Ex vivo adenovirus mediated gene transfection of human conjunctival epithelium

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Abstract

Aim—To investigate the efficacy of “ex vivo” adenoviral vector mediated gene transfection of human conjunctival epithelial cell as a possible route for gene therapy for the distribution of anti-inflammatory agents for the potential treatment of immune mediated ocular inflammatory disorders.

Methods—Human conjunctival cells (HCs) were cultured with various concentrations of recombinant adenoviral vectors carrying a reporter gene LacZ, GFP, or an immunomodulating cytokine vIL-10. vIL-10 in culture supernatant was detected by sandwich ELISA and biological activity was assessed by suppression of ConA stimulated splenocyte proliferation. X-gal and GFP expression was assessed by histochemistry.

Results—The extent of adenoviral vector mediated transfer of both reporter genes and vIL-10 was dose dependent. LacZ expression could be detected for at least 50 day after infection with multiple of infection (MOI) 200. Following AdCMVvIL-10 transduction, vIL-10 protein expression occurred between 4–6 days post-transduction, and was maintained at a detectable level for at least 1 month. Secreted vIL-10 showed biological activity, significantly inhibiting Con A induced splenocyte proliferation. Additionally, transfection of HCs with two Adv vectors, one carrying LacZ and the other carrying GFP, resulted in co-expression within a single cell.

Conclusion—These results confirm previous successful adenoviral vector mediated gene transfer to HCs and further show that expression can be maintained. Furthermore the data show HCs can secrete biologically active vIL-10 that could be developed as a strategy to suppress immune mediated disorders. The successful co-transduction of HCs as described for other tissues, opens avenues to develop a multiple target gene therapy locally.

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Compared with other tissue or organs, the development of gene therapy for ocular disease has advantages because the therapeutic effect can be observed¹ and, consequently, adenoviral vectors have been widely studied in the eye. Direct intraocular application of adenoviral mediated gene transfer can induce

expression of reporter genes in different ocular tissues, including corneal endothelium, conjunctiva, iris, lens epithelium, choroid, retina, retinal pigment epithelium, and optic nerve.^{2–5} However, although direct injection of adenoviral vector carrying the LacZ gene into the anterior chamber or vitreous cavity results in a strong expression within the ocular tissue, the long term efficacy of such an approach is limited by immune response to the adenoviral vector.^{3–6} Despite notions that the eye may protect against immune mediated inflammation, it is likely that intraocular approaches of gene transfer will induce significant immune mediated damage.⁷ For example, injection of brain gliomas with adenovirus vector expressing herpes simplex virus 1 thymidine kinase successfully inhibited syngeneic glioma growth, but also induced chronic active brain inflammation resulting in loss of myelin fibres.^{8–9} Generating alternative vectors as well as using an ex vivo approach of gene transfer may restrict any deleterious immune response. As such, the conjunctiva may be an appropriate tissue for such a therapeutic approach. Human adenovirus is highly infectious for both conjunctiva and upper respiratory tract mucosa.^{10–11} Adenovirus serotype A to E contain a conserved integrin binding motif (RGD) in their penton base proteins, which combine $\alpha\beta 5$ integrin as adenoviral receptor on the host cell,¹² such as the conjunctival epithelial, thus facilitating viral cell entry.¹³

Genetic modification of the conjunctiva with anti-inflammatory cytokines, such as vIL-10, may be of potential value in treating a variety of ocular inflammatory diseases, including uveoretinitis, corneal allograft rejection, and ulcerative keratitis. Previous work has successfully demonstrated reporter gene LacZ expression by cultured human conjunctival epithelial cells.⁴ In rodents, X-gal expression in conjunctiva persisted more than 3 weeks following subconjunctival injection of adenovirus vector.⁴

Previous studies have assumed that transgenes can be highly expressed on HCs thus providing possible therapeutic activity. The aims of this study, therefore, were to (i) assess and confirm the efficacy and duration of transgenes expressed in HCs in vitro; (ii) assess whether the persistence of secreted vIL-10 is biologically active; and (iii) assess whether transduction of HCs with more than one adenoviral vector each carrying a different transgene is possible.

