TNF Receptor Secretion after Ex Vivo Adenoviral Gene Transfer to Cornea and Effect on In Vivo Graft Survival

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PURPOSE. To explore the potential for adenovirus-mediated ex vivo gene transfer of a soluble tumor necrosis factor (TNF) receptor and evaluate the effect of transplanting the adenovirally transplanted corneas in vivo.

METHODS. Rabbit corneal segments were transfected with replication-deficient adenovirus (AdTNFR) encoding a soluble TNF receptor fusion protein (TNFR-Ig). Production of TNFR-Ig was measured by using ELISA and bioassay. Corneas were transfected ex vivo with AdTNFR and then transplanted in vivo. Survival of AdTNFR-transfected corneas was compared with that of those treated either with a null vector control adenovirus (Ad0) or nontransfected control corneas.

RESULTS. Ex vivo production of a molecule with TNF blocking bioactivity from AdTNFR-transfected corneas was demonstrated over a period of 4 weeks. Transplanted AdTNFR-transfected corneas showed a marginally increased survival time in vivo over nontransfected control corneas, but a significantly increased survival time over Ad0-treated control corneas. Ad0 treatment of corneal allografts before transplantation had a proinflammatory effect and accelerated the onset of corneal endothelial rejection.

CONCLUSIONS. Adenoviral gene transfer is an effective means of transferring a gene encoding soluble TNF-R-Ig to corneal endothelium, and ex vivo production of a biologically active secreted molecule was demonstrated for 4 weeks. However, in vivo, only a marginally increased survival was seen compared with control corneas. The introduction of this transgene using a less immunogenic vector may demonstrate prolongation of corneal allograft survival. (Invest Ophthalmol Vis Sci. 2001;42:1568–1573)

Because donor corneas can be maintained in culture for extended periods before transplantation, ex vivo gene transfer is feasible and may have useful clinical applications. One potential application is modulation of the allogeneic response to the graft. This could be achieved by local delivery of biologically active molecules from the gene-modified cornea for the prevention of rejection. Recombinant adenosinases are efficient vectors for gene transfer to the endothelium when whole-thickness corneas are incubated with virus ex vivo, resulting in high-level expression restricted to the endothelial cells with no demonstrable adverse effect on endothelial function in vivo, as determined by corneal hydration and thickness control.1–3

One potential therapeutic target for intervention is tumor necrosis factor (TNF), a proinflammatory cytokine that is frequently found during allograft rejection. In corneal allografts, TNF has been shown to be elevated in murine allografts (by using bioassay of corneal homogenates4 or an ELISA of cultured corneas5), and elevated mRNA levels have been reported in the rat.6,7 In the context of the high-risk rabbit allograft model used in this study, we have shown with a bioassay fluctuating high levels of TNF in the aqueous humor of allografts, but not in that of autografts.8 The fluctuations of TNF most probably reflect feedback mechanisms regulating TNF activity.9 The importance of TNF in graft rejection is shown in two ways. First, we have observed in some but not all animals a prolongation of graft survival after intracameral injection of a soluble fusion protein containing the TNF receptor.5 Second, Yamada et al.10 have shown that graft survival is prolonged in a minor-alloantigen–disparate combination when the recipient animals do not have the p55 (type I) TNF receptor, but do have the p75 (type II) receptor. Intriguingly, in a total-mismatch combination, p75 knockout recipients showed reduced graft survival. These data indicate that TNF may have distinct roles in corneal allograft survival, dependent on which receptor is engaged, with p55 being integral to the rejection of minor-antigen-disparate grafts.10

The data obtained showing some benefit of administration of a soluble fusion protein consisting of the p55 type I receptor for TNF coupled to a human IgG1 Fc portion (TNFR-Ig) led us to investigate whether delivery of a similar molecule by gene transfer technique would further prolong corneal allograft survival.9 We tested the ability of ex vivo adenovirus-mediated delivery of cDNA encoding TNFR-Ig to generate functional protein and to prolong graft survival in an orthotopic transplantation model. The rationale we considered was that endogenous production of soluble TNFR-Ig by the donor corneal endothelium into the anterior chamber would locally generate immunosuppression, prolong graft survival, and be more feasible in the clinical setting than intermittent injection of exogenous recombinant protein into the anterior chamber after transplantation.11

