

Forkhead Box P3 (FOXP3) mRNA Expression Immediately After Living-Donor Liver Transplant

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Abstract

Objectives: The forkhead box P3 (FOXP3) gene is considered to be the master gene of regulatory T cells. The significance of regulatory T cells in liver transplant has been investigated in previous reports, but quantitative FOXP3 messenger RNA (mRNA) expression after living-donor liver transplant has not been assessed. The objective of this study was to determine whether the human FOXP3 gene is a good marker for regulatory activity in T cells in living-donor liver transplant recipients during the immediate posttransplant period.

Materials and Methods: In peripheral blood mononuclear cells of 15 living-donor liver transplant recipients during the first month after transplant, we measured the population of CD4⁺CD25⁺ T cells using flow-assisted cell sorting and the expression of FOXP3 mRNA using real-time polymerase chain reaction.

Results: Fold induction of FOXP3 mRNA significantly increased on postoperative day 7 (3.3-fold) compared with the reference preoperative value ($P < .01$) but returned to baseline by 28 days after transplant. The population of CD4⁺CD25⁺ T cells did not change significantly. Expression of FOXP3 mRNA on days 14, 21, and 28 were lower in recipients with acute cellular rejection within 60 days after living-donor liver transplant.

Conclusions: Increased expression of FOXP3 mRNA immediately after living-donor liver transplant might

be influenced by activation of T cells including regulatory T cells and other T cells. However, after stabilization of these activation profiles, it seems likely that FOXP3 mRNA expression is associated with graft acceptance. Further studies are necessary with measurement of FOXP3 mRNA expression at appropriate sampling points.

Key words: Forkhead box P3, Regulatory T cells, Liver transplant, Acute cellular rejection, Immunosuppression

Regulatory T cells are a subset of CD4⁺ T cells with suppressive function. Because regulatory T cells constitutively express CD25 (interleukin 2 [IL-2] receptor, alpha chain), CD4⁺CD25⁺ T cells isolated from blood have been used in vitro as regulatory T cells, as initially reported using mice (1, 2). A comparable subset of regulatory T cells exists in humans, with some differences from those in mice (3, 4). This subset is reportedly related to transplant tolerance (5, 6). Demirkiran and colleagues reported that the population of CD4⁺CD25⁺ T cells in peripheral blood decreased after liver transplant and that this reduction was associated with immunosuppression (7, 8).

On the other hand, it has been reported that the forkhead box P3 (FOXP3) gene, whose defect is related to an autoimmune disease in mice (scurfy mice), is the master control gene of regulatory T cells in mice (9, 10). In addition, naive T cells transduced with the FOXP3 gene acquire suppressive function in mice (10). However, in studies of the human FOXP3 gene, Allan and colleagues reported that human naive T cells transduced with the FOXP3 gene did not show suppressive function and that FOXP3 protein is expressed in activated human effector T cells, which do not necessarily have suppressive function (11, 12). Therefore, it is necessary to determine whether or not the human

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FOXP3 gene is a good marker for regulatory activity in T cells.

In this study, we measured the population of CD4⁺CD25⁺ T cells and FOXP3 mRNA expression in peripheral blood mononuclear cells of living-donor liver transplant recipients during the immediate posttransplant period.

Materials and Methods

Fifteen recipients who underwent living-donor liver transplant at our institution between March 2005 and May 2006 were enrolled in the study. Informed consent was obtained from all recipients. And the study was approved by the ethics committee of our institute. Tacrolimus and steroids were administered to all recipients. Corticosteroids were administered during and progressively decreased after transplant. Methylprednisolone was administered intravenously at a dosage of 10 mg/kg immediately before reperfusion; at 1 mg/kg on postoperative days 1, 2, and 3; at 0.5 mg/kg on postoperative days 4, 5, and 6; and at 0.3 mg/kg on postoperative day 7. Steroid therapy was then changed to oral prednisolone at a dosage of 0.3 mg/kg. Blood sampling was performed on the day before living-donor liver transplant (day 0) and on postoperative days 7, 14, 21, and 28.

Peripheral blood was collected using EDTA tubes. Peripheral blood mononuclear cells were isolated using Ficoll-Paque (Amersham Biosciences, Piscataway, NY, USA) gradient centrifugation and washed twice in Royal Park Memorial Institute 1640 solution (Sigma, St. Louis, MO, USA).

