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• *BRIEF REPORTS* •

Adenovirus-mediated CTLA4Ig gene inhibits infiltration of immune cells and cell apoptosis in rats after liver transplantation

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Abstract

AIM: To investigate the role of adenovirus-mediated CTLA4Ig gene therapy in inhibiting the infiltration of macrophages and CD8+T cells and cell apoptosis after liver transplantation.

METHODS: The rat orthotopic liver transplantation model was applied. The rats were divided into three groups: group I: rejection control (SD-to-Wistar); group II: acute rejection treated with intramuscular injection of CsA 3.0 mg/(kg·d) for 12 d (SD-to-Wistar+CsA); groupIII: injection of 1×10^9 PFU adenovirus-mediated CTLA4Ig gene liquor in dorsal vein of penis 7 d before liver transplantation (SD-to-Wistar+CTLA4Ig). Immunohistochemistry and transferase-mediated dUTP nick-end labeling (TUNEL) were used to analyze the expression of CTLA4Ig gene in liver, infiltration of macrophages and CD8+T cells, cell apoptosis in grafts at different time-points after liver transplantation. Histopathological examination was done.

RESULTS: CTLA4Ig gene expression was positive in liver on d 7 after administering adenovirus-mediated CTLA4Ig gene via vein, and remained positive until day 60 after liver transplantation. Infiltration of macrophages and CD8+T cells in CTLA4Ig-treated group was less than in rejection control group and CsA-treated group. The apoptotic index of rejection group on d 3, 5, and 7 were significantly higher than that of CTLA4Ig-treated group. A good correlation was found between severity of rejection reaction and infiltration of immune activator cells or cell apoptotic index in grafts.

CONCLUSION: CTLA4Ig gene is constantly expressed in liver and plays an important role in inducing immune tolerance.

Key words: Liver transplantation; Adenovirus; CTLA4Ig; Apoptosis

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INTRODUCTION

Liver transplantation has become a life-saving therapeutic modality for end-stage liver diseases[1,2]. Although immunosuppressive therapy has decreased the incidence of rejection, it is still one of the main complications after liver transplantation and leads to poor results. Signal 1 through T-cell receptor (TCR)-major histocompatibility complex (MHC) plus antigenic peptide complex and signal 2 through costimulatory molecules are the bases of immune response. Interaction of CD28/CTLA-4 and B7 molecules is a major costimulatory pathway[3-6]. Binding of CD28/CTLA-4 on T cells to B7-related receptors on antigen-presenting cells (APC) can produce important antigen-nonspecific costimulatory signals critical for immune responses. Blockade of this pathway can lead to a state of antigen-specific unresponsiveness or apoptosis.

Linsley *et al*[7] bound an extra-celluar domain of mouse CTLA4 to a constant region of human IgG1 forming a fusion protein CTLA4Ig, which had high-avidity with B molecules of CD28. CTLA4Ig recombinant fusion protein can lead to a satisfactory immune result in heart, kidney and islet transplantations[8-12]. and in treatment of autoimmune diseases, such as systemic lupus erythematosus (SLE), glomerulonephritis (GN), rheumatoid arthritis (RA), *etc*[13-17]. But there are few reports about the immune effects of CTLA4Ig gene on liver transplantation. In this study, we transfected adenovirus-mediated CTLA4Ig gene into the graft liver in order to investigate the role of CTLA4Ig gene in immune tolerance through inhibiting infiltration of immune cells and apoptosis in grafts.

MATERIALS AND METHODS

Biological and chemical materials

The materials used in this study were cyclosporine (50 g/L,

Sandoz Corp, Switzerland), triphosphate nick-end labeling (TUNEL) assay kit (Promega), anti-CTLA4Ig antibody (R&D Corp, USA). ED1 and CD8 monoclonal antibodies were donated by Dr. Ren Yi of Surgery Department, Hongkong University.

