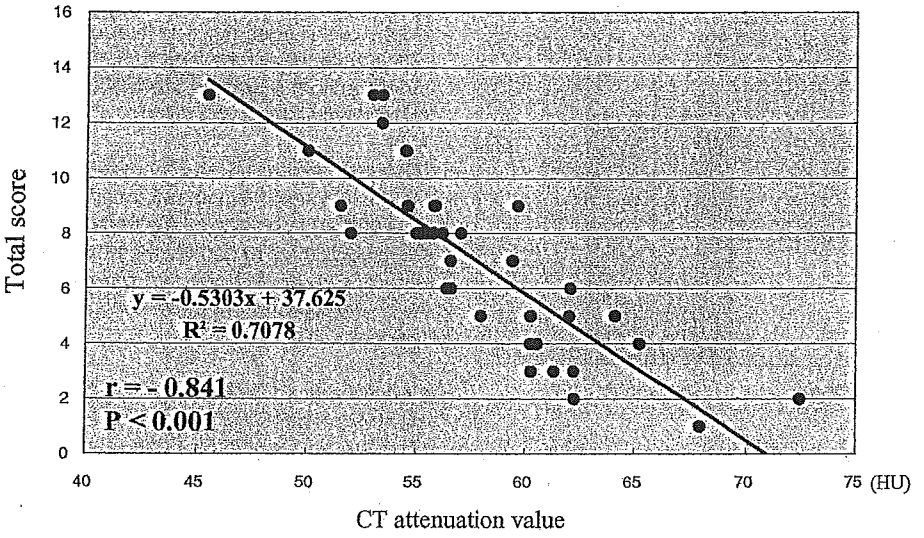


**Figure 6: Correlation between CT-AV in the graft and total score.** There was a significant negative linear correlation ($r = -0.841$, $p < 0.0001$). This result demonstrated that CT-AVs reflected the graft parenchymal damage.

Therefore, although we could perform unenhanced CT in all recipients, $^{99m}$Tc-GSA scintigraphy was only performed in seven recipients with a relatively stabilized general condition.

We managed to evaluate the translation between the CT-AVs and the objective data of $^{99m}$Tc-GSA such as the LHL15.

In the present study, there was a significant positive linear correlation between the CT-AV and LHL15 ($r = 0.803$, $p = 0.005$) in the only seven recipients.

However, due to the small number of patients who underwent $^{99m}$Tc-GSA scintigraphy, evaluation of the graft functional reserve by the correlation between the CT-AV and LHL15 alone was difficult. Therefore, we evaluated the graft functional reserve in the two CT-AV groups by conventional serum liver function tests, coagulation profiles and clinical findings.

Considering that group L was accompanied by hyperbilirubinemia, coagulopathy and massive ascites, this group was recognized as the graft dysfunction group.

Although we performed $^{99m}$Tc-GSA scintigraphy in only seven recipients, we suspected that the LHL15 deteriorated due to loss of the hepatic functioning mass in the graft, revealing a low CT-AV. It is suggested that assessment of the mean CT-AV in a graft may have the potential to be reliable for graft functional reserve.

Regardless of GV/SLV ratio, CT-AVs in liver grafts were decreased to under 60 HU in the two groups after LDLT.

CT-AVs in group H began to recover at 3 weeks after LDLT, but the low CT-AVs in group L persisted for 3 months after LDLT.

Regarding the reason for the persistent low CT-AVs in group L, six recipients died of various causes, including four graft failures, within three months after LDLT, and three recipients, who were successfully treated with stent insertions for stenosis of hepatic venous anastomoses, had persistent graft dysfunction. Therefore, we consider that the recovery of the CT-AVs in group L was delayed in comparison with group H. In fact, the CT-AVs of the remaining only two recipients in group L at 3 and 4 weeks after LDLT were 55.7 ± 3.3 HU and 57.4 ± 1.7 HU, respectively, and the CT-AVs began to recover at 4 weeks after LDLT.

Furthermore, 16 liver biopsy specimens in group B were obtained from 10 recipients in group L. Because we confirmed liver damage in group B by histological analysis, the CT-AVs were so low in group L.

The low CT-AVs (under 55 HU) in liver grafts at 1 week after LDLT are associated with graft dysfunction and fatal results, while CT-AVs over 60 HU may indicate adequate graft regeneration with allograft functional reserve.

The 1-year cumulative survival rate in group L was significantly lower than that in group H. Therefore, we suggest that CT-AVs at 1 week after LDLT may be predictive of the allograft functional reserve and the recipient prognosis.

Basically, this result indicated that several factors that decrease CT-AVs influence hepatocyte function, and induce the loss of actual functional mass, and graft dysfunction.

Graft dysfunction may have several causes, such as the graft size (1), the marginal grafts such as fatty liver (15), preservation-repurfusion injury, overperfusion due to excess portal flow (16) and elevated PVP (17), and pre-operative deteriorated condition closely related to MELD score (18).

With regard to recipient background, among the two groups, there were no significant differences in recipient age, GRWR, CIT, WIT, intra-operative blood loss or the presence of steatotic graft. However, the MELD scores in group L were significant higher than those in group H in our present study.

High pre-operative MELD scores have been shown to have an impact on the poor mortality of the recipients in several reports (18–20). Therefore, MELD scores can be used as predictors of the outcome after liver transplantation (21–23).

Regarding the maintenance of the initial graft function, the post-transplant metabolic and synthetic demands in recipients with severely damaged liver function (hyperbilirubinemia, coagulopathy) and a pre-operative deteriorated general condition (renal dysfunction, septic state, etc.) aggravated the graft metabolic function (1,24).

Furthermore, the liver grafts may be insufficiently functionated for the excessive metabolic and synthetic demands in high-risk recipients, including reduced metabolic and synthetic capacity (1).

Therefore, a pre-operative deteriorated condition with a high MELD score may impair the graft function, leading to graft dysfunction, graft failure and eventually multiple organ failure.

Accordingly, we consider that graft dysfunction accompanied by high MELD scores was responsible for the low CT-AVs in group L.

Our histological evaluations of biopsy specimens revealed that parenchymal damage was severe in the low CT-AV group (CT-AV < 55 HU) compared with that in the high CT-AV group (≥55 HU) in aspects of hepatocyte necrosis, congestion, microvesicular fat and neutrophil aggregates.

Moreover, considering that there was a significant negative linear correlation between the CT-AVs and the total scores, and the scores of hepatocyte necrosis, microvesicular fat and congestion, these occurrences may cause CT-AVs to decrease in the graft. Therefore, we consider that CT-AVs are associated with the total score, low CT-AVs may represent hepatic parenchymal damage that induces the loss of actual hepatic functional mass and graft function reserve may depend on the degree of necrosis, steatosis and congestion in the graft parenchyma, histologically.

