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Effect of recombinant adeno-associated virus mediated transforming growth factor-beta1 on corneal allograft survival after high-risk penetrating keratoplasty

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ABSTRACT

Corneal transplantation is one of the most common and successful transplant surgeries performed around the world. However, the high-risk corneal transplantation remains a high level of corneal graft failure. Gene transfer of immunomodulatory molecules is considered as one potential strategy in preventing allograft rejection. It is worthy evaluating the effects of the immunemodulating agent on corneal allograft rejection. The purpose of this paper is to investigate the effects and mechanisms of recombinant adeno-associated virus mediated transforming growth factor-beta1 (rAAV-TGF-beta1) on corneal allograft survival using a high-risk rat model after penetrating keratoplasty (PKP). The mean survival time (MST) of corneal grafts was observed and immuno-histochemical staining of TGF-beta1 and Ox-62 was performed in the study. The MST showed significant prolongation in the rAAV-TGF-beta1 group compared to the allograft group. The rejection index (RI) at day 10 revealed was significantly greater in the allograft group than that of the other two groups. Besides the increase of TGF-beta1, the expression of Ox-62 decreasing in rAAV-TGF-beta1 transplanted recipients was detected after transplantation. In short, treatment with rAAV-TGF-beta1 prolongs corneal allograft survival and inhibits the Ox-62 expression in grafts after high-risk PKP.

1. Introduction

Penetrating keratoplasty (PKP) is the most commonly performed method of corneal transplantation, and has developed approximate 80% graft survival rate [1,2]. However, immunologic rejection is still the major cause of corneal graft failure after PKP, especially in cases of high-risk recipients with a previous graft rejection, inflammation or neovascularization [3–5]. Although topical and systemic immuno-suppressive drugs have been used routinely to suppress the immune response of corneal grafts after corneal transplantation, the effectiveness is limited [6–8]. Therefore, it is necessary to find an alternative treatment or new immunosuppressive option against corneal allograft rejection.

Through local expression of therapeutic gene products in experimental transplantation, gene transfer of the immunomodulatory genes has already proved to be a potential approach to modulate the immune response [9]. It has been widely used as a strategy to prolong corneal graft survival that, immunomodulatory genes might be transfected to donor corneal endothelium prior to transplantation, resulting from the characteristics of corneal endothelium [10–12].

Since adenovirus was firstly used as viral vector for gene transfer to corneal endothelium, which has become the most common vectors in gene therapy clinical trials based on its high transduction efficiency [13]. Furthermore, studies have shown adenoviral vectors were able to transduce efficiently corneal endothelium of animals [14,15], as well as human endothelium ex vivo [16]. Our previous study indicated recombinant adeno-associated virus (rAAV) was a valuable adenovirus vector for gene transfer [17,18].

Transforming growth factor-beta1 (TGF-beta1) is a multifunctional cytokine that regulates cell growth, adhesion, and differentiation in a wide variety of cell types, including a pleiotropic effect in immune responses [19,20]. Via controlling the growth and differentiation of T cells, B cells, and dendritic cells, which are the mostly seen antigen-presenting cells (APC) associated with graft rejection, TGF-beta1 regulates immune response [19]. Therefore, there might be a potential alternative to prevent or reduce corneal graft rejection by previous transforming to corneal endothelium recurring to the immunomodulatory function of TGF-beta1.

In present study, we purpose to target TGF-beta1 gene in the corneal endothelium using adenoviral vector of rAAV prior to corneal transplantation, and evaluate its effect on modulating immune response and preventing or delaying corneal graft rejection using the high-risk rat model.

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2. Material and methods

2.1. Animals

Sixty two Sprague–Dawley (SD) and twenty Wistar male rats, weighing between 180 and 220 g, 8–`10 weeks age, were purchased from the Wuhan University Animal Laboratories, China. All animals in the study were handled in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. RAAV-TGF-beta1 vector construction and production

Rat TGF-beta1 gene was cloned from polymerase chain reaction (PCR) using the rat plasminogen cDNA as previously described [21]. PCR amplification was performed with two primers as follows: forward, 5 -GCCGAATTCATGCCGCCCTCGGGGCT GC-3 ; reverse, 5 -GCCGTCGACT CAGCTGCACTTGCAGGAG-3 (Pubmed, GenBank, NM-021578, http://www.ncbi.nlm.nih.gov/nuccore/NM-021578.2). The PCR products of vector plasmid (pSNAV2.0-IRES-EGFP-LacZa) and rat TGF-beta1 gene were cut by restriction enzyme EcoRI and SalI, and then the rat TGF-beta1 gene DNA fragment (1191-bp) was inserted into vector plasmid to construct pSNAV2.0-TGFβ1-IRES-EGFP [22]. RAAV-TGF-beta1 was produced by a three plasmid cotransfection system, whose titer (1.0×10^{12} vg ml^{-1}) were determined by dot blot hybridization.

