opacity and manifestations of mucopolysaccharidosis VII
and facilitate wound healing appears in Table 5.

The corneal clouding that gene therapy studies aim to correct
may result from excimer laser procedures, fibrin formation and
mucopolysaccharidosis VII. The difference in the etiologies,
however, leads to disparity in the pharmacological target of the
treatment.

Corneal gene therapy studies were conducted to inhibit the
development of corneal haze related to excimer laser procedures
by inducing apoptosis of activated keratocytes. The transgenes
used were dominant negative mutant cyclin G1, a survival factor
that triggers apoptosis in various proliferative cells [98], or a
suicide gene/prodrug combination of a retrovirus encoding the
HSV thymidine kinase gene with ganciclovir [159]. HSV
thymidine kinase transforms ganciclovir to a toxic metabolite in
proliferating keratocytes thereby triggering cell death [158,159].

Fibrin formation might lead to corneal opacity. A previous
study was conducted to examine the potential of recombinant
tissue plasminogen activator, a fibrinolytic enzyme, to treat
fibrin clots in the anterior chamber of the eye. However, side
effects associated with the protein administration prompted the
rationale for a corneal gene therapy study that involved the
transfer of a tissue plasminogen activator gene [6].

Corneal gene therapy studies were also performed to correct
the corneal clouding manifestations of mucopolysaccharidosis
VII. Interestingly, this is the only inherited disease with cor-
neal manifestations that was subject to corneal gene delivery,
and the transfer of the β-glucuronidase gene was hoped to
compensate for the metabolic defect. The seminal work of Li
and Davidson focused on retinal symptoms of mucopolysac-
charidosis VII, recording no significant effect of the treat-
ment on the cornea [162]. A later study concentrated on the
cornea and noted substantial reduction of storage vacuoles in
keratocytes [102].

Corneal gene therapy studies investigated the role of the
opioid growth factor receptor pathway in the regulation of cell
proliferation [160] and, in a subsequent study, wound healing
[161] in the corneal epithelium. The rationale for the studies was
based on previous works, which documented that the adminis-
tration of opioid growth factor decreased DNA synthesis and
delayed wound healing [160].

3.3. Ex vivo corneal gene delivery studies

The ex vivo studies of corneal transfection were often used
by researchers prior to in vivo evaluations of corneal graft
transplantation to optimize the genetic modification of the
donor cornea. In other cases they were simply used as a
complementary to an in vitro experiment since the ex vivo
setting better reflects the in vivo setting. Table 6 lists the vectors,
genomes and mammalian sources of cornea, which were used in ex
vivo studies. Reports of studies that include both ex vivo and in
vivo studies are not mentioned in Table 6.

In a particularly interesting ex vivo study, George et al. used
adenoviral vectors to deliver the lacZ or the CTLA4-Ig genes
to human corneas. CTLA4-Ig is a fusion protein that has
previously extended corneal graft survival in rats and mice. The
β-galactosidase transgene was expressed solely in the endo-
theлиum, probably since the endothelium or Descemet’s membrane
acted as a barrier to adenoviral infection of the stroma.
Expression of lacZ and of CTLA4-Ig reached relatively high
levels at 7 days and 28 days, respectively. It was suggested that
the difference between the transgenes might be attributed to
different kinetics of expression of secreted versus cytoplasmic
proteins. Interestingly, mRNA of lacZ was detectable for only a
short period of 3–7 days, despite existence of its DNA in the
cell for at least 56 days. It is possible that the promoter was
inactivated during that time [182].

4. Pharmaceutical aspects

4.1. Corneal gene delivery systems

An ideal corneal gene delivery system would effectively
induce gene expression in an amplitude, time span and in-
tracorneal tissue according to the clinical need. It would be
nontoxic, non-immunogenic and would have the ability to transfer
multiple genes, regardless of their size. Also desirable is a
potential for large scale, reproducible production [38] leading to
a stable pharmaceutical product.