## Conclusion

The CT-AV may be a useful parameter for assessing liver allograft functional reserve. We suggest that CT-AVs at 1 week after LDLT may be predictive of the allograft functional reserve and the recipient prognosis.

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# Role of Bcl-2 mRNA in Homeostatic Proliferation in Circulating T-Cells in Human Liver Transplant Patients after T-Cell Depletion

Hiroshi Sato,*,[1] Kazue Ozawa,† Shingo Iwata,‡ Satoshi Kaihara,§ Yasuhiro Ogura,§ Yasuhiro Fujimoto,§ Masako Ono,† Keiko Hodohara,¶ Shinji Uemoto,§ Hajime Nakamura,‖ Kenji Takai,** and Koichi Tanaka§

*Division of Bioscience, Shiga University of Medical Science; †Hepatic Disease Research Institute (HDRI); ‡Department of Surgery, Kyoto City Hospital; §Department of Transplantation and Immunology, Graduate School of Medicine, Kyoto University; ¶Blood Services Division, Shiga University of Medical Science; ‖Department of Biological Responses, Institute for Virus Research, Kyoto University; **SRL Inc., Tokyo, Japan

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***Background.*** **Prolonged T-cell depletion after liver transplantation leads to life-threatening infections. Members of the anti-apoptotic Bcl-2 gene family can maintain T-cell viability. T-cell numbers and their Bcl-2 expression following living donor liver transplantation (LDLT) were analyzed in 108 surviving and 13 deceased recipients.**

***Materials and methods.*** **Bcl-2 mRNA levels and phenotypic changes of T-cells were examined by quantitative PCR and by measuring expression of CD45RO and CCR7.**

***Results.*** **Based on the restoration of peripheral T-cell numbers, the 108 surviving recipients were classified into three groups. All recipients showed T-cell depletion, down to approximately 30% of pretransplant levels within 3 h of graft reperfusion. In Group I, the T-cell numbers were rapidly restored to pretransplant levels, within 5 days, with a rapid decrease in Bcl-2 mRNA levels immediately after LDLT. In Group II, the T-cell numbers were restored to normal levels by 19 days, with down-regulation of Bcl-2 mRNA. In Group III, the T-cell numbers were maintained at low levels for much longer, with high levels of Bcl-2 mRNA. In all three groups of recipients, there was statistically significant ($r = -0.78$) inverse correlation between T-cell numbers and Bcl-2 mRNA.**

***Conclusions.*** **For successful transplantation, homeostatic restoration of T-cells must occur as soon as possible. Evaluation of peripheral T-cell numbers and of Bcl-2 expression may have therapeutic potential in identifying those transplant patients who face increased risk of infection.** 

***Key Words:*** **human liver transplantation; T-cell number; Bcl-2 mRNA; homeostatic restoration.**

## INTRODUCTION

Living donor liver transplantation (LDLT) is now an established therapeutic modality for treating severe biliary atresia and metabolic diseases in infants, as well as irreversible terminal liver disorders in adults such as liver cirrhosis and hepatocellular carcinoma (HCC). At Kyoto University Hospital, pediatric LDLT began in 1990 [1]. By 2004 we performed more than 1000 LDLT at the institute. LDLT at our institute has increasingly been used as a remedy for terminal liver diseases in adults.

Direct or indirect alloreactive response to allogenic MHC molecules immediately after liver transplantation causes a drastic depletion of specifically alloreactive T-cells. Tolerance has been associated with many mechanisms including Th-cell immune deviation, induction of regulatory or suppressor cells, ignorance, and anergy. The most stringent tolerance mechanism is the complete deletion of T-cells. However, serious depletion of T-cells leads to life-threatening infections, which are still the most common cause of death in liver transplant recipients [2]. Restoration of the normal T-cell number, function, and repertoire diversity following T-cell depletion is necessary in homeostatic regulation to maintain life, but little is known about the mechanism of T-cell regeneration after LDLT.

The number of T-cells is determined by the homeostatic balance between proliferation of new cells and

[1] To whom correspondence and reprint requests should be addressed at Division of Bioscience, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan. E-mail: satoh@belle.shiga-med.ac.jp.



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**TABLE 1**

**Recipient Groups and Operation Profiles**

| Group (*n*) (Male/Female) | Original liver diseases (*n*) |
|---|---|
| Group I (34) 14/20 | BA (20), Alagille syndrome (2), Wilson's disease (1), PBC (3), PSC (2), FHF (1), Propioric acidosis (1), Polycystic disease (1), HCV-LC (1), HBV-LC + HCC (1), HCV-LC + HCC (1) |
| Group II (27) 17/10 | BA (6), BA, re-Tx (1), PBC (3), PSC (3), FHF (3), CTL (1), HBV-LC (1), HCV-LC (2), HBV-LC + HCC (2), HCV-LC + HCC (5) |
| Group III (61) 37/24 | BA (6) BA, re-Tx (1), Short bowel (1), Hepatoblastoma (2), Wilson's disease (1), PBC (8), PSC (1), FHF (4), Alcoholic LC (1), HCC (Caroli) (1), HBV-LC (3), HCV-LC (10), HBV-LC + HCC (6), HCV-LC + HCC (15), HBV + HCV-LC + HCC (1) |

*Note.* Values are expressed as mean ± SD.
Abbreviations: BA, Biliary atresia; PBC, Primary biliary cirrhosis; PSC, Primary sclerosing cholangitis; FHF, Fulminant hepatic failure; CTL, Citrullinemia; HBV, Hepatitis B virus; HCV, Hepatitis C virus; LC, Liver cirrhosis; HCC, Hepatocellular carcinoma.

death of senescent cells. The Bcl-2 gene family plays a central role in T-cell survival and apoptosis [3]. Synthesis of IL-2 was clearly attenuated by Bcl-2. Since IL-2 expression is essential for translocation of the nuclear factor of activated T-cells (NFAT), NFAT-mediated transactivation is impaired by Bcl-2 [4]. Survival of T-cells in appropriate numbers therefore requires the continuous expression of anti-apoptotic protein Bcl-2. Changes in Bcl-2 expression after liver transplantation are therefore a way by which activated T-cells might be selected for survival in response to alloreactive situations.

We believe that this is the first description of changes in Bcl-2 synthesis to focus on its role in T-cell homeostasis after liver transplantation. Below, the absolute number of T-cells and Bcl-2 mRNA in peripheral blood T-cells are serially analyzed from the start of allograft reperfusion until the T-cell number was once again between normal limits.

