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ORIGINAL ARTICLE

Pre-transplant risk factors predicting post-transplant cytomegalovirus infection in liver transplant recipients

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Summary

Cytomegalovirus (CMV) infection causes significant morbidity and mortality among transplant recipients. Although it is still not clear if a preemptive strategy is superior to a prophylactic strategy, many transplant programs elect for preemptive treatment for post-transplant CMV infection. In order to improve the preemptive strategy, we analyzed a series of liver recipients by means of quantitative real-time polymerase chain reaction (PCR). Ninety-one liver transplant recipients were monitored by real-time PCR for CMV, and the results were analyzed in terms of preoperative conditions. Multivariate analysis revealed fulminant hepatic failure as an underlying disease (odds ratio, 6.8; 95% CI, 1.2–39.2), while an ABO-incompatible graft (odds ratio, 5.0; 95% CI, 1.3–19.1), and a serological combination of the donor (D) being positive with the recipient (R) being negative for CMV (D+/R−) (odds ratio, 5.8; 95% CI, 1.3–26.0) were independently associated with the development of significant CMV infection. Patients with risk factors had higher peak CMV DNA concentrations than those without, and developed CMV infections faster ($P = 0.0002$). Screening of recipients according to risk factors and PCR monitoring may result in an optimization of the preemptive strategy.

Introduction

Cytomegalovirus (CMV) infection causes significant morbidity and mortality among transplant recipients [1–3]. Current therapeutic strategies for post-transplant CMV infection are generally classified into two categories, universal prophylaxis [4] and preemptive therapy [5–7]. In universal prophylaxis, antiviral agents are used for all transplant recipients. As this strategy inevitably resulted in the unnecessary exposure of uninfected patients to toxic agents, preemptive therapy was introduced. Antiviral agents are started for those who have laboratory findings indicative of active CMV infection after short-turnaround surveillance tests in preemptive therapy.

Although it is still unclear if the preemptive strategy is superior to the prophylactic strategy [8–10], many transplant programs use the preemptive one for post-transplant CMV infection [11]. The dilemma with the preemptive strategy lies in the balance between the increase in CMV disease and unnecessary treatment. As shown by The *et al.* [12], as well as Paya *et al.* [6], the preemptive strategy may be associated with an increase in CMV disease in comparison with universal prophylaxis. A recent systematic review of randomized controlled trials has demonstrated that in preventing CMV organ disease in solid organ transplant recipients, prophylaxis and preemptive strategies are beneficial, but only universal prophylaxis reduces bacterial and fungal infections and death [13].

In order to improve the preemptive strategy, we analyzed a series of liver recipients by means of quantitative real-time polymerase chain reaction (PCR). We found

several risk factors that might help to select liver recipients at risk for CMV infection or disease. CMV monitoring optimization was discussed using the risk factors.

Patients and methods

Patients

One hundred and thirty-two patients received a liver graft during the period from September 2000 to October 2001 at Kyoto University Hospital. Ninety-one of the recipients who had been evaluated for CMV infection by real-time PCR for every 4–10 days were analyzed in this study. The participants were 47 males and 44 females, and the median age at transplantation was 17.9 years (range, 0.1–67.5). Underlying diseases for transplantation were as follows: cholestatic liver diseases ($n = 35$) including biliary atresia, liver tumor ($n = 21$), liver cirrhosis ($n = 10$), fulminant hepatic failure ($n = 8$), metabolic diseases ($n = 7$), and others ($n = 10$). Among these patients, there were five patients with retransplantation. All patients received tacrolimus and low-dose corticosteroids as immunosuppressive agents, as previously described [14]. There was no significant difference in age, gender, underlying diseases, or clinical background between the original 132 recipients and the 91 target patients. For preemptive treatment of CMV infection ganciclovir 5 mg/kg/day was initiated, based on routine pp65 antigenemia performed weekly. Therapy was administered until CMV antigenemia was negative for a minimum of 14 days. The dose of ganciclovir was adjusted for creatinine clearance, if necessary. CMV serology of both the donor and the recipient was available for 77 of the 91 participants. CMV disease was defined as previously reported [15]. Treatment of CMV disease consisted of ganciclovir 5 mg/kg b.i.d. during 2 weeks, followed until clinical resolution by 5 mg/kg/day.

