Local Overexpression of Nerve Growth Factor in Rat Corneal Transplants Improves Allograft Survival

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Abstract

Nerve growth factor (NGF), a member of the neurotrophin family, is a trophic molecule originally identified and characterized by its functions in the nervous system.1,2 In addition to its trophic and top effect on nerve growth and regeneration, NGF has been suggested to play an important role in mediating and regulating the immune response. NGF is synthesized by various cells of the immune system including T cells,3 B cells,4 dendritic cells,5 eosinophils,6 and connective tissue cells such as fibroblasts.7 It has been reported that NGF modulates an immune response from a predominantly Th1 type to a Th2 type.8–10 Moreover, the presence of NGF in the anterior segment of the eye, playing an important role in tissue maintenance and wound healing, has been reported recently.11 High-affinity receptors of NGF are readily expressed on corneal tissues and are able to bind NGF.12 Recently, topical NGF treatment was found to have a profound effect on corneal wound healing while restoring corneal epithelium and improving stromal and endothelial cell function.13,14 All evidence taken together shows that NGF has—in addition to its effects on neuronal cells—pleiotropic effects on immune regulation and wound healing.

With more than 50,000 procedures per year, the cornea is the most commonly transplanted solid tissue.15–18 Immunologic rejection is still the leading cause of corneal allograft failure, especially in high-risk recipients with a history of previous graft rejection, inflammation, or neovascularization. These transplant recipients may in fact represent most patients seen in clinical centers.17,19 To avoid rejection, topical immunosuppressive therapy is routinely performed. However, the results are not satisfying, despite the cornea's location in an immune-privileged environment. In most of these patients, however, the risk of systemic immunosuppression accompanied by severe side effects may not be justified, since they do not have a life-threatening disease. Therefore, less toxic treatment protocols are desirable.

Gene therapy has the potential to modulate the allograft immune response through local expression of therapeutic gene products within the transplanted tissue.20,21 It is known that the corneal endothelium is the main target of an immune response after transplantation,15,22 which should be protected appropriately. We considered that transfer of the NGF gene into the donor corneal endothelium before transplantation may be able to modulate the host's immune response and prevent graft rejection. To test this hypothesis, we used an adenovirus (Ad)-based gene delivery system to over-express NGF, either locally (ex vivo) or systemically (in vivo) in an established rat corneal transplant model.

Herein, we report for the first-time the potential of local NGF gene therapy as a promising approach to prolong corneal allograft survival. Moreover, we show that both anti-inflammatory and antiapoptotic effects after NGF gene transfer contribute to the improved survival of gene-modified corneal transplants and provide further insight into the mechanisms of corneal graft rejection.

Keywords: corneal transplant, NGF, gene therapy, immunomodulation, corneal graft rejection, corneal endothelium.
MATERIALS AND METHODS

Animals

Inbred female rats of Dark Agouti (DA, RT.1A®) and Lewis (RT.1A®) strains weighing 200 to 250 g were obtained from Charles River (Kisslegg, Germany). Lewis rats served as recipients of DA grafts that are major histocompatibility class (MHC) I/II disparate leading to 100% rejection in untreated recipients. All animals were housed in wire-bottomed cages with controlled light-dark cycles, fed a standard laboratory diet, and given free access to tap water. Animals were handled in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, the German guidelines on the use of animals in research (Berliner Senatsverwaltung), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Line

Human corneal endothelial cells (HCECs) as a model for in vitro transduction studies were used in the study.22 The cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2% FCS, 5% glutamine, and 5% penicillin/streptomycin at 37°C and 5% CO₂.