## PATIENTS AND METHODS

### Patients and Grafts

Our study focused on 108 surviving and 13 deceased recipients who underwent LDLT. Table 1 shows profiles of the surviving recipients and donors. Written informed consent was obtained from each subject before the start of this study, which was approved by the Ethics Committee of our hospital and was in accordance with the Helsinki Declaration of 1975 as revised in 1983.

### Operations

All patients underwent standard LDLT [5]. All grafts were flushed and preserved with histidine-tryptophan-ketoglutarate solution. Table 1 shows their original liver diseases.

### Immunosuppression

Baseline immunosuppression used a combination of tacrolimus (FK506) and steroids. Target tacrolimus trough levels were initially more than 10 ng/ml, decreasing gradually to approximately 6 to 8 ng/ml a few months after LDLT. Methylpredonisolone was used for induction and was switched to oral predonisolone 1 week after LDLT. Predonisolone was gradually reduced and finally discontinued at between 3 and 6 months after LDLT, provided that graft function was maintained. ABO blood group matching between donor and recipient was limited in LDLT, because we chose only living donor candidates from among family members. In ABO-incompatible transplants, circulating anti-A or anti-B antibodies can mediate humoral rejection in recipients.

In cases of acute cellular rejection, steroid bolus pulse therapy was performed (e.g., 10 mg/kg of methylprednisolone intravenous injection for 3 days). In cases with life-threatening complications, such as posttransplant lymphoproliferative disorder, tacrolimus administration was stopped immediately. Steroids were started at the time of graft reperfusion, with an initial dose of 10 mg/kg that was gradually reduced and stopped completely by 3 to 6 months after transplantation provided that liver function was stable.

Acute rejection was diagnosed clinically and histologically according to our reported previously criteria [6]. All rejection episodes were treated using a steroid bolus injection. Diagnosis and prophylaxis of infection were the same as we have reported previously [7]. The criteria for sepsis were those defined by Bone [8].

### Tissue Typing

Serological tissue typing was performed in all patients for HLA-A, HLA-B, and HLA-DR loci.

### Flow Cytometry

Heparinized venous blood samples were obtained 1 h prior to surgery and then at 0, 1, 3, 6, 12, 36, and 120 h, and every week for 4 months following graft reperfusion. Measurements were carried out primarily on whole blood, which is more sensitive to staining than purified peripheral blood mononuclear cells. Since CD45RA and CD45RO expression are mutually exclusive, we measured only the CD45RO isoform, except when measuring cytokine for which CD45RA was used.

We used the following monoclonal antibodies to stain cell surface antigens: allophycocyanin (Coulter Immunotech, Miami, FL, USA) or PC-5 (Coulter Immunotech, Marseilles, France)-conjugated anti-CD4 or CD8, fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (Nichirei, Tokyo, Japan), TC-conjugated anti-CD45RA (Caltag Laboratories, Burlingame, CA), phycoerythrin (PE)-conjugated anti-CD3 (Coulter Immunotech, Miami, FL, USA), and PE-conjugated anti-human CCR7 (DakoCytomation, Kyoto, Japan).

For intracellular staining we used isotype-matched controls. Cells were exposed to the antibodies for 30 min at 4°C and washed twice with PBS; 5000 cells were analyzed. FITC- and PE-labeled mouse IgG were used as isotype-matched background controls. We analyzed

the stained cells on a FACS Calibur flow cytometer using three- and four-color analysis with the CELL Quest software version 3.3 (BD Biosciences, San Jose, CA).

Naive cells were defined as having $CD45RA^+$ and $CCR7^+$; effector cells were $CD45RA^+$ and $CCR7^-$. Central/memory cells were $CD45RO^+$ and $CCR7^+$, and effector/memory cells were $CD45RO^+$, $CCR7^-$ cells [9].

### Isolation of Peripheral T-Cells

To measure Bcl-2 expression more accurately in terms of protein or mRNA levels per T-cell, we used highly purified T-cells, since lymphocytes contain Bcl-2 from other mononuclear cells. The purity of $CD3^+$ T-cells ranged from 95 to 99% based on FACS analysis (Becton Dickinson, San Jose, CA). Heparinized venous peripheral blood was collected from our 25 patients at 0800 h, and from 45 healthy laboratory personnel and medical students (28 men, 17 women; aged 33 ± 19 years expressed as mean ± SD, range 4–69 years). In total, 213 venous blood samples were taken from the transplant recipients and healthy individuals. Recipients with severe leukopenia (<2000/μl) were excluded.

The blood samples were kept on ice for up to 3 h, until the mononuclear cells were ready following the use of Lymphoprep (NYCOMED, Oslo, Norway). The T-cells were purified by positive selection, since the purity and recovery of T-cells using negative selection were inadequate for Bcl-2 assays. For isolation, a High Gradient Magnetic Separation Column type LS with CD3 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) was used. mRNA was prepared immediately after the isolation. To calculate the number of cells in each T-cell subset and the total number of T-cells, the proportion of cells staining positive was multiplied by the absolute peripheral T-cell count.

### Quantification of Bcl-2 mRNA Levels

We first examined whether the changes in Bcl-2 at the protein level after liver transplantation correspond to changes at the mRNA level. We confirmed that the expression of Bcl-2 mRNA changes in parallel with Bcl-2 protein. However, variation in the Bcl-2 protein values, as measured by flow cytometry in the T-cells of nine healthy individuals, exceeded the acceptable deviation. Our laboratory recently developed a more exact PCR assay that is able to evaluate small amounts of Bcl-2 mRNA in human peripheral T-cells [10]. According to this method the levels fell in a narrow range, between 0.11 and 0.23 molecules/T-cell for healthy individuals. Furthermore, Bcl-2 mRNA showed greater changes than Bcl-2 protein following liver transplantation. Measurement of Bcl-2 mRNA is therefore appropriate when studying homeostatic restoration of T-cells after peripheral T-cell depletion in human liver transplant recipients.

T-cells were prepared from 2.5 ml of peripheral blood using CD3 microbeads. Total RNA was isolated from the T-cell with a QIAamp RNA Blood Mini Kit from Qiagen (Hilden, Germany), and T-cell cDNA was synthesized using a first-strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Bcl-2 mRNA was measured using our quantitative PCR [10]. The sequence of primer pairs used for amplification of mRNA and competitor fragments were as follows: Bcl-2 sense, 5′-CGA CGA CTT CTC CCG CCG CTA CCG C-3′; antisense, 5′-CCG CAT GCT GGG GCC GTA CAG TTC C-3′ [11].

