In vitro adenovirus mediated gene transfer to the human cornea

C F Jessup, H M Brereton, D J Coster and K A Williams

doi:10.1136/bjo.2004.061754
In vitro adenovirus mediated gene transfer to the human cornea
C F Jessup, H M Brereton, D J Coster, K A Williams

Background/aims: Replication deficient adenovirus is an efficient vector for gene transfer to the cornea. The aim was to optimise the transduction of human corneal endothelium with adenoviral vectors and to measure transgene production from transduced corneas.

Methods: Adenoviral vectors (AdV) encoding enhanced green fluorescent protein (eGFP) or a transgenic protein (scFv) were used to transfect 34 human corneas. Reporter gene expression was assessed after 72–96 hours of organ culture. The kinetics of scFv production was monitored in vitro for 1 month by flow cytometric analysis of corneal supernatants.

Results: Transduction of human corneas with high doses (5×10⁷–3×10⁸ pfu) of AdV caused eGFP expression in 12–100% of corneal endothelial cells. Corneas were efficiently transduced following up to 28 days in cold storage. Very high AdV doses (2×10⁹ pfu) reduced endothelial cell densities to 98 (SD 129) nuclei/mm² (compared to 2114 (716) nuclei/mm² for all other groups). Transgenic protein production peaked at 2.4 (0.9) µg/cornea/day at 2 weeks post-transduction, and decreased to 1.2 (0.4) µg/cornea/day by 33 days, at which time endothelial cell density had decreased to 431 (685) nuclei/mm².

Conclusion: Human corneas can be efficiently transduced by AdV following extended periods of cold storage, and transgene expression is maintained for at least 1 month in vitro.

Materials and methods
Human corneas
Human corneas were collected by the Eye Bank of South Australia for clinical transplantation, with permission from the next of kin of recently dead people. Some donor families agreed that the corneas be used for research, should they be found to be unsuitable for transplantation. Thirty-four human corneas were accordingly used in this study over a period of 28 months. No information other than the number of days that a cornea had been stored in the eye bank and the donor age was provided to the investigators. The use of corneas for research purposes was approved by the Flinders clinical research ethics committee.

Adenoviral vectors
A replication deficient E1, E3 deleted adenovirus (AdV) serotype 5 encoding enhanced green fluorescent protein (eGFP) under the transcriptional control of a CMV promoter (AdGFP) was the kind gift of Professor B Vogelstein (Johns Hopkins University, Baltimore, MD, USA). AdV encoding an anti-rat CD4 single chain antibody fragment (scFv) with a mammalian secretory leader sequence on a CMV promoter and eGFP on a separate CMV promoter was constructed. The scFv transgene was chosen because it encodes a secreted model protein that is non-toxic and does not bind to the human cornea, although it will bind specifically to rat CD4 molecules. AdV was propagated in E1A, E1B trans-complementing 293 cells. AdV was purified from transduced 293 cell culture lysates over cesium chloride density gradients and titred by the tissue culture infectious dose method. Titres of different batches varied from 3×10⁶–5×10⁹ pfu/ml.

Transduction of human corneal endothelium with AdV
Following enucleation, corneas were stored for 3–28 days at 4°C in Optisol-GS corneal storage medium (Bausch and Lomb, Rochester, NY, USA) before being made available for research. Corneas were transduced with 2×10⁶–2×10⁸ pfu/cornea AdV (estimated multiplicity of infection (MOI) 10–10 000) at room temperature for 2.5 hours in a total volume of 300 µl in HEPES buffered RPMI medium (ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 2% v/v heat inactivated (56°C, 30 minutes), fetal calf serum (FCS) (estimated MOI 6), 100 µg/ml streptomycin and 2 mM l-glutamine (all from Gibco BRL, Gaithersburg, MD, USA). Corneas were then cultured in 50 ml HEPES buffered RPMI medium (as described above) supplemented with 10% FCS and 2.5 µg/ml amphotericin B and incubated at 37°C in 5% CO₂ in air.

