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Prospects for genetic modulation of corneal graft survival

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Abstract

Irreversible immunological rejection is the major cause of clinical corneal graft failure. *Ex vivo* gene therapy directed at the donor cornea has been shown to prolong orthotopic corneal allograft survival significantly in experimental animal models including the mouse, rat, rabbit, and sheep. Transgenes effective in prolonging corneal graft survival include immunomodulatory cytokines and cytokine receptors, an inhibitor of neovascularisation, a blocker of antigen-presenting cell–T cell co-stimulation, nerve growth factor, a dominant negative regulator of apoptosis, and the enzyme indoleamine 2,3-dioxygenase. Although many viral and non-viral vectors have been shown to transduce the corneal endothelium efficiently, allograft survival has so far been prolonged only following transduction of the donor cornea with adenoviral and lentiviral vectors. The degree of graft prolongation, although promising, is still insufficient for immediate translation to the clinic. Increasing the time that the therapeutic gene is expressed in the eye with an integrative, non-immunogenic viral vector is likely to be one way to achieve long-term graft survival. Simultaneous targeting of multiple pathways of graft rejection with more than one transgene is likely to be another. We suggest that the use of an adeno-associated viral or lentiviral vector combined with multiple transgenes may provide the key to future clinical trials.
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The clinical problem of corneal allograft failure

Each year, an estimated 65 000 penetrating corneal graft procedures are performed worldwide. In Australia, which has an outcomes register that has followed over 20 000 human corneal grafts for periods of up to 22 years, the short-term outcome is excellent, with a success rate of 87% allograft survival at 1 year, but graft survival falls steadily thereafter.[1] The major reasons for corneal graft failure are rejection, corneal endothelial cell failure, infection, and glaucoma, which together account for over 70% of all cases of graft loss.[1] Irreversible rejection is clearly the important cause of graft failure, despite the long-held view that the cornea is an immune-privileged tissue in an immune-privileged site.[2,3] Immune privilege is maintained by multiple mechanisms[2] but corneal privilege is relative, and is readily subverted by neovascularisation and inflammation, which predispose a corneal graft to rejection.[4] Corneal graft rejection is immune cell-mediated, is controlled by CD4-positive lymphocytes, and is targeted primarily to the non-replicative corneal endothelium.[5] Endothelial cell loss from causes other than rejection, however, cannot be ignored.

Therapeutic options for prophylaxis and treatment of corneal graft rejection

Almost all patients receive topical immunosuppression with topical glucocorticosteroids, often over prolonged periods (typically months).[4] Concurrent systemic immunosuppression is sometimes administered, but may be associated with unacceptable morbidity and is not necessarily effective in modulating rejection.[6] HLA matching has a limited role in corneal transplantation, given continuing controversy over its clinical efficacy in improving graft survival rates.[7,8] Premorbid corneal neovascularisation, inflammatory eye disease or a history of failed previous graft will predispose a given recipient to corneal graft rejection.[1,4,5]

Within developed countries, perhaps 50% of people who might benefit from a graft will have a history of previous ocular inflammation, and in developing countries the figure may approach 75%. Such individuals are considered to be at high risk of corneal graft failure, and form the patient population for whom new therapeutic options are required. Gene therapy of the cornea may be one such option.[9]

Genetic modification of corneal allografts

Gene therapy offers attractive prospects for improving human corneal allograft survival.[9,10] Human corneas retrieved for transplantation are stored for days to weeks within eye banks, and are thus readily available for manipulation. The corneal endothelium, the primary target for *ex vivo* gene transfer to a donor cornea, is an accessible monolayer of somatic cells. Any excess vector used for gene transfer can be removed prior to transplantation, and the blood–eye barrier provides further vector sequestration from the systemic circulation. A potentially therapeutic protein can be produced locally and continuously, without the need for exogenous administration of a drug. Finally, corneal graft function can be readily assessed at the slit-lamp.

