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ORIGINAL ARTICLE

Takashi Orii
Nobuhiro Ohkohchi
Susumu Satomi
Yo Hoshino
Hiroshi Kimura

Decreasing the Epstein-Barr virus load by adjusting the FK506 blood level

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T. Orii (✉) · N. Ohkohchi · S. Satomi
Division of Advanced Surgical Science and Technology, Department of Surgery, Graduate School of Medicine, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
E-mail: orii-ltx@ya2.so-net.ne.jp
Tel.: +81-22-2753111
Fax: +81-22-2344194

T. Orii
Department of Surgery, Sendai Shakaihoken Hospital, Sendai, Japan

N. Ohkohchi
Department of Surgery, Institute of Clinical Medicine, Tsukuba University, Tsukuba, Japan

Y. Hoshino · H. Kimura
Department of Pediatrics, School of Medicine, Nagoya University, Nagoya, Japan

Abstract To prevent post-transplant lymphoproliferative disease (PTLD), the viral load must be diminished before the symptoms of Epstein-Barr virus (EBV) infection appear. Twenty-three consecutive liver transplant recipients were entered into our study to identify the characteristics of post-transplant EBV-infected patients and to clarify the correlation between the FK506 blood level and EBV load. After transplantation, EBV-DNA appeared more frequently in patients who had been seronegative before transplantation than in seropositive patients (10/13 versus 1/10; $P = 0.0014$). As for rejection, resistance to steroid pulse therapy, and FK506 trough level, there were no significant differences between patients with and without EBV infection. In patients with primary EBV infection after transplantation, there was a strong correlation ($r = 0.681$) between the FK506 level and the viral load. In liver transplant recipients, especially in those seronegative for EBV, it is necessary to check the viral load by polymerase chain reaction (PCR) carefully after liver transplantation, before any symptom appears.

Keywords Liver transplantation · Epstein-Barr virus · Post-transplant lymphoproliferative disease · Real time polymerase chain reaction · FK506 (tacrolimus)

Introduction

Post-transplant lymphoproliferative disease (PTLD), which is mainly caused by Epstein-Barr virus (EBV) infection [21], is one of the most intractable diseases affecting patients under immunosuppressive therapy after solid organ transplantation. It was reported that EBV infection was likely to occur in pediatric patients [13, 21], seronegative patients [14, 23], patients treated with FK506 [17, 27] and patients treated during steroid-resistant rejection with OKT3, antilymphocyte globulin, or antithymocyte globulin [12, 22] as a rescue therapy. Once monoclonal proliferation of EBV-infected B cells progresses to non-Hodgkin malignant lymphoma, it is necessary to suspend immunosuppressants and to administer anticancer agents. However, even if it remits, severe rejection would occur and lead to graft failure. To prevent PTLD, it is necessary to control EBV infection,

but there are few reports showing that the viral load decreases with diminishing FK506 blood levels. In this study we evaluated the correlation between FK506 trough levels and the amount of EBV-DNA in patients with EBV infection. We also investigated the possibility of controlling EBV infection.

