

## SCIENTIFIC REPORT

## In vitro adenovirus mediated gene transfer to the human cornea

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

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**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 ( $5 \times 10^7$ – $3 \times 10^8$  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 ( $2 \times 10^9$  pfu) reduced endothelial cell densities to 98 (SD 129) nuclei/mm<sup>2</sup> (compared to 2114 (716) nuclei/mm<sup>2</sup> 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/mm<sup>2</sup>.

**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.

Corneal allograft rejection is the major cause of human corneal graft failure.<sup>1</sup> Gene transfer to corneal endothelium has been shown to modulate corneal allograft rejection in a number of experimental models,<sup>2–5</sup> thereby providing proof of principle for future attempts to reduce the incidence of rejection in humans. The literature on gene therapy of the cornea has recently been reviewed.<sup>6–8</sup> Vector systems that have been used successfully to transduce reporter genes to human corneas include replication deficient adenovirus,<sup>9</sup> lentivirus,<sup>10</sup> activated polyamidoamine dendrimers,<sup>11</sup> and synthetic peptides.<sup>12</sup>

Of the currently available vectors, adenovirus appears to be the most efficient at transducing corneal endothelial cells. We were keen to explore the efficacy of adenoviral vector transfer to the human cornea in more depth.

## 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)<sup>13</sup> 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 caesium chloride density gradients and titered by the tissue culture infectious dose method.<sup>14</sup> Titres of different batches varied from  $3 \times 10^9$ – $5 \times 10^{10}$  pfu/ml.

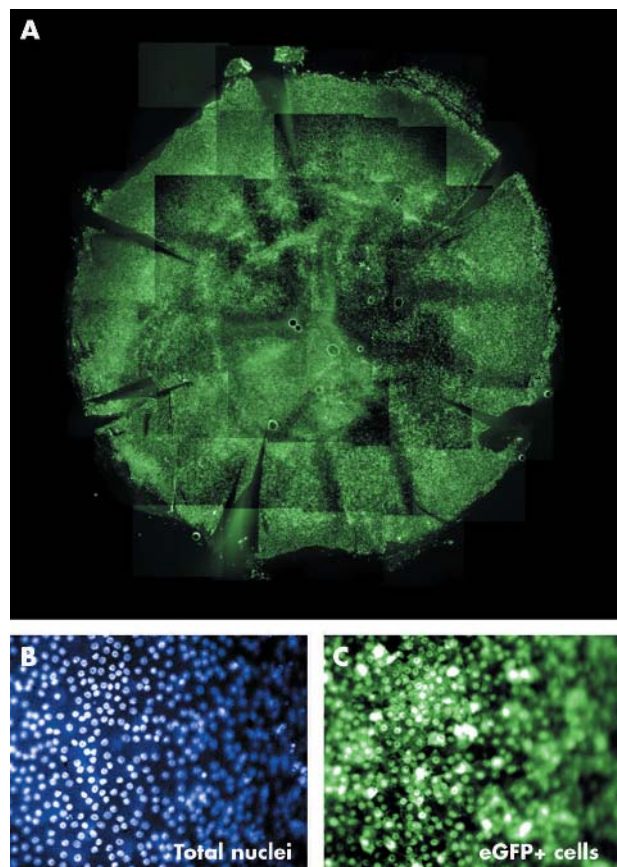
## Transduction of human corneal endothelium with AdV

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  $2 \times 10^6$ – $2 \times 10^9$  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) 100 IU/ml penicillin, 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% CO<sub>2</sub> 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

**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



**Figure 1** Reporter gene expression in AdV modified human corneal endothelium. (A) A human cornea transduced in vitro with  $2 \times 10^7$  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:  $4 \times$ ; B, C:  $20 \times$ .

the endothelium was mounted in buffered glycerol and examined at the fluorescence microscope. Five  $0.15 \text{ mm}^2$  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.<sup>13</sup> Briefly, thymocytes were incubated with  $50 \mu\text{l}$  corneal supernatants for 30 minutes at  $4^\circ\text{C}$ , 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 FACScan 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.

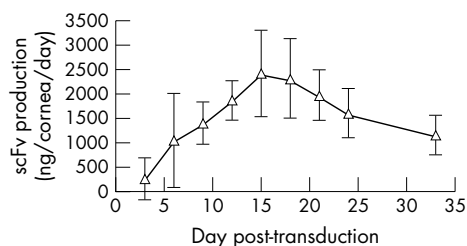
### Statistics

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 2** Production of transgenic protein by AdV modified human corneas. Human corneas ( $n = 5$ ) were transduced with high doses of AdV ( $6 \times 10^7$ – $3 \times 10^8$  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

AdV dose (MOI)*	No	pfu Adv per cornea	Donor age† (years)	Storage time‡ (days)	Endothelial cell density§ (cells/mm <sup>2</sup> )	% eGFP+ cells per cornea†§	Transduction failures¶
None	3	0	71 (6)	21 (7)	1367 (952)	0	–
Low (10)	3	$2 \times 10^6$	72 (4)	25 (3)	2720 (611)**	8 (3)	1
Intermediate (25–100)	8	$5 \times 10^6$ – $2 \times 10^7$	68 (10)	17 (6)	2195 (558)**	54 (31)	0
High (250–1500)	13	$5 \times 10^7$ – $3 \times 10^8$	72 (19)	16 (7)	2163 (669)**	78 (31)††	1
Very high (10000)	2	$2 \times 10^9$	65 (8)	27 (2)	98 (129)	64 (26)	0

\*MOI: multiplicity of infection; †Mean (SD); ‡Time in cold storage from enucleation until transduction; §As calculated from five central fields ( $0.15 \text{ mm}^2$ ) on corneal flat mounts by fluorescence microscopy; ¶Transduction failures: fewer than 2% eGFP positive cells/cornea; \*\* $p < 0.01$  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/mm<sup>2</sup>, mean 2211 (SD 644) nuclei/mm<sup>2</sup>). In contrast, very high doses of AdV significantly reduced the endothelial cell count to 98 (129) nuclei/mm<sup>2</sup>. 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/mm<sup>2</sup>).

To examine transgenic protein production, five corneas were transduced with high doses of AdV ( $6 \times 10^7$ – $3 \times 10^8$  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/mm<sup>2</sup>) but 64% (22%) of cells still expressed eGFP.

## DISCUSSION

Human corneas have previously been shown to be transduced efficiently by recombinant adenoviral vectors.<sup>9–15</sup> 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  $5 \times 10^7$  pfu and  $3 \times 10^8$  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 ( $2 \times 10^6$  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,<sup>16</sup> although reporter gene expression has been observed for up to 12 weeks in syngeneic murine corneal grafts transduced ex vivo with AdV.<sup>17</sup> 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.<sup>18</sup> The problems associated with adenoviral vectors are threefold. Firstly, they are immunogenic.<sup>19</sup> Secondly, they have been associated with at least one death after systemic administration to a human patient.<sup>20</sup> Thirdly, they are non-integrative and do not therefore produce longlasting effects, especially in cells with high mitotic potential.<sup>21</sup> Vector immunogenicity is not an insurmountable problem: the newer adenoviral vectors are very weakly or non-immunogenic,<sup>22</sup> and even first generation adenoviral vectors can be administered repeatedly to the anterior segment of the eye without serious sequelae.<sup>23</sup> Systemic administration of the very large numbers of adenovirus vector particles<sup>20</sup> 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.<sup>3</sup> 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.<sup>24</sup>

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Ethics approval: Necessary ethics committee approval was secured for the study from the Flinders Clinical Research Ethics Committee.

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