Before gene transfer designed to prolong human corneal graft survival can become a clinical reality, proof-of-principle needs to be established in appropriate experimental models. A therapeutic transgene or transgenes capable of modulating graft failure, and a safe vector-promoter system that can produce long-term local gene expression within the eye, must also be identified. In this review, we canvass the options that are available for experimental gene therapy of the cornea, and examine the prospects for future clinical applications.

Animal models of corneal transplantation for purposes of gene therapy

Gene therapy has not yet been applied to human corneal allografts. Current experience is limited to experimental models. Orthotopic corneal transplantation can be performed in many species (Table 1) including the mouse, rat, rabbit, cat, dog, pig, sheep, and monkey.[5] For the purposes of gene therapy for corneal transplants, we favour the rat and sheep models,[11,12] but the murine[13] and rabbit[14] models are also used.

Small rodent models are particularly useful because of the availability of genetically inbred strains and their ease of handling, but suffer from the disadvantage that the corneal endothelium in these species, unlike the human, is replicative. The gross anatomy of the mouse and rat eye also differs somewhat from the human, in that the crystalline lens forms a disproportionately large volume of the eye. However, the corneal graft rejection process appears similar in mice, rats, and humans, except that endothelial and epithelial rejection lines are very seldom observed in small rodents.

The sheep is a useful outbred preclinical model in which the corneal endothelium is non-replicative, and in which corneal graft rejection appears very similar at a clinical and histological level to the corresponding process in humans.[11] A new model of orthotopic corneal transplantation has recently been developed in the minipig, which may also find utility as a preclinical model.[15] Advantages of sheep and pigs are that these species are widely farmed and are reasonably easy to handle, are rarely kept as pets, are not endangered, and do not need to be anaesthetised for examination of the anterior segment.

Although regulations governing the use of genetically modified organisms vary among jurisdictions, gene-modified somatic cell tissues such as donor

Table 1 Animal models of orthotopic corneal transplantation for testing *ex vivo* gene-modified donor corneas

Species	*Ocular anatomy similar to human?*	*Corneal endothelium*	*Inbred strains/outbred*	*Rejection similar to human?*	*Handling*	*Ethical issues*
Mouse	Dissimilar[a]	Replicative	Inbred	Similar[b]	Very easy	Surmountable
Rat	Dissimilar[a]	Replicative	Inbred	Similar[b]	Very easy	Surmountable
Rabbit	Similar	Replicative[c]	Outbred[d]	Very similar	Easy	Surmountable
Cat	Similar	Amitotic	Outbred	Very similar	Difficult[e]	Pet species
Sheep	Similar[f]	Amitotic	Outbred	Very similar	Easy	Farmed species
Pig	Similar	Amitotic	Outbred	Very similar	?	Farmed species
Monkey	Very similar	Amitotic	Outbred	Very similar	Difficult	Of concern

[a]Very small eye; lens disproportionately large.
[b]Epithelial and endothelial rejection lines very rarely observed.
[c]Some replicative capacity, especially when young.
[d]Limited histoincompatibility in laboratory strains.
[e]Require substantial environmental enrichment, may be difficult to slit-lamp without anaesthetic.
[f]Eye large but ocular structures in proportion to human eye.

corneas are subject to stringent controls in most countries. The primary restrictions, once a gene-modified corneal graft has been transplanted into a non-human recipient, are that access is restricted to approved personnel wearing suitable garb and gloves, and that the animal be appropriately contained and not be permitted to escape into its surroundings. With respect to mice, rats, and rabbits, this generally means physical containment within a cage in an approved animal facility. In the context of herd animals, this means containment within an approved animal house, pen or double-fenced field. Clearly no such restrictions will be imposed upon future human recipients of gene-modified corneal grafts.

