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Original Article

Inhibitory effect of adenovirus mediated PD-L1 local gene transfection on renal transplant rejection

Bingxia Xu[1], Yunxia Zhang[2], Xuexun Chen[2], Haibo Zhang[2]

[1]*Department of Nephrology, Yidu Central Hospital of Weifang, Qingzhou, Shandong, China;* [2]*Department of Nephrology, Affiliated Hospital of Weifang Medical University, Weifang, Shandong, China*

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Abstract: Renal transplantation is currently the most effective method for renal failure treatment. Renal transplant rejection is the main factor influencing the postoperative graft survival. Study showed that adenovirus mediated PD-L1 gene transfection can inhibit T cell proliferation and activation, while its role in renal transplantation has not been elucidated. Recipient rats were randomly divided into three groups, including control group, empty vector control, and transfection group. Real-time PCR and Western blot were applied to detect PD-L1 expression. The level between receptor survival and serum creatinine (Scr) were compared. ELISA was carried out to test the levels of IFN-γ, IL-2, IL-10, and IL-6. PD-L1 mRNA and protein expression increased significantly in transfection group than that of control group ($P < 0.05$). The rats in transfection group showed obvious longer survival time and better renal function index ($P < 0.05$). Cytokine IL-10 and IL-6 elevated, while IFN-γ and IL-2 declined in transfection group than that of control group ($P < 0.05$). Donar renal adenovirus mediated PD-L1 transfection can regulate the balance of Th1/Th2 cytokines, improve receptor function, extend graft survival time, and inhibit renal transplant rejection through regulating immunosuppressive factor expression.

Keywords: Renal transplantation, adenovirus, PD-L1, cytokine, inbred rat

Introduction

Multiple factors can cause local or whole renal function injury, including long-term chronic nephritis, kidney insufficient blood supply caused by burns or trauma, etc. It further caused acute and chronic kidney failure, which leading to paruria, toxic metabolites accumulation, ion disorder, bleeding, fatigue, loss of appetite, anemia, uremia, and even other organ failure [1, 2]. With the acceleration of working rhythm, the change of social environment and living habit, the incidence of renal failure obviously increased and showed younger trend [3, 4]. At present, symptomatic treatment and dialysis are the main treatments for renal failure, whereas the best choice for terminal renal failure is renal transplantation [5]. Transplant rejection is the main problem in renal transplantation. As a result, a large fraction of patients receive too much or too little immunosuppression, exposing them to higher rates of infection, malignancy and drug toxicity, or increased risk of acute and chronic graft injury from rejection, respectively [6, 7]. However, effectively inhibit graft rejection and ensure body normal structure and function become the hotspot in renal transplantation investigation [8]. The research and development of new treatments or immunomodulatory drugs offered hope to renal transplant patients.

Recent study confirmed that genetic techniques provide new ideas for transplant rejection prevention. It was verified that transfecting immunomodulatory gene to graft can promote immune tolerance molecules expression, and further to secrete cytokines to suppress the immune response. The later played the important role of immune tolerance to suppress immune rejection and protect graft function [9, 10]. PD-L1 is an inhibitory co-stimulatory molecule that belongs to the B7 superfamily. After binding with its receptor of PD-1, two tyrosine residues in the cytoplasm combined with downstream signaling molecules to inhibit activated T cell proliferation. It also had a negative regulation function on primary immune response, memory, and effect T cells, especially on effect $CD8^+$ T cells, therefore, which can suggest its

important role in inhibiting immune rejection [11-13]. Adenovirus vector is widely applied in gene therapy with high efficiency, safety, wide host range, and high virus titer [14]. However, the role of adenovirus mediated PD-L1 gene in inhibiting renal transplant rejection was still unknown. This study intended to investigate the impact of local donor kidney transfection on renal transplant rejection through establishing orthotopic renal transplantation model of inbred rat.

