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Original Article

Adenovirus-mediated IL-10 transfection induces immune tolerance in corneal transplantation

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Abstract: Aim: This study is to investigate whether adenovirus-mediated IL-10 transfection in donor cornea can induce immune tolerance in corneal transplantation. Methods: Corneal grafts of Wistar rats were randomly divided into control group, Adenovirus-GFP group, and Adenovirus-GFP-IL-10 group. Fluorescence expression of GFP was observed at 3 h, 24 h, 48 h, and 72 h after transfection, and IL-10 level in corneal grafts was tested by Western Blot at 48 h. Then, the corneal grafts were transplanted into SD rats. The status and survival of cornea graft was observed under the slit lamp. The mRNA and protein levels of related cytokines were tested by RT-PCR and ELISA, respectively. Results: GFP fluorescence expression was initially observed at 24 h after adenovirus infection, peaked at 48 h, and then faded gradually at 72 h. IL-10 was detected at high level in corneas infected with Adenovirus-GFP-IL-10 at 48 h. Compared to control or Adenovirus-GFP groups, the survival time of transplanted cornea grafts in Adenovirus-GFP-IL-10 group were significantly prolonged ($P<0.01$). Besides, Adenovirus-GFP-IL-10 group presented mild corneal turbidity and edema and less neovascularization under the slit lamp. Additionally, in corneal grafts of Adenovirus-GFP-IL-10 group, the IFN-γ mRNA in significantly decreased, while IL-10 mRNA and IL-4 mRNA significantly increased ($P<0.01$). Additionally, in aqueous humor of Adenovirus-GFP-IL-10 group, IL-4 protein was significantly increased, while TNF-α protein was significantly reduced ($P<0.01$). Conclusions: The expression of IL-10 in donor cornea could inhibit immune rejection, thus promoting the survival of the transplanted grafts.

Keywords: IL-10, adenovirus, corneal transplantation, immune tolerance

Introduction

In China, corneal disease has become the second cause of blindness [1, 2], and corneal transplantation is the ultimate treatment to refractory corneal disease [3]. However, immune rejection after transplantation is still the main obstacle that results in corneal graft failure [4]. Currently, there are three main methods to prevent of the corneal graft rejection, including the improvement of histocompatibility between donors and recipients, the application of anti-immunity rejection drugs, and gene therapy [5, 6]. However, due to the shortages of donor cornea and serious side effects of long-term immunosuppressant drugs, the application of the first two preventive methods is restricted [7]. Meanwhile, the gene therapy, with better persistence, less drug side effects and higher safety, has become a promising treatment to corneal transplantation [8]. Carrier with high virus propagation titer is considered as the promising gene transfection vehicle [9, 10]. Adenovirus is an ideal vehicle that could carry a large number of exogenous genes into specific cells or tissues, which can provide the ideal environment for accurate translation and protein processing. Therefore, the exogenous proteins constructed into adenovirus vectors are of high expression and efficiency.

Corneal allograft rejection is a process that results from immune response. The antigen presenting cells (APC) identifies the donor's antigen and presents it to the antigen-specific T lymphocytes when the recipient receives a donor cornea. After activation by the presented antigens and the cytokines secreted by APCs, T helper cells then secretes interleukin 2 (IL-2), IFN-γ and other cytokines to induce immune rejection [11]. IL-10, mainly secreted by Th2 lymphocytes and monocytes, is the main cyto-

kine to inhibit antigen-specific T cell proliferation and secretion of pro-inflammatory cytokines, and it also inhibits the occurrence of delayed hypersensitivity in host [12, 13]. IL-10 is a potential negative regulatory factor for lymphocyte proliferation and inflammation, which is beneficial to allogeneic transplantation [12] In vitro, studies have shown that IL-10 gene transfer significantly prolonged the survival time in heart transplantation and other organs [14, 15].

In this study, we transfected the corneal grafts with IL-10 through adenovirus-IL-10 infection, and used these grafts as donors for allogeneic corneal transplantation. The role and mechanism of IL-10 in immune tolerance in corneal transplantation was investigated.

Materials and methods

Animals

Wistar Rats of either gender (n=60, 4-6 wks) were purchased from laboratory animal center of China medical university, and they served as donors. SD Rats of either gender (n=90, 4-6 wks), which served as recipients, were purchased from Liaoning medical laboratory animal center. All mice were kept in standard condition with free access to food and water. The animal experiments were conducted according to the ethical guidelines of Jinzhou Medical University.