Therapeutic transgenes

The likely usefulness of *ex vivo* gene transfer to the donor cornea, prior to transplantation, is predicated upon the assumption that expression of a given transgene by the cornea will modulate graft failure. Published reports of studies in experimental animals have already provided proof-of-principle that corneal allograft survival can be prolonged significantly by appropriate gene transfer interventions directed at the donor cornea. Expressed transgenic proteins able to modulate corneal allograft survival to a significant extent when used individually include: immunomodulatory cytokines and cytokine receptors such as interleukin 10 (IL10), interleukin 4, the p40 subunit of interleukin 12, and tumour necrosis factor-α receptor;[16–21] an inhibitor of neovascularisation, endostatin-kringle 5 fusion protein (EK5);[22] CTLA4-Ig, a blocker of antigen-presenting cell–T cell co-stimulation,[23] nerve growth factor,[24] the inhibitor of apoptosis bcl-xL,[25] and the enzyme indoleamine 2,3-dioxygenase (IDO)[26] (Table 2).

The transgenic proteins that have already shown some efficacy in experimental models of gene therapy target different pathways involved in corneal graft failure. Thus, IL10 and CTLA4-Ig influence immune sensitisation, that is, the antigen-presenting cell (APC)–T cell interaction, albeit in different ways. The multifunctional cytokine IL10 modulates dendritic cell maturation and favours the differentiation of tolerogenic APC.[27] The soluble fusion protein CTLA4-Ig binds to CD80 and CD86 on the APC, blocking effective co-stimulation[28] and increasing IDO activity in some dendritic cells.[29] IDO arrests activated T cells in G1 by catabolism of tryptophan, thereby predisposing them to apoptosis.[30] The normal cornea does express some IDO, where it may contribute to immune privilege.[31] EK5 targets corneal graft neovascularisation, a known risk factor for human corneal graft failure in multivariate analysis.[1] It probably inhibits angiogenesis by inhibiting endothelial cell proliferation and migration.[32,33] Finally, bcl-xL targets endothelial cell apoptosis by interaction with pro-apoptotic molecules such as Bax.[34] We appreciate that the mechanisms of action of these transgenes, when overexpressed in corneal endothelium, are likely to be far more complex than the brief summary provided above might indicate, but it is clear that the multiple interconnecting pathways that lead to corneal graft failure in turn provide multiple opportunities for therapeutic intervention.

Vectors for gene therapy of the corneal endothelium

Many non-viral vectors and disabled viral vectors have been used for gene transfer to the eye.[9,10] Non-viral vectors, although safe, are generally inefficient.[35] The few exceptions, for example the synthetic peptide-based

Table 2 Therapeutic transgenes reported to prolong corneal allograft survival following *ex vivo* transduction of donor cornea

Transgene	*Function of transgenic protein*	*Animal model*	*Vector*	*References*
IL10[a]	Modulates DC function	Sheep	Adenovirus	16
p40 IL12[b]	IL12 receptor antagonist	Sheep	Adenovirus	21
IL4[c]	Drives Th2 responses	Rat	Adenovirus	19
CTLA4-Ig[d]	Binds CD80, CD86; blocks co-stimulation	Mouse, rat	Adenovirus	17,18,23
EK5[e]	Targets neovascularisation	Rabbit	Lentivirus	22
NGF[f]	Accelerates wound healing	Rat	Adenovirus	24
bcl-xL	Dominant-negative regulator of apoptosis	Mouse	Lentivirus	25
IDO[g]	Arrests activated T cells in G1 by catabolism of tryptophan	Mouse	Lentivirus	26

[a]Interleukin 10.
[b]p40 subunit of interleukin 12.
[c]Interleukin 4.
[d]Cytotoxic lymphocyte antigen 4-immunoglobulin Fc fusion protein.
[e]Soluble endostatin-kringle5 fusion protein.
[f]Nerve growth factor.
[g]Indoleamine 2,3-dioxygenase.