Materials and methods

Experimental animals

24 healthy inbred male Lewis rats were selected as donor, while another 24 healthy inbred male Wistar rats were chose as receptors. All the rats were 3 months old and 300±30 g weight at SPF grade. Rats were provided by Weifang medical university. The rats were fed in SPF grade experimental animal center with constant temperature (21±1°C) and humidity (50-70%). Day/night cycle was maintained at 12 h.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Yidu central hospital of Weifang.

Main materials and instruments

Surgical instruments were bought from Suzhou medical instrument factory. Operating microscope was from Zhenjiang optical instrument co., LTD. CsA was purchased from Sigma. AdV-PD-L1 was purchased from Gene Company. HEK293 cells were obtained from ATCC cell bank. Scr detection kit was purchased from Roche. PVDF membrane was from Pall Life Sciences. Western blot related chemical reagents were from Beyotime. ECL reagent was from Amersham Biosciences. Rabbit anti-human PD-L1 primary antibody and HRP-tagged IgG secondary antibody were from Cell Signaling. RNA extraction kit, reverse transcription kit, ELISA kits for INF-γ, IL-2, IL-10, and IL-6 were from R&D. Microplate reader was from BD. DNA amplifier was from PE Gene Amp PCR System 2400. Automatic biochemical analyzer was from Beckman. Other common reagents were purchased from Sangon.

Methods

AdV-PD-L1 recombinant adenovirus vector amplification: 4×10^5/ml HEK293 cells were digested and cultured in 6 cm dish for 24 h. Then they were vaccinated with 100 μl virus liquid for 72 h. Next, the infected cells and supernatant were collected and centrifuged with 10000 g at 4°C for 20 min after repeated freezing and thawing. The supernatant was collected after filtering and stored at -80°C.

Renal transplant model: Donor rat was fasted for 12 h but not water before modeling. The rat was anesthetized with 10% chloral hydrate intraperitoneal injection and fixed on the table. The left kidney was exposed through midline incision and freed by ligating adrenal arteriovenous branches and cut the renal artery. 4°C normal saline containing heparin was perfused to renal artery through abdominal aorta. Then the donor kidney was taken out after freeing the ureter and stored at 4°C normal saline containing heparin after putting left renal vein into the Cuff tube. Receptor rat was anesthetized synchronously and fixed. The receptor kidney was removed after exposure, and the donor kidney was connected through Cuff tube. The graft change red after opening the artery and vein, and the urine flew out from ureter after 2-3 min. The rat was not restricted to eat and drink after surgery. Penicillin (10^4 IU•Kg^{-1}) intramuscular injection and CsA (2.5 mg•kg^{-1}) intraperitoneal injection were continued for 7 days after transplantation.

Grouping: The receptor rats were randomly divided into three groups with eight cases in each group. Empty vector control group, 1 ml empty vector was perfused to the donor renal artery through clipping the abdominal aorta, hepatic artery, and inferior vena cava for 3 min. Transfection group, 1 ml AdV-PD-L1 liquid (9×10^9 pfu/ml) was perfused to the renal artery for 3 min. Control group, donor kidney received no treatment, while receptor renal received CsA (2.5 mg•kg^{-1}) intraperitoneal injected for continuous 7 days after transplantation.

Sample collection: Abdominal aorta blood was collected in vacuum biochemical tube by negative pressure gathering method on d14 after transplantation. After 30 min standing at room temperature, the blood was centrifuged with 3600 rpm at 4°C for 10 min. The supernatant

Table 1. Primer sequence

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
PD-L1	GCATGACCTGCTTATGACTG	TTCGTTCCGCTCAACTCTTA

Table 2. Survival state and renal function detection

Index	Control	Empty vector group	Transfection group
Survival time (day)	15.1±8.1	16.2±7.9	35.7±15.4*
Weight (g)	221.4±52.1	237.9±46.1	355.8±41.4*
Scr (μmol/L)	89.2±12.3	87.6±11.5	55.8±11.2*

*$P < 0.05$, compared with control.

