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Influence of intravenous methylprednisolone pulse treatment on the disposition of ciclosporin and hepatic CYP3A activity in rats

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Abstract

We examined the effects of high-dose methylprednisolone (MP) on the disposition of ciclosporin (CsA) and hepatic microsomal CYP3A activity using rats. Methylprednisolone sodium succinate (MPS), a prodrug of MP, was intravenously administered as repeated doses (66.3 mg kg^{-1}) for 3 days or as a single dose. In MP-treated rats, a significant increase was observed in the total body clearance (CL_{tot}) and elimination rate constant (Ke) of intravenously administered CsA. The enzyme activities of triazolam hydroxylations and erythromycin *N*-demethylation in hepatic microsomes were also enhanced by about 50% by MP treatment, suggesting that the alteration in the CsA pharmacokinetics was due to significant induction of the hepatic CYP3A responsible for the metabolic conversion of CsA. In contrast, no significant changes in the values of CL_{tot} and Ke were found following a single treatment with MP. On the other hand, MP inhibited the CYP3A-mediated triazolam hydroxylations in a concentration-dependent manner. The difference between the in-vivo and in-vitro inhibitory behaviours of MP was attributed to the rapid elimination of MP after biotransformation from MPS because the plasma MP concentration decreased with a half-life of 15 min immediately after reaching a level close to the inhibition constant for the triazolam 4-hydroxylation reaction (32.4 μM). Although there is a general consideration that MP cannot act as an enzyme inducer at maintenance doses, the present results strongly suggest that high-dose MP is likely to interact pharmacokinetically with CsA by inducing hepatic CYP3A. These results may provide basic explanations for the clinical experience that blood CsA levels are reduced during MP pulse therapy.

Introduction

Ciclosporin (CsA), a powerful immunosuppressant, has been widely adapted to many types of organ transplantations and is also used to treat various autoimmune diseases, such as nephrotic syndrome, rheumatoid arthritis, psoriasis, atopic dermatitis, aplastic anemia, Crohn's disease and ocular Behcet's disease (Kahan 1989; Faulds et al 1993; Perico & Remuzzi 1997). Because of its narrow therapeutic window, appropriate control of the blood CsA level is essential to maintain graft survival and to exert the optimal suppression of the hypersensitive immune response while minimizing adverse side events. However, one of the most important issues regarding the clinical features of CsA is that its pharmacokinetic behaviour is influenced by concurrent medications, often leading to fluctuation or failure of therapeutic control (Campana et al 1996; Dresser et al 2000). In clinical practice, it is difficult to manage disease conditions by CsA alone, thus concomitant administration of other immunosuppressive agents is required in many cases so that they can exert their synergic effects. At the onset of episodes of acute graft rejection, graft vs host disease and immunologic relapse, high-dose steroid hormones are intravenously administered as pulse treatment to prevent further progression of the symptoms. Methylprednisolone (MP), a synthetic steroid, is commonly administered as a steroid pulse therapy regimen because it shows a five-fold potent anti-inflammatory effect with less sodium-retaining activity compared with the endogenous glucocorticoid hydrocortisone. A possible drug interaction between CsA and MP has been documented in many

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clinical studies. However, these findings remain controversial; the blood CsA level may be increased (Klintmalm & Säwe 1984; Öst et al 1985; Campana et al 1996) or decreased (Ptachcinski et al 1987; Campana et al 1996) by steroid treatment. Furthermore, there is no agreement as to the mechanism that underlies the change in blood CsA concentration.

The present study investigated whether or not an intravenous MP pulse can alter the in-vivo disposition of CsA in a rat model, and examined the effects of MP on the induction and inhibition of CYP3A, a major hepatic cytochrome P450 (CYP) species responsible for the metabolic elimination of CsA in order to elucidate the mechanisms involved in the drug interactions between these agents.

Materials and Methods

Reagents and experimental animals

CsA for injection (Sandimmun, containing 250 mg CsA per 5 mL ampoule) was obtained from Novartis Pharma KK (Tokyo, Japan). MP, methylprednisolone sodium succinate (MPS), triazolam and erythromycin were purchased from Sigma Chemical Co. (St Louis, USA). Other chemicals and solvents were of analytical grade.