Peppel et al.11 first described an engineered soluble version of TNFR. This construct was a dimer of two extracellular domains of the human type I p55 TNFR fused to a mouse IgG heavy chain. It was found to bind with higher affinity to TNF and to be a more effective TNF inhibitor than neutralizing monoclonal antibodies. A transgenic mouse line expressing the TNF receptor was later described,12 and these investigators subsequently reported the phenotypic effect of blockade of TNF activity by systemic administration of a replication-defective encoding TNFR-Ig.13 These data support the hypothesis that recombinant cytokine receptors may function as an immunomodulatory therapy. Later studies on a rabbit model of arthritis have demonstrated an anti-inflammatory effect of intra-articular injection of an adenovirus vector bearing this TNFR-Ig construct.14 In another report, tumor cells transfected with cDNA encoding TNFR-Ig have been shown to resist rejection after injection into allogeneic recipients, raising the ques-
tion of whether this strategy can be generalized from tumor to tissue transplantation.\textsuperscript{18} To address this question, we verified that donor cornea transfected with cDNA encoding TNFR-Ig produced functional protein ex vivo, and we used a rabbit corneal transplantation model to examine the effect of genetic modification of donor corneas on graft survival.

\textbf{METHODS}

\section*{Viral Extraction and Purification}

The recombinant E1-deleted type 5 adenoviral vector AdTNFR-Ig\textsuperscript{15} was kindly provided by Bruce Beutler (Howard Hughes Medical Institute, Dallas, TX) and an insertless adenoviral construct, Ad\textsuperscript{+}, was a gift of Matthew Wood, University of Oxford, Oxford, UK.\textsuperscript{16} The replication-deficient viruses, which have deletions at the EIA and EIB replication regions, were grown in the 293 cell line. The virions were harvested once the cytopathic effect of the virus (a rounding up of the cells) was observed. After further extraction, the viruses were purified by two rounds of cesium chloride (CsCl) density gradient centrifugation. Viral titers were assessed by plaque assay on the 293 cells.\textsuperscript{17}

\section*{Corneal Samples}

Outbred adult female New Zealand White (NZW) rabbits were used to provide corneas for ex vivo experiments. Full-thickness rabbit corneas were removed from animals not more than 1 hour after death, with a circular trephine blade (7.5 mm in diameter). The corneas were cut into four segments before infecting with recombinant adenovirus.

\section*{Infection and Culture Conditions for Corneas}

Rabbit corneal segments were incubated at 37°C and 5% CO\textsubscript{2} for 1 hour with 250 μl of 10% fetal calf serum (FCS) in minimum essential medium (MEM) containing 1.0 × 10\textsuperscript{6} plaque-forming units (PFU) of AdTNFR. One group of control corneas was incubated in medium containing 1.0 × 10\textsuperscript{6} PFU of Ad\textsuperscript{+} and a second group of control corneas in virus-free medium. After incubation, infected and control specimens were washed three times with MEM and maintained in MEM supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin CO\textsubscript{2} (all obtained from Gibco BRL, Paisley, UK) at 37°C and 5% in a 96-well tissue culture plate for the time course study. Culture medium was changed every 1 or 2 days by replacing with 250 μl of fresh medium and tested for production of a TFN-blocking molecule, as described later. For transplantation purposes, corneas were maintained in culture for 16 hours before grafting, to minimize risk of adherent adenovirus on the donor cornea surface being treated. Longer periods are unlikely to have any benefit and so were not looked at.

\section*{Measurement of Ex Vivo Production of TNFR-Ig Using ELISA}

The ELISA was used to measure the mouse IgG portion of the recombinant TNFR-Ig molecule. The wells of a 96-well ELISA plate were coated with 50 μl goat anti-mouse Ig (10 μg/ml; Southern Biotechnology Associates, Inc., Birmingham, AL) in 0.1 M borate-buffered saline (pH 8.2–8.4) for 1 hour at 37°C. After three washes with PBS-0.05% Tween, the plate was blocked with 50 μl 1% bovine serum albumin. The wells were then washed three times with PBS-0.05% Tween. Samples (50 μl) were added in triplicate. A murine monoclonal antibody, MR6, was used to make the standard curve (kind gift of Nesrina Imani, Imperial College School of Medicine, London, UK). After 1 hour, the plates were washed three times with PBS-0.05% Tween. A 1:1000 dilution (50 μl) of alkaline phosphatase-labeled goat anti-mouse IgG (γ-chain-specific; Southern Biotechnology Associates, Inc.) was added to each well and incubated for 1 hour. After a thorough wash with PBS-0.05% Tween, 100 μl of 1 mg/ml p-nitrophenyl phosphate (Southern Biotechnology Associates, Inc.) in substrate buffer was added to each well. The plate was incubated at 37°C in the dark until a color change to yellow was evident. The plate was then read with a microplate reader at 405 nm absorbance.