Patients and methods

Of the 27 consecutive pediatric patients who had survived for more than 1 year after living-donor liver transplantation (LDLT) carried out from July 1991 to December 1999 at Tohoku University Hospital, we examined 23 who were followed up in accordance with our strategy to control EBV infection. Serological examination of EBV was performed pre-operatively in the donor, and pre-operatively and every 3 months postoperatively, in the recipient. The diagnosis of EBV infection was established, based on seroconversion in the case of primary infection, or a more than fourfold elevated EBV-capsid antigen (VCA) IgG antibody (titer > 1:2560) or a recently converted antibody for early antigen (EADR) (titer > 1:20) in the case of reactivated infection. In the EBV-infected patients, we performed quantitative real-time polymerase chain reaction (PCR) [9] using EBV-DNA from peripheral blood mononuclear cells (PBMNC) every 3 months to monitor changes in the amount of EBV and to assess the effect of antiviral therapy [16]. Computerized tomography (CT) was performed from neck to pelvic cavity to confirm presence/absence of tumorous lesions, if necessary. We diagnosed the patients with swollen lymph nodes on the body surface detected by palpation, or with tumorous lesions detected by CT, as having PTLD. EBV-infected patients were treated with intravenous injections of ganciclovir (GCV) (10 mg/kg/day) or orally administered acyclovir (ACV) (1,500 mg/m^2/day). As a general rule, GCV was administered for 6 weeks, and ACV until reduction of EBV-DNA to levels below $10^{2.5}$ copies/μg DNA. Immunosuppression was begun with cyclosporine (CsA), azathioprine and methylprednisolone (MP), or FK506 and MP. MP was stopped 1 year after LDLT in the case of patients treated with FK506, or after 2 years in the case of patients treated with CsA, of those with an incompatible blood type or of those showing a positive T-lymphocyte crossmatch. Patients diagnosed as having rejected the graft, clinically or histopathologically, underwent steroid pulse therapy; but, among them, those with steroid-resistant rejection were treated with deoxyspergualin (DSG) or OKT3. The target FK506 trough level in the blood was 15–20 ng/ml within the first week after administration of LDLT, 12–15 ng/ml until the following week, 10–12 ng/ml until the third month, 8–10 ng/ml in the following 3 months, 5–10 ng/ml during the following 6 months, and under 5 ng/ml thereafter. The concentration of FK506 in the blood was measured by tacrolimus II microparticle enzyme immunoassay (MEIA II; Abbott Laboratories, Chicago, Ill.).

We calculated the correlation coefficient using Pearson's method, and compared observed proportions using the chi-squared test, Student's unpaired *t*-test or simple regression analysis. Calculations were done with Stat-View 4.51 software; differences of $P < 0.05$ were considered statistically significant.

Results

Post-transplant EBV-infected patients were compared with EBV non-infected patients who underwent LDLT during the same period, with regard to gender, age, conversion to positive in EBV serology before transplantation and initial immunosuppressant (Table 1).

All donors were seropositive for EBV before LDLT. Among the 11 patients who were seropositive preoperatively, only one (9.1%) developed EBV infection; on the other hand, nine of the 12 (75.0%) patients who were negative for EBV preoperatively suffered from primary EBV infection. The risk of EBV infection after LDLT was significantly high in patients who were seronegative preoperatively ($P = 0.0014$). Immunosuppression was begun with FK506 in eight of ten infected patients and in 12 of 13 non-infected patients. Three of ten EBV-infected patients, who had swollen lymph nodes in the mediastinum or retroperitoneum as confirmed by CT, we diagnosed as having PTLD, although we had no histopathological proof, because it was dangerous to excise the lymph nodes deep in the body. The lymph nodes disappeared or shrank during the administration of antiviral agents or when the dose of FK506 was tapered. Two of three patients with PTLD underwent excisional biopsies of some swollen lymph nodes in their neck, which were found to correspond to non-specific lymphadenitis, by histopathological examination. There were no cases of mortality associated with PTLD. The dose of immunosuppression was not decreased after the diagnosis of EBV infection except for the patients with PTLD.

The FK506 trough levels, rejection episodes, rescue therapy for the patients with rejection, and resistance to steroid pulse therapy are shown in Table 2. There were no significant differences in the FK 506 trough levels at any determination point between EBV-infected and non-infected patients. Six EBV-infected patients and five EBV non-infected patients experienced graft rejection. Five EBV-infected patients and five EBV non-infected patients were treated with MP. DSG was administered to three (30%) EBV-infected patients, and one (7.7%)

Table 1 Characteristics of EBV-infected and non-infected patients after liver transplantation (*LT* liver transplantation)

Characteristic	Post-transplant EBV infection		*P*
	(+)	(−)	
Number of patients	10	13	
Gender			
Male	3	6	
Female	7	7	
Age	7 Months–5 years (1.7 years ± 1.3)	9 Months–17 years (7.9 years ± 6.4)	0.0138
Seropositive before LT	1/10 (10%)	10/13 (76.9%)	0.0014
Seronegative before LT	9	3	
Immunosuppressant			
FK 506	8/10 (80%)	12/13 (92.3%)	
CsA	1 (1[a])	1	

[a]Conversion to FK 506 before postoperative seroconversion

Table 2 Concentration of FK 506 and immunological conditions in EBV-infected and EBV non-infected patients