Nucleic acid extraction

DNA samples for the real-time PCR were prepared from peripheral blood collected in EDTA-Na_2-treated tubes. After erythrolysis, the leukocytes obtained were subjected to DNA extraction using a QIAamp Blood mini-kit (Qiagen, Tokyo, Japan) as reported by Tanaka *et al.*[16]. The concentration of the extracted DNA was determined by spectrophotometry at a wavelength of 260 nm, and a 250-ng aliquot was used for the subsequent PCR assay.

Real-time PCR assay

The target region of PCR was in the immediate early (IE) gene of CMV [16]. The upstream and downstream primer sequences were 5′-GACTAGTGTGATGCTGGCCAA-3′ and 5′-GCTACAATAGCCTCTTCCTCATCTG-3′, respectively, and a fluorogenic probe (5′-carboxyfluorescein-AGCCTGAGGTTATCAGTGTAATGAAGCGCC-3′) was selected in the target region. The reaction volume was 50 μl, and contained 10 mM Tris (pH 8.3), 50 mM KCl, 10 mM EDTA, 5 mM $MgCl_2$, 100 μM of dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 0.1 μM of fluorogenic probe, 1.25 U of AmpliTaq Gold (PE Applied Biosystems, Tokyo, Japan), and DNA samples as above. Following activation of AmpliTaq Gold for 10 min at 95 °C, 50 cycles of 15 s at 95 °C and 1 min at 62 °C were carried out with a model 7700 Sequence Detector (PE Applied Biosystems). In order to improve the sensitivity and quality of the quantitation, all samples were assayed in duplicate. A threshold cycle (CT) value for each reaction was determined from the real-time fluorescence records, and compared with the positive standards described below.

Standard material and quantitation

A plasmid that contained the target region was constructed using the vector pGEM-T (Promega, Tokyo, Japan). After propagation and purification, the concentration of the plasmid was determined by photospectrometry, and the number of copies per microliter was calculated from the optical density value and plasmid molecular weight. The plasmid solution was then serially diluted and used for PCR. The concentrations were 100 000, 10 000, 1000, 100, 10, 1, and 0.1 copies/μl, and a 10-μl aliquot of each was used as standard. The copy numbers of samples were calculated from the CT values by the software program, Sequence Detector version 1.6 (Applied Biosystems). The sensitivity of this PCR was estimated from the standard curve to be approximately 10 copies/μg DNA. All PCR reactions were carried out in duplicate, and the mean of the two results was reported when both of the results were positive. When either of the tubes was positive while the other negative, the result was described as positive but <10 copies/μg DNA, provided the positive value of the pair was <100 copies/μg DNA. For statistical purposes, this qualitative positive value was assumed to be 10 copies/μg DNA. When both of the results were negative, the sample was regarded to be negative, and 0 copies/μg DNA was assigned as its value. When the two results measured were both above 100 copies/μg DNA and differed by more than 10-fold, the sample was re-examined. Any other discordant results between the two tubes were re-examined. As a peak viral load of 10 copies/μg DNA was not associated with symptomatic CMV syndrome or histologically proven disease, this low viral load was regarded as insignificant in uni- and multivariate analysis.

Ethical considerations

The present study was performed in accordance with the guidelines of the ethics committee of Kyoto University Hospital. Informed written consent was obtained before sampling and enrollment into the study.

Statistical methods

Continuous variables were compared using the Student's *t*-test or when a normal distribution could not be assumed, the Mann–Whitney's test. Categorical data were compared using the chi-squared or Fisher's exact test. A logistic regression model was used to evaluate variables found to be associated with CMV infection by univariate analysis ($P < 0.1$). To analyze the duration from transplantation until the first appearance of CMV DNA, Cox's proportional hazard regression model was used. All the analyses were performed with computer software, StatView version 5.0 (SAS Institute, Cary, NC, USA). Differences of $P < 0.05$ were considered statistically significant.

