

Original articles

Transplantation

Expression of 4-1BB molecule on peripheral blood T cells in liver transplanted patients and its clinical implication

Yun-Le Wan, Shu-Sen Zheng, Chang-Ku Jia, Ting-Bo Liang, Dong-Sheng Huang, Wei-Lin Wang, Min-Wei Li and Zhi-Cheng Zhao

Hangzhou, China

OBJECTIVE: To investigate the gene expression of 4-1BB in peripheral blood mononuclear cells (PBMCs) and its possible significance in clinical liver transplantation.

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the gene expression of 4-1BB in PBMCs from 22 patients receiving liver transplantation, 13 patients with primary liver carcinoma (PLC), and 12 healthy controls. To determine whether 4-1BB molecule is also expressed on the surface of CD4⁺ and CD8⁺ T cell, flow cytometry was used to analyse the phenotype of T cell subsets from the blood of liver transplantation patients.

RESULTS: 4-1BB mRNA was detected in PBMCs from stable survivors after liver transplantation, but almost not detected in PBMCs from PLC patients and healthy controls. Meanwhile, 4-1BB was almost not expressed on the surface of CD4⁺ and CD8⁺ T cells in healthy controls and PLC patients. A low level of 4-1BB expression, however, was found on the surface of CD4⁺ and CD8⁺ T cells from the stable survivors after liver transplantation.

CONCLUSIONS: This study demonstrates that although patients are stable after liver transplantation, effector T-cells can also be activated through the signal of 4-1BB molecule and persistent immune response to grafts. Blockage of 4-1BB/4-1BBL pathway may beneficially reduce the clinical dosage of immunosuppressive agents and prolong the survival of grafts.

(*HBPD Int* 2003 ; 2: 38-43)

Key words: 4-1BB; liver transplantation; activation of T cells

Introduction

The 4-1BB receptor in the tumor necrosis factor receptor (TNFR) superfamily is a type I membrane protein expressed on activated cytolytic and

helper T cells^[1,2] as well as NK cells.^[3] The ligand for the 4-1BB receptor is 4-1BB ligand (4-1BBL), which is expressed on antigen-presenting cells (APCs) including B cells, macrophages, and dendritic cells.^[4,5] The 4-1BB receptor can costimulate IL-2 production by resting primary T cells independently of CD28 ligation. Signals delivered to T cells by the 4-1BB receptor can induce T cells to proliferate, differentiate, and protect the cells from activation-induced cell death.^[6] Recently, Seko et al^[7] found the expression of 4-1BB costimulatory molecules on cardiac infiltrating cells and suggested that 4-1BB molecule plays a role in cell-mediated myocardial damage in patients with acute myo-

From the Department of Hepatobiliary Pancreatic Surgery, Key Laboratory of Combined Multi-Organ Transplantation, Ministry of Public Health, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China (Wan YL, Zheng SS, Jia CK, Liang TB, Huang DS, Wang WL, Li MW and Zhao ZC)

Correspondence: Yun-Le Wan, MD (Tel: 86-571-87236570; Fax: 86-571-87236570; Email: wanyl036@hotmail.com)

carditis and dilated cardiomyopathy. To investigate whether the effector T cells are still in an activated state in the blood of stable survivals after liver transplantation, we analyzed the expression of 4-1BB of peripheral blood T cells by using reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry.

Methods

Patients

From April 1993 to August 2002, liver transplantation was successfully performed in 112 patients with end-stage hepatic disease. In this series, 22 patients with stable conditions were re-examined from March to June 2002. Among them, 20 patients were male and 2 were female, aged from 35 to 58 years. Their primary diseases included cirrhosis after hepatitis B (13 patients), chronic severe hepatitis (2, one of them was accompanied with hepatorenal syndrome), primary biliary cirrhosis (2), primary hepatic carcinoma (4, two of them were associated with cirrhosis, one with diffused hepatic carcinoma, and the other with huge hepatic carcinoma, showing tumor emboli in the right branch of the portal vein), and hilar cholangiocarcinoma (1). Whole patients' blood group was the same as the donor. Basic immunotherapy was prescribed with triple-drug regimens containing cyclosporine A (CsA) or double-drug regimens containing tacrolimus (FK506). Re-examination showed that 2 of the 22 patients were complicated by biliary stenosis (one of them associated with recurrent liver cancer), which was treated by stent placement through endoscopic retrograde cholangiopancreatography (ERCP), one patient with recurrent liver cancer, 2 patients with cytomegalovirus infection, and 6 patients with HBeAg(+). According to Child-Pugh's classification of liver function, 17 of the 22 patients belonged to grade A and 5, grade B. The whole blood level of CsA was 109.78 ng/ml to 216.75 ng/ml, FK506 4.6 ng/ml to 13.6 ng/ml. Thirteen patients with primary liver carcinoma (PLC) were diagnosed pathologically after operation and 12 healthy volunteers served as controls.