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Figure 1 β -gal expression on HCs following AdCMVLacZ transfection. β -gal activity in HCs 5 days after transduction with AdCMVLacZ at MOI 25 (A), MOI 100 (B), MOI 50 (C), and MOI 200 (D). Magnification $\times 100$.

Materials and methods

All procedures involving recombinant DNA materials were performed under the guidelines of local genetic modification committee, University of Aberdeen, for the use of adenovirus.

CELL CULTURE

Dulbecco's modified Eagle's medium (DMEM, Sigma, UK) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10^{-5} M 2-mercaptoethanol (designated complete DMEM) was used for HCs culture. Human donor eyes were obtained from Amsterdam by material transmission permission. The tissue used was negative for HIV, HCV, CMV, and HBV. After rinsing with phosphate buffered saline (PBS), the conjunctiva was dissected under direct microscopy, and then cut into 2×2 mm² pieces. After two washes in PBS, the conjunctiva was placed in a 35 mm² culture dish, and digested with 0.25% trypsin (Difco, UK) for 2 hours at 37°C. Digestion was stopped by adding complete DMEM. Cells were suspended in 5 ml medium and seeded in 35 cm flasks and cultured at 37°C with 5% carbon dioxide. Cells were propagated to 70% confluence and used for transfection between fifth and sixth passage.

ADENOVIRAL VECTORS

Adenoviral vectors expressing green fluorescence protein (GFP) and β -galactosidase (LacZ) vectors were obtained from Quantum Company (Canada). The viruses were deleted for both E1 and E3 regions and so lacked the

ability to replicate in non-permissive cells. Adenovirus encoding vIL-10 was constructed by Dr Emri Kovesdi as previously described.¹⁴⁻¹⁶ In brief, the BCRF-1 coding gene was flanked at the 5' end with the promoter of the human cytomegalovirus and at the 3' end with SV40 polyadenylation sequence. All viruses were tested and found to have replication competent (RCA) levels of less than $1:1 \times 10^7$ plaque forming units (PFU). Viral titres were determined by optical density at 260 nm (1 U = 10^{12} viral particles).

TRANSDUCING HUMAN CONJUNCTIVAL CELL WITH ADENOVIRAL VECTOR

Petri dishes of 35 mm or chamber slides were used to transduce HCs with AdCMVLacZ and AdCMVGFP, and 75 cm flask for AdCMVvIL-10. Ad1307 a null vector deficient of cDNA¹⁵ was used as a control. When HCs were 70% confluent, adenoviral vector was added at different multiples of infection (MOI). Viral infection was carried out at 37°C for 4 hours, and then complete DMEM was added to the cells. Cultures were maintained in a humid atmosphere of 5% carbon dioxide at 37°C.

When transfecting with adenoviral vectors, 75 mm culture dishes were seeded with 2×10^4 HCs. After the cells adhered to the wells (about 3 hours), cells were washed with DMEM to remove non-adherent cells, and then 2 ml fresh complete DMEM was added. The cells were challenged with two adenoviral vectors (AdCMVLacZ and AdCMVGFP) simultaneously. The cells were stained with X-gal at 24 hours

Figure 2 Time course of β -gal expression after AdCMVLacZ transduction. HCs were transfected with AdCMVLacZ at different MOI. At selected times, the β -gal expression were determined via X-gal staining. Results showed that the maximal expression at MOI 50 was seen at days 2 post-transfection and day 5 for MOI 100 and MOI 200. β -gal expression was maintained for at least 50 days at MOI 200.

and 48 hours and viewed by fluorescence microscopy. X-gal positive cells, GFP positive cells, and double positive cells were counted in the same field at $\times 100$ magnification. A total of 100 cells/field were counted.

