Effects of Intestinal and Hepatic Metabolism on the Bioavailability of Tacrolimus in Rats

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Purpose. Tacrolimus, an immunosuppressive agent, has poor and variable bioavailability following oral administration in clinical use. We investigated the contribution of intestinal metabolism to the first pass effect of tacrolimus in rats.

Methods. Tacrolimus was administered intravenously, intraportally or intraintestinally to rats. Blood samples were collected over a 240-min period, and blood tacrolimus concentrations were measured. The extraction ratios of tacrolimus in the intestine and liver were investigated. In addition, the metabolism of tacrolimus in the everted sacs of the small intestine was examined.

Results. The rate of absorption of tacrolimus in the intestine was rapid, and tacrolimus was almost completely absorbed after intestinal administration. The bioavailability of tacrolimus was about 40% and 25% after intraportal and intraintestinal administration, respectively, indicating that tacrolimus is metabolized in both the intestine and the liver. In addition, tacrolimus was significantly metabolized in the everted sacs of the rat intestine.

Conclusions. The present study suggested that the metabolism of tacrolimus in the intestine contributes to its extensive and variable first pass metabolism following the oral administration.

KEY WORDS: tacrolimus; bioavailability; metabolism; intestine; pharmacokinetics.

INTRODUCTION

Tacrolimus is a macrolide lactone with potent immunosuppressive properties, and has been in clinical use as prophylaxis against organ rejection after liver and renal transplantation. Blood tacrolimus concentrations in patients have to be kept within a narrow therapeutic range (from 5 to 20 ng/ml) due to its side effects such as nephrotoxicity and neurotoxicity (1). However, tacrolimus shows large intra- and interindividual pharmacokinetic variability. Especially, it shows large variability in bioavailability after oral administration (2). Therefore, monitoring of blood tacrolimus concentrations is necessary for the optimization of dosage regimens.

Tacrolimus is extensively metabolized in the liver, and its bioavailability is relatively poor (mean of around 25%) after oral administration (1,3). Cytochrome P450 (CYP)3A is involved in the metabolism of tacrolimus (4). It has been shown that CYP3A is abundant in rat and human enterocytes as well as in the liver (5). Enterocyte CYP3A was suggested to be involved in the first pass metabolism of tacrolimus. Furthermore, drug interactions in the intestine between CYP3A substrates have been reported (6,7). On the other hand, tacrolimus is also a substrate of P-glycoprotein, which has also been shown to be abundant in rat and human enterocytes (5). Therefore, enterocyte P-glycoprotein may decrease the oral bioavailability of tacrolimus. However, the extent to which the metabolism and/or exsorption by proteins contribute to the poor and variable oral bioavailability of tacrolimus remains to be determined.

In this study, to clarify the respective contributions of the liver and intestine to the first pass effect of tacrolimus, we examined the time course of changes in blood tacrolimus concentrations following intraintestinal, intraportal and intravenous administration. In addition, we examined the metabolism of tacrolimus in the rat intestine using an *in vitro* everted sac experiment.

MATERIALS AND METHODS

Materials

Tacrolimus injection solution (Prograf® injection, 5 mg/ml) was obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Sodium pentobarbital was purchased from Abbott (Chicago, IL). Cyanoacrylate glue was obtained from Sankyo Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

Animals

Male Wistar rats, each weighing 210–260 g, were used. Prior to the experiments, the rats were housed in a temperature- and humidity-controlled room with free access to water and standard rat chow. The animal experiments were performed in accordance with *The Guidelines for Animal Experiments of Kyoto University.*

Experimental Protocol for Bioavailability Study

Rats (seven or eight per group) were anesthetized with 50 mg/kg sodium pentobarbital. Supplemental doses of pentobarbital were administered as required. Body temperature was maintained with appropriate heating lamps. The femoral artery was cannulated with a polyethylene tube (SP-31, Natsume Seisakusho, Tokyo, Japan) for blood sampling. The jugular vein was cannulated with a polyethylene tube (PE-10, Becton Dickinson and Co., Parsippany, NJ) for intravenous infusion of tacrolimus. To adjust the solution for intravenous infusion to a dose of 1.0 mg/kg, 200 μl/kg tacrolimus injection solution (Prograf®) was diluted with saline. The solution was infused over a 1-hr period (2.2 ml/hr) via the jugular vein by means of an automatic infusion pump (Natsume Seisakusho, Tokyo, Japan). In a separate experiment, the abdominal cavity was opened via a midline incision, and a catheter with a 26G needle was carefully fixed with cyanoacrylate glue into the portal vein. To adjust the solution for intraportal infusion to a dose of 1.0 or 0.3 mg/kg, 200 or 60 μl/kg of tacrolimus injection solution (Prograf®) was diluted with saline respectively. The solution was infused over