Reagents

The following antibodies were used as reagents for flow cytometric analysis in vitro assay. Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies (mAbs) specific for human surface antigens included anti-CD4 (IgG1k clone RPA-T4), anti-CD8 (IgG1k clone RPA-T8), anti-CD3 (IgG1k clone UCHT1), phycoerythrin (PE)-conjugated anti-CD25 (IgG1k clone M-A251), anti-4-1BB (IgG1k clone 4B4-1), and FITC or PE-conjugated mouse IgG1k (clone MOPC-21) as isotype controls, all purchased from BD Pharmingen, San Diego, CA, USA. Revert-AidTM M-MuLV reverse transcriptase and TaqDNA polymerase were obtained from Promega, USA.

Peripheral blood mononuclear cell preparation

Five ml heparinized peripheral blood was sampled from healthy volunteers, patients with PLC and those after liver transplantation. In order to isolate peripheral blood mononuclear cells (PBMCs), 5 ml heparinized blood was diluted at a ratio of 1:1 with phosphate balanced solution (PBS) containing 0.6% Na₃-citrate and layered over a 5 ml ficoll cushion. After centrifugation (20 min, 700 × g), the interface containing PBMCs was collected and washed two times with PBS. The precipitate contained approximately 25% monocytes and 75% lymphocytes.

RT-PCR analysis of gene expression levels

Total RNA was isolated from PBMCs by using trizol (Gibco BRL Life Technologies, Breda, the Netherlands) according to the manufacturer's protocol. Briefly, the isolated PBMCs were resuspended in 1 ml trizol. They were mixed with 500 μl chloroform and incubated for 10 min on ice. After centrifugation for 10 min (1000 × g), the RNA in the upper phase was precipitated using isopropanol. To minimize the loss of RNA during isopropanol precipitation, 100 mg glycogen was added as a carrier. The RNA pellet obtained after centrifugation for 20 min (10 000 × g) was washed with 70% ethanol, and dissolved in water. And then it was quantified by OD_{26/28} determination, and

visualized in an ethidium bromide-stained agarose gel.

cDNA was prepared by reverse transcription of total RNA (2 μg) using Monloney murine leukemia virus (M-MuLV) reverse transcriptase. Two micrograms of total RNA, and 2 μg of oligodT (25 ng/ml) were incubated for 10 min at 72 $^{\circ}\text{C}$ in a final volume of 11 μl ; 1 μl of reverse transcriptase (200 u/ μl), 2 μl of dNTP mixture and 5 μl of reverse transcriptase buffer (5 \times) were added to final volume of 25 μl and incubated for 90 min at 42 $^{\circ}\text{C}$. cDNAs were stored at -20 $^{\circ}\text{C}$.

RT-PCR methodology was utilized to identify 4-1BB mRNA. cDNA used as template was checked in respect to the human β_2 -MG amplification. The following primers (Shanghai Sangon, China) were used:

β_2 -MG sense primer: 5'-CCAGCAGAGAATG-GAAAGTC-3'; β_2 -MG antisense primer: 5'-GAT-GCTGCTTACATGTCTCG-3'; 4-1BB sense primer: 5'-TCAGGACCAGGAAGGAGTGT-3'; 4-1BB antisense primer: 5'-AACGGAGCGTGAGGAABAAC-3'.

Using these primers, fragments of 240 and 414bp were expected to result from amplification of β_2 -MG and 4-1BB cDNAs, respectively.