Male Sprague–Dawley rats were obtained from Japan SLC Inc. (Hamamatsu, Japan). The rats were acclimated for at least 3 days before assignment to experimental groups at 7–9 weeks of age (200–300 g). The animals were housed in mesh-floored cages in accommodation maintained at $23 \pm 2\,^{\circ}C$ with a relative humidity of $55 \pm 10\%$ and a 12-h light/dark cycle, with free access to normal diet and water except when fasted overnight before sacrifice. The rats used in this study were handled in accordance with the Guidelines for Animal Experimentation of Shiga University of Medical Science, and the experimental protocol was approved by the Animal Care and Use Committee of this institution.

Treatment with MP

Because of the low solubility of MP in aqueous fluid, MPS, which is a water-soluble esterified prodrug of MP, is administered as an MP substitute in clinical practice. Intravenously administered MPS is rapidly converted to MP in the body by enzymatic hydrolysis of the succinyl moiety. Thus, MPS was intravenously injected to rats to emulate the clinical protocol of steroid pulsing. MPS was dissolved in saline at a final concentration of $66.3\,mg\,mL^{-1}$, and was injected into the tail vein at doses of $66.3\,mg\,kg^{-1}$ (equivalent to $50\,mg\,kg^{-1}$ MP) for three consecutive days (designated as MP-treated rats) or $66.3\,mg\,kg^{-1}$ once. Control rats were administered vehicle alone at the same time. In the experiment of MP pharmacokinetics, rats fasted overnight were given a single dose of MPS ($66.3\,mg\,kg^{-1}$) via the jugular vein. Blood samples were withdrawn using heparinized sample tubes without restriction from the opposite side of the jugular vein under light ether anaesthesia at 0.25, 0.5, 1, 2, 4 and 6 h after MPS administration. Plasma samples were separated by centrifugation and were stored at $-20\,^{\circ}C$ until analysed.

Intravenous administration of CsA

CsA solution (0.1%) for injection was prepared by diluting Sandimmun with physiological saline. Rats were fasted overnight prior to CsA administration. MP-treated rats received an intravenous dose of CsA solution ($1\,mg\,kg^{-1}$) over a period of approximately 5 s through the jugular vein 24 h after the last MPS injection, considering the time-lag until onset of enzyme induction following MP exposure. The same CsA dose was given immediately after MPS injection to rats administered MPS as a single dose. Blood collection and handling of the rats were performed by the same procedure as described above, except that sampling was carried out 0.25, 0.5, 1, 2, 4, 6, 9 and 12 h after CsA administration. Whole blood samples were stored at $-20\,^{\circ}C$ until analysed.

Preparation of rat hepatic microsomes

The rats were sacrificed by bolus injection of KCl solution under heavy anaesthesia with ethyl ether. Livers from untreated and MP-treated rats were perfused with ice-cold 1.15% KCl, then homogenized in the same salt solution (1:4, w/v) using a Potter-type, Teflon-glass motor-driven homogenizer. The homogenate was centrifuged at $10\,000 \times g$ for 15 min, and the supernatant fractions were centrifuged at $105\,000 \times g$ for 60 min to obtain microsomes. The pellet was suspended in 100 mM sodium/potassium phosphate buffer (pH 7.4) at an appropriate concentration. The protein concentration was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard.

Biochemical analysis

Triazolam α- and 4-hydroxylations (Kronbach et al 1989; Kanamitsu et al 2000) and erythromycin *N*-demethylation (Watkins et al 1989; Wang et al 1997) have been established as metabolic probes of hepatic CYP3A in rodents and humans. The enzyme reaction was performed in 1 mL of a mixture of an NADPH-generating system consisting of 0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, $2\,IU\,mL^{-1}$ glucose-6-phosphate dehydrogenase, 70 mM sodium/potassium phosphate buffer (pH 7.4), microsomes ($0.2–0.5\,mg\,mL^{-1}$) and substrate (50–200 μM triazolam or 2.5 mM erythromycin) in the presence or absence of MP and MPS. The reaction was started by adding the microsomal suspension, and the incubation was conducted at $37\,^{\circ}C$ with constant shaking for 20 min. The reactions were stopped by addition of 2 mL of acetonitrile (triazolam hydroxylations) and 300 μL of 15% trichloroacetic acid (erythromycin *N*-demethylation). The activities of triazolam α-hydroxylation and triazolam 4-hydroxylation were measured according to the HPLC method previously reported (Kanamitsu et al 2000). Erythromycin *N*-demethylase activity was estimated