Corneal Transplantation and Definition of Graft Rejection

Orthotopic corneal transplantations were performed as reported previously.23 Briefly, all animals were anesthetized by intramuscular injection of a mixture of ketamine (90 mg/kg, Ketavet; Pharmacia GmbH, Erlangen, Germany) and xylazine (7.5 mg/kg, Rompun 2%; Bayer Vital GmbH, Leverkusen, Germany), diluted in saline, during the surgical procedure. Before surgery 1% atropine sulfate drops (Ciba Vision, Welfing, Germany) were topically applied to dilate the pupil. The recipient and donor right cornea were trephined with a 3.0- or 3.5-mm trephine, respectively, and excised with Vannas scissors. The donor graft was sutured into the recipient bed with running suture (10-0 Mersilene; Ethicon, Hannover, Germany). The suture was not removed. After transplantation, antibiotic ointment (ofloxacin, Floxal; Mann Pharma, Berlin, Germany) was applied immediately to the eye. Animals with surgical complications, such as intraocular hemorrhage or cataract, were excluded. Corneal transparency was analyzed for their mRNA expression profile: proinflammatory cytokines (TNF-α, IFN-γ), anti-inflammatory cytokines (IL-4, IL-10), family of proteins involved in the response to apoptosis (proapoptotic genes Bax and Bad, anti-apoptotic genes Bcl-2, Bcl-xL, and Bag-1, respectively). At day 12 after transplantation, recipients of gene-modified grafts (AdNGF or AdCTLAIg) were killed and grafts and cervical draining lymph nodes were harvested to analyze mRNA expression profiles as described elsewhere.28,29 All samples were normalized regarding their β-actin content. All reactions were performed on a sequence detection system (Prism 7700; Applied Biosystems [ABI], Weiterstadt, Germany).

Ex Vivo and In Vivo Gene Transfer Experiments

For ex vivo gene transfer, HCECs were placed in 24-well plates at a density of 2 × 10⁴/well. Twenty-four hours later, transduction was performed for 30 minutes at room temperature with a virus-to-cell ratio of 1:5. Transduction was confirmed by Western blot analysis and the presence of the recombinant adenovirus AdNGF in the supernatant. For systemic administration of recombinant Ad, 1.0 × 10⁹ pfu of AdNGF or AdCTLA4Ig in 500 µL PBS was injected intraperitoneally into transplant recipients one day before transplantation. An Adβ-Gal construct served as the control vector.

Detection of NGF in Cell Culture Supernatants

NGF protein in culture supernatants was detected by using an ELISA kit specific for NGF, according to the manufacturer’s instructions (R&D Systems, Wiesbaden, Germany). The detection limit was 20 pg/mL.

Detection of CTLA4Ig in the Serum

Sera from blood of animals receiving systemic AdCTLA4Ig gene therapy were analyzed for CTLA4Ig protein content by anti-human IgG ELISA, according to the manufacturer’s instructions (Seramun, Dolgenbrodt, Germany). The detection limit was 15 ng/mL.

Detection of Anti-Ad Antibodies in the Serum

Serum samples from different gene therapy groups were analyzed for the presence of IgG anti-Ad antibodies by specific antigen ELISA, according to the manufacturer’s instructions (Virotech, Rüsselsheim, Germany). Sera were diluted 1:100, and an anti-rat IgG-POD conjugate was used for the specific detection of anti-Ad antibodies. Sera from naïve animals or from animals treated systemically with recombinant Ad expressing other transgenes was used as negative or positive controls, respectively.

Quantitative Real-Time RT-PCR for the Analysis of mRNA Expression Profiles

To investigate immunologic and antiapoptotic changes occurring within the transplants and involved secondary lymphoid tissues in the early postoperative phase, the following parameters were quantified for their mRNA expression profile: proinflammatory cytokines (TNF-α, IFN-γ), anti-inflammatory cytokines (IL-4, IL-10), family of proteins involved in the response to apoptosis (proapoptotic genes Bax and Bad, anti-apoptotic genes Bcl-2, Bcl-xL, and Bag-1, respectively). At day 12 after transplantation, recipients of gene-modified grafts (AdNGF or Adβ-Gal) were killed and grafts and cervical draining lymph nodes were harvested to analyze mRNA expression profiles as described elsewhere.28,29 All samples were normalized regarding their β-actin content. All reactions were performed on a sequence detection system (Prism 7700; Applied Biosystems [ABI], Weiterstadt, Germany).