The most common ophthalmic drug delivery system, topical
eye-drops [203], would also be a convenient delivery system of
genomes to the cornea. Advantages of eye-drops over petrolatum-
based ointment, another common ophthalmic delivery system,
Only a few studies of corneal gene transfer utilized gene delivery systems other than a standard solution. In one such study an intrastromal lamellar pocket was created in rats to implant a dehydrated form of a polyethylenimine–DNA complex. The dual advantage of the implantation is the control over the dose as well as the anatomical site of transfection [156]. A different study extended the contact time of the viral vector with the murine stroma to a few minutes by inserting a sponge soaked with 50 μl of the viral solution to a wound created by lamellar keratotomy [102].

Many of the traditional DNA vectors of gene therapy have been tested in corneal gene transfer (see Tables 1–5). These include viral vectors such as adenovirus, adeno-associated virus, lentiviruses and retroviruses, and the non-viral vectors of naked DNA, cationic lipoplexes, polyethylenimine and MIDGE. Recently the use of RNAi duplexes [146] and a plasmid DNA encoding shRNA in the cornea were reported [142]. Generally, viral vectors find substantially more common use in gene therapy than non-viral vectors [36,40]; however, corneal gene delivery studies do not follow that pattern, as viral vectors were used in approximately 47% of the studies, see Fig. 2.

The choice of the vector is a major factor in the strategy to rationally design a corneal gene delivery system. Various vectors have different inherent traits and past experiences, both inside and

are their ease of use and the fact that they do not blur the vision [204]. An additional lesson to learn from ocular drug delivery systems in the development of corneal gene delivery systems relates to the concentration of the formulation. It is preferable to adjust the dosing to a small drop size of 5–15 μl [203] compared to a standard drop size of approximately 50 μl [205] because higher volumes result in spillage of the solution over the lid margin onto the skin, due to the blinking reflex [204]. The small drop size is related to the fact that the tear film volume is 7–10 μl [27] and the eye can momentarily hold up to 30 μl [205].

![Fig. 2. The gene vectors and methods used in corneal gene delivery studies in vivo (1994–2006). Data were compiled from studies mentioned in Tables 1–5, studies involving extra-ocular modes of administration [99,112–116] and additional studies [107,109–111]. For studies that involved experiments with two types of vectors [146,163,174,175], both vectors were taken into account.](image-url)
outside the cornea, can assist in this decision. Some components of vectors can also confer tissue selectivity and enable activation and deactivation of gene expression. Rational design of the delivery system does not obviate the need to optimize the DNA dose as was demonstrated by corneal gene delivery studies.

The importance of understanding the inherent characteristics of the vector for the design of corneal gene delivery systems was exemplified when the inability of retroviruses to transduce non-dividing cells [39] highlighted the need to test other gene vectors for corneal gene therapy. This was due to the hypothesis that retroviral vectors are not expected to be useful in transducing human corneal endothelium [3,159] and stroma [171].

Consistent with previous findings, viral vectors are generally more efficient than the non-viral vectors in transducing their genes [38] to the cornea in vitro and in vivo. For instance, adenovirus was substantially more efficient than cationic lipoplexes in producing expression of the lacZ gene in ovine corneal endothelial monolayers [206]. Following administration to rabbit stroma via a lamellar flap, aden-associated virus yielded higher expression of the lacZ gene and chloramphenicol acetyl transferase in keratocytes than cationic lipoplexes [174].

In comparison to naked DNA, administration of polyplexes offers the potential advantages of enhancing the transgene expression [207] and the biological effect [208]. These traits were recently demonstrated in corneal gene therapy when intrastromal injection of albumin-DNA nanoparticles extended Flt23K expression and substantially reduced the development of neovascularization [154].

Tissue selectivity can be achieved by the use of tissue-specific promoters that can be cloned into the viral vector or the plasmid DNA. This methodology has been successful in obtaining exclusive gene expression in corneal stroma [175] and epithelium [62].

Control of the activation and time span of gene expression can be obtained by the use of inducible promoters that are triggered by the presence of an additional agent that is released endogenously under certain physiological conditions (e.g., ischemia) or is administered exogenously [209]. In corneal gene therapy an E-selectin, tumor necrosis factor-inducible, promoter was studied ex vivo, since tumor necrosis factor appears in aqueous humor during corneal graft rejection. Expression of chloramphenicol acetyl transferase was conditional on the presence of tumor necrosis factor in the experimental media [185].