by colorimetric measurement of liberated formaldehyde based on the Hantzsch reaction (Nash 1953). It was confirmed that the enzyme reactions showed linearity with respect to protein concentration and incubation time.

Measurements of blood CsA concentration and plasma MP concentration

CsA concentration in whole blood was determined by a fluorescence polarization immunoassay technique with the TDxFLx system using monoclonal antibody according to the manufacturer's instructions (Abbot Laboratories, Tokyo, Japan). The cross-reactivities with the metabolites of CsA were 19.4% for AM9 and 6.7% for AM1, and less than 5% for other metabolites. There were no cross-reactivities with MP and MPS at concentrations of at least 100 mg L^{-1}.

MP concentration in plasma was measured using the method proposed by McWhinney et al (1996) with modifications. A 100 μL aliquot of plasma sample was diluted with 5 mL of 0.1 M HCl following the addition of 500 ng dexamethasone (internal standard), and then loaded onto the Sep-Pak Plus C_{18} extraction cartridges (Waters, Milford, MS). They were washed under reduced pressure with 3 mL of each in the following order: 100 mM sodium bicarbonate, 15% methanol, hexane and distilled water. The desired portion was eluted with 5 mL of diethyl ether. The co-eluted aqueous fraction was discarded, and the ether layer was evaporated to dryness at 37 °C. The residue was reconstituted in 150 μL of the mobile phase and a 50 μL aliquot was subject to HPLC. The chromatographic assembly (Shimadzu, Kyoto, Japan) consisted of an LC-10A solvent-delivery pump, an SPD-10A UV detector and a C-R6A Chromatopac data integrator. The analytes were separated on a Cosmosil C_{18} column (150 × 4.6 mm, Attest) using a mixture of methanol/tetrahydrofuran/distilled water (6:25:65 v/v%) as the mobile phase. The mobile phase was delivered at a flow rate of 0.7 mL min^{-1}, and the column effluent was monitored at 254 nm. The retention times of MP and the internal standard were 15.8 min and 18.6 min, respectively.

Calculation of pharmacokinetic parameters of CsA after intravenous administration to rats

The standard pharmacokinetic parameters of CsA were obtained by the model-independent moment method using the computer program MOMENT(EXCEL) (Tabata et al 1999). The elimination rate constant at the terminal phase (Ke) was determined by linear regression of the log-linear portion of plots of blood concentration against time. The total body clearance (CL_{tot}) was determined by dividing the intravenous dose (1 mg kg^{-1}) by the area under the CsA concentration in whole blood vs the time curve after administration (AUC). The AUC was calculated from the time zero to the last sampling point by the linear trapezoidal rule, with the addition of the correction term by extrapolation to infinity using the ratio of the last measured concentration to the calculated Ke value. The apparent volume of distribution at steady state (Vd_{ss}) was estimated by multiplying CL_{tot} and the ratio of the area under the moment curve of the blood CsA concentration to the AUC.

Calculation of pharmacokinetic parameters of MP after intravenous administration of MPS to rats

On the assumption of rapid and complete conversion of MPS to MP, the Ke and volume of distribution (Vd) of MP were calculated by fitting a one-compartment open model using a nonlinear least-squares regression analysis program (Yamaoka et al 1981). The elimination half-life ($t_{1/2}$) was calculated to be 0.693 Ke^{-1}. The peak MP concentration in plasma was estimated by dividing the intravenous MPS dose (66.3 mg kg^{-1}, equivalent to 50 mg kg^{-1} MP) by the Vd.

Statistical analysis

The results are expressed as means ± s.d. Statistical comparison of the data between the two groups was made using Student's *t*-test, and the level of significance (*P*) was set at < 0.05.