Analysis of Apoptotic Cell Loss of Corneal Endothelial Cells after Ex Vivo AdNGF Gene Transfer

Corneal allografts after local AdNGF and AdCTLA4Ig gene therapy were harvested and assessed for apoptosis by analyzing DNA-fragmentation (TUNEL-assay; Roche, Mannheim, Germany). Normal DA rat corneas were stained as background, and black nuclear staining as positive apoptosis staining. The staining was analyzed with a light microscope. Slight brown nuclear staining was seen as background, and black nuclear staining as positive apoptosis staining. The same experimental procedure has been performed for the analysis of endothelial cell integrity of corneal transplants after combined CTLA4Ig/NGF gene therapy.

Statistical Analysis

Graft survival was analyzed using the Kaplan-Meier survival method. The log-rank test was used to compare graft survival times at Mantel-Haenszel survival analysis. Because some data were not normally distributed, and equality of variance could not be assumed, nonparametric statistical tests (Mann-Whitney and Kruskal-Wallis) were used to compare mean survival times. P < 0.05 was defined as statistically significant. Quantitative real-time RT-PCR data were analyzed using...
RESULTS

Effect of Local AdNGF Gene Therapy on Corneal Allograft Survival

First, we determined whether corneal cells express NGF after transduction with AdNGF 3 days after gene transfer. NGF was detected in high amounts in culture supernatants of AdNGF-transduced HCECs (655.8 ± 108.3 pg/mL, n = 4; 46.8 ± 7.63 pg/mL in control cells, n = 2) and rat corneas (6881.2 ± 1099.9 pg/mL, n = 4; not detectable in control corneas, n = 2). To investigate the effects of local overexpression of NGF on allogeneic corneal transplant survival, grafts were transduced ex vivo with AdNGF and transplanted into MHC class I/II disparate recipients. During the first week after transplantation, all grafts had slight stromal edema but then recovered and remained clear until rejection. Transplantation of DA corneal grafts to Lewis recipients resulted in a rejection rate of 100% in both control groups receiving either no treatment (mean survival time [MST] 13.1 ± 0.3 days, n = 16) or local Adβ-Gal gene therapy (MST, 13.3 ± 0.3 days, n = 6) without a statistically significant difference (P = 0.71; Fig. 1A). In contrast, graft survival was significantly prolonged after ex vivo AdNGF gene transfer (MST, 16.8 ± 1.4 days, n = 16) compared with both no treatment (P = 0.002) and local Adβ-Gal gene transfer (P = 0.03). Moreover, local (ex vivo) AdNGF gene therapy resulted in the generation of several grafts that survived indefinitely (>70 days), indicating the efficiency of this approach. In contrast, systemic treatment with AdNGF (1.0 × 10⁹ pfu/animal) was less effective (MST, 15.2 ± 1.0 days, n = 5), and no prolonged graft survival was observed, although the difference between systemic Adβ-Gal therapy (MST, 13.2 ± 0.3, n = 6, P = 0.03) was statistically significant (Fig. 1B). The NGF concentration in the serum of AdNGF treated animals on day 7 after transplantation was measured by specific ELISA. Results obtained varied between undetectable levels and 188 pg/mL (n = 5), which was similar to the control.

Effect of Local AdNGF Gene Therapy on Th1/Th2 and Pro-/Antiapoptotic mRNA Expression Pattern in Draining Lymph Nodes and Corneal Allografts at Day 12 after Transplantation

To understand better the graft-protective mechanisms of local AdNGF gene therapy, the mRNA expression profile of pro-/anti-inflammatory (Th1/Th2) cytokines and pro-/antiapoptotic genes in cervical draining lymph nodes and corneal allografts from transplant recipients was analyzed by quantitative RT-PCR. For these experiments, two additional series of transplantations were performed (local AdNGF and local Adβ-Gal gene therapy, n = 4 each). On day 12 after transplantation, recipients of gene-modified grafts were killed, and tissue samples were collected as described previously.29 To investigate changes in the mRNA expression profile after AdNGF gene therapy, the mean values from samples of local Adβ-Gal gene therapy group were set as 1, and