Assessment of transduction of human corneas with AdV
To quantify the number of cells expressing eGFP, corneas were fixed in buffered formalin and counterstained with 300 µl of 10 µg/ml Hoechst 33258 nuclear dye (Sigma Chemical Co, St Louis, MO, USA) for 30 minutes at room temperature. Corneas were dissected through the stroma and To quantify the number of cells expressing eGFP, corneas were fixed in buffered formalin and counterstained with 300 µl of 10 µg/ml Hoechst 33258 nuclear dye (Sigma Chemical Co, St Louis, MO, USA) for 30 minutes at room temperature. Corneas were dissected through the stroma and

Abbreviations: AdGFP, replication deficient E1, E3 deleted adenovirus serotype 5 encoding GFP; AdV, adenovirus based vector; eGFP, enhanced green fluorescent protein; FCS, fetal calf serum; MOI, multiplicity of infection; PBS, phosphate buffered saline; pfu, plaque forming units; scFv, single chain antibody fragment

www.bjophthalmol.com
Gene transfer to human corneas

Original magnification: A: 4× positive cells in the same field were detected under blue light (C). endothelial cell nuclei were detected under ultraviolet light (B) and eGFP microscope and digitally collated. Hoechst 33258 stained corneal AdV encoding eGFP. The image was captured at the fluorescence USA). Fluorescence was measured on a FACScan flow R-phycoerythrin conjugate (Molecular Probes, Eugene, OR, (DakoCytomation, Carpinteria, CA, USA) and streptavidin-antibody (Sigma), biotinylated anti-mouse antibody described in detail elsewhere.13 Briefly, thymocytes were cytometry on rat thymocytes, which are CD4 positive, as Secreted transgenic anti-rat CD4 scFv was detected by flow of endothelial cell nuclei and eGFP positive cells recorded. central fields were examined for each cornea and the number examined at the fluorescence microscope. Five 0.15 mm2 the endothelium was mounted in buffered glycerol and examined at the fluorescence microscope. Five 0.15 mm2 central fields were examined for each cornea and the number of endothelial cell nuclei and eGFP positive cells recorded. Secreted transgenic anti-rat CD4 scFv was detected by flow cytometry on rat thymocytes, which are CD4 positive, as described in detail elsewhere.13 Briefly, thymocytes were incubated with 50 µl corneal supernatants for 30 minutes at 4°C, followed sequentially by anti-polyhistidine monoclonal antibody (Sigma), biotinylated anti-mouse antibody (DakoCytomation, Carpinteria, CA, USA) and streptavidin-R-phycoerythrin conjugate (Molecular Probes, Eugene, OR, USA). Fluorescence was measured on a FACSscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). To calculate the amount of functionally active transgenic protein produced by an AdV transduced human cornea, serial dilutions of purified scFv (CSL Ltd, Melbourne, Victoria, Australia) were tested against rat thymocytes by flow cytometry and the data used to create a standard curve. ScFv concentrations in corneal supernatants were then estimated using the linear portion of the curve.

Statistics
Data from dosage groups were compared by one way ANOVA with Tukey-Kramer multiple comparisons post hoc tests, and relations were analysed by univariate linear regression and the Spearman rank correlation test.

RESULTS
Twenty-nine corneas from donors with a mean age of 70 (SD 14) years were stored in standard eye bank conditions for a mean of 18 (7) days before being transduced with different doses of AdV. Following Hoechst 33258 staining and surgical removal of the epithelium, eGFP positive endothelial cells were detected in corneal flat mounts (fig 1). The percentage of eGFP positive corneal endothelial cells is documented in table 1. Two transduction failures (<2% of endothelial cells expressing eGFP) were seen, one each in the low and high AdV dosage groups. The efficiency of transduction varied among individual corneas. The best efficiency of 78% (31%) was observed in the high dose group. There was no significant difference in transduction efficiency between groups treated with intermediate and high doses of AdV. Efficiency of transduction in these corneas did not correlate with donor age, endothelial cell density or time in storage before transduction (p>0.05). Endothelial cell densities did not correlate with donor age or storage time before transduction (p>0.05) (data not shown). At 72–96 hours post-transduction, corneal endothelial cell

Figure 1  Reporter gene expression in AdV modified human corneal endothelium. (A) A human cornea transduced in vitro with 2×107 pfu AdV encoding eGFP. The image was captured at the fluorescence microscope and digitally collated. Hoechst 33258 stained corneal endothelial cell nuclei were detected under ultraviolet light (B) and eGFP positive cells in the same field were detected under blue light (C). Original magnification: A: 4×; B, C: 20×.

Figure 2  Production of transgenic protein by AdV modified human corneas. Human corneas (n = 5) were transduced with high doses of AdV (6×107–3×108 pfu per cornea) encoding a transgenic protein (scFv) and maintained in organ culture in vitro. Supernatants were sampled regularly and assayed for scFv levels by flow cytometry on rat thymocytes. Protein production rates were calculated as ng per cornea per day. Points represent mean (SD).