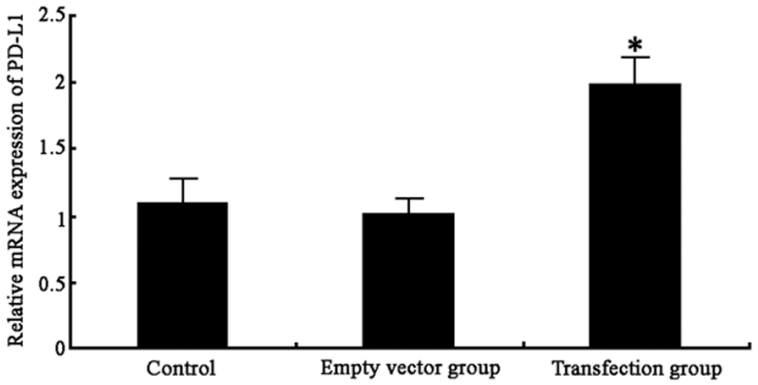

Figure 1. PD-L1 mRNA expression in kidney tissue. *$P < 0.05$, compared with control.

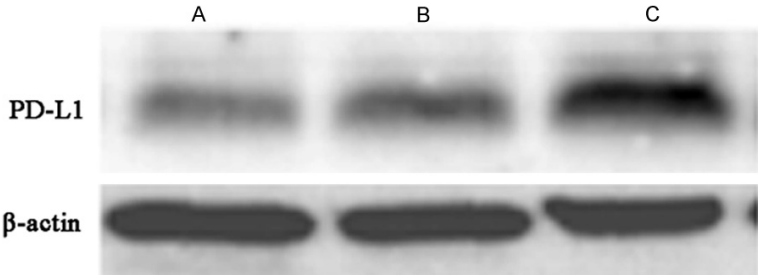

Figure 2. PD-L1 protein expression in kidney tissue.

was stored at -20°C, and the Kidney tissue was stored at -80°C.

Physical index detection: Survival time, weight, and physiological status were observed. Scr level was tested by automatic biochemical analyzer.

Real-time PCR: Total RNA was extracted by Trizol according to the manual and reverse transcripted to cDNA. The primers were designed by Primer 6.0 and synthetized by Invitrogen (**Table 1**). Real-time PCR was applied to detect target gene. The condition was 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as reference. Gene expression level was calculated by $2^{-\Delta Ct}$ method.

Western blot: Kidney tissue was grinded in liquid nitrogen and treated with RIPA for 15-30 min. Then the tissue was crushed by ultrasonication at 5 s×4 and centrifuged with 10000 g at 4°C for 15 min. The supernatant was moved to a new Ep tube for Western blot. Total protein was separated by 10% SDS-PAGE and transferred to PVDF membrane. After being blocked by 5% skim milk for 2 h, the membrane was incubated with PD-L monoclonal antibody (dilution 1:1000) at 4°C overnight. After being washed by PBST, the membrane was further incubated with secondary antibody (dilution 1:2000) for 30 min. At last, the membrane was treated by ECL for 1 min and developed under X-ray. Protein image processing system software and Quantity one software were applied to analyze the band density. All the experiments were repeated for four times.

The level of IFN-γ, IL-2, IL-10, and IL-6 in serum was detected with LISA method according to the kit instruction. Linear regression equation was drawn according to the standard substance concentration and corresponding OD value. Sample concentration was calculated based on linear regression equation.

Statistical analysis: All the statistical analysis was performed on SPSS 19.0. Measurement data was presented as mean±standard deviation. ANOVA and LSD test were used for mean value comparison. $P < 0.05$ was considered as statistical significance.