### Statistical Analysis

We determined bivariate correlations using Spearman rank correlation. Pairwise comparisons between groups were performed using Tukey–Kramer adjustment for multiple comparisons. We used the statistical software package StatView 5 (Abacus Concepts, Berkeley, CA). All values in the text (but not tables) are presented as mean ± SEM. All statistical tests were two-sided, and we defined significance as $P < 0.05$.

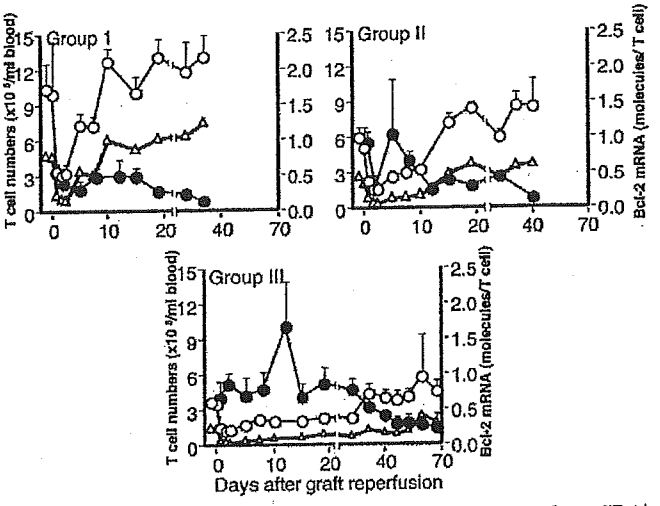


**FIG. 1.** Changes with time of peripheral T-cell number, $CD4^+$ naive cells, and Bcl-2 mRNA expression in Group I, II, and III recipients after liver transplantation. Open circle, open triangle, and closed circle, respectively, represent T-cell number, $CD4^+$ naive cells, and Bcl-2 mRNA expression.

## RESULTS

### Three-Group Classification

Based on the capacity for restoration of peripheral T-cells, the 108 surviving recipients were classified into three groups. Group I recipients had restored their T-cells to within normal limits in not more than 5 days, Group II by 19 days, and Group III recipients took longer (see Table 1 and Fig. 1). Acute rejection occurred in 27 of 121 recipients; in these patients, T-cell numbers were restored by steroid bolus injection. The time points during rejection were excluded from Fig. 1. Preoperative numbers of peripheral T-cells and naive T-cells decreased progressively from Group I to Group III recipients. There was no difference between the groups in the counts of central/memory and effector/memory T-cells, however.

In all recipients, T-cell numbers fell drastically within 6 h of graft reperfusion. In Group I recipients the T-cell numbers fell to $3.5 \times 10^5$ cells/ml blood (32% of pretransplant levels), in Group II recipients to $1.8 \times 10^5$ cells/ml blood (31%), and in Group III to $1.4 \times 10^5$ cells/ml blood (33%). Table 2 shows preoperative profiles of $CD4^+$ and $CD8^+$ T-cells. The absolute numbers of $CD4^+$ and $CD8^+$ naive cells decreased progressively from Group I to Group III recipients, but there was no significant difference between the three groups in relative proportion, although it was slightly higher in Group I. In $CD4^+$ and $CD8^+$ T-cells, the absolute numbers of central/memory, effector/memory, and effector cells did not differ across the groups. The $CD4^+/CD8^+$ ratio was higher in Group I than in other groups and healthy individuals.

**TABLE 2**

**Preoperative Peripheral T-Cell Number and Profiles of $CD4^+$ and $CD8^+$ T-Cells of Three Groups**

| Group | (*n*) | Age (yrs) | T-cell number ($\times 10^6$/ml blood) | CD4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Naive ($\times 10^6$/ml) | (%) | Central/memory ($\times 10^6$/ml) | (%) | Effector/memory ($\times 10^6$/ml) | (%) | | |
| I | (33) | 18 ± 22 | 10.45 ± 9.40 | 3.49 ± 3.76 | 65.63 ± 17.46 | 0.48 ± 0.37 | 17.26 ± 13.05 | 0.13 ± 0.10 | 3.99 ± 3.11 | | |
| II | (27) | 35 ± 20 | 6.05 ± 3.68 | 1.02 ± 0.75 | 41.79 ± 22.63 | 0.95 ± 0.84 | 33.90 ± 19.58 | 0.36 ± 0.57 | 10.63 ± 9.01 | | |
| III | (61) | 42 ± 19 | 3.70 ± 2.72 | 0.73 ± 0.90 | 42.08 ± 20.97 | 0.38 ± 0.35 | 29.54 ± 15.68 | 0.16 ± 0.18 | 12.24 ± 11.27 | | |
| Healthy individuals | (45) | 33 ± 19 | 14.65 ± 6.66 | 2.87 ± 2.51 | 42.95 ± 16.85 | 1.49 ± 0.63 | 26.56 ± 8.91 | 0.66 ± 0.49 | 11.51 ± 7.09 | | |
| | | | | CD8 | | | | | | | |
| Group | | | | Naive ($\times 10^6$/ml) | (%) | Central/memory ($\times 10^6$/ml) | (%) | Effector/memory ($\times 10^6$/ml) | (%) | Effector cell ($\times 10^6$/ml) | (%) | $CD4^+/CD8^+$ |
| I | | | | 1.37 ± 1.23 | 66.70 ± 21.66 | 0.03 ± 0.03 | 3.32 ± 3.79 | 0.06 ± 0.06 | 5.72 ± 9.45 | 0.41 ± 0.66 | 15.16 ± 10.84 | 2.86 ± 2.09 |
| II | | | | 0.60 ± 0.51 | 41.37 ± 22.62 | 0.09 ± 0.07 | 7.23 ± 6.09 | 0.18 ± 0.22 | 10.52 ± 8.00 | 0.34 ± 0.25 | 24.74 ± 10.86 | 2.26 ± 1.57 |
| III | | | | 0.38 ± 0.54 | 37.09 ± 26.99 | 0.04 ± 0.03 | 5.78 ± 5.63 | 0.07 ± 0.08 | 10.63 ± 9.96 | 0.27 ± 0.29 | 29.72 ± 20.41 | 2.24 ± 1.71 |
| Healthy individuals | | | | 1.51 ± 1.30 | 37.78 ± 17.70 | 0.20 ± 0.12 | 6.01 ± 3.49 | 0.48 ± 0.39 | 13.72 ± 10.39 | 0.72 ± 0.49 | 21.00 ± 10.77 | 1.78 ± 0.52 |

*Note.* Values are expressed as mean ± SD.