DETECTION OF β -GALACTOSIDASE ACTIVITY

At different time points, cells were washed with PBS and immediately fixed with 0.05% glutaraldehyde for 15 minutes at room temperature. Cells were rinsed thoroughly with PBS three times. β -galactosidase activity was assayed by reacting the cells with 1 mg/ml 5-bromo-4-chloro-3 indolyl β -gal-D-galactoside (X-gal, Sigma, UK) in a solution containing 50 mM $K_3Fe(CN)_6$, 50 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS at 37°C for 4 hours or overnight.

ELISA ASSAY FOR vIL-10

Supernatants from cultured cells were collected every other day for 30 days. After the supernatants were removed, flasks were washed twice with 2 ml DMEM, and then 2 ml complete DMEM was added. Samples were stored at -20°C until use. mAb specific for human and viral IL-10, vIL-10 standard and biotinylated anti-human and anti-viral IL-10 mAb were purchased from Pharmingen (San Diego, USA).

vIL-10 was determined by sandwich ELISA assay. Wells of flat bottom (Nunc, Denmark) microtitre plates were coated with 100 ng of mAb specific for human and viral IL-10 in carbonate buffer, pH 9.0 at 4°C overnight. Following two washes with PBS-0.05% Tween 20, the plates were blocked with 200 μl PBS-10% BSA for 2 hours at room temperature (RT). After three washes with washing buffer, 100 μl of rHuIL-10 standard or cultured supernatants were added and plates were incubated at RT for 2 hours. After five washes, 100 μl of biotin conjugated rat anti-human and antiviral IL-10 mAb was added to each well. After 1 hour of incubation at RT, the plates were washed and incubated at RT with an avidin-peroxidase conjugate. After seven washes, the wells were visualised by adding 100 μl of substrate solution (2642KK, PharMingen, USA) for 30 minutes at RT in the dark. The reaction was stopped by adding 50 μl 2N H_2SO_4 and the plates were read on microplate reader at wavelength 450 nm (Dynatech RR5000, USA).

Figure 3 Green fluorescence protein (GFP) expression following HC transfection with AdCMVGFP.

Figure 4 Transduction of HCs with two adenoviral vector carrying *E coli LacZ* and green fluorescence protein (GFP). (A and B) Dual expressing cells. (C and D) β -gal negative and GFP positive cell. β -gal expression is mainly nuclear compared with wider distribution of GFP expression. Magnification $\times 400$.

SPLENOCYTE PROLIFERATION ASSAY

Splenocytes were isolated from C57BL/6 mice. Conditioned medium was made as follows: culture supernatant from HCs transduced with or without AdCMVIL-10 at day 4, 6, 8, and 10 were passed through a 0.2 μ l filter. Splenocytes 2×10^6 in 100 μ l were seeded in triplicate in a 96 well round bottom plate in the presence or not of 5.0 μ g/ml ConA (Sigma, UK), 100 μ l of test conditioned medium and/or a neutralising antibody to vIL-10 at concentration 200 ng/ml (Pharmingen, San Diego, CA, USA). Cultures were cultivated for 48 hours, after which 1 μ l Ci 3 H thymidine was added to each well for a further 18 hours. 3 H thymidine incorporation was quantified using a Beckman scintillation counter. Results were expressed as the mean CPM (SD) of triplicate cultures.

Figure 5 Simultaneous transduction of HCs with AdCMVLacZ and AdCMVGFP. The results are a representative experiment which show that incidence of dual expression was increased with greater MOI.

STATISTICS

Data are represented as mean value (SD). Statistical analysis was carried out using the Student's *t* test.

Results

ADENOVIRAL VECTORS EFFECTIVELY TRANSFER EXOGENOUS GENE TO HUMAN CONJUNCTIVAL CELL

Figure 1 shows 95% of HCs exhibited an intense blue staining at 5 days following infection at MOI 200. More than 70% of the cells at day 20, and 24% of cells at day 50 post-transfection, remained X-gal positive. At varying multiples of infection (MOI 50), relatively high levels of LacZ expression in HCs (34%) were still observed at day 2 post-transfection (Fig 2), although expression rapidly diminished. HCs appeared more sensitive to AdCMVGFP transfection. At MOI 100, nearly 100% of cells showed strong GFP expression (Fig 3).