Student’s t-test for mean ± SD, and the expression differences between different groups were calculated.
the mean values from samples of local AdNGF gene therapy group are shown as expression change (x-fold; Fig. 2). Local AdNGF gene therapy led to a reduction of IFN-γ mRNA expression (more than fivefold; Fig. 2A) in the draining lymph node. Moreover, increased IL-4 mRNA expression (>45-fold; Fig. 2B) was observed in samples from corneal grafts. These results indicate that NGF gene therapy is able to modulate the mRNA expression profiles of both Th1 and Th2 cytokines.
Moreover, we explored whether local AdNGF gene therapy protects the allogeneic graft itself through induction and up-regulation of proapoptotic (Bax and Bad) and anti-apoptotic (Bcl-2, Bcl-xl, Bag-1) molecules. We showed that local AdNGF gene therapy upregulates Bag-1 mRNA expression, whereas Bax mRNA is downregulated (Fig. 2C). This strategy resulted in an ~15-fold increase in the Bag-1/Bax ratio and in an ~7-fold increase in the Bcl-2/Bax ratio in corneal grafts compared with the control on day 12 after transplantation.

**Effect of Local AdNGF Gene Therapy on Apoptosis of Corneal Endothelial Cells In Vivo**

Next, we investigated whether local AdNGF gene therapy modulates apoptotic cell loss of corneal endothelial cells after allogeneic transplantation. The number of apoptotic endothelial cells was counted by analyzing DNA fragmentation (TUNEL-assay) in randomly assigned corneal allografts after AdNGF (opacity grade 1) or Adβ-Gal (opacity grade 3 and 4, respectively) gene therapy (Fig. 3, representative micrographs are shown). Untreated corneal specimens from contralateral eyes of graft recipients served as an additional control for the intact endothelial cell layer (Fig. 3A). Almost all endothelial cells were lost and most of the remaining cells were apoptotic (Fig. 3B) in corneal allografts receiving local Adβ-Gal gene therapy. Of note, only a few endothelial cells were lost and only some of the remaining cells were apoptotic (Fig. 3C) in corneal allografts receiving local AdNGF gene therapy. We further calculated the proportion of viable versus apoptotic cells in the remaining cells by vision area (400× magnification).
value was 1:171 in untreated corneas (0.5%), 11:14 in Ad\textsubscript{A/D-H9252-Gal}-treated grafts (78.6%), and 22:95 in AdNGF-treated grafts (23.2%; Fig. 3D).

Effect of Synergistic CTLA4Ig/NGF Gene Therapy on Corneal Allograft Survival and Intragraft mRNA Expression of Markers of Inflammation

To evaluate whether the inhibition of immune responses directed against the gene-modified transplants further improves the survival of allogeneic corneal transplants, we induced systemic immunosuppression by administering a soluble CTLA4Ig molecule known to interfere with the T-cell activation cascade by blocking costimulation\textsuperscript{26,29}. An adenoviral construct expressing CTLA4Ig (1 \times 10^{9} adenoviral particles per recipient) was injected intraperitoneally into graft recipients 1 day before transplantation (day -1). On day 0, corneas were harvested from organ donors, gene modified ex vivo as described earlier, and transplanted into MHC class I/II-disparate recipients. Corneas transduced with a reporter gene (Ad\textsubscript{A/D-H9252-Gal}) served as the control. Synergistic application of CTLA4Ig/NGF (systemic/local) gene therapy (n = 4) lead to a significantly prolonged survival of corneal allografts that was superior to both single local NGF (P = 0.0029) and single systemic CTLA4Ig gene therapy (P = 0.0133)\textsuperscript{29} (n = 6, Fig. 4A). Most of these transplants (6/7) did not show any signs of rejection throughout the whole observation period (70 days). In contrast, transplantation of corneal grafts that had been transduced ex vivo with a reporter gene (β-Gal; n = 4) did not alter the rejection kinetics of the single application of CTLA4Ig gene therapy.

In addition, we found that synergistic application of CTLA4Ig/NGF profoundly reduced signs of inflammation, as indicated by intragraft mRNA expression of CD3, CD25, TNF-α, IFN-γ, IL-4, IL-10 (n = 7 for combined application of CTLA4Ig/NGF) when compared with no treatment, systemic CTLA4Ig and local NGF gene therapy on day 12 after transplantation, respectively (Fig. 4B).