Animals, surgical procedure and experimental groups

Inbred male *Wistar* and *Sprague-Dawly* (SD) rats weighing 200-250 g were purchased from Shanghai Experimental Animal Center. Under ether inhalation, orthotopic rat liver transplantation was performed with Kamada's two-cuff technique[18]. SD rats were selected as transplant donors and Wistar rats served as recipients that were randomly divided into 3 groups (21 pairs in each group): groupI: rejection control (SD-to-Wistar), group II: acute rejection treated with intramuscular injection of CsA 3.0 mg/(kg/d) for 12 d (SD-to-Wistar+CsA), group III: injection of 1×10^9 PFU adenovirus mediated CTLA4Ig gene liquor in dorsal vein of penis 7 d before liver transplantation (SD-to-Wistar+CTLA4Ig). On d 1, 3, 5, 7 and 12 post-transplant, 3 rats were randomly selected from each group for sample harvesting; another 6 rats in each group were bred for the observation of common situation and survival time.

Histopathological examination

Grafted liver samples were fixed in 10% buffered formalin and embedded in paraffin. Five micrometer thick sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphologic changes and severity of acute rejection by the Kemnitz's standard[19].

Immunohistochemical method

The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using respective antibodies to CTLA4Ig (dilution: 1:100), ED1 (dilution: 1:200) and CD8 (dilution: 1:200). Yellow or yellow- brown staining in cellular membrane or cytoplasm was a sign of being positive. ED1-positive and CD8-positive cells of portal area were counted as the mean number of positive cells in 10 randomized hyper-visual fields.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay

Transferase-mediated dUTP nick-end labeling (TUNEL) staining was used in the examination of apoptosis. Positive control slides were treated with DNase-1 and negative controls were stained in the absence of terminal deoxynucleotidyl transferase enzyme. Yellow- brown nuclei with nuclear condensation in stained cells were considered as TUNEL positive. Apoptotic index (AI) was counted as the mean number of apoptotic cells in 10 randomized hyper-visual fields of one section[20].

Statistics analysis

All data were expressed as mean±SD. The analysis of variance (ANOVA) was used for comparison between groups. Pearson's correlation analysis was used between parameters. Calculations were performed with SPSS for Windows Release 11.0 (SPSS Inc., Chicago, USA) statistical software. P less than 0.05 was considered statistically significant.

RESULTS

Survival time

The mean survival time of SD-to-Wistar group was 13.17±2.79 d, which was much shorter than that of SD-to-Wistar+CsA group and SD-to-Wistar+CTLA4Ig group (both over 60 d). The difference among the 3 groups were statistically significant ($P<0.01$).

Expression of CTLA4Ig gene in liver

CTLA4Ig gene expression was positive in liver on d 7 after administering adenovirus-mediated CTLA4Ig gene via vein, and remained positive until d 60 after liver transplantation. The common SD rat liver showed negative expression of CTLA4Ig gene (Figure 1).

Infiltration of immune cells

The detection of ED1-specific antigen in cytoplasm of mononuclear cells/macrophages by anti-ED1 antibodies was as follows. In SD-to-Wistar group, there was obvious infiltration of ED1-positive cells in portal area on d 5 after liver transplantation; the number of ED-positive cells was reduced in portal area but increased significantly in hepatic lobules on d 7. In SD-to-Wistar+CTLA4Ig group, the number of ED1-positive cells was low and did not show obvious changes at any time point. There was statistically significant difference between the SD-to-Wistar+CTLA4Ig group and the other two groups on d 3, 5, 7, and 12 after transplantation ($P<0.01$). The number of CD8-positive T cells in SD-to-Wistar+CTLA4Ig group was much lower than

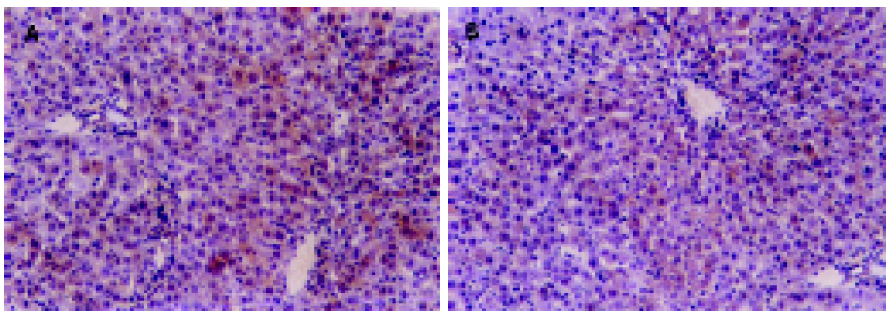

Figure 1 Immunohistochemical staining of CTLA4Ig in liver. A: on d 7 after administering adenovirus-mediated CTLA4Ig gene via vein (×200); B: on d 60 after liver transplantation in CTLA4-Ig-treated group (×200).