Results

Effects of MP treatment on in-vivo CsA pharmacokinetics

Figure 1 shows the blood CsA concentration–time courses in MP-treated and control rats after intravenous

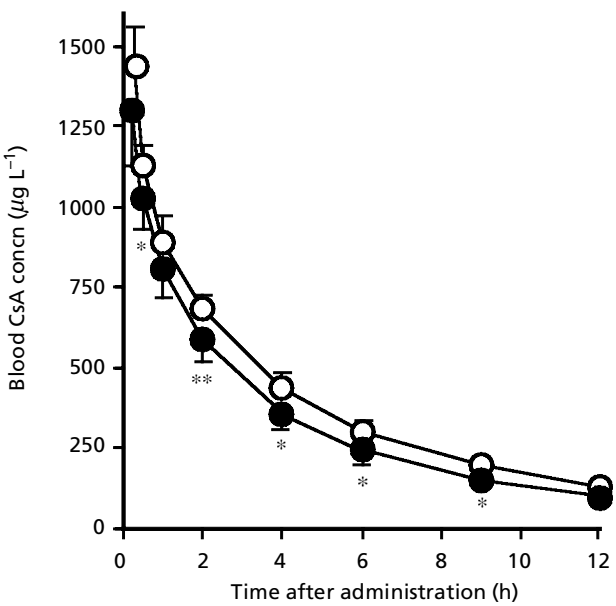

Figure 1 Blood concentration–time profiles of CsA after intravenous administration to MP-treated and control rats. Rats were intravenously administered MPS (66.3 mg kg^{-1}) for 3 days (MP treatment) prior to the injection of CsA (1 mg kg^{-1}). Control rats were administered vehicle alone instead of MPS. Symbols: ○, control rats; ●, MP-treated rats. Each point with a bar represents the mean ± s.d. of seven experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ vs the corresponding value of control group.

Table 1 Pharmacokinetic parameters of CsA intravenously administered to MP-treated and control rats.

Parameters	Control	MP treatment
Ke (h^{-1})	0.20 ± 0.01	0.23 ± 0.02*
AUC ($h\,mg\,L^{-1}$)	5.52 ± 0.39	4.60 ± 0.49**
CL_{tot} ($L\,kg^{-1}\,h^{-1}$)	0.18 ± 0.01	0.22 ± 0.03**
Vd_{ss} ($L\,kg^{-1}$)	0.95 ± 0.07	1.03 ± 0.13

Pharmacokinectic parameters were calculated according to a model-independent moment analysis. Each value represents the mean ± s.d. of seven determinations. $^{**}P < 0.01$, $^{*}P < 0.05$.

administration of CsA. The means of the blood CsA levels of MP-treated rats were lower than those of the control rats at each sampling point. The pharmacokinetic parameters in the two groups are shown in Table 1. In MP-treated rats there were significant increases in Ke and CL_{tot} and decreases in the AUC compared with those in control rats. However, the Vd was unchanged.

When the rats were concomitantly administered MPS as a single dose, the blood CsA concentrations tended to rise in the early phase after administration, then approached the levels in the control rats during the terminal phase. There were no significant differences in Ke, CL_{tot} and Vd between the two rat groups (data not shown).

Inducing and inhibitory effects of MP on CYP3A-dependent drug-metabolism

To examine whether or not the pulsed MP can induce CYP3A in the rat liver, the activities of triazolam α-hydroxylase, triazolam 4-hydroxylase and erythromycin *N*-demethylase in hepatic microsomes obtained from MP-treated rats were compared with those of control rats. As shown in Figure 2, these enzyme activities were significantly enhanced by treatment with MP pulse.

To assess the inhibitory potencies of MP and MPS for hepatic CYP3A activities, triazolam hydroxylation reactions were carried out in the presence or absence of these steroids using untreated rat liver microsomes. As shown in Figure 3, MP inhibited both activities in a concentration-dependent manner, while inhibition by MPS was trivial. The inhibition constants (K_i) of MP were calculated to be $72.6 \pm 8.4\ \mu M$ (n = 4) for triazolam α-hydroxylation and $32.4 \pm 3.8\ \mu M$ (n = 4) for triazolam 4-hydroxylation by analysis of Dixon plots.