We next attempted to investigate whether HCs could be infected with two adenoviral vectors carrying independent reporter genes LacZ and GFP. Cells were seeded into 75 mm petri dishes at 1×10^4 /ml, where cells were transfected with adenoviral vectors at 70% confluence. The results showed that both reporter genes could be expressed in the same cell. At MOI 100 for each vector, 37% cells were dual expressing, and 67% dual expressing cells were observed at MOI 200 cell (Figs 4 and 5). The percentage of GFP expression (both single and dual expressing cells) was significantly higher than X-gal expression ($p < 0.01$). No GFP positive cells were observed in non-transfected HCs.

Figure 6 vIL-10 concentration in cultured supernatant of HCs transfected with AdCMVvIL-10. The peak secretion occurred at day 6 post-transfection at both MOI 100 and MOI 200. Although vIL-10 levels declined thereafter, detectable levels were maintained for at least 30 days.

VIL-10 TRANSDUCED HCS SECRETE BIOLOGICALLY ACTIVE VIL-10

Culture supernatants collected every other day for 30 days from HCs transduced with AdCMVvIL-10 at MOI 200 were assayed by sandwich ELISA. Results are shown in Figure 6. AdCMVvIL-10 infected HCs produced significant quantities of vIL-10 for over 30 days. Maximal secretion occurred at days 4–6 (170 (13) ng/ml), after which expression decreased but persisted at detectable levels for 30 days (39 (5.3) ng/ml).

To assess biological activity, vIL-10 transduced HCs culture supernatant (conditional medium) inhibition of ConA stimulated splenocyte proliferation was determined. The results showed (Fig 7) that secreted vIL-10 significantly inhibited splenocyte proliferation, and anti-vIL-10 mAb reversed vIL-10 mediated suppression of splenocyte proliferation (CPM 9245 (964) v 20049 (1886), $p < 0.01$). No difference in splenocyte proliferation was found in extent of Con A stimulation between the groups where normal supernatant, normal supernatant plus blocking anti-vIL-10 mAb or additional 100 μ l medium was added ($p > 0.05$).

Discussion

In this study HCs were successfully transduced by adenoviral vectors containing LacZ, GFP and vIL-10. Our results show that HCs genetically modified with AdCMVLacZ, strongly express X-gal in a dose dependent manner and that X-gal expression persisted for up to 50 days at MOI 200. These data confirm recent reports of successful gene transfer to conjunctival cells. In previous reports, this route was potentially more feasible than corneal epithelium.^{4–17} Such differences may be related to the distribution of adenoviral receptor, although we have not formally tested this, and direct comparisons of the ability of both routes to suppress immune mediated disorders, including EAU and corneal graft rejection, and the use of other vectors are needed. As these data show that HCs can not only stably express target gene

Figure 7 Supernatant of AdCMVvIL-10 transduced HCs significantly inhibited Con A stimulated lymphocyte proliferation. (A) Control groups for splenocyte proliferation. A = medium alone. B = Con A stimulated medium plus conditioned medium from non-infected HCs plus blocking anti-vIL-10 mAb. C = Con A stimulated medium plus conditioned medium from non-infected HCs; and D = medium plus Con A. Values represent mean CPM (SD). (B) Con A stimulated splenocyte proliferation was inhibited by supernatant of AdCMVvIL-10 transduced HCs throughout days of detectable vIL-10 secretion (see Fig 6). Figure represents mean CPM (SD) for splenocyte proliferation in presence of conditioned medium from AdCMVvIL-10 transduced HCs (vIL-10 supernatant) and conditioned medium plus blocking anti-vIL-10 mAb (anti-vIL-10 mAb). Peak inhibition occurred day 8 post-transfection, that was reversed when IL-10 activity was neutralised with anti-vIL-10 mAb block ((CPM 9245 (964) v 20 049 (1886), $p < 0.01$). Thereafter, degree of inhibition gradually decreased. By day 32, there was no difference in splenocyte proliferation between controls and AdCMVvIL-10 transduced HCs ($p > 0.05$).