Table 1  Efficiency of transduction of human corneal endothelium by AdV encoding eGFP

<table>
<thead>
<tr>
<th>AdV dose (MOI)*</th>
<th>No</th>
<th>pfu Adv per cornea</th>
<th>Donor age† (years)</th>
<th>Storage time‡ (days)</th>
<th>Endothelial cell density† (cells/mm²)</th>
<th>% eGFP+ cells per cornea§</th>
<th>Transduction failures*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>0</td>
<td>71 (6)</td>
<td>21 (7)</td>
<td>1357 (922)</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Low (10)</td>
<td>3</td>
<td>2×10⁶</td>
<td>72 (4)</td>
<td>25 (3)</td>
<td>2720 (611)</td>
<td>8 (3)</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate (25–100)</td>
<td>8</td>
<td>5×10⁶–2×10⁷</td>
<td>68 (10)</td>
<td>17 (6)</td>
<td>2195 (558)</td>
<td>54 (31)</td>
<td>0</td>
</tr>
<tr>
<td>High (250–1500)</td>
<td>13</td>
<td>5×10⁷–3×10⁸</td>
<td>72 (19)</td>
<td>16 (7)</td>
<td>2163 (669)</td>
<td>78 (31)</td>
<td>1</td>
</tr>
<tr>
<td>Very high (10000)</td>
<td>2</td>
<td>2×10⁹</td>
<td>65 (8)</td>
<td>27 (2)</td>
<td>98 (129)</td>
<td>64 (26)</td>
<td>0</td>
</tr>
</tbody>
</table>

*MOI: multiplicity of infection; †Mean (SD); ‡Time in cold storage from enucleation until transduction; §As calculated from five central fields (0.15 mm²) on corneal flat mounts by fluorescence microscopy; *Time in cold storage from enucleation until transduction; §As calculated from five central fields (0.15 mm²) on corneal flat mounts by fluorescence microscopy; *p<0.01 compared to very high dose group; ††p<0.05 compared to low and no dose groups.
densities were not significantly different among untreated corneas or corneas treated with low, intermediate, or high doses of AdV (range 564–3763 nuclei/mm², mean 2211 (SD 644) nuclei/mm²). In contrast, very high doses of AdV significantly reduced the endothelial cell count to 98 (129) nuclei/mm². Since these corneas received high volumes of AdV stocks (40 μl in a total 300 μl transduction volume) it was considered that the AdV vehicle itself (10% glycerol in PBS), rather than the AdV particles, may have damaged corneal endothelium. However, three corneas in the high dosage group received greater volumes of AdV stock (50–100 μl in a total 300 μl transduction volume) and did not have reduced endothelial cell densities (2383 (222) nuclei/mm²).

To examine transgenic protein production, five corneas were transduced with high doses of AdV (6 × 10⁷–3 × 10⁸ pfu/cornea) encoding secreted scFv protein and production was monitored in vitro. Supernatants were sampled regularly and assayed by flow cytometry (fig 2). Protein production peaked at 2 weeks post-transduction at 2.4 (SD 0.9) μg per cornea per day, and declined to 1.2 (0.4) μg per cornea per day by day 33. After 33 days in organ culture following transduction, corneas contained enlarged endothelial cells and had reduced endothelial cell densities (431 (685) nuclei/mm²) but 64% (22%) of cells still expressed eGFP.

**DISCUSSION**

Human corneas have previously been shown to be transduced efficiently by recombinant adeno viral vectors.4 9 In this study, human corneas were efficiently transduced by AdV following up to 28 days cold storage and transgene expression in vitro was maintained for at least 1 month. The efficiency with which endothelium was transduced varied, but did not depend on endothelial cell density, suggesting that factors other than multiplicity of infection are important. High doses of between 5 × 10⁷ and 3 × 10⁸ pfu AdV per cornea resulted in transgene expression in 12–100% of corneal endothelial cells, with no decline in cell density relative to corneas receiving no or low (2 × 10⁶ pfu/cornea) doses of AdV. Very high doses of AdV (MOI: 10 000) reduced corneal endothelial cell density, probably as a result of viral toxicity. Transgenic protein production peaked at 2 weeks post-transduction at 1.4–3.3 μg per cornea per day. The decrease in production rate after 2 weeks probably resulted from a loss in endothelial cell number during extended organ culture. Long term transgene expression in vitro does not necessarily translate into prolonged expression in vivo,9 although reporter gene expression has been observed for up to 12 weeks in syngeneic murine corneal grafts transduced ex vivo with AdV.9 In the context of using a gene therapy approach to modulate corneal graft rejection, indefinite transgene expression may not be required.