Pharmacokinetics of MP after intravenous administration of MPS

The plasma MP concentration–time curve following intravenous injection of MPS is shown in Figure 4. The peak MP level was presumed to attain about $30\ \mu M$ just after MPS administration. However, MP concentration after intravenous administration showed an extremely rapid decline with a mean $t_{1/2}$ of 15.6 min, and decreased below the limit of quantification 4 h after administration.

Discussion

When administering MP as pulse therapy, an extremely high short-term dose is generally given, unlike the dose for maintenance therapy. Steroid hormones were suggested not only to have the potential to induce hepatic CYP3A enzymes (Schuetz & Guzelian 1984; Pichard et al 1992; Watanabe et al 1998) but also to act as a substrate of CYP3A itself (Varis et al 1998, 1999; Lebrun-Vignes et al 2001). It is, therefore, of practical importance to examine

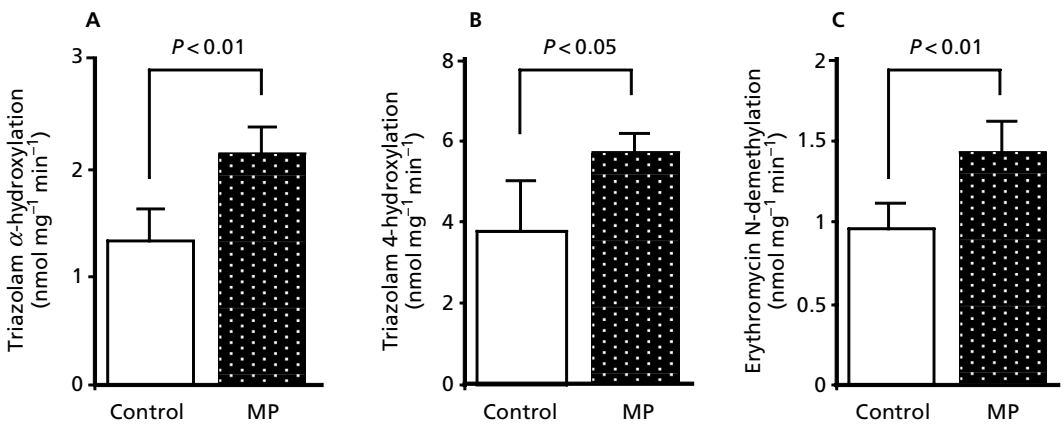

Figure 2 Changes in the activities of triazolam α-hydroxylation (A), triazolam 4-hydroxylation (B) and erythromycin *N*-demethylation (C) in hepatic microsomes by MP treatment. Rats were intravenously administered MPS ($66.3\,mg\,kg^{-1}$) for 3 days (MP treatment). Control rats were administered vehicle alone instead of MPS. The animals were killed 24 h after the last injection for preparation of microsomes. Enzyme reactions were carried out at substrate concentrations of $200\ \mu M$ (triazolam) and 2.5 mM (erythromycin). Microsome concentrations were $0.2\,mg\,mL^{-1}$ for triazolam hydroxylations and $0.5\,mg\,mL^{-1}$ for erythromycin *N*-demethylation, respectively. Each column with a bar represents the mean ± s.d. of four experiments.

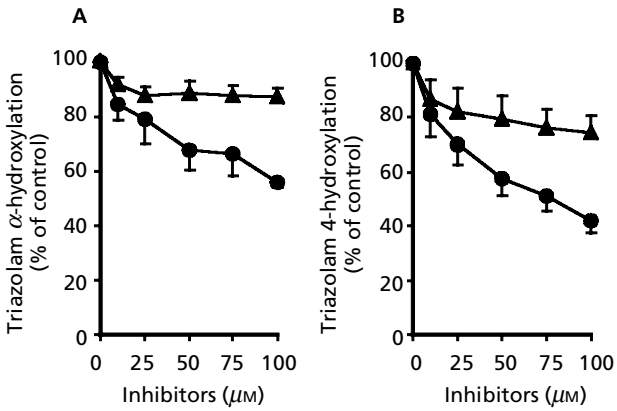

Figure 3 Inhibitory effects of MP and MPS on triazolam α-hydroxylation (A) and triazolam 4-hydroxylation (B) in hepatic microsomes. Microsomal preparation was obtained from untreated rats. Enzyme reactions proceeded for 20 min using 0.2 mg mL^{-1} microsomes at a triazolam concentration of 200 μM. Symbols: ●, MP; ▲, MPS. Each point with a bar represents the mean ± s.d. of four microsomal preparations, and was expressed as the percentage of the corresponding activity in the absence of inhibitors.

