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Research paper

Effect of lentivirus-mediated gene silencing, targeting toll-like receptor 2, on corneal allograft transplantation in rats

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ABSTRACT

Aim: The present work aims to assess the effectiveness of lentiviral vector (LV) mediated Toll-like receptor 2 (TLR2) gene silencing in the survival of transplanted corneal allografts, against immune rejection, in rats.

Methods: LV mediated TLR2 small interference RNA (SiRNA) with enhanced green fluorescent protein (eGFP) [LV-TLR2-siRNA-eGFP] was realised and transfected to both rat corneal epithelial (EC) and stromal cells (SC). Multiplicity of infection (MOI) was optimized for transfection efficiency using flow cytometric (FCM) analysis. Viability of transfected cells and the success rate of TLR2 gene silencing were respectively determined by CCK-8 assay and western blot assay. The in-vivo experiments were subjected to a penetrating keratoplasty (PK) performed between host Sprague Dawley (SD) and donor Wistar/SD rats, randomly dividing them into 4 groups including the allograft, isograft, allograft treated with LV-eGFP (LV blank control) and allograft treated with LV-TLR2-siRNA-eGFP (LV treated group). The rejection index (RI) was then recorded under a slit lamp, every day following surgery. Expression of the TLR2 and Myeloid differentiation primary response gene 88 (MyD88) were detected by using immunohistochemistry on day 9 post-surgery, whereas grafts's TLR2 and MyD88 mRNA were determined on day 5, 9, and 14 post-surgery performing RT-PCR and, normal rat corneas were included as additional controls.

Results: Transfected cells showed the strongest eGFP expression when MOI was 200 with an efficiency of 77.5% for EC and 76.3% for SC. CCK-8 assay, as measured at 72 and 96 h post transfection, showed no significant changes in the cell viability (both EC and SC) between the transfected and the control group ($p > 0.05$, $p > 0.05$). Western blot results demonstrated a successful down regulation of TLR2 expression by LV-TLR2-SiRNA-eGFP, in both EC and SC. In vivo, compared to allograft group, LV treated group demonstrated less edema, opacity and neovascularization. Immunohistochemical analysis revealed a lower expression of TLR2 and MyD88 in isograft and LV treated group as compared to allograft group. TLR2 and MyD88 mRNA were detected in all grafts, and increased over time. With its highest expression in allograft group (peak on day 9), the mRNA expression for TLR2 and MyD88 showed a significant difference amongst the groups, on both day 9 and 14 ($p < 0.001$).

Conclusions: LV mediated TLR2 siRNA could effectively down regulate the TLR2 expression via RNA interference and prolong the survival of corneal grafts, although not necessarily able to prevent the rejection.

1. Introduction

Penetrating keratoplasty (PK) is a standard corneal transplantation surgery for treating irreversible corneal diseases (Coster et al., 2014; Medsinge et al., 2014). Although PK holds the highest success rate among all categories of organ transplantations, immune rejection is still a primary threat to the survival of the transplanted corneal grafts (Niederkorn, 2013; Perez et al., 2013; Niederkorn and Larkin, 2010). Corneal transplantation is a complicated procedure initiated by local tissue injury associated with tissue harvest and implantation which could lead to a cascade release of potential Toll-like receptor (TLR) ligands (Goldstein, 2006; Daniel et al., 2014; Martins et al., 2014; Mastoridis et al., 2015). As acknowledged, TLRs are considered to be a "bridge" linking the innate and adaptive immunity (Goldstein et al., 2003). Among all the TLRs, TLR2 is found to be most widely expressed and capable of recognizing most varieties of pathogens (Borrello et al., 2011). Its action consist in adjusting immune reaction by activation the signal adaptor protein MyD88 (Thakur et al., 2015).

Researchers have discovered that TLR2, also plays an important part in the immune rejection of transplanted organs (Alegre et al., 2008). In

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our previous studies, we have observed an increased expression of TLR2 along with its downstream adaptor MyD88 during corneal graft rejection after PK, which decreased after glucocorticoid (Tobradex) and TLR2 mAb treatment, suggesting that TLR2/MyD88 pathway might play an essential role in the immune rejection of corneal grafts post transplantation (Bai et al., 2011; Zheng et al., 2015). As a follow up to this finding, in this present study, we blocked the TLR2/MyD88 pathway, using gene silencing technique, in order to observe its influence on the survival of corneal grafts which further strengthens our understanding of its critical effects.

RNA interference (RNAi) is a post-transcriptional regulatory pathway that specifically results in silencing target gene and thus its functions (Presloid and Novella, 2015). Specific siRNA or short hairpin RNA (shRNA) for a target gene, carried by LV, could effectively suppress the gene expression either by degrading mRNA or blocking mRNA translation. According to previous studies, ocular tissues and cells, both in vivo and ex vivo could be effectively transfected by using Lentiviral vector (Balaggan and Ali, 2012). Because of its creditable abilities like high expression, consistent targeting of various post-mitotic ocular cells and few occurrence of associated intraocular inflammation, LV could efficiently mediate stable intraocular gene transfer. In this study, we proceed to the fabrication of a specific LV, carrying TLR2 siRNA with the aim to investigate the efficiency of LV in the down regulation of TLR2/MyD88 expression and its effect on immune rejection of transplanted corneal allografts in rat, and were able to successfully transfected rat corneas.