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ABBREVIATIONS: HPLC, High-performance liquid chromatography; EIA, Enzyme Immunoassay; *CL*, Total clearance; V_1, Volume of the central compartment; *Q*, Intercompartmental clearance; V_{ss}, Volume of distribution; *F*, Bioavailability; AUC, Area under the blood concentration-time curve; KHBB, Krebs-Henseleit bicarbonate buffer.

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a 1-hr period (2.2 ml/hr) via the portal vein by means of an automatic infusion pump. Blood samples were drawn before drug administration and 15, 30, 60, 65, 75, 90, 120, 180, and 240 min after the start of intravenous and intraportal administration of tacrolimus (3).

In a separate experiment, the abdominal cavity of rats fasted for 18 hours was opened via a midline incision, and the upper site of duodenum was ligated twice with silk sutures (4-0 Nescosuture®, Nihon-shoji, Osaka, Japan). To adjust the solution for intraintestinal injection to a dose of 1.0 mg/kg, 200 μl/kg of tacrolimus injection solution was diluted with saline. The solution was injected (2 ml/kg) into the middle part of the duodenum. Blood samples were drawn before the drug administration and 15, 30, 45, 60, 75, 90, 120, 180, and 240 min after injection of tacrolimus. At the end of the 240-min experimental period, the entire small intestine was removed by cutting across the upper end of the duodenum and the lower end of the ileum. The small intestine was washed out with 5 ml of saline for quantitation of tacrolimus remaining in the intestinal lumen. In addition, we confirmed that the mucosal site of the intestine did not show sores or ulceration under this experimental condition.

Metabolism *In Vitro* Everted Sacs of the Small Intestine

Rats fasted for 18 hours were anesthetized with 50 mg/kg sodium pentobarbital. The abdominal cavity was opened via a midline incision, and the small intestine was removed. The small intestine was washed out with saline, and 10-cm everted sacs of duodenum were prepared by the technique of Wilson and Wiseman (8). The buffer used was Krebs-Henseleit bicarbonate buffer (KHBB) containing sodium chloride (115 mM), potassium chloride (4.7 mM), calcium chloride (2.5 mM), monobasic potassium phosphate (1.2 mM), magnesium sulfate (1.2 mM) and sodium bicarbonate (25 mM). The solution was continuously bubbled with 95% O_2-5% CO_2. For inhibition of metabolism of tacrolimus, the everted sacs were preincubated in KHBB containing 5 mM glucose and 20 μM miconazole, an inhibitor of CYP3A, for 15 min. The everted sacs containing KHBB in the serosal side were incubated for 60 min at 37°C in 30 ml KHBB containing 5 mM glucose and 500 nM tacrolimus as methanol solution (final 3.3% methanol) with and without 20 μM miconazole. Sixty minutes after the addition of tacrolimus, the buffer solution of the mucosal side and the serosal side were collected and the intestinal tissue was homogenized with 9 volumes of KHBB for the quantitation of tacrolimus.

Assay of Tacrolimus

Samples (100 μl) were mixed with a precipitation reagent (200 μl) consisting of methanol (50%, v/v), ethylene glycol (30%, v/v), water (20%, v/v) and zinc sulfate (100 mM), and centrifuged for 10 min at 10,000 rpm. The supernatant (180 μl) was fractionated by HPLC. The HPLC system was composed of a pump (LC-9A, Shimadzu Corporation, Kyoto, Japan), guard filter (Sumipax filter PG-ODS, Sumika Chemical Analysis Service, Ltd., Osaka, Japan), an analytical column (ChemcoPak, Chemcosorb 5-ODS-H, 4.6 × 150 mm, Chemco Scientific Co., Ltd., Osaka, Japan), and a spectrophotometric detector (SPD-2A, Shimadzu Corporation). The mobile phase consisted of methanol and water (80:20, v/v), the flow rate was 1 ml/min, and column temperature was 60°C. The retention time of tacrolimus was determined with a UV spectrophotometric detector after injection of 1.8 μg tacrolimus into the column. The fraction corresponding to unchanged tacrolimus was collected between 4.7 and 6.7 min, evaporated with an Automatic Environmental Speed Vac® System (Savant Instruments, Inc., Farmingdale, NY), and reconstituted with 100 μl of drug-free whole blood. The samples were quantitated by an EIA method (IMx®, Dainabot Co., Ltd., Tokyo, Japan) (9).