Results

The 91 patients enrolled in this study were examined by real-time PCR at intervals of <10 days for 8–90 days (mean, 26.3) post-transplant. The duration from transplantation until the first appearance of CMV DNA in peripheral blood was 1–60 days (mean, 23.1; median, 21.0) post-transplant. The mean, the median, and the peak concentrations of all CMV DNA quantified were 810, 10, and 45 000 copies/μg DNA, respectively.

Among the 91 recipients forty had a significant CMV infection defined by a peak copy number above 10/μg DNA. Searching for preoperative risk factors of significant CMV infection, the following variables were evaluated by univariate analysis: age, gender, underlying diseases, an ABO incompatible graft, and a serologic combination of positive donor (D+) and negative recipient (R−). Five recipients without CMV serologic tests were assumed to have no risk factors in terms of serology. As summarized in Table 1, significant preoperative risk factors associated with CMV infection were fulminant hepatic failure as an underlying disease, an ABO incompatible graft, and D+/R−.

Multivariate analysis using a logistic regression model, based on the variables identified by the univariate analysis at the $P < 0.1$ level, revealed that fulminant hepatic failure, ABO incompatibility, and D+/R− were independently associated with the development of significant CMV infection (Table 2).

These risk factors were then evaluated in patients with CMV disease. Five of 91 patients had been diagnosed with CMV disease in spite of preemptive strategy based on antigenemia. All five had more than one risk factor (Table 3). As 31 recipients had more than one risk factor before operation, the frequency of patients with CMV disease among those with risk factors was 16.1%. Peak CMV titers of the five patients with CMV disease were more than 1000 copies/μg DNA. The peak CMV titers were significantly higher in the patients with CMV disease (median, 1300 copies/μg DNA; $n = 5$) than those of the patients with risk factors who did not develop disease (median, 67 copies/μg DNA; $n = 26$; $P < 0.0001$).

In terms of significant CMV infection, 25 recipients of the 31 (80.6%) patients with one or more CMV risk factor experienced significant CMV infection, whereas 15 of the 60 patients (25.0%) with no risk factors experienced significant CMV infection ($P < 0.0001$). Figure 1 shows the cumulative incidence of the period required until the first positive result of CMV DNA among patients with and without risk factors. Those patients with CMV risk

Table 1. Univariate analysis of risk factors for CMV infection.

Candidates for risk factors	No. patients with CMV infection ($n = 36$)	All others ($n = 55$)	*P*-values
Underlying disease			
FHF ($n = 8$)	6	2	0.0320
Retransplantation ($n = 5$)	3	2	0.3806
CLD ($n = 35$)	16	19	0.3426
LC($n = 10$)	4	6	>0.9999
Blood type			
ABO incompatible ($n = 10$)	10	0	0.008
Serology			
D+/R− ($n = 10$)	7	3	0.0465
Age at surgery			
>30 year ($n = 36$)	14	22	>0.9999
<1 year ($n = 15$)	8	7	0.2326

CMV, cytomegalovirus; FHF, fulminant hepatic failure; CLD, cholestatic liver disease; LC, liver cirrhosis; D+/R−, donor seropositive for CMV and recipient seronegative.

Table 2. Multivariate analysis of risk factors for CMV infection.

Risk factors	*P*-value	Odds ratio	95% CI
Underlying disease			
FHF	0.0329	6.769	1.168–39.213
Blood type			
Incompatible	0.0191	4.978	1.300–19.060
Serology			
D+/R−	0.0228	5.756	1.276–25.970

CMV, cytomegalovirus; FHF, fulminant hepatic failure; D+/R−, donor seropositive for CMV and recipient seronegative.

Table 3. Characteristics of the recipients with CMV disease.

Case	Gender	Age at transplantation	Onset of infection (POD)	Peak of CMV DNA (copies/μg DNA)	Risk factors	Site of disease*
A	M	1 month	10	1300	FHF	Liver
B	M	2 years	27	1300	D+/R−†	Intestine
C	F	30 years	20	3700	FHF	Liver
D	M	10 months	34	1300	Incompatible‡ and D+/R−†	Liver
E	M	48 years	28	45 000	Incompatible‡	Lung

CMV, cytomegalovirus; POD, post-operative day; FHF, fulminant hepatic failure.
*Case A, clinical diagnosis of CMV disease; other cases, pathologically defined CMV disease.
†D+/R−, donor seropositive for CMV and recipient seronegative.
‡Incompatible, incompatible blood type combination between recipient and graft.