2.3. Alkali-induced corneal neovascularization

Corneal neovascularization (CNV) model was induced all in right eyes of forty-six SD rats (recipient). In brief, SD rats were anesthetized with an intramuscular injection of 25 mg/kg of ketamine hydrochloride. Alkali injuries to the rat eyes were induced by exposure to a 3 mm diameter disk of filter paper soaked in 1 N NaOH for 30 s, followed by being rinsed with 20 ml of sterile saline. Injured corneas were then evaluated daily by slit lamp bio-microscopy. 14 days after alkali injury, forty SD rats (about three quarters of all SD rats) were selected following experiments, whose superficial vessels had grown into cornea at least 2 mm into the cornea.

2.4. Corneal transplantation

The animals were divided into groups as follows: rAAV-TGF-beta1 group (n = 20), allograft group (n = 20) and isograft group (n = 16). SD rats with CNV were served as recipient from Wistar (n = 20) rats in the rAAV-TGF-beta1 group and allograft group. Donor corneas supported by Wistar rats, were excised using a 3.5 mm diameter trephine and curved with vannas scissors. Anesthesia was performed as described previously. For transfection of corneal grafts, rAAV-TGF-beta1 stock solution was diluted to a concentration of 1.0×10^{10} vg ml^{-1} in DMEM/F12 medium with 2% fetal calf serum (FCS) in rAAV-TGF-beta1 group, whereas corneal grafts were immersed in DMEM/F12 medium with 2% FCS in allograft group, both of which were placed in carbon dioxide incubator, at 37 °C for 30 min.

The surgical procedure used was based upon the rat orthotopic keratoplasty model of Williams and Coster [23]. The recipient bed was prepared by 3.25 mm diameter trephine in the similar way. The donor corneas were immediately applied to the bed and attached with eight interrupted sutures (10-0 nylon sutures, Mani, Tochigi, Japan). To protect the transplant, blepharorrhaphy was performed by means of two interrupted sutures (6-0 nylon sutures), which remained in place for 48 h, and antibiotic ointment (Ofloxacin, Floxal TM, Mann Pharma, Berlin, Germany) was applied in the palpebral fissure. All grafted eyes were opened for clinical evaluation after 48 h and transplant sutures were removed carefully after 7 days. Animals with surgical complications such as intraocular hemorrihage or cataract were excluded.

2.5. Definition of graft rejection

Corneal transparency as an indicator of corneal endothelial function and graft rejection was evaluated daily. The criteria for the allograft scoring was as follows (Table. 1) [24]. The evaluated value of which was recorded as rejection index (RI) and rejection was considered only if RI ≥ 5, or graft opacity reaches 3. Sixteen rats were evaluated by mean survival time (MST) followed up to 28 days in rAAV-TGF-beta1 and allograft groups, respectively. Ten rats were included in isograft group. The animals were discarded when infection occurred in the cornea, and follow up stopped when graft failure occurs. The transparency of corneal grafts was always assessed by the same observer.

2.6. Immunohistochemistry

At day 7, 14 after transplantation, the corneas of three rats per group were taken for immunohistological analysis. The corneas were enucleated, fixed with formalin solution and imbedded in paraffin, then cut into 4 μm sections for immunohistochemical staining. The primary antibodies of rabbit anti TGF-beta1 polyclonal antibody (Boster, Wuhan, China) and mouse anti rat Ox-62 monoclonal antibody (Serotec, Oxford, UK) were incubated overnight at 4 °C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min before biotinylated anti-rabbit and anti-mouse were applied as secondary antibodies and avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories, USA) served as the third reagent. Negative control sections were incubated in the absence of the primary antibody. The medial area of corneal section was obtained and evaluated by HMIAS-2000 high definition color medical image analysis software, on the expression of TGF-beta1 and OX-62 in each group.

2.7. Statistical methods

SPSS 13.0 statistical package was used for statistical analysis. Corneal graft survival of recipients was compared using Kaplane–Meier survival curve and the clinical scoring data were compared among various groups by means of one-way ANOVA. All data were expressed as mean ± standard deviation (mean ± SD). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Corneal graft survival

During the first week after transplantation, slight stromal edema was observed in all grafts, then disapeared until rejection in isograft and rAAV-TGF-beta1 groups. However, in allograft group, the new vessels began to migrate into the grafts and the rejection line

Table 1
Clinical Scoring Scheme for the index of corneal Graft Rejection [24].

Type/Score	Clinical finding
Graft opacity	
0	No opacity
1	Slight opacity, details of iris clearly visible
2	Some details of iris no longer visible
3	Marked opacity, pupil still recognizable
4	Total opacity
Graft edema	
0	No edema
1	Moderate edema
2	Diffuse marked edema with thickening
Graft neovascularization	
0	No vessels
1	Vessels appearing in the corneal bed (25%)
2	Vessels appearing in the graft periphery (50%)
3	Vessels extending deeper (75%)
4	Vessels extending to the center