Table 3 Viral vectors for use in *ex vivo* transduction of donor corneal endothelium

Vector	*Size of expression cassette (kb)*	*Integrative*	*Efficiency*	*Immunogenicity*	*Safety in humans*
Adenovirus	8	No	High	High	Probably safe; some concerns
AAV	<5	Yes	Low	Low	Probably safe
Lentivirus	8	Yes	High	Low	Probably safe

vector described by Fabre and co-workers,[36] produce only short-term gene expression, of the order of a few days up to a week, in ocular tissues. The majority of studies exploring gene transfer to corneal endothelium have used replication-deficient adenoviral vectors. For example, we reported that *ex vivo* adenoviral vector-mediated gene transfer of interleukin 10 or the p40 subunit of interleukin 12 to donor corneal endothelium significantly prolonged ovine corneal allograft survival in two-thirds of recipients, in the absence of topical steroid immunosuppression.[16,21] The occurrence of graft rejection correlated with loss of transgene expression, a not unexpected outcome given that non-integrative vectors were used.

Adenoviral vectors transduce corneal endothelial cells very efficiently, but are associated with problems including relatively short-term transgene expression consequent upon the episomal nature of the vector, and significant immunogenicity.[37] Long-term expression over weeks to years post-operatively is likely to be necessary for any transgene designed to prolong corneal allograft survival, given the slow but steady attrition rate of human corneal grafts. It is not clear that adenoviral vectors are the most appropriate choice for future trials in human corneal transplantation, although there remain some proponents[38] and the issues have not been fully resolved.

Other choices for viral vectors for transducing the cornea include adeno-associated viral (AAV) and lentiviral vectors (Table 3). Both integrate into the host genome. AAV vectors transduce ocular cells efficiently,[39] and are being used in human trials of gene therapy for retinal disorders.[40] Although their small expression cassette is a relative disadvantage,[41] AAV vectors have an excellent safety profile because the wild-type virus is not known to be pathogenic in humans.[42] Lentiviral vectors[43] are another option because of their failure to induce an immune response after intraocular delivery,[38] their wide tropism for cells of the anterior segment, and their ability to induce long-term gene expression in ocular cells.[44–47] An additional advantage is their relatively large expression cassette,[41] important if a large transgene or multiple transgenes need to be inserted. Indefinite corneal graft survival in all recipients of gene-modified corneal allografts has not been achieved with any vector system-single transgene combination tested to date, but it is probably fair to say that lentiviral vectors have so far produced the best results in animal models.[22,25]

The safety of gene therapy for the cornea

How safe is gene therapy for corneal transplantation? Non-viral vectors are very likely to be safe, provided toxicity for corneal endothelium can be avoided. The systemic administration of high doses of an adenoviral vector has been associated with one death in a human gene therapy trial for ornithine transcarbamylase deficiency.[48] Subsequent analysis of low and intermediate doses of adenoviral vectors in humans has suggested that such doses are safe.[49] Intra-articular administration of an AAV vector has been temporally associated with the recent death of a patient with rheumatoid arthritis, although the gene therapy intervention may not have caused the death.[50]

The integrative capacity of AAV and lentiviral vectors generates the specific safety concern of insertional mutagenesis. Integration of AAV vectors into the genome is probably semi-random, but with some preference for transcriptional start sites and CpG islands.[42] However, new vectors that target specific, safe integration sites are under development.[42] Lentiviruses are non-oncogenic viruses, and modern vectors are deleted for the viral enhancer sequences that are responsible for oncogene activation by replication-competent oncogenic retroviruses.[41,51] Further, the patterns of integration of lentiviruses[48] differ from those of the oncogenic gammaretroviruses that have thus far been associated with development of T-cell leukaemia in paediatric patients treated by gene therapy for X-linked severe combined immunodeficiency disease.[52] The available evidence would suggest that lentiviral vectors are inherently much safer than gammaretroviral vectors.[51]

Conclusions

Ex vivo gene therapy directed at the donor cornea has been demonstrated to prolong corneal allograft survival significantly in experimental animal models, but the results, although promising, are still insufficient for immediate translation to the clinic. Increasing the time

that the therapeutic gene is expressed in the eye is likely to be one key to achieving long-term graft survival. Simultaneous targeting of multiple pathways with more than one transgene is likely to be another. We suggest that the use of an AAV or lentiviral vector combined with multiple transgenes may provide an appropriate way forward.

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