Evaluation of Interleukin-2 mRNA in Whole Blood as a Parameter for Monitoring Cyclosporine Pharmacodynamics

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Inhibition of cytokine production is the main immunosuppressive effect of cyclosporine (CsA), which is widely used in organ transplantation. Pharmacodynamic (PD) assay for evaluating the inhibition of interleukin-2 (IL-2) production for each patient could provide a more appropriate dosing regimen. We measured the suppression of IL-2 mRNA expression in whole blood following the addition of a range of CsA concentrations by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method. Individual CsA sensitivity on the IL-2 mRNA expression was assessed with healthy subjects both in vitro and ex vivo. We also evaluated it in pre-transplant patients before taking immunosuppressive drugs. Sigmoid Emax model was used to analyze the relationship between CsA concentration and IL-2 mRNA expression. The assay was completed within 8 h. The concentration that resulted in IC50 showed high reproducibility and specificity among the healthy subjects (p<0.005, n=5). Ex vivo study indicated similar inhibition profiles to those of in vitro studies (n=3). The values of IC50 obtained from patients (n=22) also showed large variations and were significantly lower than those from healthy subjects (p<0.05). Semi-quantitative RT-PCR was considered to be a rapid and reliable assay. Our data imply that measurement of IL-2 mRNA levels in whole blood could be valuable in monitoring CsA PD in transplant patients.

Key words cyclosporine; pharmacodynamics; interleukin-2

Although a progressive decline in the incidence of acute rejection is observed with the use of increasingly powerful drug combinations, the incidence of subclinical acute rejection still occurred between 5 and 15%,1 and long-term transplant recipients have still a high rate of morbidity and early mortality.2 Additionally, serious adverse events are frequent in the first months posttransplantation including mainly opportunistic infections that occur in immunosuppressed patients.3

Cyclosporine (CsA) is a potent immunosuppressive compound used widely in organ and bone marrow transplantation and autoimmune disease. Since there is considerable inter- and intra-individual variation in its pharmacokinetics (PK), CsA is dosed according to therapeutic drug monitoring schemes based on its PK. But PK of CsA does not directly relate to the pharmacological effects on immune cells in vivo because of the genetic heterogeneity of human immune system among patients and the clinical response to CsA may vary substantially even in patients with similar CsA concentrations. If the immunosuppressive levels vary substantially even in patients with similar CsA concentrations, the clinical response to CsA may relate to the pharmacological effects on immune cells. Several markers, including calcineurin activity, cytokine production, cytokine mRNA, T cell surface antigen expression and lymphocyte proliferation have been proposed to assess the PDs on the immune systems in individual patients.4–9 To our knowledge, however, adequate PD indices to define the CsA response in individual patients have not been established, and there are no markers with wide clinical application.

In this study an assessment of individual CsA PD was performed by measuring the levels of interleukin 2 (IL-2) mRNA expression in whole blood following the addition of a range of CsA concentrations in vitro and ex vivo in healthy subjects. Pre-transplant blood was also obtained from recipients of kidney transplants before taking immunosuppressive agents. We compared CsA PD in the recipients to that in healthy subjects to evaluate clinical efficacy for PD index.

MATERIALS AND METHODS

Healthy Subjects Written informed consent was obtained from all healthy subjects and renal transplant patients who participate in this study. No subject had clinically significant abnormalities on routine laboratory tests. The study protocol was approved by the Ethics Committee of the Nagoya University Hospital.

In Vitro Studies To determine the optimal time points for stimulation of lymphocytes in whole blood, venous blood was obtained from four healthy subjects into heparin-containing tubes. One milliliter whole blood cultures were incubated at 37 °C for 1, 2, 4, 6 and 8 h in falcon 14 ml polycarbonate tubes (Becton-Dickinson, Franklin Lake, NJ, U.S.A.) with 40 ng phorbol 12-myristate 13-acetate (PMA) and 4 μg calcium ionophore. Twenty milliliters of venous blood was drawn four times at intervals of one or more week from five healthy subjects into heparin-containing tubes. Before stimulation of the cells, 5 different concentration mixtures of CsA were added to whole blood to obtain final CsA concentrations of 0, 100, 250, 400, 600 and 800 ng/ml for 30 min at 37 °C in falcon tubes to determine the relationship between CsA concentrations and the inhibitory response of IL-2 mRNA expressions. Samples were then incubated at 37 °C with 40 ng PMA and 4 μg calcium ionophore for 2 h.