Benefits of replication defective adenoviral vectors are that they are relatively easy and comparatively safe for laboratory workers to handle, can be grown to high titre, and can accommodate large transgenes.11 The problems associated with adenoviral vectors are threefold. Firstly, they are immunogenic.11 Secondly, they have been associated with at least one death after systemic administration to a human patient.12 Thirdly, they are non-integrative and do not therefore produce longlasting effects, especially in cells with high mitotic potential.12 Vector immunogenicity is not an insurmountable problem: the newer adenoviral vectors are very weakly or non-immunogenic,23 and even first generation adenoviral vectors can be administered repeatedly to the anterior segment of the eye without serious sequelae.12 Systemic administration of the very large numbers of adenovirus vector particles24 that appear to be required to correct systemically acting gene defects may not be necessary for genetic modification of the cornea, assuming that a therapeutic effect can be achieved by ex vivo treatment of the donor cornea before surgery. Finally, relatively short term expression of transgenes within the eye may be sufficient to modulate an allograft response in the longer term.9 The loss or silencing of episomal elements may actually improve vector safety, given that there is no risk of the insertional mutagenesis that has bedevilled recent clinical trials using integrative vectors.24

**ACKNOWLEDGEMENTS**

We thank Mrs M Philpott and Mrs B McGrath in the Eye Bank of South Australia, and the families of some eye donors for the provision of human corneas considered to be unsatisfactory for clinical transplantation for this research study.

**Authors’ affiliations**

C F Jessup, H M Brereton, D J Coster, K A Williams, Department of Ophthalmology, Flinders University, Adelaide, Australia

**Correspondence to:** Dr Keryn Williams, Department of Ophthalmology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia; keryn.williams@flinders.edu.au

**Accepted for publication 1 January 2005**

**REFERENCES**

Clinical Evidence—Call for contributors

Clinical Evidence is a regularly updated evidence-based journal available worldwide both as a paper version and on the internet. Clinical Evidence needs to recruit a number of new contributors. Contributors are healthcare professionals or epidemiologists with experience in evidence-based medicine and the ability to write in a concise and structured way.

Areas for which we are currently seeking authors:
- Child health: nocturnal enuresis
- Eye disorders: bacterial conjunctivitis
- Male health: prostate cancer (metastatic)
- Women’s health: pre-menstrual syndrome; pyelonephritis in non-pregnant women

However, we are always looking for others, so do not let this list discourage you.

Being a contributor involves:
- Selecting from a validated, screened search (performed by in-house Information Specialists) epidemiologically sound studies for inclusion.
- Documenting your decisions about which studies to include on an inclusion and exclusion form, which we keep on file.
- Writing the text to a highly structured template (about 1500–3000 words), using evidence from the final studies chosen, within 8–10 weeks of receiving the literature search.
- Working with Clinical Evidence editors to ensure that the final text meets epidemiological and style standards.
- Updating the text every six months using any new, sound evidence that becomes available. The Clinical Evidence in-house team will conduct the searches for contributors; your task is simply to filter out high quality studies and incorporate them in the existing text.
- To expand the topic to include a new question about once every 12–18 months.

If you would like to become a contributor for Clinical Evidence or require more information about what this involves please send your contact details and a copy of your CV, clearly stating the clinical area you are interested in, to Klara Brunnhuber (kbrunnhuber@bmjgroup.com).

Call for peer reviewers

Clinical Evidence also needs to recruit a number of new peer reviewers specifically with an interest in the clinical areas stated above, and also others related to general practice. Peer reviewers are healthcare professionals or epidemiologists with experience in evidence-based medicine. As a peer reviewer you would be asked for your views on the clinical relevance, validity, and accessibility of specific topics within the journal, and their usefulness to the intended audience (international generalists and healthcare professionals, possibly with limited statistical knowledge). Topics are usually 1500–3000 words in length and we would ask you to review between 2–5 topics per year. The peer review process takes place throughout the year, and our turnaround time for each review is ideally 10–14 days.

If you are interested in becoming a peer reviewer for Clinical Evidence, please complete the peer review questionnaire at www.clinicaledvidence.com or contact Klara Brunnhuber (kbrunnhuber@bmjgroup.com).