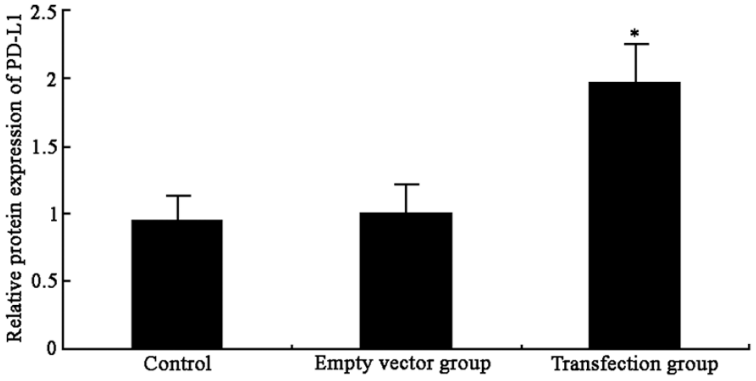

Figure 3. PD-L1 protein expression analysis in kidney tissue. *$P < 0.05$, compared with control.

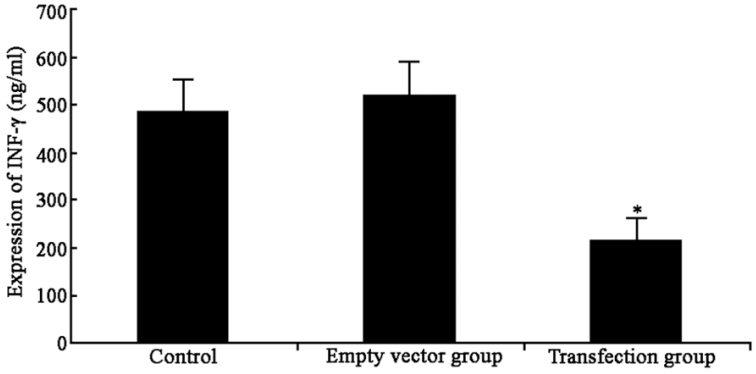

Figure 4. Serum IFN-γ expression comparison. *$P < 0.05$, compared with control.

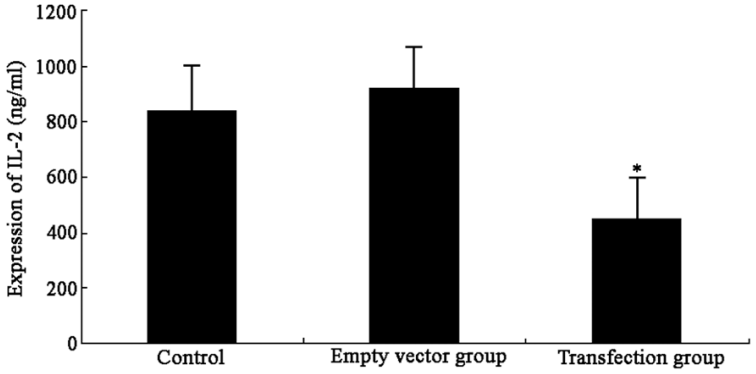

Figure 5. Serum IL-2 expression comparison. *$P < 0.05$, compared with control.

Results

Detection of survival state and renal function

Survival state, average survival days, weight, and Scr in each group were observed. It was showed that the rats presented good mental state, including luster hair, normal diet, activity, and urine output after AdV-PD-L1 treatment. They appeared paruria, depression, and death at 18th day after transplantation. The rats in empty vector group and control group showed abnormal reaction at 10th day after transplantation. The average survival time and weight obviously increased in transfection group compared with control ($P < 0.05$). Of which the longest survival time was 50 days in transfection group. Scr level in transfection group was significantly lower than that in control group ($P < 0.05$). No statistical difference was observed between empty vector group and control group (**Table 2**).

Comparison of PD-L1 mRNA expression

Real time PCR revealed that PD-L1 mRNA expression in kidney tissue elevated significantly after AdV-PD-L1 treatment ($P < 0.05$), while no statistical difference was found between empty vector group and control group (**Figure 1**).

PD-L1 protein expression in kidney tissue

Western blot was applied to observe PD-L1 protein expression in kidney tissue. Similar with PD-L1 mRNA result, PD-L1 protein expression in kidney tissue up-regulated significantly after AdV-PD-L1 treatment ($P < 0.05$). No statistical difference was observed between empty vector group and control (**Figures 2** and **3**).