Data Analysis

The pharmacokinetic parameters of tacrolimus after intravenous infusion were estimated using the software package NONMEM (double precision NONMEM Version IV and PREDPP Version III) (10). The model was parameterized in terms of the total clearance (CL), volume of the central compartment (V_1), intercompartmental clearance (Q), and the volume of distribution (V_{ss}) using the PREDPP subroutines, ADVAN3 and TRANS3, for the two-compartment model. The apparent clearance values (CL/F) expressed by the CL and bioavailability (F) after intraportal infusion and intraintestinal injection were calculated from dose/area under the blood concentration-time curve (AUC). The AUC after intraportal infusion and intraintestinal injection were calculated using the linear trapezoidal rule and extrapolated to infinity by adding the ratio of the last measurable tacrolimus concentration to the terminal disposition rate constant. The F values after intraportal infusion (F_{portal}) and intraintestinal injection ($F_{intestinal}$) were calculated from the CL/F values after intraportal and intraintestinal administration, respectively.

Statistical Analysis

Values are expressed as mean ± S.E. Statistical significance of differences between mean values was calculated using the non-paired t-test.

RESULTS

Pharmacokinetics of Tacrolimus in Rats

Fig. 1 shows the mean blood concentration of tacrolimus following intravenous infusion at a dose of 1.0 mg/kg in rats. The kinetics of tacrolimus in rats can be adequately described by a two-compartment model with a rapid initial distribution phase followed by a slower disposition phase. Table 1 shows the pharmacokinetic parameters of tacrolimus after intravenous infusion. The mean CL value was 42.7 ml/min/kg, and the V_{ss} was very large (5.22 l/kg). Fig. 1 also shows the mean blood concentrations of tacrolimus following intraintestinal administration at 1.0 mg/kg in rats. The blood tacrolimus concentrations after intraintestinal injection were significantly lower than those after the intravenous infusion. The time to peak concentration after intraintestinal administration was from 15 to 30 minutes, indicating that the absorption of tacrolimus was very rapid. The amount of tacrolimus in the intestine after the 240-min intraintestinal injection experiment was small (0.2% of dose). Table 2 shows the pharmacokinetic parameters of tacrolimus after intraintestinal administration. The mean $F_{intestinal}$ was low (26.2%). Under these experimental conditions, the mucosal site

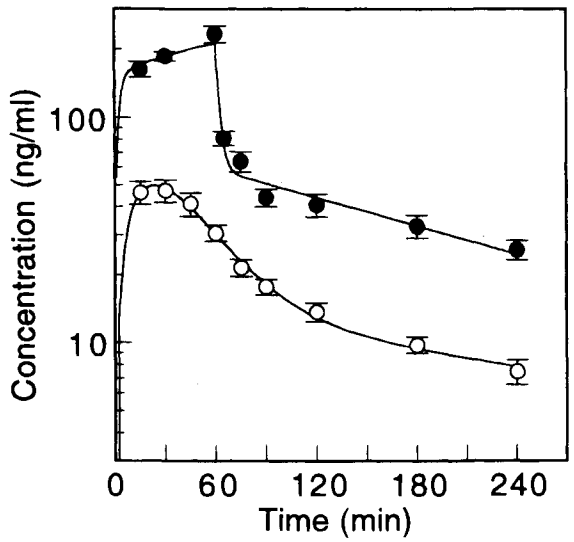

Fig. 1. Blood tacrolimus concentrations measured by HPLC-EIA following 60-min intravenous (closed circles, n = 8) or intraintestinal (open circles, n = 7) administration at a dose of 1.0 mg/kg to normal rats. Each point is the mean ± S.E.