Postoperative month	Post-transplant EBV infection		*P* value
	(+) Trough level of FK 506 [ng/ml]	(–) Trough level of FK 506 [ng/ml]	
1	11.76 ± 4.25	9.92 ± 3.04	0.329
3	8.09 ± 1.28	8.63 ± 2.71	0.6
6	7.96 ± 1.87	6.84 ± 1.56	0.212
12	6.22 ± 1.12	6.95 ● 1.85	0.375
Rejection episodes			
(+)	6/10 (60%)	5/13 (38.5%)	0.305
(–)	4	8	
Rescue therapy			
(+)	5/10 (50%)	5/13 (38.5%)	0.58
(–)	1	0	
Steroid-resistance			
(+)	3/10 (30.0%)	1/13 (7.7%)	0.162
(–)	2	4	

EBV non-infected patient with steroid-resistant rejection, but the difference was not significant ($P = 0.161$).

Figure 1 shows the changes in the amount of EBV-DNA in PBMNC, as measured by real-time PCR, in relation to the FK506 trough levels in eight (patients a, b, c, d, e, f, g and h) EBV-infected patients. Only patient h was seropositive to EBV preoperatively. In patients b and c, the FK506 levels and EBV-DNA changed in parallel. In the other patients, the changes in EBV-DNA generally corresponded to the FK506 level. The maximum EBV load in patients b, c and g, who were diagnosed as having PTLD, was $10^{3.88} \pm 10^{0.63}$ copies/μg DNA, which was not significantly different from that in other patients ($10^{3.33} \pm 10^{0.68}$ copies/μg DNA).

Figure 2 shows the strong positive correlation ($r = 0.681$, $P < 0.0001$) between the amount of EBV-DNA and the trough level of FK 506 in the same blood sample obtained every 3 months after diagnosis of EBV infection, in seven patients who had been seronegative before transplantation. The regression line was calculated as follows:

$$\log_{10}(EBV - DNA) = 1.83 + 0.24 \times (\text{FK506 trough level.})$$

The compatibility of this regression function was found to be significant by simple regression analysis ($P < 0.0001$); $10^{2.5}$ copies/μg DNA (open square along the ordinate), represents the cut-off value of symptomatic patients with EBV infection [9], and corresponds to 2.79 ng/ml of the FK506 trough level (open square along the abscissa).

Discussion

Post-transplant EBV infection, that does not relate to rejection, rescue therapy, or steroid-resistant rejection [14, 22], was reported to depend on age [13] and seropositivity [14, 20] for EBV at transplantation. In general, as people age, their chances of being infected by EBV increase; therefore, in our study, the age of EBV-infected patients after liver transplantation was significantly higher than that of EBV non-infected patients. Moreover, the FK506 trough levels within 1 year after transplantation did not differ significantly between EBV-infected and EBV non-infected patients. These results suggested that pre-transplant seronegativity was the most risky factor for post-transplant EBV infection.

EBV, which always infects B cells expressing CD21 (CR2, B2), the receptor of EBV [25], induces immortalization of the B cells. Latently, EBV-infected B cells (EBV-B cells) are found in the blood and lymphoid organs of the vast majority of adults, and are restricted by EBV-specific cytotoxic T lymphocytes (EBV-CTLs) and natural killer (NK) cells, because the proteins of EBV – EBNA 2 and LMP 1 – act as strong antigens in normal and healthy immune individuals [7, 10]. However, immunosuppressants such as FK506 inhibit these CTLs and NK cells, contributing thereby to the expansion of EBV-B cells and the development of polyclonal or oligoclonal lymphoma [6, 8]. Furthermore, monoclonal proliferation of the EBV-B cells would progress to non-Hodgkin malignant lymphoma. Therefore, it is important to diagnose primary and reactivated EBV infection in the early stage, that is, when only infection or proliferation of EBV is taking place [19], and to reduce immunosuppressants without allowing a severe rejection, which may lead to graft failure.

There are several reports showing that PTLD could be successfully treated by reducing the dose of immunosuppressants and administering antiviral agents in most patients [3, 4, 14, 27]. However, there are few reports demonstrating that the EBV load actually decreases when the blood level of immunosuppressants diminishes. In this study, we found a clear positive correlation between the FK trough level and the amount of EBV-DNA, although the seven seronegative patients investigated had different backgrounds; that is, they differed with regard to administration of steroids or other medications, presence/absence of other infections and/or complications, etc. Therefore, if patients with the same background were evaluated, the correlation coefficient (r) would be close to 1.0. At any rate, it is apparent that the FK506 blood level influences the EBV load. Baldanti et al. described a patient in whom the EBV load changed parallel to the level of FK506 [2], just as in the eight patients whose data are shown in Fig. 1.