\section*{Measurement of TNFR-Ig by Blocking on TNF Bioassay}

The L929 mouse fibrosarcoma cell line (kindly donated by Tom Evans, Imperial College Medical School) was used to assess TNF activity by bioassay.\textsuperscript{18} Cells were maintained in RPMI medium with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin (all from Gibco). L929 cells were suspended at a concentration of 5 × 10\textsuperscript{5} per milliliter and 100 μl (3 × 10\textsuperscript{4} cells) per well plated out onto a 96-well plate (Nunclon; Nalge-Nunc International, Roskilde, Denmark). After incubation overnight at 37°C and 5% CO\textsubscript{2}, the cells were sensitized with 25 μl actinomycin D (Sigma Chemical Co., Poole, UK) at a concentration of 8 μg/ml. Samples were diluted in L929 medium, and 37.5 μl added to wells in triplicate. Recombinant rat TNF (Serotec, Oxford, UK) at a concentration of 20 ng/ml was then added in a volume of 37.5 μl medium to each well. After overnight incubation at 37°C and 5% CO\textsubscript{2}, the medium was discarded and the plate washed with 200 μl RPMI without additives. Cells were fixed in 10% formyl saline for 10 minutes, using 50 μl per well. The fixative was discarded and the cells stained with 50 μl Gram’s crystal violet solution (BDH Laboratory Supplies, Poole, UK). The plate was then washed four times with 200 μl distilled water. The absorbance at 560 nm was recorded. A standard curve was constructed from known dilutions of TNFR-Ig protein (kindly donated by John Isaacs, St. James Hospital, Leeds, UK; and Geoff Hale, Therapeutic Antibody Center, University of Oxford, Oxford, UK). In some experiments, the recombinant rat TNF was replaced with aqueous humor from an eye containing an allograft.

\section*{Rabbit Corneal Transplantation}

Adult female NZW rabbits of more than 2.5 kg body weight were used as recipients of corneal grafts from adult female Dutch Belted donors. Rabbits were obtained from Harlan Olac, Ltd., (Bicester, UK). All transplants were performed on the right eye only. Vascularization was induced in the recipient eye by insertion of 80 silk sutures approximately 3 weeks before transplantation. Vascularization was scored by assessing vessel growth across the cornea in each quadrant, with scores of 1, 2, 3, and 4 indicating 25%, 50%, 75%, and 100% vascularization, respectively. All NZW corneas had a vascularization score of at least 8 before transplantation. Full-thickness 8-mm-diameter donor corneal grafts were inserted into prevascularized NZW corneas, by using a continuous 10-0 nylon suture and a 7.5-mm-diameter recipient bed. Control autografts were performed after vascularization of NZW corneas (as for an allograft) by removing a corneal disc 8 mm in diameter, rotating it through 180°, and then suturing it in position. This was thought to control for surgical trauma, while not introducing alloge-

\section*{Postoperative Assessment and Diagnosis of Rejection}

After surgery, rabbits were examined every other day at the slit lamp. Onset of endothelial rejection was taken as the first day on which an endothelial line of rejection was observed. Statistical comparison between groups was performed using the Mann–Whitney test with correction for multiple comparisons.

\section*{Pachymetry}

Central corneal thickness was recorded using a handheld ultrasonic pachymeter (Humphrey 885; Carl Zeiss, Oberkochen, Germany). After application of one drop of amethocaine 1% (Chauvin Pharmaceuticals, Romford, UK), thickness was recorded. The mean of three readings was taken. Corneal thickness is an indirect measure of the action of the corneal endothelial pump mechanism and, as such, is an objective
measure of corneal endothelial function. At the time of transplantation, no corneas were more than 600 μm thick. Thickening of the graft beyond this point was therefore taken as an indication of graft failure. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

Titration of Virus

To determine the concentrations of the AdTNFR virus needed for efficient transfection of rabbit corneas ex vivo, corneal specimens were incubated for 1 hour at 37°C and 5% CO₂ in corneal medium containing varying concentrations of AdTNFR. After 2 days, the medium was replaced. On day 4 the medium was collected and assayed for its ability to block a TNF assay. As shown in Figure 1 the production of enough TNFR-Ig to block at least 5 ng/ml TNF concentration was achieved with 10⁶ PFU, and this concentration was therefore selected for the ex vivo studies. Because we have measured TNF in rabbit aqueous from rejecting corneal allografts to be at this level, this amount of blocking was considered to constitute an acceptable level of TNFR-Ig production. As a positive control, a large excess of TNFR-Ig protein (50 μg/ml) was also shown to block TNF activity. A negative control sample from corneas trans- fected with 10⁵ PFU Ad0 per corneal specimen at day 4 after transfection showed no effect on TNF activity.