There is often a need to identify an optimal DNA dose, since increasing doses have shown a direct correlation with the magnitude of gene expression or knock-down, and also with pharmacological effect and side effects. For example, the percentage of corneal surface area of lacZ gene expression following intrastromal injection of naked DNA increased from 0.6%±0.1% to 40.5%±7.5% as doses increased from 0.05 ng to 1800 ng, respectively [16]. Elevated doses (1–4 μg) of shRNA plasmids complexed with cationic liposomes were administered intrastromally to target matrix metalloproteinase-9. This treatment enhanced the corresponding gene knock-down in the murine cornea [142]. Higher doses (5–100 μg) of naked DNA encoding IFN-α1 were administered topically to scarified corneas, resulting in increased survival of mice following HSV-1 challenge [143]. Monkeys injected intracamerally (i.e., to the anterior chamber) with high (10^9 plaque-forming units) doses of adenovirus encoding green fluorescent protein developed strong inflammatory reactions, which were not observed at lower doses [17]. A connection between increased adenovirus dose and the appearance of side effects was also reported by Larkin et al. [119]. These examples clearly illustrate the importance of optimizing the DNA dose.

Finally, the issue of toxicity is related to the design of corneal gene delivery systems in that gene vectors substantially vary in their safety profile, and as expected, some of them are not devoid of corneal side effects. It is important to note that in the eye even a mild inflammatory response might impair vision [21]. When used in the cornea, intrastromal injection of naked DNA [16] and both topical and intracameral administrations of lipoplexes [164] have yielded no side effects. Viral vectors are generally more toxic than non-viral vectors [210]. In the cornea, 3 studies involving intracameral injections [1,3] or topical administration [14] of adenovirus were unable to detect cytopathic effects. Ex vivo transduction of corneal graft using adenovirus has shown that postoperative inflammation was not exacerbated by the administration of adenovirus [18].

However, a few studies did exhibit mild to severe inflammation following intracameral administration of adenovirus [17,120]. In one such study by Borras et al., the inflammatory response that appeared in histologic examination demonstrated no gross external manifestations such as redness or irregular tearing [12]. This highlights the importance of awareness of potential side effects and cytotoxicity in the design and evaluation of corneal gene delivery systems.

4.2. Modes of administration of corneal gene delivery systems

The mode of administration of corneal gene delivery systems has a considerable impact on the study outcomes and applicability. When conducting research in corneal gene delivery, the first decision that should be made regarding the mode of administration is whether a systemic or an ocular mode of administration is preferred. In the latter case, a choice can be made between topical and invasive administration. Among the invasive modes of administration, there are several alternatives for the site of injection. Furthermore, there are additional invasive procedures that were used in preclinical studies to enhance corneal gene transfer.

When comparing systemic to ocular modes of gene administration, it is clear that the latter carries the inherent advantages of local delivery i.e., the ability to achieve enhanced local expression levels and therapeutic effects combined with a lower probability of systemic adverse effects [211]. Thus, numerous studies involving corneal gene delivery, as seen in Tables 1–5, have used transfer to ocular tissues such as the anterior chamber, the vitreous body and the surface of the eye i.e., the cornea and conjunctiva.

However, there are a number of studies that have employed non-ocular gene administration to correct corneal diseases. Most studies using nasal [116,138,144], but not vaginal [144] or intramuscular [88,138], administration to prevent HSK were successful, although to a lesser extent than ocular administration.
Systemic gene therapy that treated corneal manifestations of mucopolysaccharidosis VII was successful in neonate mice [114] and neonate dogs [112,113]. However, no pathological correction was observed in adult mice, possibly due to the blood-retinal barrier preventing transduction of adenovirus and uptake of the transgene, β-glucoronidase. It was suggested that immaturity of the blood-retinal barrier in the neonates may have enabled the ocular adenoviral vector transduction [114].

Several studies describe the unequivocal success of corneal gene delivery following peripheral administration. Sakamoto et al. describe intramuscular administration of adenovirus encoding soluble transforming growth factor β receptor to treat corneal opacity and neovascularization. This treatment resulted in elevated transgene levels in the supernatant of the homogenized eye, similar to systemic gene expression [99]. Pardridge et al. identified lacZ transgene expression in the cornea following intravenous injection of pegylated immunoliposomes targeting the mouse retina [115]. Ghiasi et al. succeeded in immunizing mice against corneal HSV-1 infection using intramuscular injections (primary and 2 boosters) of naked DNA encoding a cocktail of 5 of the viral glycoproteins [131]. Larkin et al. and Ritter et al. noted a substantial prolongation of corneal graft survival following systemic administration of adenovirus encoding CTLA4-Ig or viral IL-10, respectively, in comparison to ex vivo transduction of corneas prior to transplantation [79,119].