Amplification using a PTC-200 DNA engine (MJ Research, USA) was achieved by adding 2 μl of cDNA to a PCR mixture containing 20 pmol of each primer for 4-1BB, 2.5 μl of Taq polymerase, 1 μl of 25 mmol dNTPs, 1.2 μl of 25 mmol MgCl_2 and 10 \times PCR buffer in a final volume of 25 μl . For β_2 -MG amplification, 1 μl of each primer at a 1:8 diluted concentration to 4-1BB primers was used for reaction. After an initial denaturation for 4 min at 94 $^{\circ}\text{C}$, samples were subjected to 30 cycles of amplification: annealing 45 s at 59 $^{\circ}\text{C}$, extension 60 s at 72 $^{\circ}\text{C}$, and denaturation 45 s at 94 $^{\circ}\text{C}$. PCR products (8 μl) were analyzed on 1.5% agarose gel containing ethidium bromide using Kodak DNA Analyses (Gibco BRL, USA) with Kodak digital science 1S 2.0 software. The expression level of 4-1BB mRNA was described as the ratio of 4-1BB/ β_2 -MG $\times 100$.

Flow cytometric analysis

Staining and flow cytometric analysis were performed as described previously.^[8] Briefly, 100 μl of heparinized peripheral blood was incubated in tubes (12 mm \times 75 mm) with monoclonal antibody at room temperature for 15 min to 30 min. Another 100 μl of heparinized peripheral blood incubated with FITL or PE-conjugated mouse IgG1k (clone MOPC-21) was used as negative isotype control. Erythrocytes were lysed in turn with ImmunoPrep A, B, and C haemolytic solution on Coulter Q-Prep (Beckman-coulter, USA). Alignment was checked using immunocheck beads (Beckman-coulter). All results were obtained using EPICS[®] XL FACScan (Beckman-coulter) with system[™] software. Forward- and side-scatter settings were gated to exclude red cells and debris, and 1×10^4 cells were analyzed for each determination.

Statistical analysis

Data were expressed as $\bar{x} \pm s$ (standard deviations). Statistical analysis was performed using one-way ANOVA with SPSS 10.0 software. *P* values ≤ 0.05 were considered significant.

Results

Detection of 4-1BB mRNA in PBMCs

As expected, amplification of 4-1BB mRNA and β_2 -MG mRNA from PBMCs stimulated by PHA (Sigma, USA.) led to 414bp and 240bp fragments respectively (data not shown). 4-1BB mRNA expression was not detected in the sample from 8 healthy controls (66.6%, 8/12) and 7 patients with PLC (53.8%, 7/13), while faint expression of 4-1BB mRNA was found in another 4 healthy volunteers and 6 patients with PLC. The median of 4-1BB mRNA expression level in each group was 0, whereas 4-1BB mRNA was detected in all sample from stable survivals of liver transplantation (median of expression level was 44.79. *P* < 0.05, comparison with healthy controls and PLC patients respectively using Kruskal-Wallis H Test) (Figs. 1 and 2).

Analysis of 4-1BB expressed on CD4⁺ or

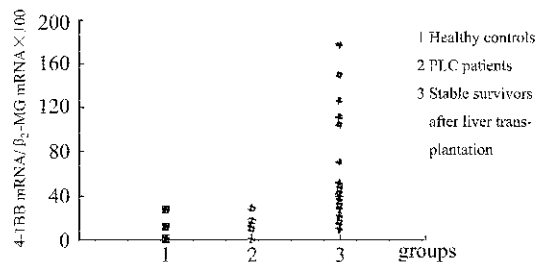


Fig. 1. Comparison of 4-1BB mRNA expression level among healthy controls, PLC patients and stable survivors after liver transplantation.

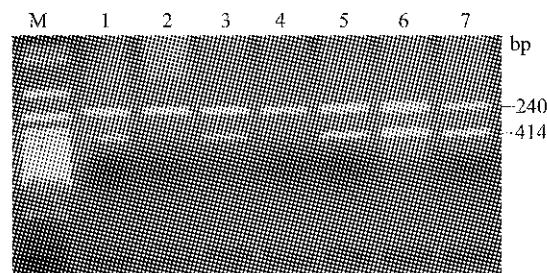


Fig. 2. RT-PCR analysis of 4-1BB mRNA in PBMCs. M: GenRuler™ 100bp DNA Ladder; Lanes 1 and 2: healthy controls; Lanes 3 and 4: PLC patients; Lanes 5 to 7: stable survivors after liver transplantation.