Reagents

Adenovirus-GFP and Adenovirus-GFP-IL10 was constructed by Shanghai JiKaiJi chemical technology co., LTD (Shanghai, China). Fetal Bovine Serum was purchased from Hyclone (Logan, UT, USA). RPMI-1640 medium was purchased from GIBCO (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR kit was purchased from Takara (Tokyo, Japan). Primers were synthesized by Takara. TNF-α ELISA kit and IL-4 ELISA kit was purchase from Bio-Swamp (Novusbio, USA).

The culture of corneal graft and transfection of recombinant adenovirus

Mydriasis was performed in Wistar rats under anesthesia in sterile conditions. The corneas were collected under microscope and then were randomly divided into three groups. The control group was cultured in RPMI-1640 medium. The GFP group was cultured in RPMI-1640 medium with recombinant adenovirus containing Adenovirus-GFP. The IL-10 group was cultured in RPMI-1640 medium containing recombinant Adenovirus-GFP-IL-10. At 3 h, 24 h, 48 h, and 72 h after transfection, respectively, the fluorescence of GFP was observed by fluorescence microscope to evaluate transfection efficacy.

Western blot analysis

At 3 hours after transfection, the corneal graft samples of the three groups were lysated and total proteins were extracted. The protein concentration was detected by CBA kit. Then, the proteins were separated by the SDS-PAGE and transferred to PVDF membrane. After blocking, the primary antibody was added and incubated at 4°C overnight. After washing by PBST for 3 times, the secondary antibody was added and incubated at room temperature for 1 h. β-actin was used as an internal control. The membrane was developed by enhanced chemiluminescence plus reagent.

Rat model for penetrating keratoplasty

The SD rats were divided into 3 groups. The right eye was used for corneal transplantation. The planting beds were made by using 3.0 mm diameter corneal trephine. After transfected by adenoviruses for 3 h, the grafts were then sewed in the planting bed. Then the postoperative rats were eye-dropped with tropic amide and chloramphenicol every day. The corneal sutures were not removed. At 12 days after the keratoplasty, the right eye enucleation was performed in 6 rats of each group. The aqueous humor was collected by limbal puncture. The eyes of 3 SD rats in control group (6 eyes) were used as a negative control.

Observation the status of postoperative cornea by slit lamp microscope

The postoperative eyes were observed under the slit lamp microscope every day to check the occurrence of immune rejection. Based on the method of Larkin et al., [6] three indicators of turbidity, edema, and neovascularization were used for scoring standards. The scores of the three indicators were defined as rejection index (RI). Immune rejection was defined when RI ≥5 or turbidity index reached 3. The survival for corneal grafts was recorded at the same time.

Table 1. Primer ssequences.

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'	Product length
IL-4	TGATGGGTCTCAGCCCCCACCTGGC	CTTTCAGTGTTGTGAGCGTGGACTC	378 bp
IL-10	ACTGCTATGTTGCCTGCTCTTACT	GAATTCAAATGCTCCTTGATTTCT	318 bp
INF-γ	ACACTCATTGAAAGCCTAGAAAGTCTG	ATTCTTCTTATTGGCACACTCTCTACC	432 bp
β-actin	TCCTCCTGAGCGCAAGTACTC	GCTCAGTAACAGTCCGCCTAGAA	150 bp