Table 1 Comparison in the infiltration of macrophage and CD8-positive cells in portal area (mean±SD)

Group	Post-operative time (d)				
	1	3	5	7	12
Macrophage					
Group I	2.11±1.54	20.13±8.34	106.75±22.56	49.82±14.74	29.22±8.69
Group II	2.42±1.68	10.78±3.70[a]	29.64±10.60[b]	33.25±12.44[b]	21.78±7.17[a]
Group III	1.90±1.45	3.50±2.37[b,d]	5.31±2.66[b,d]	2.45±2.50[b,d]	2.90±2.38[b,d]
CD8-positive cells					
Group I	1.25±1.04	57.43±19.64	87.29±23.44	130.83±31.61	60.67±19.71
Group II	2.13±1.64	5.11±3.02[b]	14.88±7.02[b]	8.79±6.65[b]	28.86±8.03[b]
Group III	1.22±1.48	5.60±2.55[b]	6.00±2.31[b,c]	2.18±1.83[b,d]	3.22±2.91[b,d]

[a]P<0.05, [b]P<0.01 *vs* Group I; [c]P<0.05, [d]P<0.01 Group II *vs* Group III.

Table 2 Comparison apoptosis index after transplantation (mean±SD)

Group	Post-operative time (d)				
	1	3	5	7	12
Group I	2.70±2.50	14.30±11.61	22.10±7.59	14.50±12.80	11.30±10.71
Group II	2.60±3.78	2.00±1.63[b]	2.80±1.87[b]	4.90±5.57[b]	6.50±6.93
Group III	2.30±2.58	2.10±1.79[b]	3.20±2.66[b]	2.40±1.96[b]	3.90±4.23[a]

[a]P<0.05, [b]P<0.01 *vs* GroupI.

that in Wistar-to-SD group at any time point except for on d 1 and 3 after transplantation. Compared with SD-to-Wistar+CsA group, there was a difference on d 5, 7, and 12 after transplantation. The results are shown in Table 1.

Apoptosis

The AI of SD-to-Wistar+CTLA4Ig group was much lower than that of SD-to-Wistar group on d 3, 5, and 7 after transplantation. The difference was statistically significant. But there was no substantial difference between SD-to-Wistar+CTLA4Ig group and SD-to-Wistar+CsA group at any time- point. The data indicated that AI correlated significantly with the number of infiltration CD8-positive cells in port area (r = 0.882, P<0.01). The results are shown in Table 2.

Histopathological examination

Rejection grade of SD-to-Wistar+CTLA4Ig group was significantly lower than that of Wistar-to-SD group on d 3, 5, 7, 12 and after transplantation (P<0.01) and lower than that of SD-to-Wistar+CsA group on d 12 after transplantation (P<0.05). The rejection grade correlated significantly with the number of macrophages/CD8-positive cells in portal area and AI (r = 0.696, P<0.01; r = 0.924, P<0.01; r = 0.914, P<0.01). The results are shown in Table 3.

Table 3 Comparison in rejection grade after transplantation (mean±SD)

Group	Post-operative time (d)				
	1	3	5	7	12
Group I	0,0,0	2,1-2,2	3,2-3,2-3	3,2-3,3	3,3,2-3
Group II	0,0,0	0,0,0[a]	0-1,0-1,1[a]	1,0-1,1[a]	1,1,1-2[a]
Group III	0,0,0	0,0-1,0[a]	0,0-1,0[a]	0,1,0-1[a]	0,0,1[a,b]

[a]P<0.05, *vs* Group; [b]P<0.01 Group II *vs* Group III.

DISCUSSION

CTLA4Ig is a fusion protein formed in an extracelluar domain of CTLA4 and a constant region of human IgG1. This protein has high avidity with B7-1/2 molecules and blocks the co-stimulatory signal pathway from APC to antigen specific T cells and inhibits immune tolerance[21,22]. The study by Hayashi *et al*[23] revealed that CTLA4Ig gene begins to express in liver on d 7 after administering adenovirus-mediated *CTLA4Ig* gene and remains positive for about 3 mo in blood. But they did not study the effects of CTLA4Ig after transplantation. We found that *CTLA4Ig* gene was positive in liver after injection in dorsal vein of penis with adenovirus-mediate gene just once and remained positive after transplantation. The rejection grade of Wistar-to-SD group increased rapidly 3-5 d after transplantation. This period is called "crisis period"[24], which differed in SD-to-Wistar+CsA group because of the strong inhibiting rejection effects of CsA. In contrast, there was no crisis period in+CTLA4Ig group at any time point and the rejection was inhibited effectively with the result the survival time prolonged significantly.