### Relation Between T-Cell Regeneration and Bcl-2 mRNA in the Three Groups

We have analyzed the numbers of peripheral T-cells at various time points until restoration of T-cells was complete following transplantation in each of our 108 surviving recipients, together with measurements of Bcl-2 mRNA in 25 recipients (Fig. 1). The time points during acute rejection are excluded from Fig. 1. In Group I recipients in which T-cell numbers were back to normal within 5 days, the Bcl-2 mRNA levels transiently but significantly increased to 0.68 ± 0.19 (range 0.23–1.2) molecules/T-cell at 12 h, then fell rapidly to 0.40 ± 0.09 at 24 h, and normalized within 12 days. In Group II recipients, the T-cell numbers decreased rapidly and drastically to 1.94 ± 0.39 (range 0.26–4.7) × $10^5$ cells/ml blood at 12 h postoperatively; the Bcl-2 mRNA increased rapidly and peaked at 1.00 ± 0.14 (range 0.24–2.04) molecules/T-cell at 12 h and 1.19 ± 0.23 at 24 h. In Group III recipients, the recovery in the number of T-cells remained incomplete even after 40–60 days. The Bcl-2 mRNA remained at considerably higher levels than the norm for healthy individuals.

From 3–12 h after graft reperfusion, there was a significant ($P < 0.005$) difference in T-cell numbers between Group I recipients and those in Groups II and III, but there was no significant difference ($P = 0.189$) in Bcl-2 mRNA levels at 12 h between Group I and Group II or Group III recipients. However, a significant difference ($P < 0.01$) was found in Bcl-2 mRNA levels at 24 h between Group I and Groups II and III.

The extent of T-cell depletion and Bcl-2 overexpression at 12 h after graft reperfusion did not differ significantly ($P = 0.10$ in T-cell number; $P = 0.31$ in Bcl-2 mRNA) between the nine younger recipients (≤28 years old: 7.63 ± 2.58 in T-cell number; 0.74 ± 0.18 in Bcl-2 mRNA) and the 10 older recipients (>40 years old: 2.83 ± 1.30 in T-cell number; 0.99 ± 0.16 in Bcl-2 mRNA).

Relationship in the number of peripheral T-cells and their Bcl-2 mRNA levels were examined at all time points after the operation in the 19 surviving transplant recipients (Fig. 2) except during acute rejection. The T-cell numbers were negatively correlated with the Bcl-2 mRNA levels ($r = -0.78$).

### T-Cell Numbers and Bcl-2 mRNA Levels in Posttransplant Life-Threatening Infections

Thirteen of the 121 recipients undergoing LDLT deceased, all of severe infection apart from two cases due to operative technical errors. Fig. 3 shows the time courses of three representative recipients who died of infectious complications. All displayed Bcl-2 overexpression was consistent with marked reductions in T-cell numbers. These recipients did not experience homeostatic restoration of T-cells.

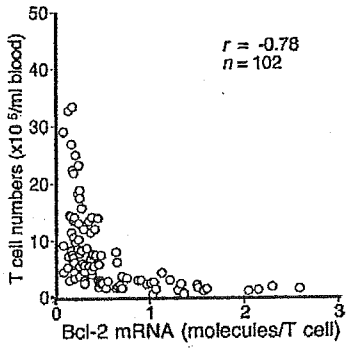


FIG. 2. Number of peripheral T-cells *versus* Bcl-2 mRNA levels at all observation times in 25 recipients.

First, a 53-year-old man underwent LDLT under HCC with HBV-liver cirrhosis. The ABO combination was incompatible. Bcl-2 mRNA levels increased within 24 h of transplantation to 0.67 molecules/T-cell, and T-cells decreased drastically to $0.44 \times 10^5$ cell/ml blood (Fig. 3, left). The Bcl-2 mRNA level remained elevated at 0.83–1.60 molecules/T-cell for 28 days, and T-cells remained low at $0.2–0.5 \times 10^5$ cells/ml blood for 14 days, followed by a slight increase to $1.6 \times 10^5$ on postoperative day 28. The condition of this recipient was complicated by cerebral bleeding at 7 days post-transplantation and by gastrointestinal bleeding at 13 days. At about 20 days, a fever (39°C) developed, with leukocytosis (>30,000 cells/$\mu$l) and elevated C-reactive protein (CRP, 17.9 mg/dl). The patient died of multiple organ failure on postoperative day 37, with bacterial infections.

Second, a 64-year-old man underwent LDLT for liver cirrhosis (HCV). His Bcl-2 mRNA levels exceeded 0.75 molecules/T-cell for about 2 months from 8 days post-operative, consistent with a marked decrease of T-cells to less than $0.5 \times 10^5$/ml (Fig. 3, middle). The patient's condition was complicated by bacterial infection, with a high bilirubinemia (>30 mg/dl), leukocytosis (>10,000/$\mu$l), high CRP level (10.0 mg/dl), fever (38°C), and septic shock. He died of multiple organ failure 63 days after transplantation.

Third, a 38-year-old female underwent LDLT for subacute fulminant hepatitis. The Bcl-2 mRNA concentration in T-cells increased markedly to 1.24 molecules/T-cell, accompanied by a decrease in T-cell numbers to $0.25 \times 10^5$ cells/ml blood at postoperative day 14 (Fig. 3, right). At 28 days postoperative, the Bcl-2 mRNA concentration had fallen to a normal level, with a slight increase of T-cells to about $2.8 \times 10^5$ cells/ml blood. However, hepatic function had deteriorated seriously; the bilirubin level was as large as 19.3 mg/dl. A retransplantation (Re. Tx.) was performed. Within several days the Bcl-2 mRNA concentration increased rapidly to 2.27 molecules/T-cell, and the T-cell population fell markedly to $0.46 \times 10^5$ cells/ml blood. The patient's clinical conditions were as follows: bilirubin, 22.8 mg/dl; leukocytosis, 42,200/$\mu$l; fever, over 40°C; CRP, 4.5 mg/dl. Two weeks after retransplantation, she died of severe pulmonary infection and multiple organ failure associated with septic shock.

## DISCUSSION

### Characteristics of LDLT-Induced T-Cell Depletion

Homeostatic regeneration of T-cells after peripheral T-cell depletion is impaired in patients severely infected with human immunodeficiency virus (HIV)-1 [12], following bone marrow transplantation [13, 14], and after intensive chemotherapy and irradiation for residual neoplastic disease [15, 16]. The profound depletion of T-cells observed after liver transplantation is markedly different from these depletions, however. Based on the present study, we propose that the following mechanism underlies the depletion of T-cells immediately after liver transplantation. First, large numbers of donor passenger leukocytes migrate into the recipient secondary lymphoid organs, lymph nodes, and spleen. The activated T-cells, which differentiate into effector T-cells capable of immediate effector function, migrate into the graft where they react with the large size of liver allograft and its large component of passenger leukocytes. This leads to overstimulation of recipient T-cells which end up dying by apoptosis due to Fas (CD95)–FasL interactions, termed activation-induced apoptosis [17, 18].