Blocking of TNF Produced during Corneal Allograft Rejection

The level of TNF in a sample of rabbit aqueous taken from a rabbit that underwent corneal allograft rejection was found by bioassay to be 1.266 ng/ml. Supernatant from a quarter of rabbit cornea transfected 2 days previously ex vivo with 10⁶ PFU AdTNFR was shown to completely block the TNF-mediated L929 cell lysis (data not shown).

Kinetics of Production of TNFR-Ig

The kinetics of ex vivo production of a biologically active TNF blocking molecule was investigated by testing sequential samples from quartered rabbit cornea samples incubated in 250 μl medium in a 96-well tissue culture plate over a period of up to 33 days. The TNFR-Ig product is soluble and was released into the tissue culture medium. The medium was changed on a 1- to 2-day basis and cumulative production of TNFR-Ig measured.

Active TNFR-Ig was measured with a blocking bioassay. An ELISA was also used to test for the mouse IgG1 portion of the TNFR-Ig molecule. Results of both tests demonstrated the kinetics of production shown in Figure 2. Control supernatants from Ad0-transfected rabbit corneas and nontransfected corneas did not show TNFR-Ig production, either by bioassay or ELISA.
Effect of Transfection on Corneal Allograft Survival

Dutch Belted rabbit corneas were transfected with $1.5 \times 10^7$ PFU of AdTNFR (or control Ad0) per whole cornea. This amount of virus had been optimized for in vivo use in a previous study.1 The corneas were incubated for 3 hours and then cultured in virus-free medium overnight before transplantation. The animals were divided into three groups: untreated allografts ($n = 14$), AdTNFR-treated allografts ($n = 8$), and Ad0-treated allografts ($n = 6$).

After transplantation, the allografts were assessed on a 1- to 2-day basis for clinical signs of corneal allograft rejection, by monitoring the appearance of an endothelial rejection line (Fig. 3A) and corneal allograft thickening (Figs. 3B, 4). Time to appearance of an endothelial rejection line was significantly longer in AdTNFR-treated corneas when compared with Ad0-treated grafts (Fig. 3A; $P = 0.0262$), although there is no statistical difference compared with untreated grafts ($P = 0.4682$). Treatment with Ad0 significantly shortened graft survival as determined by time to endothelial rejection, when compared with untreated animals ($P = 0.0185$).

When graft survival was monitored using ultrasonic pachymetry as an assessment of endothelial cell function, it could be seen that in control animals the corneal graft thickened immediately after transplantation as a reaction to surgical trauma (Fig. 4A). After a few days, it thinned to 400 to 500 µm, associated with increased corneal transparency. At approximately 2 weeks after transplantation the corneal graft thickened as a consequence of impaired endothelial cell function. A similar picture was seen in Ad0-treated animals (Fig. 4B). In grafts treated with AdTNFR there was a delay in the onset of rejection-associated thickening (Fig. 4C). We took a threshold of 600 µm to indicate graft failure (based on the observation that no grafts were more than 600 µm thick before transplantation). Marginally significant prolongation of graft survival was seen between AdTNFR- and Ad0-treated corneas ($P = 0.0939$) and between AdTNFR-treated corneas and untreated control allografts ($P = 0.0832$).

Of note is that during the early period after grafting (0–10 days) there was no obvious difference in the pachymetry data.
from the three groups in Figure 4, indicating that viral transfection had little direct effect on early endothelial function after transplantation.

Tissue sections taken from all three groups after onset of rejection showed similar mononuclear cell infiltrates in graft stroma and extensive damage to graft endothelium (data not shown). These indicate that once the process of graft rejection has been initiated there are no morphologic findings that differentiate between the treatment groups and control grafts.