Effect of Synergistic CTLA4Ig/NGF Gene Therapy on Apoptosis of Corneal Endothelial Cells In Vivo

To investigate whether combined application of CTLA4Ig/NGF has any synergistic effect on corneal endothelial cell survival in vivo, corneas were harvested at day 12 after transplantation (n = 4) and stained for apoptotic cell death. Whereas apoptotic cell loss is partly inhibited in AdNGF gene-modified corneas (Fig. 3C), synergistic application of CTLA4Ig/NGF profoundly prevents apoptotic cell loss (Fig. 5B, representative photographs are shown). Moreover, the structure of the endothelium is kept almost intact due to this therapeutic regimen. These data are also confirmed by the clinical scoring of the corneas, as there were three corneas with grade 0 (no signs of rejection)
and 1 cornea with grade 1 (few signs of inflammation/rejection).

**Detection of CTLA4Ig Protein and Anti-Ad Antibodies in the Serum of Animals Receiving Systemic AdCTLA4Ig Treatment**

To confirm that synergistic application of CTLA4Ig/NGF leads to significant expression of the CTLA4Ig protein in the serum, samples were taken at day 7 after transplantation (day 8 after intraperitoneal injection of CTLA4Ig) and analyzed for apoptotic cells by TUNEL-staining. (A) Untreated DA corneas, (B) corneal allografts from synergistic CTLA4Ig/NGF gene therapy. Representative images are shown. Magnification X400.

NGF gene therapy has been shown to have therapeutic effects in various diseases both in preclinical and phase-1 studies. Of note, gene transfer with neurotrophins has also been investigated in models of eye diseases (e.g., photoreceptor or retinal epithelial cell death). However, gene therapy using neurotrophins with the purpose of modulating corneal allograft rejection has not been investigated. The application of neurotrophins in cornea transplantation may be of great importance, however, because it is known that the cornea is one of the most densely innervated tissues in the body. This indicates the need of neurotrophic factors for nerve regeneration (e.g., after cornea transplantation). Recently, it has been demonstrated that the topical application of NGF increases corneal nerve regeneration after photorefractive kerectomy. In our study, in contrast to systemic expression of NGF, local (ex vivo) modulation of cultured corneas improved the outcome of allogeneic grafts. Most genetic modifications to prevent corneal graft rejection require a systemic expression of the therapeutic protein, although few reports on successful prevention of graft rejection using local gene transfer have been published. It has been speculated that the local expression of the therapeutic gene may be limited to a few days, which may be insufficient to prevent rejection. Downregulation of promoter activity (e.g., mediated by soluble factors in the eye), choice of gene therapy vector for transgene delivery, or immune-mediated elimination of transduced cells have been discussed. However, we showed that the local gene therapeutic approach was more successful in the prevention of allograft rejection than systemic expression of NGF (Fig. 1). To identify the mechanisms how NGF improves graft survival, we found that NGF has anti-inflammatory effects on graft-infiltrating and lymph nodes cells and has antiapoptotic effects on corneal endothelial cells. As shown in Figure 2A, the expression of proinflammatory cytokines (e.g., IFN-γ) was downregulated, whereas the mRNA expression of antiapoptotic molecules (Bag-1) was upregulated (Fig. 2C). This conclusion is further supported by our data concerning the mRNA expression profiles of pro-/antiapoptotic proteins in the graft. We found that mRNA transcription of proapoptotic molecules (Bax) was downregulated, whereas the mRNA expression of antiapoptotic molecules (Bag-1) was upregulated (Fig. 2C).