The lowest level of T-cell-depletion at 12 h postoperative, for instance, varied considerably between the three groups, from 0.26 to $23.44 \times 10^5$ cells/ml blood. However, the extent of T-cell depletion in all three groups was approximately 30% of pretransplant levels (Fig. 1). Alloreactive T-cells are known to constitute about 1–10% of all T-cells, and the present value was prominently high. The total T-cell numbers of the three groups before transplantation were 28–80% of healthy individuals ($14.7 \times 10^5$), indicating that a considerable number of the recipients' peripheral T-cells had accumulated in the periportal field due to liver disorders such as primary biliary cirrhosis or chronic hepatitis C [19, 20]. This may be why the three groups displayed high proportions of alloreactive T-cells.

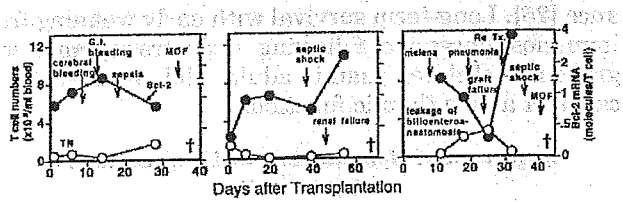


FIG. 3. Variation with time of T-cell numbers and Bcl-2 mRNA levels in three typical recipients who died after transplantation. MOF: multiple organ failure; G.I. bleeding: gastrointestinal bleeding; Re. Tx: retransplantation; †: deceased.

### Homeostasis-Driven T-Cell Regeneration in the Three Groups

We found a marked difference in the pattern of homeostatic restoration of T-cells following T-cell depletion in the three groups (Fig. 1). It is likely that the homeostatic restoration following T-cell depletion in Group I recipients took place promptly through the export of large numbers of recent thymic emigrant T-cells (RTEs) into the periphery. In contrast, in recipients in Groups II and III there was gradual restoration of T-cell number after depletion, coupled with a slowing down or even cessation of naive T-cell export from the thymus, leaving an expanded population of new memory cells.

The T-cell depletion following graft reperfusion causes a deficit that induces homeostatic proliferation [21, 22]. The thymus plays a key role in the repopulation of the various T-cell subsets. In particular, the regeneration of $CD4^+$ T-cells and of naive T-cells depends on thymopoiesis [22, 23]. Because of progressive thymic involution, adult patients show a persisting $CD4^+$ lymphopenia. Various factors affect T-cell regeneration after T-cell depletion by LDLT. Humans suffer an age-associated decline in thymic function that begins as early as young adulthood and contributes considerably to a decrease in the number of naive $CD4^+$ T-cells [23].

Also, various immunosuppressive measures inhibit T-cell proliferation by enhanced apoptosis or cell-cycle inhibition. It has long been known that circulating lymphocytes are markedly reduced by high doses of prednisone in man [24]. However, recently prednisone has generally been administered at its minimal dose (less than 0.3 mg/kg) as a daily maintenance level in view of the steroid-enhancing effect of tacrolimus, and the prednisone levels involved in the present study did not have a significant effect on T-cell counts.

Most importantly, T-cell diversity and population size should be maintained simultaneously. So long as export of RTEs in the periphery continues, the T-cell receptor (TCR) repertoire in Group I recipients remains diverse and the pool size is maintained. In contrast, reliance on peripheral expansion in Group II and III recipients must cause a progressive loss of repertoire diversity, leading to possible impairment of immune response. Intact functioning of the thymus is also necessary for rapid and stable induction of tolerance [25]. Long-term survival with early weaning from immunosuppression following liver transplants was greater in children than in adults [26], apparently because of active thymic functions

### Regulation of Homeostatic T-Cell Regeneration by Bcl-2 mRNA

In the present study, homeostatic regeneration of peripheral T-cells was inhibited when Bcl-2 mRNA levels increased to more than 0.5 molecules/T-cell (Fig. 2). This suggests that high levels of Bcl-2 expression inhibit progression of the cell cycle. On the other hand, although IL-2 produced by activated T-cells acts as the primary progression factor in the mid $G_1$ phase [4, 27], IL-2-production in all subsets except effector/memory T-cells was similarly inhibited after LDLT in our three groups (data not shown).

Under suboptimal growth conditions, Bcl-2 promotes exit into quiescence and delays entry of cells from quiescence into the usual cycle. Conversely, when Bcl-2 expression had fallen to normal levels or below, the T-cells of transplant recipients underwent homeostatic proliferation. Down-regulation of Bcl-2 levels in T-cells could shorten the $G_0 \rightarrow$ S-phase transition time, allowing the cell to progress rapidly through the rest of the cycle [4]. The return of overexpressed Bcl-2 to normal levels was associated with homeostatic restoration of T-cells.

In normal individuals the turnover of naive T-cells is slow compared with the memory pool, in which cells turn over more rapidly [28]. It has recently been suggested that naive T-cells undergo homeostatic proliferation, which is driven by low-affinity interactions with self-MHC molecules loaded with self-peptides [29, 30]. Unlike naive T-cells, the memory pool no longer requires continuous contact with self-antigen-stimulation for survival. The memory T-cell compartment is maintained independent of antigens by cytokine-stimulated or antigen-stimulated proliferation [31]. Hence, although the mechanisms controlling homeostatic proliferation of naive and of memory cells are fundamentally different, T-cells must in both cases proliferate by progressing through the cell cycle.

Under conditions of T lymphocytopenia, the TCR signals and cytokine- or antigen-stimulation evidently cause the T-cell cycle to progress through down-regulation of Bcl-2, resulting in substantial replenishment of the T-cell pool. It is therefore likely that subtle up- or down-regulation in the level of Bcl-2 is central to any commitment to entry into the cell cycle in homeostasis-driven regeneration of naive and memory T-cells.

### T-Cell Depletion and Bcl-2 mRNA in Posttransplant Life-Threatening Infection

It has been suggested that depletion of circulating lymphocytes contributes to lethality due to sepsis [32]. However, the immune response to sepsis in liver transplant recipients is very different from that in most other septic patients. All 13 deceased recipients are characterized by profound depletion and Bcl-2 mRNA overexpression of peripheral T-cells (Fig. 3). The immunosuppressive cascade has a greater—and catastrophic—effect on transplant recipients than on conventional septic patients. They developed serious infections and could not begin the homeostatic restoration of T-cells.