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To assess the inhibitory effect of other immunosuppressive compounds on IL-2 mRNA expression, samples from a healthy subject were incubated with mycophenolic acid (MPA; 0, 10, 25, 50, 75, 100 μg/ml) or prednisolone (0, 100, 250, 500, 750, 1000 ng/ml) in the same manner as described above. Whole blood levels of MPA and prednisolone were within or slightly excess over the levels in clinical setting.

Ex Vivo Study To determine the relationship between the IL-2 mRNA levels and CsA concentration in whole blood in vitro and ex vivo, three of the five healthy subjects received a single 8 mg/kg dose of CsA (Neoral, Novartis Pharma K.K.). Venous blood was drawn before and 4 and 6 h after the intake of CsA. CsA levels in blood samples were analyzed using fluorescence polarization immunoassay with TDX analyzer (Abbott Laboratories Chicago, IL, U.S.A.).

Patients Twenty-two patients (19 men and 3 women, ages 20 to 68 years) who underwent adult-to-adult living-related renal transplantsations at Nagoya Daini Red Cross Hospital were enrolled in this study. Five days before the transplantation venous blood was collected into heparin-containing tubes as pre-transplant blood to assess CsA PD.

Sample Preparation and Quantification of IL-2 mRNA Level IL-2 mRNA level in whole blood was quantified by TaqMan reverse transcription PCR as described previously with some modification. mRNA was extracted from blood using the QIAamp RNA Blood Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer’s instructions. The reverse transcription reaction was performed by incubating a reaction mixture containing 600 ng RNA, 2.5 μmol of random hexamer primer, 20 U of reverse transcriptase, 6 U of RNase inhibitor, and 2.5 μmol dNTP in a total of 15.23 μl reaction buffer at 48 °C for 30 min, and at 95 °C for 5 min. Obtained cDNA was stored at −20 °C until use in real-time PCR assay.

Quantitative, real-time multiplex PCR was performed using TaqMan assay and PCR amplification in an ABI-PE prism 7700 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan). TaqMan probe and primers for IL-2 and β-actin genes, were obtained from Applied Biosystems. The reaction mixture containing IL-2 and housekeeping gene primers and probes, distilled water and 2 Taq-Man Universal PCR Master Mix was prepared and dispersed in a 96-well MicroAmp Optical Tube (Applied Biosystems). To each optical tube, cDNA was added to give a final volume of 25 μl. Real-time PCR assay was done under the following universal conditions: 2 min at 50 °C, 10 min at 95 °C, 50 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. The PCR amplification cycle at which the reporter dye fluorescence passed the selected baseline (C’) was determined by real-time monitoring of fluorescent emission after cleavage of sequence specific probes by nuclease activity of Taq polymerase. All reactions were performed in triplicate. The level of IL-2 mRNA was determined by normalization to that of β-actin genes, using the comparative C’ method for relative quantitation with IL-2 mRNA levels as described in detail elsewhere. 11)

Data Analysis The PD model describes the relationship between the CsA concentration and the expression of IL-2 mRNA levels. We assume that this relationship is nonlinear and can be described by the inhibitory sigmoid $E_{max}$ model as follows:

$$E = E_{max} - (E_{max} - Bottom) \times \frac{C^\gamma}{IC_{50}^\gamma + C^\gamma}$$

where $E$ is the intensity of the effect (the expression levels of IL-2 mRNA), $E_{max}$ and Bottom are maximal and minimal effect values, $C$ is the concentration of CsA, $IC_{50}$ is the concentration that results in 50% inhibition, and $\gamma$ is the Hill coefficient that affects the slope of the curve. Each parameter was determined using non-linear regression curve fitting and analyzed using Prism 4 for windows software (GraphPAD Software Inc., San Diego, CA, U.S.A.). Data obtained from healthy subjects were analyzed by one-way analysis of variance (ANOVA) using the same software with above. Intra-individual variations were determined by calculating the mean, standard deviation and coefficient of variance (CV) from fourth whole blood preparations of each individual. The statistical significance of the $IC_{50}$ levels between healthy subjects and renal transplant patients was analyzed using the nonpaired t test. Differences were considered significant at $p<0.05$.