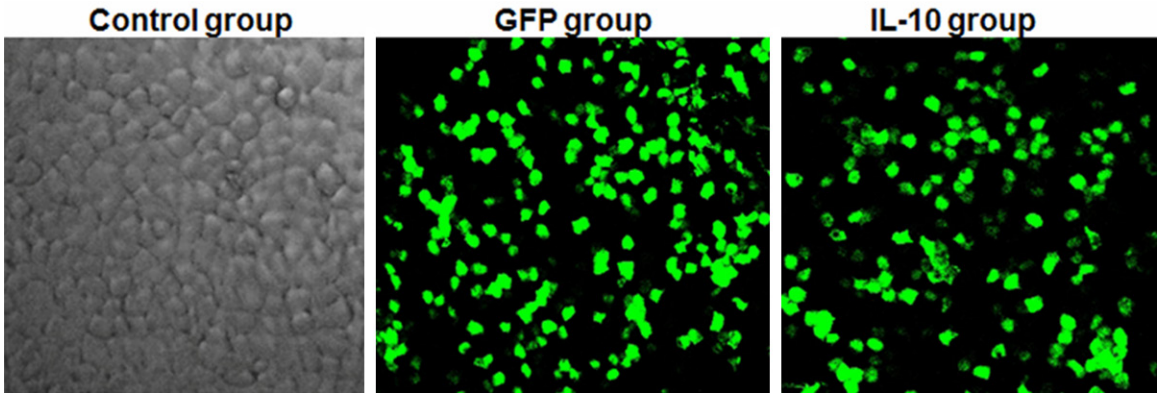

Figure 1. Observation of GFP fluorescence at 48 h after infection. Corneal grafts were uninfected (control group), or infected with GFP-Adenovirus and Adenovirus-GFP-IL-10. Representative images at 48 hrs after transfection were shown. Magnification: ×200.

The survival time was defined as the time period from corneal transplantation to rejection.

Semi-quantitative PCR

Total RNAs were extracted by Trizol reagent. Then the RNA was reverse transcribed into cDNA. The samples were analyzed by using semi-quantitative PCR (semi-qPCR) with the following specific primers shown in **Table 1**. Real-time PCR was performed using the SYBR Premix Ex Taq kit (Takara, Japan) with an ABI Fast 7500 Real-Time PCR System.

ELISA

The levels of IL-4 and TNF-α were measured by ELISA kits. Briefly, aqueous humor was collected 12 days after the operation. IL-4 and TNF-α were measured as the Manufacturer instructions. OD450 was recorded by spectrophotometry. The corresponding concentrations of cytokines in different samples were calculated by standard curves.

Statistical analysis

SPSS19.0 statistical analysis software was applied for data processing. Data were expressed as mean ± SD. The results of each group were compared using one-way ANOVA and LSD test. $P<0.05$ was considered statistically significant.

Results

The infection efficiency of adenovirus in corneal grafts

The green fluorescence in infected corneas was observed under microscope to evaluate the virus infection efficacy. Within 3 h, no obvious florescence was detected in all of the three groups. After 24 h, a small amount of fluorescent was expressed in the Adenovirus-GFP and Adenovirus-GFP-IL-10 groups, and the fluorescence reached the peak at 48 hours (**Figure 1**). Then, the fluorescence gradually subsided at 72 h. Thus, the corneal endothelial cells were successfully infected with Adenovirus-GFP and Adenovirus-GFP-IL-10.

The IL-10 level in corneal grafts

Western Blotting was performed to detect the IL-10 levels in corneal grafts. Obvious IL-10 expression was observed in the Adenovirus-GFP-IL-10 group at 48 h (**Figure 2A**). The relative

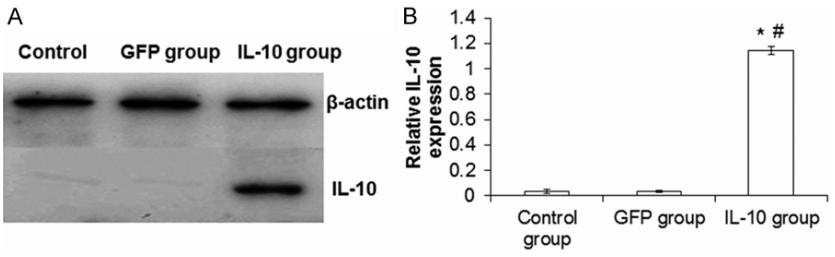

Figure 2. Analysis of IL-10 expression in corneal graft. Corneal grafts were uninfected (control group), or infected with Adenovirus-GFP and Adenovirus-GFP-IL-10. Three hours after infection, IL-10 expression in corneal graft was detected by Western blot. Representative results and quantitative results were shown on the left and right, respectively. *Compare with control group, $P<0.01$; #Compare to GFP group, $P<0.01$.