Recipients at this stage are therefore susceptible to viral, fungal, and bacterial infections; the longer this stage lasts and the greater the T-cell depletion, the greater the vulnerability.

## CONCLUSIONS

T-cell numbers decreased drastically within 6 h of graft reperfusion (Fig. 1). In Group I, the depleted T-cell numbers returned to normal within 5 days, whereas in Group III the restoration of T-cells to normal took much longer. The absolute number of $CD4^+$ naive cells greatly increased in Group I, but only a slight increase was observed in Group III. In Group II these changes were intermediate between Group I and III. Since the $CD4^+/CD8^+$ ratio was higher than in other groups (Table 2), the prompt restoration of the T-cells in Group I may be due to an export of RTEs into the periphery [22]. Groups II and III, in contrast, may show persistent $CD4^+$ lymphopenia, which depends on progressive thymic involution. Fig. 2 shows that the peripheral T-cell number and the Bcl-2 mRNA level are negatively correlated. All of the deceased patients were characterized by profound depletion and Bcl-2 overexpression by peripheral T-cells (Fig. 3).

It is therefore likely that regulation of the level of Bcl-2 is crucial for the regeneration of naive and memory cells. For successful liver transplantation it would therefore be necessary to stimulate homeostatic restoration of T-cells as early as possible. Monitoring of the number of peripheral T-cells and of Bcl-2 expression holds great therapeutic potential for showing the balance between immunosuppression and homeostatic regeneration of T-cells, and for identifying the subgroup of liver transplant recipients who face an increased risk of infection.

## ACKNOWLEDGMENTS

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分担研究報告書

# 生体肝移植後のＣ型肝炎再発予防を目指したステロイド剤不使用による免疫抑制療法に関する研究

分担研究者　木内 哲也　名古屋大学大学院医学系研究科 病態外科学講座教授

研究要旨：　分担研究者の施設で行われた HCV 関連疾患に対する肝移植治療症例を対象に、周術期の血中ウイルス動態と移植肝における生化学的/組織学的 HCV 肝炎再燃の動態を解析し、個々の病態と免疫抑制強度、および免疫抑制剤選択の影響、さらに抗ウイルス治療の効果を検討した。

## A. 研究目的

本邦における 4,000 例近い生体肝移植症例のうち、2,000 例以上が成人に対して行われており、Ｃ型肝炎ウイルス(HCV)関連の肝硬変・肝細胞癌症例はその約４分の１を占めている。健康保険適用拡大後は、年間 100 例以上の HCV 陽性症例が、生体ドナーを中心とした肝移植を受けていると推定される。

欧米の脳死全肝移植における経験と同様に、肝移植後早期のウイルス血症再燃はほぼ必発であり、移植肝感染による急性肝炎を経て慢性化し、放置すると自己肝の場合よりも速い経過で肝硬変・肝不全へと進行する。一方で、肝繊維化の進行速度や、胆汁鬱滞型の急性増悪は生体肝移植で高い可能性が示唆されている。

本研究では、HCV 関連疾患に対する生体肝移植治療の周術期における末梢血液中 HCV 動態と生化学的および組織学的肝炎再発の動態を解析し、班研究の主目的である「ステロイド剤不使用による免疫抑制療法」を含め、免疫抑制剤の選択や使用量、さらに抗ウイルス療法の介入によって移植後Ｃ型肝炎再発制御の可能性を探ることを目的とした。

## B. 研究方法

平成 15 年６月より平成 18 年１月までの期間に分担研究者の施設にて行われた HCV 抗体陽性の肝硬変に対する肝移植症例 14 例を対象に、周術期から慢性期における血中 HCV-RNA

動態と生化学的および組織学的肝炎再発の経過、さらに抗ウイルス療法の効果を検討し、免疫抑制療法の選択と病態の影響を検討した。症例は男性 9 例女性 5 例で、年齢中央値 58.3(49.6-62.3)歳、9 例に肝細胞癌を合併、2 例が血液型不適合移植、1 例の脳死全肝移植の他は肝右葉を用いた生体肝移植であった。移植前 CPT score、MELD score の中央値はそれぞれ 12 (6-12)、18.8 (7.1-28.1)、移植後追跡期間中央値は 5(0-23)か月であった。

14 例中 8 例はタクロリムス(Tac)±リンパ球合成阻害剤、6 例はシクロスポリン(CsA)で免疫抑制導入を行った。

(倫理面への配慮)

当該施設における生体肝移植および脳死肝移植は、医学部倫理委員会の監視の元にそれぞれの適応判定委員会の承認を受け、さらに「ステロイド剤不使用による免疫抑制療法」については、附属病院治験管理委員会へ申請し、承認を得た。

C. 研究結果

現時点で、「ステロイド剤不使用による免疫抑制療法」についての無作為対照試験に組み入れられる症例はなかった。移植後に 4 例を劇症型急性拒絶(2 週)、誤嚥に起因する難治性肺炎(3 か月)、インターフェロン(IFN)治療に伴う慢性拒絶(4 か月)、胆汁鬱滞性 HCV 再燃に伴う敗血症(4 か月)で失った。

14 例の HCV の内、2 例が genotype 2a、他は 1b であった。移植前の血中 HCV-RNA 量は中央値 464 (2.7-4240) KIU/ml、その後の経過は移植術中無肝期 10.4 (0.5 未満-54) KIU/ml、1 日目 0.9 (0.5 未満-31) KIU/ml、7 日目 20 (0.5 未満-4,650) KIU/ml、1 か月では 1,690 (0.5 未満-10,000) KIU/ml, 2 か月(未治療例) 9,780 (86-42,100) KIU/ml、3 か月(未治療例) 2,955 (325-30,600) KIU/ml と変化した。血中の HCV-RNA は移植後 1.5(1-50)日で陽性となり、42 (3-109)日で最高値 6,010 (49-46,000) KIU/ml を示した。

移植前の血中 HCV-RNA が少ないほど無肝期のウイルス量は減少し、移植後の再出現も遅れる傾向が見られた。免疫抑制強度の強い血液型不適合症例では、ウイルス血症出現もまたその後の増殖も速い傾向が認められ、Tac と CsA では初期のウイルス増加は前者が大きいが、慢性期のウイルス量は後者が大きい傾向が認められた。また、脳死全肝移植症例では、移植前ウイルス量も少なかったが、血中ウイルス検出時期が 50 日と大きく遅延した。

大量ステロイド投与による拒絶治療を要した症例がほとんどなかった

ため、そのウイルス動態への影響は明らかでなかったが、移植肝の機能が著しく低下した症例では、血中のウイルス増殖も抑制される傾向が認められた。

血中にウイルスが再出現してから少し遅れて ALT 優位の肝機能異常が出現し、1か月程度で組織学的な急性肝炎(A1-2)が認められ、その後個々の速度で繊維化が緩徐に出現した。