Total RNA (0.5 µg) from the peripheral blood mononuclear cells was reverse transcribed with a high capacity complementary DNA (cDNA) archive kit (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reaction (RT-PCR) was performed for FOXP3 or 18s ribosomal RNA (rRNA) using Pre-developed TaqMan Assay Reagents (Applied Biosystems) with primer pairs FOXP3 forward 5'-TGCTCCTCTTCTTCCTTGAAC-3' and reverse 5'-GGGCGTGGGCATCCA-3' with TaqMan MGB probes (Assays-on-demand TM gene expression assay) for 5'-6FAM-ATCCGCTGGGCCATCCTGGAGGCT-C-3' (FOXP3), and specific primer pairs and a TaqMan MGB probe for 18s rRNA (TaqMan Pre-Developed Assay Reagents Human 18s rRNA, Applied Biosystems). Real-time PCR was carried out in an Applied Biosystems PRISM 7000 Sequence detection system

with cycling of 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Fold induction was determined by using $\Delta\Delta Ct$ method and was expressed as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct_{FOXP3} - Ct_{18s})_{sample} - (Ct_{FOXP3} - Ct_{18s})_{reference}$. For each patient, the FOXP3 mRNA fold induction of samples from postoperative days 7, 14, 21, and 28 was divided by the FOXP3 fold induction on day 0, and the values were reported as the relative FOXP3 fold induction.

Fresh peripheral blood mononuclear cells were stained with fluorescently labeled antibodies, CD3-allophycocyanin (Biosciences Pharmingen, San Diego, CA, USA), CD4-phycoerythrin (Biosciences Pharmingen), and CD25-fluorescein isothiocyanate (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) for 30 minutes at 4°C. The cells were washed twice with phosphate-buffered saline containing 2% bovine serum albumin and 0.2% azide and fixed in 0.5 mL 2% paraformaldehyde in phosphate-buffered saline. The percentage of CD3⁺CD4⁺CD25⁺ cells in peripheral blood mononuclear cells was analyzed with a flow cytometer (FACS Calibur; Becton Dickinson Biosciences).

The serum trough level of tacrolimus and the concentration of total bilirubin were measured on day 0 and postoperative days 7, 14, 21, and 28. Acute cellular rejection was confirmed by histologic examination of liver biopsies using the Banff classification (13), with a rejection activity index of 4 or more defined as rejection.

The Mann-Whitney test and 1-factor repeated measures analysis of variance were used for statistical analyses. Significant differences identified by analysis of variance were followed by Ryan's method for post hoc comparisons. *P* values less than .05 were considered statistically significant.

Results

The age of the 15 recipients at the time of living-donor liver transplant ranged from 6 months to 65 years (Table). The group consisted of 7 patients with biliary atresia, 5 with hepatitis C virus-related disease, and one each with familial amyloid polyneuropathy, primary biliary cirrhosis, and hepatitis B virus-related disease. Total bilirubin decreased gradually after living-donor liver transplant (Figure 1a), and all recipients had good graft function in the early postoperative period.

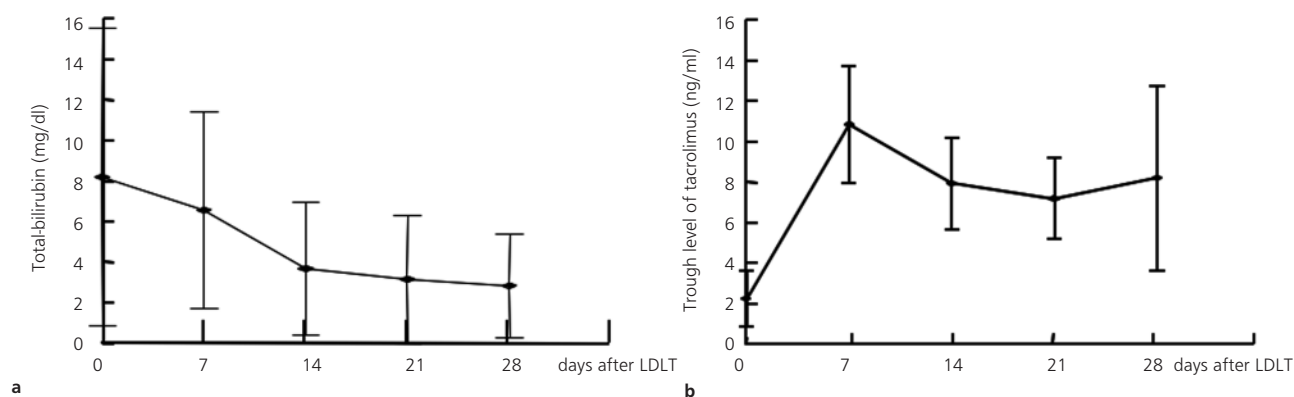


Figure 1. Total bilirubin levels and trough level of tacrolimus in the first 28 days after living-donor liver transplant. The bars show the standard deviation. **Figure 1a.** Mean total bilirubin level decreased gradually after living-donor liver transplant. **Figure 1b.** Trough levels of tacrolimus were maintained between 8 and 12 ng/mL.