Table 1. Analysis of T cells phenotypes of PBMCs from healthy controls, PLC patients and stable survivors undergoing liver transplantation ($\bar{x} \pm s$)

Group	n	CD4	CD8	CD4 ⁺ /CD8 ⁺	CD3	CD4 ⁺ 4-1BB ⁺	CD8 ⁺ 4-1BB ⁺	CD3 ⁺ CD25 ⁺
Healthy controls	12	32.51 ± 6.12	28.32 ± 7.34	1.18 ± 0.22	70.11 ± 9.14	2.5 × 10 ⁻³ ± 6.21 × 10 ⁻³	1.67 × 10 ⁻³ ± 5.78 × 10 ⁻³	1.17 ± 0.27
PLC patients	10	37.37 ± 10.70	23.16 ± 6.34	1.84 ± 1.15	62.96 ± 6.32	6 × 10 ⁻³ ± 1.26 × 10 ⁻²	5 × 10 ⁻³ ± 1.08 × 10 ⁻²	1.96 ± 0.88
Liver transplantation	22	27.95 ± 9.76 [▲]	29.80 ± 11.85	1.55 ± 2.13	70.19 ± 11.74	0.24 ± 0.24 ^{◆◆}	0.20 ± 0.34 ^{◆◆}	2.01 ± 1.29 [▼]

[▲] versus PLC group, $P < 0.05$; ^{◆◆} versus healthy and PLC group; $P < 0.01$, [▼] versus healthy group, $P < 0.05$.

CD8⁺ T cells and CD25 molecules on CD3⁺ T cells

To determine whether 4-1BB molecule is involved in immune response after liver transplantation, flow cytometric analysis was used to analyse T cell phenotypes, expression of 4-1BB molecules on CD4⁺ or CD8⁺ T cells, and CD25 molecules on CD3⁺ T cells.

Percentage of CD8⁺ T cell and CD3⁺ T cell, as well as the ratio of CD4 to CD8 revealed no difference among the three groups ($P > 0.05$) (Table 1). However, the percentage of CD4⁺ T cell was significantly lower in the stable liver transplantation group than in PLC group ($P < 0.05$). But the percentage of CD3⁺ CD25⁺ T cell in stable liver transplantation group was significantly higher than in healthy control group ($P < 0.05$). 4-1BB molecule was almost not found on the surface of CD4⁺ and CD8⁺ T cell in the PLC and healthy control groups. But a low expression of 4-1BB molecules just as its mRNA expression was detected on the surface of CD4⁺ and CD8⁺ T cell in the stable liver transplantation group versus the healthy and PLC groups, respectively ($P < 0.01$).

Discussion

Orthotopic liver transplantation has become a well-established modality for treatment of end-stage liver disease.^[9] Progress has been made in the last 2 decades since the first OLT was performed for a child in 1963. The development of new immunosuppressive agents and the refinement of surgical techniques have improved the 1-year survival rate from 20% to 30% in the 1970s to 80% to 90% in the 1990s, and the 5-year survival rate up to 60%. Nevertheless, almost all of the patients who underwent liver transplantation should receive permanent and regular treatment of immunosuppressive agents. CsA withdrawal has been reported to result in initial improvement of renal function and blood pressure, though the benefits were unsustainable over time. The high incidence of cellular rejection (50%) and ductopenic rejection (25%) resulted in the death of two patients.^[10,11] B7/CD28 pathway plays a critical role in T cells activation during the allograft rejection, but immunosuppressive drugs such as CsA or FK506 can adequately inhibit the CD28 T-cell activation.^[12] The incidence of acute rejection after liver transplantation is up to 50% to