A study by Rouse et al. emphasized the advantages of the ocular over the systemic mode of administration. An approach of VEGF RNAi was used to study the suppression of HSK lesions and corneal neovascularization by comparing subconjunctival with intravenous injection. In order to increase the effectiveness of the intravenous injection of the siRNA duplexes, which were used in the subconjunctival injection, TargeTran™ was compounded with the RNAi duplexes and the dose was increased 4-fold. Combining these means the systemic administration achieved an equivalent anti-angiogenic effect and a HSK clinical score as local administration [146].

A major challenge for the ocular mode of administration is to obtain substantial gene expression by topical administration, the preferred ocular mode of administration for therapeutic agents [176,212]. After application, the hydrodynamics of blinking and tear flow immediately remove the delivery system from the corneal surface [130,176]. It was also suggested that the tight junctions of the epithelium and Bowman’s membrane, might act as a barrier for penetration of the adenovirus [102].

Among the studies that evaluated topical administration to the naïve eye, in contrast to the scarified eye, only those studies from 1996 by Matsuo et al. using a low dose (0.8μg) of cationic lipoplexes [163,164] achieved corneal transgene expression. Other studies using cationic lipoplexes [176], adenovirus [14,102] and naked DNA [88,137,163] reported no detectable reporter gene expression. One study exemplified the usefulness of eye-drops in treating a disease model of unscarified, naïve cornea. Application of naked DNA to the conjunctival sac has yielded partial immunization against HSV-1, consequently inhibiting stromal, but not epithelial, keratitis [130]. However, it is important to note that even very low transgene expression can yield successful immunization [208].

Methods were examined to augment corneal gene expression following topical administration. One way to accomplish this is to change the delivery system. For instance where naked DNA has yielded negative results, cationic lipoplexes successfully yielded expression of the lacZ gene [163]. An alternative, which was used in a majority of studies involving HSK, is to scarify the cornea. This procedure was shown to enhance and improve the consistency of gene expression [88], although Carr et al. noted that it has only limited relevancy for the clinical setting [143]. Studies that have found no transgene expression following topical instillation, and continued experiments using invasive ocular administration i.e., intracameral [102] or intravitreall [176] injections, have resulted in substantial expression. The limited success of topical administration to the naïve eye may explain the propensity to use invasive modes of administration in preclinical studies. These include intrastromal, intralimbal, subconjunctival, intracameral and intravitreal injections. It should be mentioned that repeated subconjunctival and intravitreal injections of drugs to patients were associated with morbidity e.g., vitreous hemorrhages, endophthalmitis and cataract [205,212].

The ocular mode of administration is correlated with the intracameral location of gene expression since the site of micro-injection controls the type of cells that receive the transgene [179]. Therefore, as exemplified from various studies (see Table 1), intracameral and intravitreal injections yielded gene expression in the endothelium, and intrastromal injections produced gene expression in the stroma, and in some cases in the epithelium. Studies outlying this pattern of gene expression describe lipoplexes administered intracameraly or intravitreally yielding transgene expression in the epithelium, and in the former case also in the endothelium [164]; and adenovirus administered intracameraly yielding transgene expression in both endothelium and epithelium [179]. The specific intracorneal layer where the gene was expressed was unrelated to the vector type or animal species used.

From the reports using physical techniques to transfer reporter genes to the cornea it is apparent that electroporation [5,21,171] had no effect on the location of expression i.e., the gene was expressed according to the injection site, and that gene gun delivered the transgene to the epithelium [61,62]. It was suggested that gene gun technology is inappropriate to transfect stromal keratocytes due to their dense collagenous matrix [62].