Th1 cytokine expression in serum

ELISA was performed to evaluate the expression of Th1 cytokine IFN-γ and IL-2 in serum. The results revealed that serum IFN-γ and IL-2 levels decreased significantly after AdV-PD-L1 therapy compared with control group ($P < 0.05$).

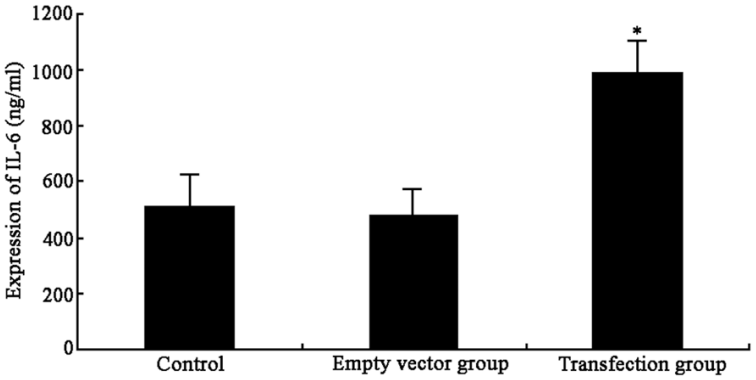

Figure 6. Serum IL-6 expression comparison. *$P < 0.05$, compared with control.

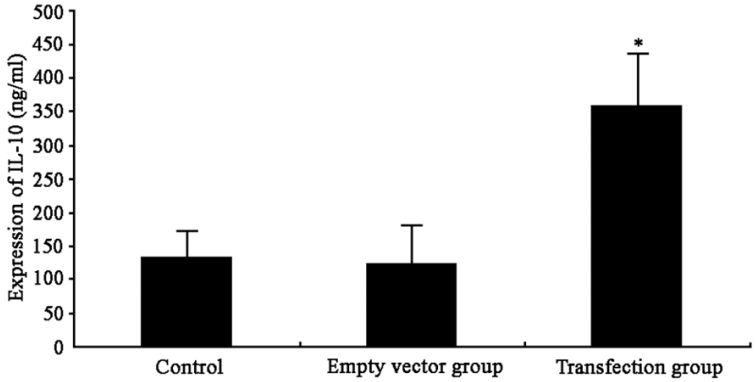

Figure 7. Serum IL-10 expression comparison. *$P < 0.05$, compared with control.

Their levels in empty vector group were similar with control group (**Figures 4** and **5**).

Th2 cytokine expression in serum

ELISA was applied to test the levels of Th2 cytokine IL-6 and IL-10 in serum. The results revealed that the levels of IL-6 and IL-10 in serum increased significantly after AdV-PD-L1 therapy compared with control group ($P < 0.05$). Their levels in empty vector group were similar with control group (**Figures 6** and **7**).

Discussion

Following transplantation (TX) of renal organs, a large proportion of patients have transplant rejection reaction. It is important to be able to perform precise and valid measurements or estimates of renal function, glomerular filtration rate (GFR), renal tubular secretion and morphological detection in renal transplantation patients after taking immunosuppresants. Cano H et al. [15] found that the intrarenal resistance index (RI) is a valuable marker to determine graft function and related vascular complications. It reveals a strong correlation with Scr levels measured days after transplant. Wang YT et al. [16] found that new advances in magnetic resonance imaging (MRI) technology have enabled the calculation of various renal parameters that were previously not feasible to measure noninvasively. Diffusion-weighted imaging provides information on renal diffusion and perfusion simultaneously, with quantification by the apparent diffusion coefficient, the decrease of which reflects renal function impairment. Pan P et al. [17] analyzed the diagnostic value of serum cystatin C and creatinine for evaluation of glomerular filtration function in renal transplant patients using a meta-analysis, the results showed that there were significant correlations among cystatin C, creatinine and GFR. Cystatin C had more sensitivity but less specificity than creatinine for evaluation of GFR. Cystatin C had strong ability in diagnosing renal function after renal transplant and ruling out diagnostic efficacy. Reznichenko A et al. [18] also studied that SSC22A2 is associated with tubular creatinine secretion and bias of estimated GFR in renal transplantation.