but also vIL-10, further investigation as a possible ex vivo gene therapeutic approach for management of inflammatory eye disease following a similar strategy described for other tissues is warranted.¹⁸

vIL-10, the IL-10 homologue of Epstein-Barr virus, has anti-inflammatory activity similar to human IL-10. vIL-10 downregulates MHC class II, ICAM-1, and B7 expression on macrophages and dendritic cells,¹⁹ inhibits T cell activation and alloreactivity, and cell adhesion molecule expression^{20–21} thus suppressing inflammation. Additionally, vIL-10 suppresses proinflammatory cytokines (TNF- α and IL-1 β) production.²² As such, results have shown that transfer of vIL-10 by adenoviral vectors or retroviral vector prolonged organ graft survival and greatly reduced leucocyte infiltration into cartilage in experimental arthritis.^{23–27} These data show that AdCMVvIL-10 transduction of HCs resulted in an early high peak of biologically active vIL-10 that was maintained despite reduced levels of vIL-10 for over 20 days.

Ocular inflammation and corneal allograft rejection are complex pathophysiological processes that involve multiple mediators such as cytokines, chemokines, and growth factors.²⁸ However, blockade of one inflammatory cytokine or induction of cytokine antagonist may not be adequate to suppress inflammatory responses or induce a prolonged therapeutic effect. Using AO to PVG rat orthotopic keratoplasty model, Torres *et al* recently demonstrated that injection of murine IL-10 subconjunctivally did not prolong corneal allograft survival and may have even have accelerated rejection.²⁹ An arguable future approach, therefore, is to target two or more regulators of the immune response. There has been success by blocking more than one co-stimulatory signal via CTLA4-Ig and anti-CD40L mAb, where both suppression and long term tolerance was induced.³⁰ Technically, however, achieving this by gene therapy is problematic. Although it is difficult to clone more than one target gene in one vector because the capacity of insertion is limited, there has encouragingly been progress made in generating multiple gene transfer within a single vector.^{31 32} In addition, intratumoral co-injection of two adenoviral vectors one encoding IFN- γ inducible protein-10 and the other encoding IL-12 resulted in marked antitumoral synergy.³³ Similarly, successful antitumoral activity has been demonstrated with concomitant adenoviral delivery of IL-2 and IL-12.³⁴

Our present data showed that both reporter genes could be expressed in HCs, although the efficacy of expression appeared dependent upon the reporter gene. As it is clear that two simultaneous adenoviral vectors can mediate independent gene delivery to the same cell, further development of dual vector delivery to resident ocular surface cells will potentially lead to the development of long term immunomodulatory strategies. Previous reports⁴ have shown successful Adv vector gene delivery to HC, and indirectly commented on potential cytopathogenicity by noting increased IL-6, IL-8, and ICAM-1 expression that could in turn be suppressed by concomitant topical steroid therapy. It is likely, however, that despite the efficiency of Adv vector gene delivery, the generation of Adv vector used in these studies will not be clinically pertinent because of the potential inflammatory response.³ However, as this extraocular route can secrete immunomodulatory products it may provide a strategy to limit intraocular damage while maintaining a therapeutic effect. In conclusion, these data show that transduction of HCs with recombinant adenoviral vectors can result in high levels of stable target gene expression with biological activity and simultaneous transduction of HCs with two reporter genes results in dual gene expression within one cell. Such an approach warrants further investigation to elicit any putative clinical application, particularly for ocular surface disorders, including corneal allograft rejection and non-infectious inflammatory disease.

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