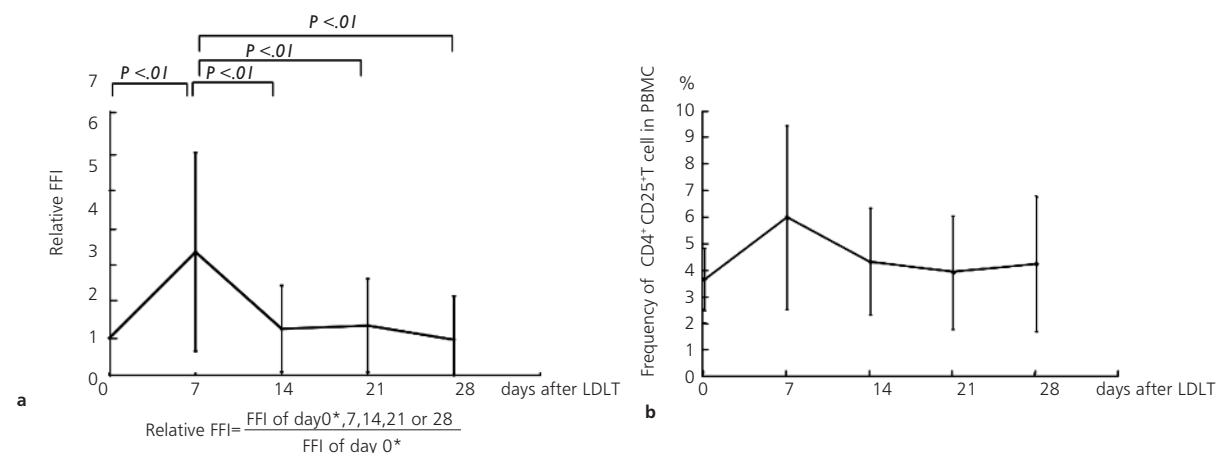


Figure 2. Mean relative FOXP3 fold induction and mean frequency of CD4⁺CD25⁺ T cells in the first month after living-donor liver transplant. **Figure 2a.** Mean relative FOXP3 fold induction on postoperative day 7 was significantly higher than the reference preoperative level ($P < .01$). **Figure 2b.** There were no significant differences between the sampling points ($P = .19$) for the proportion of CD4⁺CD25⁺ T cells. The bars show the standard deviation.

Abbreviations: FOXP3, forkhead box P3; mRNA, messenger RNA; rRNA, ribosomal RNA; *day 0 = the day immediately before living-donor liver transplant.

Table. Characteristics of 15 LDLT Patients

Patient	Disease	Age, y	Sex	ACR within 60 days	FFI peak on day 7
1	BA	0	F	-	+
2	BA	0	M	+(POD37)	+
3	BA	4	M	-	-
4	BA	16	M	-	-
5	BA	22	F	-	+
6	BA	23	F	-(POD40)	+
7	FAP	24	F	-	-
8	BA	32	M	-	+
9	HCV	46	M	+(POD23)	+
10	PBC	49	M	-	+
11	HCV	53	M	-	-
12	HBV	54	F	+(POD20)	+
13	HCV	56	F	-	+
14	HCV	61	F	-	-
15	HCV	65	M	-	-

Abbreviations: ACR, acute cellular rejection; BA, biliary atresia; FAP, familial amyloid polyneuropathy; FFI, forkhead box P3 (FOXP3) messenger RNA fold induction; HBV, hepatitis-B-virus-related disease; HCV, hepatitis-C-virus-related disease; LDLT, living-donor liver transplant; PBC, primary biliary cirrhosis; POD, postoperative day.

Trough levels of tacrolimus were maintained between 8 and 12 ng/mL (Figure 1b). Although the trough level of tacrolimus reached a peak on postoperative day 7, it was not significantly different from the values at other sampling points.

Mean relative FOXP3 fold inductions at each sampling point are shown in Figure 2a. Mean relative FOXP3 fold induction was significantly increased on postoperative day 7 (3.3-fold) compared with the reference preoperative value ($P < .01$); it returned to baseline by 28 days after transplant. This relative FOXP3 fold induction peak on postoperative day 7 was observed in 9 of the 15 patients (Table). Mean population of CD4⁺CD25⁺ T cells increased on postoperative day 7, but this was not significantly different compared with the other sampling points ($P = .19$; Figure 2b).