Acute rejection, characterized by infiltration of mononuclear cells, is a kind of immune response through CD8-positive cytotoxic T cells (CTL) with the assistance of CD4-positive T cells recognizing specific antigen. Intragraft infiltration and gathering of specific immune cell sub-group in circulation are the key to immune injury. In our study, infiltration of macrophages and CD8-positive T cells in portal area significantly increased from the 3rd day after transplantation and reached a peak on d 5 and 7 in Wistar-to-SD group. In contrast, the number of infiltrating immune cells was much lower in SD-to-Wistar+CTLA4Ig group. This phenomenon is consistent with allograft pathologic representation under rejection reaction. These data indicate that CTLA4Ig can block co-stimulatory signal pathway, inhibit infiltration of immune cells, alleviate CTL-induced

injury and induce immune tolerance. As is known, transplantation operation causes chemotaxis and infiltration of mononuclear cells/macrophages in one direction. In SD-to-Wistar+CTLA4Ig group, the number of post-operative infiltration macrophages in portal area was very small and did not show obvious changes at any time point, suggesting that hepatocytes construct and secret CTLA4Ig recombinant fusion protein after transfection of *CTLA4Ig* gene, increase local CTLA4Ig dosage and transport it into circulation via hepatic sinusoid. CTLA4Ig protein is combined with B7 molecule on the surface of mononuclear cells/macrophages, and leads to some biological changes of these cells, such as low-adhesion and low-movement or other unknown changes, thus decreasing the number of infiltrating macrophages in graft liver and affecting the ability of macrophages to present antigen. It is probably the mechanism of CTLA4Ig-induced immune tolerance through the changes of biologic characteristics of macrophages. Based on this hypothesis, we believe that CTLA4Ig concentration in circulation should reach a sufficient level before transplantation; but some authors suggested that CTLA4Ig protein is administrated 2 d after operation, although the peak of migration to lymphoid nodes around allografts of APC occurs on d 2 after operation[25].

Apoptosis, after liver transplantation, is related with post-operative survival time[26]. Dresske *et al*[27] reported that apoptotic level in immune tolerance group is much lower than that in rejection group. Memon *et al*[28] found that some patients develop allograft dysfunction approximately 7 d following an initial normal graft function, which is characterized by marked apoptosis of hepatocytes. This phenomenon is known as seventh- day syndrome. Apoptosis of hepatocytes is regarded as an important mechanism and an index of rejection. In our series, AI of Wistar-to-SD group increased quickly from the 3rd d and reached a peak on the 5th d, keeping a parallel relationship with rejection grade and infiltration of CD8-positive cells. This result is in agreement with that of Mignon[29]. The AI of SD-to-Wistar+CsA group and SD-to-Wistar+CTLA4Ig group were much lower than that of Wistar-to-SD group, indicating an obvious protection of CsA and CTLA4Ig expression in hepatocytes. The common situation of SD-to-Wistar+CTLA4Ig group was better than that of SD-to-Wistar+CsA group, because CTLA4Ig inhibited T cells with antigen specificity[30] and decreased the infiltration and activation of lymphocytes, mononuclear cells/macrophages, whereas CsA held back the whole immune system non-specifically. Adenovirus-mediated *CTLA4Ig* gene does not need repeated injection of recombinant protein and is safe because adenovirus cannot recombine with human genes.

In conclusion, *CTLA4Ig* gene is constantly expressed in liver via single intravenous injection into rats using adenovirus as vector and remains positive after transplantation. Adenovirus-mediated *CTLA4Ig* gene therapy can inhibit infiltration of immune activator cells and apoptosis in grafts, thus prolonging the survival of recipients. CTLA4Ig inhibits the ability of marophage chemokinesis via its ligation with B7 expressed on the surface of marophages, and plays an important role in inducing immune tolerance. Adenovirus-mediated *CTLA4Ig* gene therapy is a satisfactory therapy against rejection after liver transplantation.

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