Additional means of modifying the mode of administration to enhance corneal gene transfer in preclinical studies include using invasive procedures for improving the accessibility of the delivery system to the stroma. For example, Perez et al. created a small tunnel from the corneal epithelium to the anterior stroma using a 33-gauge needle [175]. Mohan et al. produced a corneal flap to expose the corneal bed prior to instillation of the viral solution [174]. Kitajima et al. created a stromal pocket to implant a hydrated hydrogel that has a size of 1 × 2 mm and a thickness of 0.2 mm, and was pre-incubated with antisense or sense oligonucleotides [181]. Okuyama et al. formed a lamellar keratotomy using a microscalpel and subsequently inserted a sponge soaked with viral solution into the wound for a few
minutes. Lamellar keratotomy does not usually cause scars that impair vision. In fact, lamellar keratoplasty, a more invasive procedure, is executed repeatedly in the clinic [102].

Sakamoto et al. highlight the importance of not interfering with the transparency of the central cornea [6,171] as under this condition it is possible to maintain good vision even if the peripheral cornea is hazy [21]. Challa et al. report that the specific technique used for intracameral injection determines whether corneal epithelial neovascularization will develop, presumably due to endothelial damage [177]. These issues pertain to various modes of administration.

4.3. Animals used in corneal gene delivery studies

Mice are by far the most commonly used animal species in experimental gene therapy, in general [213], and their use is also more common than other species in experimental corneal gene therapy, see Fig. 3A. The animal species and model chosen for a preclinical study affect its chances of success and relevancy. The ultimate decision regarding the choice of animal is a compromise between factors, which vary in their importance for research of different corneal diseases. To assist in this decision for corneal gene therapy, the following considerations can be taken into account, and some examples follow:

4.3.1. Inter-species variability in the anatomy, physiology and immunology of the eye

Examples include eye size, immunological response and the ability of the corneal endothelium to replicate. The fact that the rabbit eye is similar in size to the human eye was mentioned as an advantage in using the rabbit to study corneal gene delivery [12]. In contrast, the small size of the murine eye was pointed out as a disadvantage in efforts to follow clinical signs of ocular disease progression. Topical immunological challenge in rats is difficult due to the unusual structure of their conjunctiva [214]. The corneal endothelium is mitotic in rodents [18,75] while in rabbits it is potentially mitotic [23]. However, since its replicative capacity is low [185,192] some researchers consider it to be virtually amitotic [215]. The corneal endothelium is amitotic in humans [18,23], sheep [18], cats [18,23] and monkeys [23]. This is particularly germane to corneal gene therapy studies where the endothelium plays a significant role e.g., corneal graft rejection.

4.3.2. The existence of an animal strain that mimics human pathology

Mucopolysaccharidosis VII mice [102,112], cats [112] and dogs [112,113] develop a disease that is similar to human mucopolysaccharidosis VII in many features. Such mice and dogs were used in gene therapy studies that also evaluated the effect of the treatment on the cornea [102,112–114,162].

4.3.3. The disease pattern and symptoms in the animal model in comparison to humans

The fact that HSK lesions in mice following primary infection mimic the human disease was noted by Rouse et al. as the primary reason for the popularity of murine model in preclinical studies of HSK [216]. The process of corneal graft rejection in sheep is similar to that in humans [217]. In mice and rats, unlike humans, the process is acute [75] and rejection lines in rats are infrequent. In rabbits the process is slow and there is the need to accelerate graft rejection by induction of pregraft neovascularization and inflammation [217]. It was suggested that this procedure changes the disease progression from a model of low-risk to high-risk transplantation in humans [75]. A different example is a model of corneal haze in rabbits where the symptoms peak 2 to 3 weeks post-treatment, which is significantly faster than in humans [159].

4.3.4. Past experience in corneal gene delivery studies of a connection between managing a specific disorder and the use of a particular animal species

When relating the disease treated to the animal species utilized it is apparent that almost all corneal gene therapy studies treating HSK have used mice, see Fig. 3B.
5. Conclusions and future perspectives

The fact that there are various immunological reagents available for scientific work with mice [214] has contributed to the use of this animal species in transplantation [217] and in HSV-1 [145] studies. There are also cytokine probes and cell-surface markers available for sheep, presumably due to their economic importance [217].

4.3.5. The availability of analytical methods, including investigational reagents, to conduct the study in a particular animal species

Corneal gene therapy studies yielded positive outcomes in the field of corneal gene therapy. A challenge remains to lead to the prevalence of invasive ocular administration. The design of advanced delivery systems that prolong the contact time of the vector with the surface of the eye may enhance transgene expression, thereby enabling non-invasive administration.