But how to overcome the transplant rejection reaction to extend renal transplant patient's survival time is still a urgent problem to solve. Though the application of immunosuppressants increase the survival rate, which complications including infection, tumor, and chronic rejection still are increasingly prominent. Recent research suggested that gene therapy can be used to replace immunosuppressants, which has brought a new hope for kidney transplant patients [19, 20]. Bring gene to graft through vector, which can be promoted to pro-

duce related protein, and further to play local immunosuppression role can prevent systemic immunosuppression caused by traditional immunosuppressor [21]. Therefore, this study selected inbred rat to investigate the mechanism of gene transfection in treatment renal transplantation.

This study chose inbred Lewis rat and Wistar rats as donor and receptor model, respectively. Since different inbred rats had different major histocompatibility complex (MHC) and minor histocompatibility complex (mHC), which may cause acute rejection. Therefore, it was widely applied in experimental study of organ transplantation acute rejection [22]. At present, it was reported that adenovirus vector perfusion via artery got high transfection efficiency in liver and heart transplantation [23]. Programmed death ligand 1 (PD-L1) negative regulated T and B cells dependent immune response through mediating T cell activity. It was confirmed to reduce heart transplant rejection and prolong the survival time of islet transplantation in mice [11, 12]. This study intended to analyze PD-L1 effect in regulating renal transplantation rejection in inbred rat. The results showed that PD-L1 gene and protein expression obviously increased in adenovirus mediated PD-L1 gene transfection group, which suggested that adenovirus mediated PD-L1 transfection had obtained good effect. In addition, the rats in transfection group showed significantly longer survival time, better life quality, and less Scr compared with control, it indicated that adenovirus mediated PD-L1 gene therapy can effectively improve the kidney function, survival time, and quality of life.

This study discovered that cytokines IL-10 and IL-6 elevated, while IFN-γ and IL-2 declined in transfection group. The imbalance of Helper T lymphocytes (Th) and cytokines in graft is the crucial factor leading to acute immune rejection [24, 25]. After antigen stimulation, T cells were mainly differentiated to Thl and Th2 subgroups. Th1 cells secreted cytokines INF-γ and IL-2, while Th2 cells secreted IL-6 and IL-10. Subgroup and cytokines inhibited each other, thus the cell differentiation and secretion were restricted. However, a large amount of Th1 type cytokines appeared in transplanted organ during the course of acute rejection process. On the other hand, Th2 type cytokines were mainly appeared in the graft during the course of immune tolerance process. Th2 type cytokines can suppress immune reaction and induce immune tolerance by inhibiting lymphocytes maturation and differentiation. Furthermore, some cells can long-term specifically decrease immune rejection through cytokines. Our study suggested that adenovirus mediated PD-L1 gene transfection can negatively regulate the proliferation of activated T cells , inhibit the synthesis and secretion of Th1 cytokines, and block graft rejection.

To sum up, our investigation confirmed that adenovirus mediated PD-L1 transfection can upregulate PD-L1 gene expression, regulate Th1/Th2 cytokines balance, improve receptor renal function, prolong graft survival time, and suppress graft rejection. It provides theoretical and treatment basis for targeting PD-L1 in renal graft rejection.

But future studies are required to test targeting PD-L1 that tailoring immunosuppression on the basis of results offered by the potential molecular target leads to better outcomes than current standard clinical practice.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xuexun Chen, Department of Nephrology, Affiliated Hospital of Weifang Medical University, 2428 kuiwen Yuhe Road, Weifang 261041, Shandong, China. Tel: +86-15966081167; Fax: +86-0536-3081608; E-mail: chenxuexund@sina.com

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