**DISCUSSION**

TNF is secreted in response to inflammatory stimuli by a variety of somatic cells but primarily by activated macrophages and T cells. It has multiple effects in inflammatory responses, including upregulation of adhesion molecules on inflammatory cells and endothelium, expression of major histocompatibility complex (MHC) class I and II molecules, stimulation of leukocyte migration, and cytolyis of target cells. Biological agents that block the activity of such proinflammatory cytokines as TNF have become the subject of much interest. These include cytokine-specific antibodies, receptor antagonists, and soluble receptors. As in other in vitro studies, we have shown that soluble TNFR-Ig blocks the cytopathic effect of recombinant TNF in the L929 bioassay, indicating that the soluble receptors can bind to their ligand and reduce interaction of TNF with its cellular receptors. The biological effect of transfer of cDNA encoding soluble TNFR-Ig has been demonstrated in a number of in vivo models. These include increased susceptibility to Listeria infection in a murine model after systemic injection of adenoavirus vectors, reduction of leukocyte infiltration in experimental rheumatoid arthritis after intraarticular injection of adenovirus vectors, and prevention of tumor allograft survival after transfection of tumor cells in vitro.

In the context of corneal allograft rejection, blocking TNF activity may be effective both at inhibiting the induction of the alloreponse and in preventing direct toxicity to the graft. This has been highlighted by the differential effects seen in p55 and p75 TNF receptor knockout animals. Because the fusion protein used in this study would block any action of TNF, we were unable in this study to discriminate between selective effects on different TNF receptors.

As determined by the interval from transplantation to onset of endothelial rejection, we found that donor corneas transfected with AdTNFR had significantly longer survival before the appearance of an endothelial cell rejection line than Ad0-transfected control corneas. However, corneas transfected with the vector control Ad0 had significantly shorter survival until endothelial rejection than did untreated control corneas. This implies that the adenovirus leads to a more rapid response to the donor cornea. However, there was a significant difference in survival of corneas infected with Ad0 and AdTNFR, and because these viruses differ only in the cDNA construct encoding TNFR-Ig, this suggests that AdTNFR can attenuate the alloreponse.

It was interesting to note that Ad0 transfection did not significantly affect endothelial cell function as measured by graft pachymetry, compared with nontransfected control corneas. This suggests that the virus itself is not toxic to the corneal endothelium, but is immunogenic, inducing lymphocyte recruitment. The observation of marginal prolongation of endothelial cell function in AdTNFR-transfected grafts over both nontransfected and Ad0-transfected corneas suggests the production of TNFR-Ig protein may have some corneal endothelial protective effect in the immunologic environment during corneal allograft rejection.

In this study we investigated a high-risk model of rejection, in which the graft bed was vascularized before surgery. We did not investigate a low-risk setting because, in this model, graft rejection is seen in only approximately 10% of rabbits. In any case, given the success rate of corneal grafting in patients with low risk of rejection, there is no clinical requirement for novel therapy in this setting.

This report highlights some of the toxic and immunogenic effects of virus protein expression, and it is unlikely that the viruses used in this study will be used in the clinical setting of corneal transplantation. In previous work we have not noted any inflammatory response to adenoviral vectors in the rabbit; however, other investigators have seen significant inflammatory responses after intracameral administration of adenovirus. One potential solution is the development of helper-dependent adenoviral vector, in which the viral protein coding sequences are completely eliminated (so-called gutless adenovirus). These have potential to combine the efficiency of adenovirus vectors with reduced cellular immune responses to viral-encoded proteins.

Although increased immunogenicity is the most likely explanation for the reduced graft survival after Ad0 transduction, there was a risk of contamination of the adenovirus used in these experiments by low levels of replication-competent adenovirus, caused by recombination in the 293 cells used to prepare viral stocks. This is unlikely to have occurred to a significant extent, in that we did not see early endothelial damage or loss of epithelial cells due to wild-type viral infection. Furthermore, no significant cytotoxic effect was found with noncomplementing cell lines (data not shown).

The culture conditions used in these experiments are almost identical with those used for maintenance of human corneas before transplantation. The production of functionally active protein indicates that corneal endothelium can be transfected with genes of therapeutic interest during periods of storage, and ex vivo incubation with recombinant virus obviates many of the safety concerns surrounding in vivo administration of viral vectors. The results of the experiments in an in vivo transplantation model demonstrate that adenoviral vectors can deliver genes directly into the anterior chamber of the eye and that expression of these gene products has the potential to produce therapeutic biological effects. Further investigation of less immunogenic vectors for immunomodulatory gene transfer is now in progress.

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**References**