70%,^[13-15] and chronic rejection is still the main cause of death in liver recipients who survived more than 3 years.^[16] This suggests that although patients are prescribed permanent and regular treatment of immunosuppressive agents, effector T-cells can also be activated through the signal of other molecules and immune response to grafts permanently. Cytomegalovirus infection or other complications may induce a strong immunoresponse to grafts or grafts rejected under this condition.^[17]

The 4-1BB receptor in the TNFR superfamily is a type I membrane protein expressed on activated cytolytic and helper T cells^[1,2] as well as NK cells.^[3] The ligand for 4-1BB receptor is 4-1BB ligand (4-1BBL), which is expressed on APCs including B cells, macrophages, and dendritic cells.^[4,5] Recent report^[18] indicates that under the condition of repeated Ag-stimulation, down-regulated expression of CD28 molecule on activated T cells leads to activation-induced cell death (AICD), while very few 4-1BB molecules may supply sufficient costimulatory signals to sustain T cells activation, and inhibit AICD.^[6] Interaction of 4-1BB with 4-1BBL plays an important role in sustaining T cells activation, amplifying cytotoxic T lymphocyte (CTL) response, as well as inducing IL-2 production in the complete absence of a signal through CD28 molecule.^[5,19] Analysis of 4-1BBL knockout mice has shown that 4-1BBL^{-/-} mice as well as CD28^{-/-} mice reject skin allografts as rapidly as wild mice, whereas mice lacking both CD28 and 4-1BBL show a delay in skin allograft rejection.^[20] Using anti-4-1BB mAb infusion has demonstrated that 4-1BB/4-1BBL interaction can amplify the proliferation of CD8⁺ T cells, and augment GVHD-induced lethality and allogeneic BM rejection mediated by either CD4⁺ or CD8⁺ donor T cells.^[21] These findings suggest that 4-1BB signal plays an important role in the cellular immune response after allo-transplantation.

In this study, 4-1BB mRNA expression was almost not detected in the samples from healthy controls and patients with PLC, but was detected in those from stable survivors after liver transplantation. To further investigate that expression of 4-1BB molecules on CD4⁺ and CD8⁺ T cells, flow cytometer was used to analyze the phenotype of T

cells. The results showed that 4-1BB molecule was almost not found on the surface of CD4⁺ and CD8⁺ T cell in the PLC and healthy control groups, indicating tumor immune escape associated with 4-1BB signals. A recent study^[22] demonstrates that immunomodulatory gene therapy with 4-1BB ligand can induce long-term remission of liver metastases in a mouse model and augment CTL response against tumor, making 4-1BB/4-1BBL pathway a promising target for anti-tumor immuno-therapy.^[22,23] Moreover, a significant higher percentage of CD3⁺ CD25⁺ T cell, and a relatively low expression of 4-1BB molecules on the surface of CD4⁺ and CD8⁺ T cell were found in stable survivors after liver transplantation, suggesting that although patients are stable after liver transplantation, effector T-cells can also be activated through the signal of 4-1BB molecule and persistent immune response to grafts.

In summary, the data of this study show that 4-1BB molecule is not expressed on peripheral blood T lymphocytes from healthy controls and PLC patients, but induced to expressed on CD4⁺ and CD8⁺ T cells with a relatively low level by allograft antigen. The data also suggest that although patients are stable after liver transplantation, effector T-cells can also be activated through the signal of 4-1BB molecule and exert a persistent immune response to grafts. Blockage of 4-1BB/4-1BBL pathways may beneficially reduce the clinical dosage of immunosuppressive agents and prolong the graft survival.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

- 1 Kwon BS, Weissman SM. cDNA sequences of two inducible T-cell genes. *Proc Natl Acad Sci USA* 1989; 86:1963-1967.
- 2 Pollok KE, Kim YJ, Zhou Z, et al. Inducible T cell antigen 4-1BB; analysis of expression and function. *J Immunol* 1993;150:771-781.