In our attempts to improve the survival rate of corneal grafts expressing NGF, we investigated the potential of synergistic immunosuppressive therapies. Because it is known that alloantigen-specific T cells are the key mediators of allograft rejection, we blocked T-cell activation and proliferation by application of a soluble CTLA4Ig chimeric molecule. This approach has been shown to prevent the rejection of allogeneic grafts effectively, in various models of organ transplantation, including the cornea. A combination of local NGF gene
transfer, together with systemic expression of CTLA4Ig significantly improved the survival of allogeneic corneal grafts (Fig. 4A). This highlights the importance of the immune system in corneal graft rejection. Because of this synergistic approach, the integrity of the endothelial cell layer was almost completely preserved, as it was in untreated contralateral corneas (Fig. 5B). Moreover, the mRNA expression profile of proinflammatory molecules in grafts from animals treated systemically with CTLA4Ig was profoundly reduced in grafts at day 12 after transplantation (Fig. 4B). Of note, CTLA4Ig protein was detected in the serum of treated animals until the end of the observation period (day 42). Whether this is essential to the maintenance of high levels of systemic immunosuppression or it can be reduced or even withdrawn is currently under investigation. A local increase in immunosuppression as mediated by combined gene transfer of NGF and CTLA4Ig was not effective, since coexpression of these molecules did not prolong the survival of allogeneic corneal grafts (data not shown). We do not believe that the application of a single agent (NGF) will be sufficient to achieve long-term survival in animals (and probably in humans as well). Therefore, we investigated supportive anti-inflammatory gene therapy using CTLA4Ig (Fig. 4). Combined application of local NGF and systemic immunosuppression by CTLA4Ig, which is now approved by the FDA for the treatment of rheumatoid arthritis,43 leads to long-term survival. Whether this approach can be used in humans must be carefully evaluated. However, with respect to local NGF treatment, recent studies for the therapy of wound healing after surgery or treatment of ulcers in humans support this strategy.11,44,45

FIGURE 6. Detection of CTLA4Ig protein and anti-Ad antibodies in the serum by specific ELISA. An ELISA specific for human IgG was used to detect CTLA4Ig protein in the serum of rats from different systemic gene therapy groups. Each bar represents an individual animal. (A) No CTLA4Ig was detected in naive animals (n = 4) and graft recipients treated systemically with Adβ-Gal (n = 6). In contrast, CTLA4Ig protein was detected in animals receiving systemic CTLA4Ig gene therapy: systemic AdCTLA4Ig, 1 × 10⁹ pfu/animal (n = 6), three out of six animals29; combined application of AdCTLA4Ig/β-Gal gene therapy (n = 4); and combined application of CTLA4Ig/NGF gene therapy (n = 7; 6/7). (B) Expression of CTLA4Ig protein was detected in animals receiving combined application of CTLA4Ig/NGF gene therapy throughout the whole observation period (until day 42 after transplantation). Moreover, the presence of anti-Ad antibodies in the serum was analyzed by specific ELISA. Each bar represents an individual animal. (C) In naive (untreated) animals, only very low levels of anti-Ad antibodies were detected. High titers (OD > 3.0) were found in Adβ-Gal-treated animals. In contrast, only low titers of anti-Ad antibodies were detectable in all animals receiving systemic CTLA4Ig gene therapy (systemic AdCTLA4Ig, 1 × 10⁹ pfu/animal, n = 6; combined application of CTLA4Ig/β-Gal gene therapy, n = 4; combined application of CTLA4Ig/NGF gene therapy; n = 7). (D) Low levels of anti-Ad antibodies were detected in animals receiving combined application of CTLA4Ig/NGF gene therapy throughout the whole observation period (until day 42 after transplantation).
Adenoviral vectors have been shown to transduce the corneal endothelium efficiently and have been selected in this study as the carrier of the therapeutic gene. However, both cellular and humoral immune responses directed against the transduced tissues or the vector itself may limit the success of the therapy.4,5 Because of our immunosuppressive regimen, the generation of anti-Ad antibodies was dramatically reduced over the whole observation period. Furthermore, markers of T-cell activation were profoundly reduced, indicating that the inhibition of immune-mediated processes is crucial for the long-term acceptance of gene-modified corneal transplants. These data demonstrate that adenoviral vectors could be used as a gene delivery system to prevent corneal graft rejection. However, the application of less immunogenic gene therapy vectors (e.g., adenoassociated viruses or lentiviral vectors expressing NGF) must be carefully evaluated.

In summary, we have shown that NGF gene therapy significantly prolongs the survival of corneal allografts by mediating both anti-inflammatory and antiapoptotic effects. NGF therapy could therefore be an interesting approach for the prevention of corneal graft rejection in humans.

Acknowledgments
The authors thank Sabine Jyrch for technical assistance and thank Adrienne Gorman and Afshin Samali (Department of Biochemistry, National University of Ireland, Galway) for critically reading the manuscript.

References