According to the regression line shown in Fig. 2, $10^{2.5}$ copies/μg DNA of EBV-DNA, which was reported to be the average number of copies detected in symptomatic patients with EBV infection [9], corresponded to about 3.0 ng/ml of the FK506 trough levels in patients who

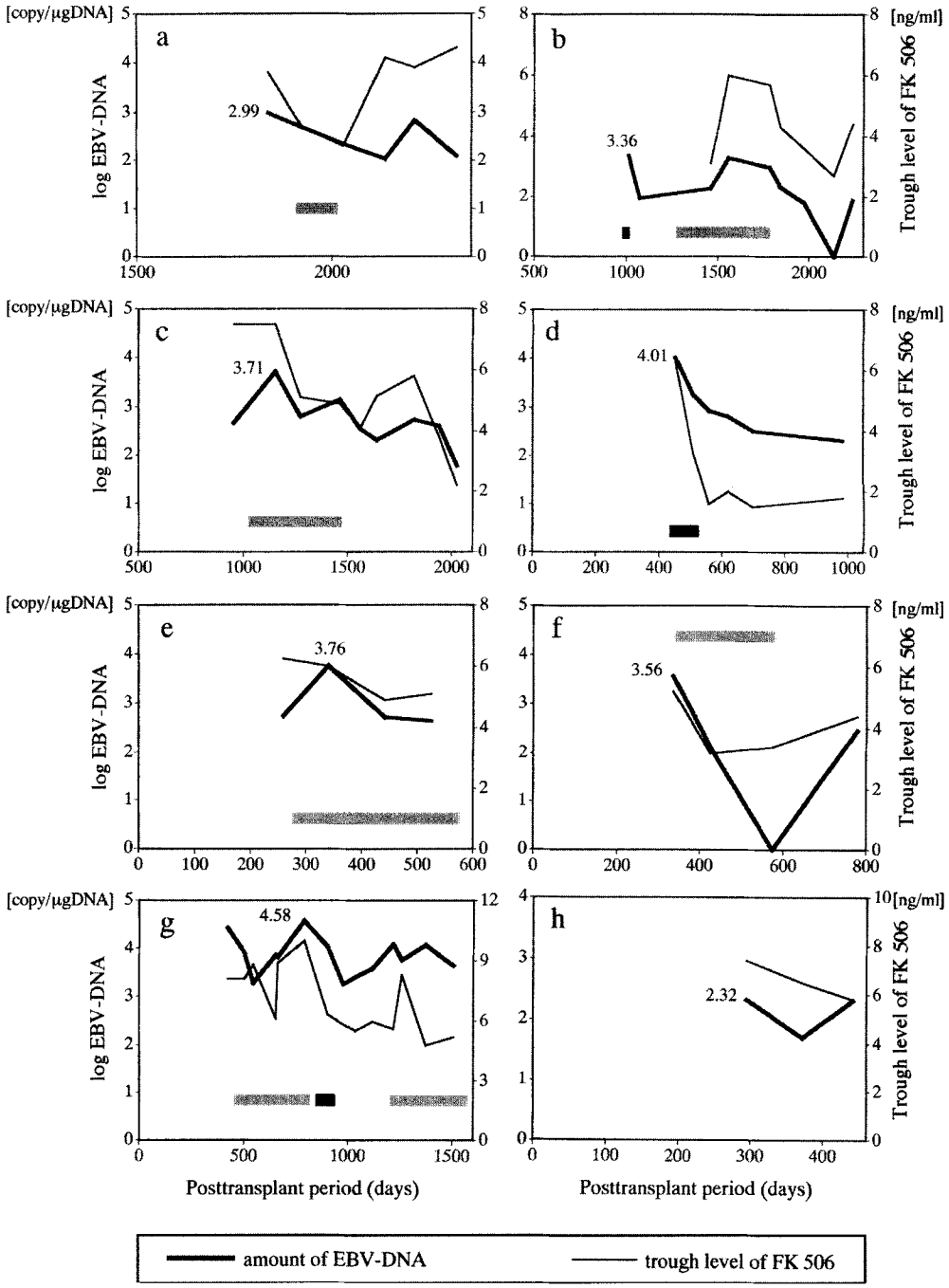

Fig. 1 Changes in the amount of EBV-DNA and the trough level of FK 506. *The letters (a, b, c, d, e, f, g and h) at the top of each graph* correspond to the patients described in the text. *The left and right ordinates of each graph* represent the logarithm of the amount of EBV-DNA and the trough concentration of FK 506, respectively. *The abscissa* represents the time course after LDLT. *The value underlined in each graph* indicates the maximum level of EBV-DNA. *Black bar* Duration of treatment with ACV, *gray bar* duration of treatment with GCV

were seronegative before undergoing transplantation. In other words, symptomatic EBV infection would develop if the FK506 trough level were to increase to 3.0 ng/ml, which indicates that our protocol of immunosuppression may place patients at a high risk of developing symptomatic EBV infection within a year of liver transplantation. Thus, it is important to diagnose EBV infection in the early stage by regular check ups, followed by prompt suitable treatment.

When EBV infection becomes symptomatic, antiviral therapies using antiviral agents, such as ACV or GCV [4, 5, 11, 22], interferon alpha [15] and gamma-globulin [1] were reported to be effective to some extent; however, the efficacy of antiviral agents in our series was not confirmed, because the majority of EBV-infected B cells were shown to be in the immortalized phase, not in the lytic phase. Theoretically, the presence of an antiviral agent might prevent lytically EBV-infected B cells from

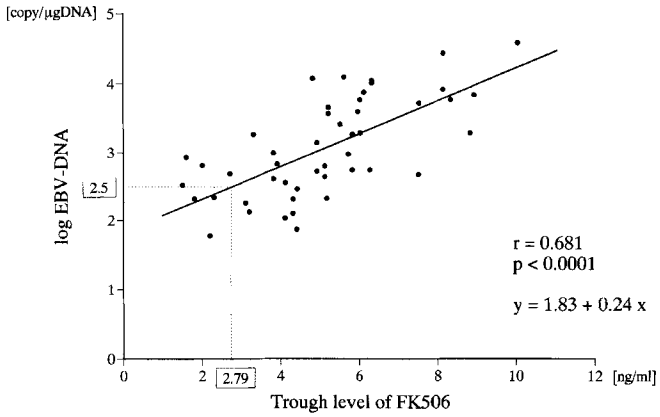