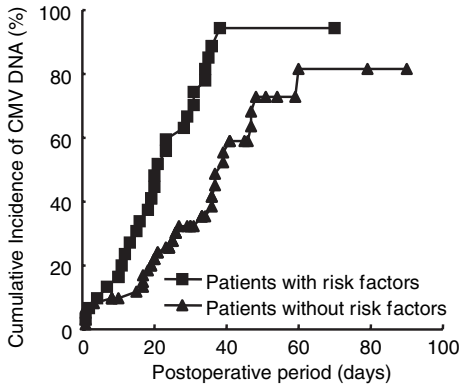

Figure 1 Comparison of cumulative incidence of the period required until the first positive result of cytomegalovirus (CMV) DNA among patients with and without risk factors. Those patients with CMV risk factors had a positive result significantly faster than patients without risk factors ($P = 0.0002$).

factors had a positive result significantly faster than patients without risk factors ($P = 0.0002$).

Discussion

Cytomegalovirus infection is the most common viral infection, and it still requires significant resources in terms of prevention and treatment even today. Our analysis revealed that significant CMV infection or CMV disease was predicted before operation by three risk factors; fulminant hepatic failure as an underlying disease, an ABO-incompatible graft, and a lack of pre-existing immunity to CMV combined with transplantation of a CMV-positive organ.

Few studies have considered risk factors other than a lack of immunity (i.e. D+/R−) when analyzing the quantitative results of CMV detection among liver recipients [7,17,18], even though there are numerous studies on preemptive therapy [17,19–25]. As our recipients frequently received an ABO-incompatible graft from living donor in comparison with Western countries, our result deemed to depict it as a risk factor not reported yet. Older age (>30 years) achieved significance in univariate analysis ($P = 0.02$) but was marginally significant in multivariate tests ($P = 0.06$) [26]. Age was not a risk factor for CMV infection in our study.

The reason why we were able to extract these factors: to find out or retrieve seems better may be related to the assay method. PCR has been proved to be more sensitive than antigenemia in many reports, and it becomes positive faster than antigenemia (data not shown). PCR also had a better dynamic range for quantification than antigenemia (data not shown). Although our patients were preemptively treated with ganciclovir after a positive test based on pp65 antigenemia, PCR might have lead to a better and faster detection of CMV infection.

Finding the risk factors and using them to select patients at risk is an effective strategy. As these factors were known before surgery, it is efficient to follow these recipients for CMV-related complications. Although there were recipients with CMV disease in our series, the incidence of CMV disease may be decreased with improving CMV detection methods. That is, to monitor these recipients more frequently (e.g. twice a week by antigenemia) or to use a more sensitive test like PCR (e.g. once a week by PCR). Razonable *et al.* [27] reported that high-risk (CMV D^+/R^-) patients may require more frequent surveillance than the once-weekly strategy. Furthermore, routine use of the preemptive approach to CMV in liver transplant patients may be the therapeutic strategy of choice to prevent CMV-related post-transplantation complications, minimize the development of ganciclovir-resistant CMV, and decrease the cost of CMV prevention strategies [28]. As one of the requirements for successful preemptive treatment strategies is the early detection of CMV infection, we consider that it is preferable to test all patients with a sensitive method, start effective therapy in

those positive for CMV DNA, and examine patients with risk factors more frequently.

In our study, the mean post-transplant monitoring time of CMV DNA titers was 26.3 days. This might be short when monitoring CMV disease post-transplantation. Therefore, we could not fully monitor CMV DNA titers for the first 4 months after the transplantation when liver transplant recipients are generally considered to be at highest risk for CMV diseases.

In summary, risk factors for CMV infection were found by monitoring liver recipients by means of real-time PCR. Selection of patients using risk factors coupled with PCR monitoring may result in an optimization of preemptive strategy.

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