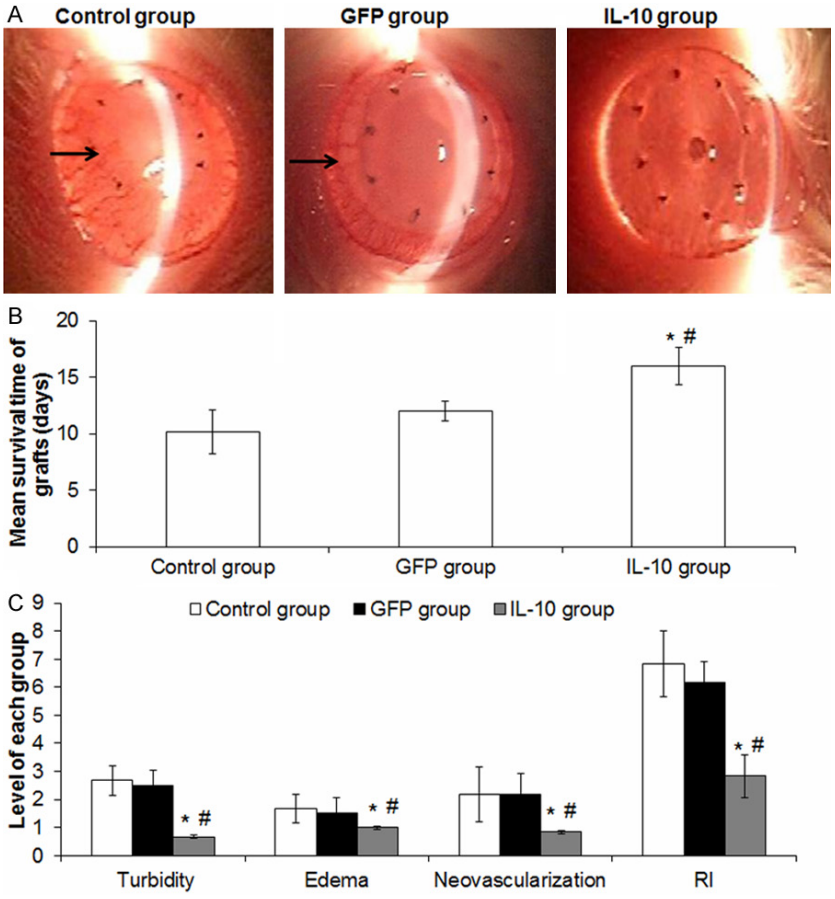

Figure 3. Observation of transplanted corneal graft. SD rats were used as recipients and corneal grafts from control group, Adenovirus-GFP group and Adenovirus-GFP-IL-10 group were transplanted. At day 12 after transplantation, the corneas were observed under slit lamp. A. Representative images were shown. Arrows indicate neovascularization. B. Survival of corneal grafts in each group. *Compare with control group, $P<0.01$; #Compare to GFP group, $P<0.01$. C. The analysis of turbidity, endema, neovascularization, and rejection index (RI) of each group. *Compare with control group, $P<0.01$; #Compare to GFP group, $P<0.01$.

expression of IL-10 was evaluated by gray value ratio. The result showed that IL-10 was highly expressed in the cornea after Adenovirus-GFP-IL-10 infection for 48 h ($P<0.05$) (**Figure 2B**).

The growth and survival of corneal graft

The growth and survival of corneal graft in each group was evaluated. At 12 day after transplantation, the corneal was turbid in control group and Adenovirus-GFP infected group (**Figure 3A**). Edema and neovascularization were also observed under the slit lamp. However, the corneal was transparent in Adenovirus-GFP-IL-10 infected group (**Figure 3A**). Besides, no obvious edema or neovascularization was observed in Adenovirus-GFP-IL-10 group. Compared with control group (10.17± 1.94 days) or Adenovirus-GFP group (12.00± 0.89 days), the survival period for corneal graft in Adenovirus-GFP-IL-10 group (16.00±1.6 days) was significantly longer ($P<0.05$) (**Figure 3B**). Furthermore, the turbidity, edema, neovascularization, and RI of each group were evaluated (**Figure 3C**), which showed that the Adenovirus-GFP-IL-10 group had significantly lower levels of turbidity, edema, neovascularization, and RI than control group and Adenovirus-GFP group ($P<0.05$). These results suggest that IL-10 could induce transplantation tolerance and prolong the survival period for corneal grafts.