2. Materials and methods

2.1. Fabricating lentiviral vector; encoded with rat toll-like receptor 2 gene

Rat TLR2 gene was cloned by means of PCR, using the rat cDNA sequence (CTAAGAGATACTAACTTGG). PCR amplification was performed with two primers: forward, 5-CCGGAATTCATGAATGCTGCGGCAGAAGCC-3 and reverse, 5- CGCGGATCCCTAGTGAGGCGCCACATCCTGCT-3. The PCR products of vector plasmid (pLVX-eGFP-IRES-Neo) and rat TLR2 gene were cleaved by restriction enzyme EcoRI and BamHI. The rat TLR2 gene DNA fragment was inserted into vector plasmid to construct pLVX-eGFP-TLR2-IRES. LV-TLR2 was synthesized by a three plasmid co-transfection system. The titer of the vector (1×10^9 TU/ml) was determined by dilution counting method. 293T cells were infected with 50 μl of filtered lentivirus supernatant, in each well of a 24-well plate, in the presence of 5 μg/ml polybrene (GenePharma, Suzhou, China).

2.2. MOI optimization and transfection efficiency detection

Corneal tissues were detached from SD rats, post sacrifice, and cut into small pieces. EC and SC were isolated and cultured separately, allowing them to adhere to the culture dishes and were later divided into two groups/cell: EC-control and EC-LV, SC-control and SC-LV. While normal growth was allowed to the cells in the control group, the EC-LV and SC-LV groups were transfected with LV–TLR2-eGFP-siRNA at 4 different multiplicity of infection (MOI): 10, 50, 100 and 200.

Cells were then observed 72 h post transfection under a phase-contrast fluorescence microscope to evaluate eGFP expression followed by a cytofluorometric analysis of eGFP positive cell suspension, in order to determine the transfection efficiency.

2.3. CCK-8 assay

EC and SC were both divided into 3 culture groups; normal cells without any treatment, LV-blank transfected cells, LV-TLR2-siRNA transfected group with MOI 200. Viability of cells, seeded at 5×10^3 cells/well in 96-well plate, were detected using a CCK-8 assay just before transfection, considered as 0 h, and at 72 and 96 h, post transfection. In brief, cells were seeded into 96-well plates at density of 5×10^3 cells/well and cultured. 10 μl of CCK-8 dye was added to each group at specified time points, followed by incubation at 37 °C for about 2 h. A plate-reader was used to determine the post incubation absorbance, which was measured at 490 nm. All assays were performed in triplicates.

2.4. Western blot analysis

Three different culture groups were established in each type of cell. Group1: normal cells − with no intervention; group 2: LV-blank − cells transfected with blank LV; group3: LV-TLR2-siRNA − cells transfected with LV-TLR2-siRNA. Proteins (50 mg) extracted from the cells from each group, were subjected to electrophoresis on a 12% SDS–PAGE and then transferred onto PVDF membranes. After blocking with 5% non-fat milk, for 1 h, the membranes were washed 3 times with TBST, for 5 min each, followed by overnight incubation with polyclonal antibodies, at 4 °C, against TLR2 and GAPDH (1:1000 dilution in 5% non-fat milk, Boster, Wuhan, China), diluted in TBST. GAPDH served as the control. After washing 3 times in TBST, membranes were incubated with secondary HRP conjugated anti-mouse IgG antibody (Boster, Wuhan, China) for 1 h. Post incubation, the membranes were rinsed 3 times with TBST and 1 time in TBS, for 5 min each, before visualizing the blots under enhanced chemiluminescence reagent (Millipore, USA). Experiment was repeated 3 times. The results were quantified by capturing the exposed X-ray film image.

2.5. Animals and anesthesia

Sprague Dawley (SD) and Wistar rats (weighing 180–220 g) were obtained from the Experimental Animal Center, Southern Medical University. SD rats served as hosts, accepting corneal grafts from either SD rats (isograft) or Wistar rats (allograft). SD rats without any intervention served as normal control.

All the animals were anesthetized by intraperitoneal injection of 3% pentobarbital (1.5 ml/kg), prior to any procedures. All experimental procedures were conformed to the ARVO Statement for the “Use of Animals in Ophthalmic and Vision Research” and were approved by the Medical Ethics Committee of Southern Medical University.

2.6. Animal surgery and post-surgery follow up

Three experimental groups were included: isograft group, allograft group (graft treated with PBS), LV-blank control (allograft treated with LV-eGFP) and LV treated group (grafts transfected with LV-TLR2-siRNA). 20 μl of LV-eGFP/LV-TLR2-siRNA was injected to the conjunctival sac after surgery using a hammilton microsyringe. Penetrating orthotopic keratoplasty was performed, unilaterally on the right eye, as described in our previous study (Bai et al., 2011). Briefly, a 3.5-mm of the cornea was excised from its central zone from the donor, using Vannas scissors and sutured on to the host graft bed (3.0-mm diameter), with 8–10 interrupted 10-0 nylon sutures. Antibiotic ointment was applied to the corneal surface before suturing the eyelid.

All postoperative grafts were examined by slit-lamp microscopy, every other day, to determine the rejection index (RI) based on opacity, edema and neovascularization. As per Holland criteria, the grafts showing a score RI ≥ 5 were acknowledged as rejected, as previously explained (Balaggan and Ali, 2012). 8 specimens, from each experimental group, were evaluated for mean survival time (MST) by following them up to the day they were declared failure/rejected. All animals displaying signs of hemorrhage, synechia or cataract, during or after the surgery, were excluded from the study and were relatively replaced complemented in time.

2.7. Immunohistochemistry

On the 9th postoperative day, operated eyeballs from animals of all