Fig. 2 Relationship between viral load in PBMNC and the FK506 trough levels in EBV-infected patients

undergoing replication of their DNA [26]. Therefore, in this study, we can conclude that there were no notable effects of ACV or GCV on the EBV load in PBMNC.

Recently, some treatments against EBV-infected B cells have been reported to prevent PTLD [24]. Reynaud-Gaubert et al. [18] reported that administration of anti-CD20 monoclonal antibody to a patient with EBV-associated B cell lymphoma of the nasopharynx occurring 6 months after lung transplantation, resulted in a complete response and was well tolerated, with minimal side effects. Zompi et al. [28] also reported that anti-CD20 monoclonal antibody (rituximab) was administered to two patients with polymorphic PTLDs, who underwent rapid complete remission without any side effects. Khanna et al. [8] developed a protocol to activate autologous EBV-specific CTL lines from patients with primary EBV-infection, and indicated that these CTLs consistently showed strong EBV-specificity in spite of concurrent immunosuppressive therapy; therefore, CTLs might be of value against PTLD, under a high dose of CTL-suppressive agents such as FK506 or CsA. These innovating therapies are expected to be more effective than the common therapies of today, but must be confirmed in a larger cohort of liver transplant recipients suffering from symptomatic EBV infection or PTLD.

In the present study, we demonstrated that reduction of the FK506 blood level was associated with a decrease of the EBV load after liver transplantation. In order to render EBV infection asymptomatic, the FK506 blood level must be kept under 3.0 ng/ml; at a lower blood level, however, it is liable to place the recipient at a high risk of rejection. Thus, it is necessary to check the viral load strictly for at least 1 year after liver transplantation.

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