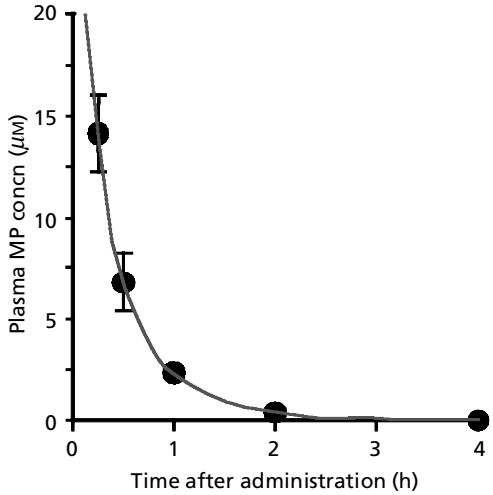

Figure 4 Plasma concentration–time profile of MP after intravenous administration of MPS to rats. MPS (66.3 mg kg^{-1}) was injected to untreated rats and the plasma MP concentration was serially measured up to 4 h after administration. Each point with a bar represents the mean ± s.d. of five experiments.

the possible pharmacokinetic interaction between the pulsed MP and CsA because CsA is not a drug with a high hepatic extraction ratio (Akhlaghi & Trull 2002) and hepatic CYP3A is involved in the metabolic elimination of intravenously administered CsA.

In the present study it was confirmed that the MP pulse treatment was capable of enhancing CsA elimination without altering its systemic distribution, based on the aspect of change in the pharmacokinetic parameters in MP-treated rats. As shown by the marked enhancements of microsomal triazolam α-hydroxylation/4-hydroxylation and erythromycin *N*-demethylation, the increase in in-vivo clearance of CsA was strongly supported by significant induction of CYP3A in the hepatic microsomes. The degree of elevation of hepatic microsomal CYP3A activities was prominent compared with that of the change in CsA disposition. CsA undergoes extensive metabolism, resulting in the formation of three primary metabolites, two monohydroxylated products (AM1 and AM9) and one *N*-demethylated product, across most mammal species, including rats and humans (Maurer et al 1984; Whalen et al 1999). Although CYP3A2 serves as a major catalyst of CsA biotransformation, CYP subfamilies other than CYP3A enzymes are also responsible for the metabolic conversion of CsA in rats (Prueksaritanont et al 1993; Brunner et al 1996), as in humans (Christians & Sewing 1995). It has been reported that AM1 and AM9 are present at concentrations ranging from only 5 to 20% of unchanged CsA in whole blood at the trough level when rats received subcutaneous injection of CsA (Brayman et al 1988). Based on the relatively high specificity of monoclonal anti-CsA antibody used in the present assay system, interference by these metabolites with CsA measurement was considered to be negligible in control rats. However, if blood levels of the metabolites were increased due to enzyme-induction by MP pretreatment, the blood CsA concentration would be slightly overestimated in MP-treated rats, suggesting that a difference in the actual CsA concentration between MP-treated and control groups should be further enlarged although the degree might be small. A slight disparity in the degree of change between CYP3A activity and CsA pharmacokinetics is therefore probably attributable to the multiplicity of CYP species involved in CsA metabolism and the presence of metabolites marginally affecting CsA measurements. Yokogawa et al (2002) reported that intraperitoneal application of dexamethasone to rats led to a marked increase in hepatic CYP3A2 at both the mRNA and protein levels in dose- and duration-dependent manners, and consequently decreased blood CsA concentration. This finding is in good agreement with the present observations, with regard to change in the CsA disposition and its relation to the inductive effects of glucocorticoids on hepatic microsomal CYP3A. However, in clinical practice it was reported that neither single (32 mg) nor chronic (8 mg day^{-1}) administrations of oral MP produced a significant enhancement of CYP3A4-dependent triazolam metabolism (Villikka et al 2001), implying that a low-to-moderate dose of MP is not a potent CYP3A4 inducer. Inductive effects on hepatic CYP3A appear to vary with steroids, as shown in experiments using primary cultures of hepatocytes where the effect of MP on biosynthesis of CYP3A protein was less potent than that of dexamethasone (Schuetz & Guzelian 1984; Pichard et al 1992). In these previous studies, the inducibility of CYP3A was assessed by continuous contact of steroids to cultured hepatocytes. However, MP is generally administered at greater than 500 mg as a single intravenous dose in pulse therapy, while the therapeutic dosage of dexamethasone is below one-tenth that of MP. Although the detailed mechanism of induction of hepatic CYP3A remains unclear, converging intravenous exposure of MP should be able to efficiently induce hepatic CYP3A because there is a dose-