The importance of choosing an optimal animal model for corneal gene therapy was highlighted in a discussion in the United States Department of Health and Human Services, National Institutes of Health regarding an application to use a retroviral vector in a clinical study to treat superficial corneal opacity and scarring. A concern that addressed the preliminary studies, which aimed at supporting the application, was that these might require additional preparative work in their husbandry as a part of a corneal gene delivery study.

4.3.6. Past experience in husbandry of the animals under study in research facilities

Viral vectors carry the risk of eliciting pronounced immune responses although their use is known to yield stronger gene expression that expands over a longer time span, when compared to use of non-viral vectors. All of these traits were exemplified in corneal gene therapy studies and should be taken into account in future studies. Moreover, some of the new vectors that are constantly being designed are (a) safer; (b) have the potential to improve the gene transfer process, thereby achieving an enhanced effect, and (c) allow control over activation and deactivation of gene expression by the use of inducible promoters. For example, episomal plasmids that replicate during the cell cycle and segregate into daughter cells, prolong the transgene expression in comparison to traditional plasmids [222]. High-capacity adenovirus not only extends the transgene expression but is also safer than previously available adenoviral vectors [223]. The repertoire of pharmacologically-induced promoters that are triggered by clinically available agents was expanded [224]. Implementation of new vectors in corneal gene delivery studies is expected to yield valuable results.

4.3.7. Modifications and improvements of existing animal models that are suggested in contemporary publications

Harvey et al. described a high-reactivation HSV-1 strain that following infection of the rabbit cornea causes scarring on days 35–58 post-infection in the majority of animals. Since the animals were free of scarring on day 30, the authors suggested that this model mimics recurrent episodes of human HSK that include neovascularization and scarring. HSV-1 strains with low-reactivation phenotypes yielded significantly less scarring [218]. It should be noted that while murine models involving reactivation of HSV-1 have been reported, the most common murine model for HSK involves a primary infection [216].

The importance of choosing an optimal animal model for corneal gene therapy was highlighted in a discussion in the United States Department of Health and Human Services, National Institutes of Health regarding an application to use a retroviral vector in a clinical study to treat superficial corneal opacity and scarring. A concern that addressed the preliminary studies, which aimed at supporting the application, was “whether higher rates of haze in the rabbit make it an inadequate animal model of the condition” [219].

5. Conclusions and future perspectives

Along with tissue engineering [220,221], gene therapy approaches stand on the front line of advanced biomedical research to treat blindness arising from corneal diseases, which are second only to cataract as the leading cause of vision loss [220]. The significance and prevalence of corneal diseases were the driving force behind the studies in corneal gene therapy that have created a rich body of evidence in the literature, which can guide future studies and opportunities in corneal gene therapy.

Topical delivery to the eye is the most convenient way of ocular gene delivery. However, the challenge of obtaining substantial gene expression following topical administration has led to the prevalence of invasive ocular administration. The design of advanced delivery systems that prolong the contact time of the vector with the surface of the eye may enhance transgene expression, thereby enabling non-invasive administration.

 Clearly, significant progress has been accomplished in the field of corneal gene therapy. A challenge remains to characterize the kinetics of corneal gene expression with the
emphasis on understanding the barriers and rate limiting steps underlying the gene transfer processes. Information about the nature of the interaction between corneal cells and the gene vector will assist in the design of optimized delivery systems. This will facilitate the advance from preclinical to clinical studies, which have not yet been conducted for corneal gene therapy. The goal of translating corneal gene delivery systems to pharmaceutical products is yet to be achieved.

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References


Glossary

CMV: cytomegalovirus

CTLA4-Ig: cytotoxic T lymphocyte antigen 4-immunoglobulin

gD: HSV-1 glycoprotein D

HSK: herpetic stromal keratitis

HSV: herpes simplex virus

IFN: interferon

IL: interleukin

MIDGE: minimalistic immunologically defined gene expression

RNAi: RNA interference

shRNA: short hairpin RNA

Th1: T-helper 1

Th2: T-helper 2

VEGF: vascular endothelial growth factor