- 3 Melero I, Johnston JV, Shufford WW, et al. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell Immunol* 1998;190:167-172.
- 4 Pollok KE, Kim YJ, Hurtado J, et al. 4-1BB T-cell antigen binds to mature B cells and macrophages, and costimulates anti- μ -primed splenic B cells. *Eur J Immunol* 1994;24:367-374.
- 5 DeBenedette MA, Shahinian A, Mak TW, et al. Co-stimulation of CD28⁻ T lymphocytes by 4-1BB ligand. *J Immunol* 1997;158:551-559.
- 6 DeBenedette MA, Chu NR, Pollok KE, et al. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its up-regulation on M12 B lymphomas by cAMP. *J Exp Med* 1995;181:985-992.
- 7 Seko Y, Ishiyama S, Nishikawa T, et al. Expression of tumor necrosis factor ligand superfamily costimulatory molecules CD27L, CD30L, OX40L and 4-1BBL in the heart of patients with acute myocarditis and dilated cardiomyopathy. *Cardiovasc Pathol* 2002;11:166-170.
- 8 National Committee for Clinic Laboratory Standards. Clinical applications of flow cytometry: quality assurance and immunophenotyping of peripheral blood lymphocytes. NCCLS Document H42-T 1992;12:1-15.
- 9 Zheng SS, Huang DS, Wang WL, et al. Orthotopic liver transplantation in treatment of 77 patients with end-stage hepatic disease. *HBPD Int* 2002;1:8-13.
- 10 Te HS, Schiano TD, Conjeevaram HS, et al. Long-term follow-up of liver transplant recipients undergoing cyclosporine withdrawal. *Transplant Proc* 2001;33:2874-2877.
- 11 Sandborn WJ, Hay JE, Porayko MK, et al. Cyclosporine withdrawal for nephrotoxicity in liver transplant recipients does not result in sustained improvement in kidney function and causes cellular and ductopenic rejection. *Hepatology* 1994;19:925-932.
- 12 Charco R, Caragol I, Urban S, et al. CD28 regulation in liver transplant recipients treated with two different immunosuppressive regimens. *Transplant Proc* 1999;31:3365-3366.
- 13 Fang SD, Zhang ZW. Comparison of acute cellular rejection between living donor liver transplantation and cadaveric liver transplantation. *Natl Med J China* 2001;81:1092-1094.
- 14 Fung J, Starzl T. Prophylactic use of OKT3 in liver transplantation: a review. *Dig Dis Sci* 1991;36:1427-1330.
- 15 Yang ZF, Wang WL, Lu CM, et al. Acute rejection in hepatic allograft. *Chin J Organ Transplant* 1999;20:237-240.
- 16 Pruthi J, Medkiff KA, Esrason KT, et al. Analysis of causes of death in liver transplant recipients who survived more than 3 years. *Liver Transplant* 2001;7:811-815.
- 17 Martelius TJ, Blok MJ, Inkinen KA, et al. Cytomegalovirus infection, viral DNA, and immediate early-1 gene expression in rejecting rat liver allografts. *Transplantation* 2001;71:1257-1261.
- 18 Kim YJ, Kim SH, Mantel P, et al. Human 4-1BB regulates CD28 co-stimulation to promote Th1 cell responses. *Eur J Immunol* 1998;28:881-890.
- 19 Chu NR, DeBenedette MA, Stiernholm BJ, et al. Role of IL-12 and 4-1BB ligand in cytokine production by CD28⁺ and CD28⁻ T cells. *J Immunol* 1997;158:3081-3089.
- 20 DeBenedette MA, Wen T, Bachmann MF, et al. Analysis of 4-1BB ligand-deficient mice and of mice lacking both 4-1BB ligand and CD28 reveals a role for 4-1BB ligand in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J Immunol* 1999;163:4833-4841.
- 21 Blazar BR, Kwon BS, Panoskaltis-Mortari A, et al. Ligation of 4-1BB (CDw137) regulates graft-versus-host disease, graft-versus-leukemia, and graft rejection in allogeneic bone marrow transplant recipients. *J Immunol* 2001;166:3174-3183.
- 22 Martinet O, Ermekova V, Qiao JQ, et al. Immunomodulatory gene therapy with interleukin 12 and 4-1BB ligand: long-term remission of liver metastases in a mouse model. *J Natl Cancer Inst* 2000;92:931-936.
- 23 Melero I, Shufford WW, Newby SA, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Natl Med* 1997;3:682-68.

Received September 17, 2002

Accepted after revision November 21, 2002