RESULTS

In Vitro Studies To determine the optimal time points for stimulation of lymphocytes, the analyses of PMA and calcium ionophore-stimulated whole blood sample over the time were performed. As shown in Fig. 1, IL-2 mRNA expression was induced as an early response with the peaks between 2 and 4 h in 4 healthy subjects. From the results of this study, whole blood was stimulated by 2h incubation with PMA and calcium ionophore after the incubation of mixtures of CsA.

We performed in vitro IL-2 mRNA quantifications in whole blood from 5 healthy subjects. IL-2 mRNA levels were decreased in accordance with the increase in CsA level as shown in Fig. 2 (Figure 2 shows the data of 2 subjects.). Mean $IC_{50}$ values of 5 individual subjects (each was determined by 4 experiments) ranged from 173 to 340 ng/ml, and were significantly different among the subjects using one-way ANOVA as shown in Fig. 3 and Table 1. CV values of $IC_{50}$ for 5 subjects ranged from 16 to 24%. Although the parameter of $IC_{50}$ showed a good reproducibility and high specificity, the CV values of $E_{max}$ and Hill coefficient exhibited large variations among healthy subjects ($E_{max}$ 25 to 73%, Hill coefficient; 18 to 42%) but were significantly different (one-way ANOVA).

We evaluated the inhibitory effects of MPA and prednisolone on IL-2 mRNA expression (Fig. 4). These immuno-

![Fig. 1. IL-2 mRNA Expression Levels in Whole Blood (1 ml) Following Stimulation by 40 ng PMA and 4 μg Ca Ionophore for 1, 2, 4, 6 and 8 h. Each bar represents individual subjects.](image-url)
suppressive compounds had no inhibitory effects on the IL-2 mRNA expression at the doses examined.

**Ex Vivo Study** To assess whether the inhibition levels of IL-2 mRNA expression in *ex vivo* are correlated with those in *vitro*, three subjects received a single 8 mg/kg dose of CsA. In this *ex vivo* study, inhibition profiles similar to those in *vitro* studies for IL-2 mRNA expression were found in both subjects as shown by filled circles in Fig. 2.

Patients Individual CsA PD was also assessed by adding a range of CsA concentrations to pre-transplant blood obtained from 22 recipients of kidney transplants from living-related donors. Levels of IL-2 mRNA expression were dose-dependently inhibited by CsA (data not shown). IC50 values obtained from patients were compared with those from healthy subjects (Fig. 5). IC50 obtained from patients ranged from 85.6 to 322.7 ng/ml (mean ± S.D.; 168.7 ± 58.6) and was significantly lower than in healthy subjects.

### DISCUSSION

Until now many biomarkers have been proposed, but there has been very little information about association to clinical or histopathologic outcome data. Most recent report, Boleslawski et al. reported that mean frequencies of CD28 and CD38 expressing T cells were significantly higher in patients with acute rejection, but there was no difference between patients with or without infection in 52 recipients. Although this method may be effective for the prevention of acute rejection, it can’t predict bacterial and cytomegalovirus infection occurred by overimmunosuppression.

#### Table 1. Parameters of Sigmoid $E_{\text{max}}$ Model in Healthy Subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Subject A</th>
<th>Subject B</th>
<th>Subject C</th>
<th>Subject D</th>
<th>Subject E</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{max}}$</td>
<td>21286±10787</td>
<td>5581±1382</td>
<td>6046±1553</td>
<td>12298±8941</td>
<td>8873±4448</td>
</tr>
<tr>
<td>CV (%)</td>
<td>50.7</td>
<td>24.8</td>
<td>25.7</td>
<td>72.7</td>
<td>50.1</td>
</tr>
<tr>
<td>IC50 (ng/ml)</td>
<td>259.4±61.6</td>
<td>281.2±45.7</td>
<td>199.2±44.8</td>
<td>172.7±34.2</td>
<td>339.7±54.7</td>
</tr>
<tr>
<td>CV (%)</td>
<td>23.7</td>
<td>16.2</td>
<td>22.5</td>
<td>19.8</td>
<td>16.1</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>2.70±0.47</td>
<td>4.03±0.92</td>
<td>2.92±1.23</td>
<td>2.20±0.56</td>
<td>4.24±1.09</td>
</tr>
<tr>
<td>CV (%)</td>
<td>17.7</td>
<td>22.7</td>
<td>42.3</td>
<td>25.5</td>
<td>25.8</td>
</tr>
<tr>
<td>Bottom</td>
<td>160.6±321.1</td>
<td>99.3±20.8</td>
<td>0.0±0.0</td>
<td>5.1±10.1</td>
<td>23.6±29.8</td>
</tr>
<tr>
<td>CV (%)</td>
<td>200.0</td>
<td>121.6</td>
<td>0.0</td>
<td>200.0</td>
<td>126.0</td>
</tr>
</tbody>
</table>

Data are mean±S.D., n=4. $p$ value of $E_{\text{max}},$ IC50, Hill coefficient, Bottom were <0.05, <0.005, <0.05, 0.53, respectively.