胆汁鬱滞型再燃の場合を除いて、F1以上の繊維化が確認され、血中ウイルス量のピークを過ぎたことを確認してから IFN/リバビリン(RVB)による抗ウイルス療法を行った。結果的に5例に中央値 97 (52-270)日で治療が開始され、ウイルス学的反応は全例に認められたが、早期に治療を開始した1例に慢性拒絶を合併した。3例で HCV-RNA が陰性化したが、内1例は再出現した。治療終了時の陰性化確認例はまだない。

D. 考察

現在までの結果から、移植前重症肝不全症例において移植後に強い免疫抑制療法が行われた場合の胆汁鬱滞型 HCV 再燃のリスクが改めて示唆された。また、同時に移植後早期の IFN 治療に伴う難治性拒絶発生のリスクも確認された。

肝移植の術中においては、血中の HCV-RNA が出血などの因子によって減少した後、最大のウイルス源である自己肝が取り除かれた後、緩やかな自然減衰を示し、移植肝再灌流時に残ったウイルスが移植肝に吸い込まれてさらに減少し、見かけ上短時間の血中消失期を経て(移植肝再灌流時にウイルス量の多い場合には消失に至らず)、移植肝の中で増殖したウイルスがその後急速に血中に放出され、この次点で移植肝の再感染が起こることが示唆された。移植肝への吸収速度や移植肝内でのウイルス増殖速度には、相当の個体差があり、移植肝の機能や大きさも関与していることが推測され、また、脳死肝移植では生体肝移植に比べてそれが遅れることが示唆された。

肝移植後ステロイド剤不使用の影響に関しては情報が得られなかったが、強い免疫抑制がウイルス動態に影響を与える可能性、また、カルシニューリン抑制剤(Tac, CsA)の選択が、生化学的/組織学的再燃は別として、少なくとも肝移植周術期の血中ウイルス動態には影響を与える可能性が示唆された。

肝移植後生化学的・組織学的 HCV 肝炎再燃後の IFN/RVB 治療は、脾機能亢進症状の遷延下でも、ウイルス学的には少なくとも一定の効果を示すことが示唆されたが、血中ウイル

ス陰性化持続状態(sustained viral response)の獲得までに至ることがどの程度可能であるかについては明らかにならなかった。

E. 結論

HCV 関連疾患に対する生体肝移植後において、移植肝の HCV 肝炎再燃速度は速く、移植前重症例や免疫抑制強度の強い症例はよりハイリスクとなる。一方で、早期 IFN 治療に伴う難治性拒絶合併の危険は、頻度は高くないもののひとたび発生すると重篤化しやすい。肝移植術前の血中ウイルス量と術中無肝期の残存ウイルス量、さらに移植直後の血中からいったん消失する時間と肝内で増殖したウイルスが血中に放出されるタイミングとは、互いに密接に関係している可能性がある。移植後の免疫抑制療法の選択のみならず、こうしたウイルス動態に基づいた周術期の多面的対応を行うことが、移植肝における HCV 再燃制御の鍵となる可能性が示された。

F. 健康危険情報

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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

別紙４

研究成果の刊行に関する一覧表レイアウト

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# Initial Dosage Adjustment for Oral Administration of Tacrolimus Using the Intestinal MDR1 Level in Living-Donor Liver Transplant Recipients

S. Masuda, M. Goto, M. Okuda, Y. Ogura, F. Oike, T. Kiuchi, K. Tanaka, and K. Inui

ABSTRACT

The role of intestinal P-glycoprotein (encoded by the *MDR1/ABCB1* gene) and/or metabolic enzyme CYP3A4 for tacrolimus therapy was examined in recipients of living-donor liver transplantation (LDLT), under the hypothesis that these proteins are factors for pharmacokinetic variability. The intestinal mRNA expression level of MDR1 and CYP3A4 was evaluated by real-time polymerase chain reaction (PCR), using the upper jejunum from a part of the Roux-en-Y limb for biliary reconstruction at LDLT. For 7 days postoperatively, good inverse correlation was found between the tacrolimus concentration/dose (C/D) ratio and the intestinal mRNA level of MDR1 ($r$ 0.776), but not of CYP3A4 ($r$ 0.096), in the 46 cases. After classifying the patients according to median of the intestinal MDR1 mRNA expression, the oral dose of tacrolimus in the high-MDR1 group was approximately twofold higher than in the low-MDR1 group ($P$ .001), whereas its trough level was similar between the two groups. In addition, the correlation between the intestinal MDR1 mRNA level and the tacrolimus C/D ratio was confirmed with a larger population ($r$ 0.645, n 104). Using the regression line between the intestinal MDR1 mRNA level and tacrolimus C/D ratio, we could prospectively predict the individual C/D ratio of tacrolimus immediately after LDLT. Known genetic variations of the *MDR1* gene had no effect on intestinal MDR1 mRNA level and tacrolimus C/D ratio in LDLT patients. This suggests that the intestinal mRNA level of MDR1 is a useful molecular marker for determination of the personalized oral dose of tacrolimus in recipients of LDLT immediately after surgery.

LIVING-DONOR liver transplantation (LDLT) with the immunosuppressant tacrolimus is a principal life-saving therapy for patients with end-stage liver failure. Because the success of the transplantation depends on a delicate balance between immunosuppression and rejection, the maintenance of adequate levels of blood tacrolimus is critical. Tacrolimus is principally metabolized by cytochrome P450 (CYP) 3A subfamilies in the liver. Recently, the contribution of active secretion by P-glycoprotein (the product of the *MDR1/ABCB1* gene) and the metabolism by CYP3A expressed in enterocytes have been acknowledged as factors influencing the bioavailability of tacrolimus. In the present study, we examined the role of intestinal P-glycoprotein and/or CYP3A4 for tacrolimus therapy in LDLT recipients, under the hypothesis that these proteins are factors in pharmacokinetic variability.

## MATERIALS AND METHODS

The upper jejunum was obtained from a part of the Roux-en-Y limb for biliary reconstruction at LDLT.[1] The intestinal mRNA expression level of MDR1 and CYP3A4 was evaluated by real-time

From the Department of Pharmacy, Kyoto University Hospital, Kyoto University, Kyoto, Japan (S.M., M.G., M.O., K.I.); and Department of Transplantation and Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan (Y.O., F.O., T.K., K.T.).

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Address reprint requests to Dr Ken-ichi Inui, Department of Pharmacy, Kyoto University Hospital, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan.E-mail: inui@kuhp.kyoto-u.ac.jp.

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