dependency in the enzyme-inducing effects. In fact, it was demonstrated that blood concentrations of the anticancer agent paclitaxel (Monsarrat et al 1998) and the immunosuppressant tacrolimus (Shimada et al 2002), both of which undergo CYP3A-dependent metabolism, were also decreased by combined administration of high-dose MP. The present results therefore provide a fundamental explanation for a significant decrease in the blood CsA levels encountered in clinical practice when co-administered with an MP pulse regimen.

The other interesting drug interaction caused by a high dose of MP is that MP might act as a competitive inhibitor of CsA metabolism, since MP and CsA share the common CYP3A enzymes for their biotransformation. Despite the possibility of metabolic interference by MP with CsA, the disposition of CsA was virtually unaffected by co-administration of MP. In the experiment using hepatic microsomes, MP inhibited triazolam hydroxylations in a concentration-dependent manner, while the degree of inhibition by MPS was insignificant. With respect to the in-vivo MP disposition, the plasma MP concentration probably reached a level close to the K_i values for triazolam 4-hydroxylation (32.4 μM) after being converted from MPS, but the MP produced is eliminated from the systemic circulation with a $t_{1/2}$ of about 15 min. Thus, the disagreement between the in-vivo and in-vitro inhibitory effects of MP on CYP3A activities is ascribed to the rapid removal of MP in the blood circulation, despite having a potential to metabolically compete with other CYP3A substrates. In addition, the MP concentration in the vicinity of the active centre of CYP3A enzyme may be lower than that in the bloodstream, since the protein-unbound fraction of MP in serum is about 20% (Ebling et al 1986). The pharmacokinetic property of MP after intravenous administration of MPS in humans is similar to that observed in rats (Al-Habet & Rogers 1989). In this regard, the occurrence of an interaction between MP and CsA is unlikely to be based on the high K_i value (210 μM) of MP for CYP3A4-mediated CsA oxidase activity in human hepatic microsomes (Pichard et al 1992). However, several clinical studies have reported that blood CsA concentration is markedly increased by concurrent treatment with the high-dose MP (Klintmalm & Säwe 1984; Öst et al 1985). The reason for this phenomenon is not clear, but may be attributed to methodological issues in determining blood CsA concentration by nonspecific radioimmunoassay. Unlike monoclonal anti-CsA antibody, which has only a minor analytical interference, polyclonal antibody has been suggested to cross-react to a significant extent with polar metabolites of CsA accumulated by CYP3A4 induction, leading to marked overestimation in the CsA measurements (Campana et al 1996).

Conclusions

In the present study using rats we demonstrated that pulsed MP can increase the systemic clearance of intravenously administered CsA, and that enhancement of CsA elimination is closely related to the induction of hepatic CYP3A. These results provide evidence that the mechanism of interaction between high-dose MP and intravenous CsA is likely to involve hepatic CYP3A induction rather than its inhibition. As intestinal CYP3A and P-glycoprotein are well known to play central roles in the first-pass elimination of CsA in the absorption process, we consider that it is also important to examine further the pharmacokinetics of orally administered CsA during MP pulse treatment.

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