The recipients were divided into 2 groups according to whether they developed acute cellular rejection within 60 days after living-donor liver transplant (Table). The 4 patients with acute cellular rejection all had peaks of relative FOXP3 fold induction on postoperative day 7, compared to 5 patients with FOXP3 peaks of 11 patients without acute cellular rejection. Relative FOXP3 fold induction on postoperative days 14, 21, and 28 showed a tendency to be lower in the 4 patients with acute cellular rejection than in the 11 patients without acute cellular rejection (day 14: $P = .093$, day 21: $P = .057$, day 28: $P = .16$, Figure 3). The population of CD4⁺CD25⁺ T cells did not show any significant differences between the acute cellular rejection group and the group without acute cellular rejection (data not shown).

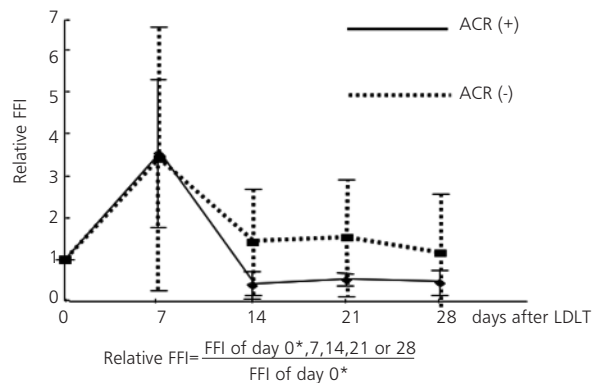


Figure 3. Mean relative FOXP3 fold induction in recipients with and without acute cellular rejection.

Mean relative FOXP3 fold induction on postoperative days 14, 21, and 28 showed a tendency to be lower in the 4 patients with acute cellular rejection (+) than in the 11 patients without acute cellular rejection (-).

Abbreviations: FOXP3, forkhead box P3; *day 0 = the day immediately before living-donor liver transplant.

Discussion

The FOXP3 gene is considered to be the master control gene of regulatory T cells in mice; however, it is debated whether the human FOXP3 gene also controls the activity of regulatory T cells because there are some differences between the mouse and human FOXP3 genes (14). Therefore, it is important to evaluate whether the FOXP3 gene can be a marker for immunologic status in living-donor liver transplant recipients. Although the population of CD4⁺CD25⁺ T cells did not show any significant changes during the first month after living-donor liver transplant in our study, FOXP3 mRNA was

significantly increased on postoperative day 7 and subsequently returned to preoperative values.

However, the mechanism underlying these results is unclear. Morgan and colleagues found in an in vitro study that both activated CD4⁺CD25⁺ T cells and activated CD8⁺ T cells expressed FOXP3 mRNA (15). They also reported that the production of FOXP3 in human cells, unlike in murine cells, is greatly affected by activation of various types of T cells. Moreover, recent studies have found that CD25⁺ T cells activated by anti-CD3/CD28 monoclonal antibody expressed FOXP3 protein and that these T cells did not show any suppressive function (12, 16). In the clinical setting, it is conceivable that the increase in FOXP3 mRNA expression on postoperative day 7 reflected the activation of T cells, not only of regulatory T cells but also of other T cells, as part of the immune response that occurs after transplant. Moreover, this peak of FOXP3 mRNA expression may not necessarily be related to the regulatory function against allograft, because all 4 recipients who developed acute cellular rejection within 60 days after living-donor liver transplant had the relative FOXP3 fold induction peak on postoperative day 7.

Average trough level of tacrolimus increased on postoperative day 7 (although not to a statistically significant degree). This indicates that this high concentration of tacrolimus stabilized immune system reactivity and therefore allowed FOXP3 mRNA expression to return to preoperative values. The FOXP3 fold induction on postoperative days 14, 21, and 28 was lower in the patients who developed acute cellular rejection within 60 days after living-donor liver transplant. This finding suggests that the change in FOXP3 fold induction after stabilizing T-cell activation immediately after living-donor liver transplant is associated with graft acceptance.

We have used intracellular staining and fluorescently labeled antibodies to identify FOXP3 protein in previous studies (17) and obtained reliable results for assessing the relation between regulatory T cells and immunologic tolerance in living-donor liver transplant recipients. Serial and dynamic analysis of FOXP3 protein levels before and after living-donor liver transplant, including the time of rejection episodes, is necessary in future studies.

In conclusion, FOXP3 mRNA expression increased immediately after living-donor liver transplant, probably because of immune reaction

with T cells, which was not associated with immunologic tolerance. However, the subsequent decrease in FOXP3 mRNA expression after stabilizing T-cell activation might be associated with graft acceptance. We must investigate appropriate sampling points for measuring FOXP3 mRNA expression to determine whether it can be used as a marker for graft acceptance after living-donor liver transplant.

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