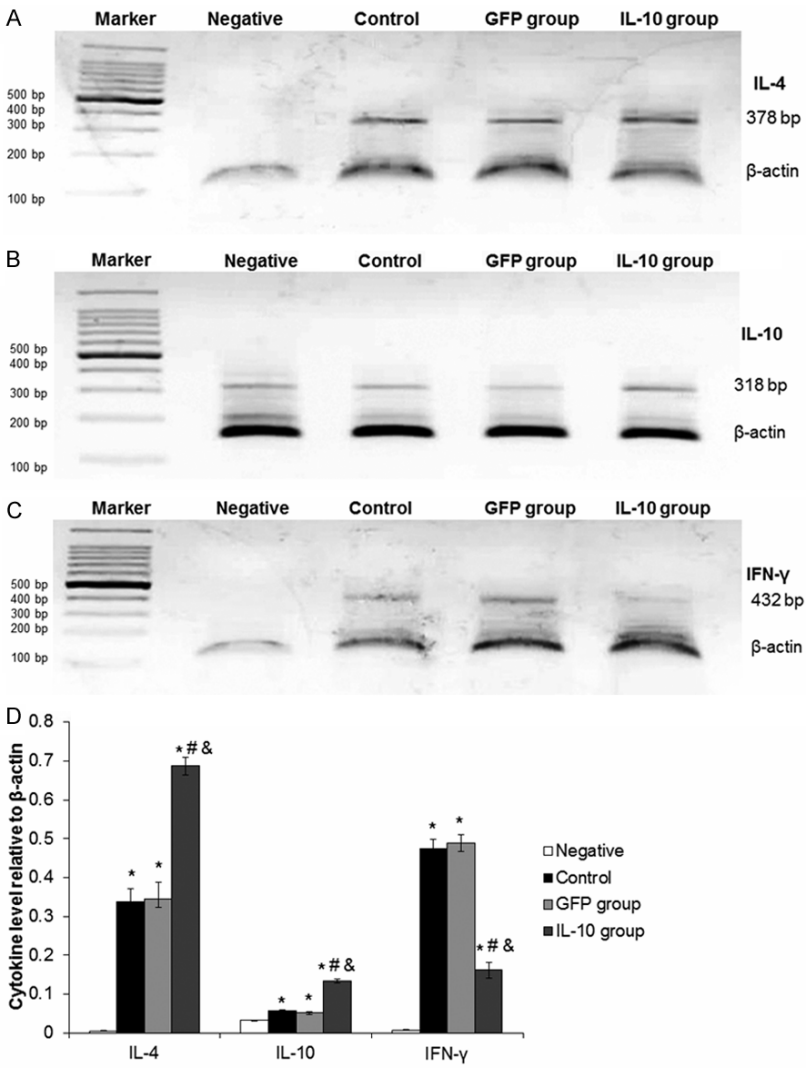

Figure 4. Detection of IL-4, IL-10 and IFN-γ in corneal graft. Levels of IL-4, IL-10 and TNF-α in corneal graft were detected with RT-PCR. A. IL-4. B. IL-10. C. IFN-γ. D. Cytokines level in each group at day 12 postoperation. *Compare with negative control, *P<0.05*; #Compare with control group, *P*<0.01; &Compare with GFP group, *P<0.01*.

The IL-4, IL-10 and IFN-γ mRNA level in postoperative eyes

To detect the level of IL-4, IL-10 and IFN-γ mRNA level in postoperative eyes, semi-quantitative PCR was performed. The representative electrophoresis results for target genes were shown in **Figure 4A-C**. As shown in **Figure 4D**, in negative control rats, the expression of IL-4, IL-10 and IFN-γ in the cornea of was low. In contrast to the control group, IL-4 and IL-10 levels in Adenovirus-GFP-IL-10 group were significantly higher (P<0.05). However, IFN-γ was significantly lower (P<0.05). These results suggest that both Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4 and IL-10) are involved in immune rejection.

IL-4 and TNF-α protein level in aqueous humor

The IL-4 and TNF-α protein levels in aqueous humor were tested by ELISA. As shown in **Figure 5**, IL-4 protein level in aqueous humor was significantly higher in Adenovirus-GFP-IL-10 group, compared with control group and Adenovirus-GFP group (P< 0.05). However, the TNF-α protein level in aqueous humor of Adenovirus-GFP-IL-10 group was significantly lower than control group and Adenovirus-GFP group (P<0.05) (**Figure 5**). These results indicate that IL-10 may regulate the level of IL-4 and TNF-α to inhibit corneal graft rejection.

Discussion

Qian et al. [16] used adenovirus-mediated gene transfer in cornea for mouse corneal transplant, and the green fluorescence in corneal endothelial cells could last for 12 weeks. Douglas G et al. [17] used adenovirus and lentivirus carrying IL-10 to infect sheep cornea, and the results suggested that the transfection efficiency of adenovirus was significantly higher than lentivirus vectors. In this study, we used adenovirus as a vector to mediate IL-10 transfection into cornea *in vitro*. The results showed that visible fluorescence was detected at 24 h after infection, and peaked at 48 h, then the fluorescent gradually reduced at 72 h after infection. At the same time, we detected IL-10 in transfected corneal grafts by Western Blot. IL-10 level increased in Adenovirus-GFP-IL-10 group. These results indi-