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**Fig. 2.** Effect of CsA on IL-2 mRNA Expression in Whole Blood *in Vitro* (○) and *ex Vivo* (●)

IL-2 mRNA expression was assayed in the presence of CsA added in whole blood and plotted as a percentage of the control (no CsA added). *Ex vivo,* After receiving 8 mg/kg dose of CsA (Neoral), venous blood samples were drawn after 4 and 6 h.

**Fig. 3.** Box Plots of IC50 in Five Healthy Subjects: (Whiskers) Tenth and 90th Percentiles; (Low and Upper Limits of Boxes) 25th and 75th Percentiles; (Lines within Boxes) Median Values

The mean values of IC50 were significantly different among the subjects ($p<0.005$ with one-way ANOVA). Each box plot was determined by 4 experiments.

CsA. In this *ex vivo* study, inhibition profiles similar to those in the *in vitro* studies for IL-2 mRNA expression were found in both subjects as shown by filled circles in Fig. 2.

**Patients** Individual CsA PD was also assessed by adding a range of CsA concentrations to pre-transplant blood obtained from 22 recipients of kidney transplants from living-related donors. Levels of IL-2 mRNA expression were dose-dependently inhibited by CsA (data not shown). IC50 values obtained from patients were compared with those from healthy subjects (Fig. 5). IC50 obtained from patients ranged from 85.6 to 322.7 ng/ml (mean±S.D.; 168.7±58.6) and was significantly lower than in healthy subjects.
Fig. 5. Comparison of IC$_{50}$ Values between Healthy Subjects (n=5) and Renal Transplant Patients (n=22)

IC$_{50}$ values were significantly different between groups (p<0.05). Each value in healthy subjects was determined by 4 experiments.

The most effective immunosuppressive strategies in organ transplantation are based on the inhibition of IL-2 signal by calcineurin inhibitors, and the introduction of these drugs has resulted in a marked improvement in graft survival. However, treatment with these drugs is associated with serious adverse effects and infection at overdoses and with rejection at insufficiency. Since PK properties of these drugs show high variability and these drugs have a narrow therapeutic index, therapeutic drug monitoring is necessary to optimize the treatment. However, there are some concerns about the linear relationship between the blood concentration and inhibitory effects on IL-2 signal among individual patients. Therefore, many investigators have tried various methods to measure PD to find an adequate marker.

Recently, the monitoring of cytokine gene transcription using a quantitative real-time PCR technique has been described and a highly sensitive and reliable detection system is available to facilitate the in vitro and ex vivo quantification of basal cytokine mRNA expression.$^{12,13}$ Especially the determination of cytokine mRNA levels in whole blood is faster and more economical than that in peripheral blood mononuclear cells (PBMC). Quantification of mRNA levels in PBMC is hampered by mRNA degradation, which may occur during the Ficoll–Hypaque density gradient centrifugation. The separation of T cells from a physiological environment is likely to have profound modifying effects on T cell function and even cause pre-activation.$^{14}$ In addition, the drug concentration to spike the cells is far from the clinical doses. These reasons make it much more difficult to use in routine practice.

The IL-2 mRNA expression in whole blood incubation with PMA and calcium ionophore was decreased gradually after 4 or 6 h (Fig. 1). These results were in good agreement with the previous report, although they were stimulated with anti-CD3/anti-CD28 and added tacrolimus.$^{12}$ Accordingly, the whole blood samples were incubated for 2 h with PMA and calcium ionophore in the present study.