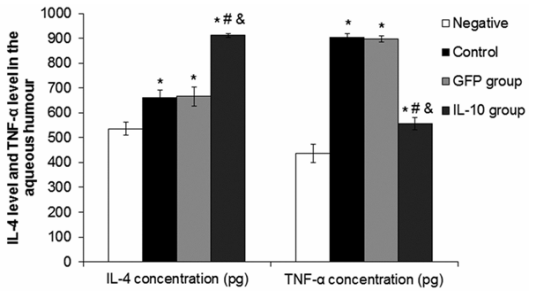

Figure 5. IL-4 level and TNF-α level in the aqueous humour of each group. Levels of IL-4 and TNF-α were detected by ELISA. *Compare with negative control group, $P<0.01$; #Compare with control group, $P<0.01$; &Compare with GFP group, $P<0.01$.

cated that Adenovirus-GFP-IL-10 successfully infected the corneal endothelial cells *in vitro*.

Whether the application of IL-10 gene transfection in cornea could promote the graft survival in corneal transplant is still controversial. Gong et al. [18] showed that topical application of IL-10 did not prolong corneal graft survival time, in contrast, systemic application of IL-10 significantly prolong corneal graft survival time. However, Douglas G et al. [17] found that the survival for corneal graft infected by IL-10 gene in lentivirus was significantly longer, suggesting that topical application of IL-10 gene transfection played an important role in suppressing immune rejection and in inducing immune tolerance. In the present study, we found that topical application of IL-10 prolonged the survival of corneal grafts. Besides, IL-10 alleviated the turbidity, edema and neovascularization of transplanted corneas. Our results were consistent with those by Douglas G et al., suggesting that local IL-10 gene transfection could inhibit graft rejection.

By inhibiting secretion of IL-2 and TNF-α, IL-10 inhibits the body's delayed hypersensitivity thereby [19]. Besides, IL-10 could affect NK cell activity by inhibiting IFN-γ production. Such biological characteristics indicate that IL-10 is beneficial for allogeneic transplant. IFN-γ plays an important role in immune reactions. IFN-γ improves the ability of APCs to amplify the function of IL-2 by inducing helper T cells to express IL-2 receptor. On the other hand, IFN-γ inhibits Th2 cells to secrete Th2 inhibitory factors (mainly IL-4, IL-10, etc.), which induce immune tolerance [20, 21]. IL-4 plays an important role in the induction of immune tolerance, mainly through promoting Th2 cell differentiation, inhibiting proliferation of Th1 cells, and reducing Th1 cells to secrete IL-2 and IFN-γ [22, 23]. TNF-α is co-secreted by both Th1 and Th2 cells, which is a positive regulate factor for inflammatory immune response [24]. Studies have shown that TNF-α binding to specific receptors after the allogeneic keratoplasty induced positive feedback on helper T cells to promote monocyte-macrophage cells and Langerhans cells to invade the cornea [25, 26]. TNF-α also induces the production of interleukin by corneal epithelial and stromal cells, and, increases the expression of MHC I and MHC II antigens in corneal endothelial cells and stromal cells, thereby inducing the immune responses [27, 28]. Rayner et al. [29] found higher TNF-α mRNA and biologically active TNF-α proteins in aqueous humor after corneal transplantation in rabbits, indicating that TNF-α plays a role in the induction of immune rejection. In order to investigate the mechanism underlying the inhibitory effect of Adenovirus-GFP-IL-10 on immune rejection, the levels of IFN-γ, IL-4, IL-10, and TNF-α at 12 days after transplantation were detected in this study. The results showed that after IL-10 transfection, the expression levels of IFN-γ and TNF-α were significantly reduced, while the expression levels of IL-4 and IL-10 were significantly increased. These results indicate that Adenovirus-GFP-IL-10 transfection may induce increased levels of IL-4 and IL-10 and decreased levels of IFN-γ and TNF-α to induce immune tolerance and inhibit corneal rejection.

In summary, adenovirus mediated transfection of IL-10 into corneal graft prolonged the survival time for corneal graft, indicating that local IL-10 application inhibits immune rejection and induces immune tolerance. Our findings may provide a theoretical basis and experimental evidence for gene therapy of corneal graft transplantation by IL-10. However, the graft had no long-term survival after IL-10 transfection. This may be due to the complex immune responses involved in corneal allograft rejection.

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Disclosure of conflict of interest

None.

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