The inhibition of IL-2 mRNA expression was closely correlated with the CsA concentrations as shown in Fig. 2. The dose-dependent suppressions in IL-2 mRNA expression (IC$_{50}$) showed inter-individual variation but good reproducibility within healthy subjects (Table 1). Mean IC$_{50}$ values were comparable to those reported by others in healthy subjects by measuring calcineurin phosphatase activity$^{15}$ and IL-2 concentration in whole blood.$^{16}$ Furthermore, the relationship between CsA concentration and IL-2 mRNA expression level in vitro was closely correlated with the ex vivo data in the blood of healthy subjects who had received CsA as shown in Fig. 2. These results indicate that the in vitro method using IL-2 mRNA expression accurately reflects the ex vivo data and supports our hypothesis that measurement of IL-2 mRNA in whole blood may be useful in monitoring CsA PD in patients.

Previous studies investigated the degree of immunosuppression in activated lymphocytes by measuring the cytokine mRNA level at only one point,$^{12,13}$ however the quantification of cytokine mRNA expression levels vary according to blood sampling technique or cell separation.$^{17}$ In our study the parameter of $E_{max}$, which represents IL-2 mRNA expression without CsA shows large intra-individual variation as shown in Table 1 (CV; 25 to 73%), indicating that only one point quantification of IL-2 mRNA expression is difficult to evaluate the biological effects of the drug and monitoring of the allogeneic response. To avoid such shortcomings in previous studies, we determined IC$_{50}$ value from the added CsA concentration and IL-2 mRNA expression levels which was calculated from several points. Therefore the IC$_{50}$ value is a useful parameter for monitoring CsA PD, independent on blood sampling technique or cell separation.

Large-scale clinical trials using Neoral C2 monitoring have demonstrated low acute rejection rates and good tolerability with a low adverse event profile, suggesting the clinical usefulness of C2 monitoring of CsA.$^{18}$ The results in single center studies suggest that an upper C2 limit of 800 ng/ml is appropriate in renal transplant patients who are receiving triple therapy of CsA, prednisolone and mycophenolate mofetil at more than 1 year after the transplantation.$^{19,20}$ In our study, the IL-2 mRNA expression levels were fully inhibited by CsA at >800 ng/ml and little additional response were observed. These results indicated that CsA at concentrations more than 800 ng/ml is not necessary for the inhibition of IL-2 expression as reflected by the fact that widespread clinical use of C2 levels to optimize therapy in patients has been developed empirically.

MPA and prednisolone had no inhibitory effects on the IL-2 mRNA expression as shown in Fig. 4. MPA is a selective inhibitor of inosine monophosphate dehydrogenase, the rate-limiting enzyme needed to produce guanosine. Inhibition of its enzyme leads to the depletion of intracellular guanosine triphosphate stores slowing proliferation of lymphocytes and monocytes.$^{21}$ We can confirm MPA do not possess the inhibitory effects on the IL-2 mRNA expression. A previous study by Hodge et al. reported that the production of IL-2 by stimulated T cells was suppressed by prednisolone in a dose-dependent manner and that IL-2 production was inhibited 42% by 3600 ng/ml and 21% by 36 ng/ml prednisolone.$^{22}$ We could not confirm the inhibitory effect of prednisolone on IL-2 mRNA expression at concentrations of less than 1000 ng/ml. Both studies used whole blood but they assayed only intracellular IL-2 by a flow cytometric analysis after staining with fluorescent-conjugated antibodies in the presence of Brefeldin A, which acts as a golgi blocker and inhibits intracellular IL-2 transport. The precise reasons for the discrepancy between the two studies remain unclear, and fur-
ther studies are needed on this matter.

The values of IC$_{50}$ obtained in the patients were significantly lower than those in healthy subjects. We have previously indicated that hematocrit levels affect the sensitivity to CsA and the values of IC$_{50}$ on IL-2 mRNA expression are reduced from 310 to 55 ng/ml when hematocrit levels were decreased from 50 to 20%.23) Fourteen out of 22 transplant patients have been on hemodialysis for more than one year. Presumably, these patients had low hematocrit levels, which may explain the difference between the patients and healthy subjects.

The method presented herein has potential advantages as compared to previous studies. Indeed, the results can be obtained without requiring special analytical equipment such as flow cytometry within 8 h. Furthermore, the values of IC$_{50}$ can be performed good reproducibility (CV; about 20%).

CONCLUSION

Our data imply that measurement of IL-2 mRNA levels in whole blood by a real-time PCR method could be valuable for monitoring CsA PD in transplant patients. It requires only a small amount of blood and takes only half a day to obtain the results. Further clinical data will be required concerning the relationship between the values of IC$_{50}$ and clinical events including rejection and infection.

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