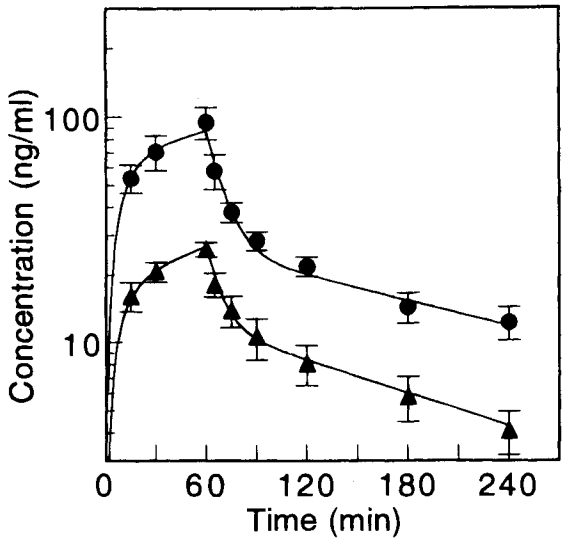

Fig. 2. Blood tacrolimus concentrations measured by HPLC-EIA follwing 60-min intraportal administration (n = 8) at a dose of 1.0 mg/kg (circles) or 0.3 mg/kg (triangles) to normal rats. Each point is the mean ± S.E.

of the intestine did not show sores or ulceration even after the 240-min experimental period.

Fig. 2 shows the mean blood concentrations of tacrolimus following intraportal infusion at 1.0 mg/kg or 0.3 mg/kg in rats. The slope of the terminal phase after intraportal infusion was not different from that after the intravenous infusion. The initial distribution phase after intraportal infusion was slower than that after intravenous infusion (Fig. 1 and Fig. 2). Table 2 also shows the pharmacokinetic parameters of tacrolimus after intraportal infusion. The *CL/F* was dose-independent, and consequently, the F_{portal} value was also dose-independent. The F_{portal} values were about 39.8%–41.3%, which were higher than that after intraintestinal injection.

Table 1. Pharmacokinetic Parameters After Intravenous Infusion in Normal Rats

CL (ml/min/kg)	V_1 (l/kg)	*Q* (ml/min/kg)	V_{ss} (l/kg)
42.7 ± 2.4	0.253 ± 0.003	60.5 ± 1.3	5.22 ± 0.34

Note: Results are mean ± S.E. of eight rats.

Table 2. Apparent Clearance and Bioavailability in Normal Rats

Administration	Dose (mg/kg)	*CL/F* (ml/min/kg)	*F* (%)
Intraintestinal	1.0	163 ± 18	26.2
Intraportal	1.0	107 ± 13	39.8
Intraportal	0.3	104 ± 20	41.3

Note: Results are mean ± S.E. of seven or eight rats per group.

In Vitro Metabolism of Tacrolimus by Everted Sacs of the Intestine

The amount of tacrolimus in the serosal side was less than 0.5% (detection limit) of the applied amount after 60 min incubation of everted sacs of the rat small intestine with 500 nM tacrolimus solution both in the presence and absence of miconazole. Table 3 shows the % of applied amount of tacrolimus in the mucosal side, the serosal side and in the tissue. In addition, the amount of metabolized tacrolimus is shown as % of that applied in Table 3. Without miconazole, the amounts of tacrolimus in the mucosal side and in the tissue after 60-min incubation were 69.4% and 7.6% of the amount applied, respectively. This result indicated that 23% of the applied amount of tacrolimus was metabolized during the 60-min incubation period. On the other hand, the amounts of tacrolimus in the mucosal side and in the tissue after 60-min incubation with miconazole were 85.4% and 15.3% of the amount applied, respectively. Therefore, about 100% of the amount of tacrolimus applied was recovered after 60-min incubation with miconazole, indicating that this agent completely inhibited the metabolism of tacrolimus in the small intestine.

Table 3. Amount Remaining After Incubation and Metabolism of Tacrolimus by the Everted Sac Method

	miconazole (−)	miconazole (+)
% of remained after 60 min		
Mucosal side	69.4 ± 2.7	85.4 ± 5.2[a]
Tissue	7.6 ± 1.6	15.3 ± 1.6[a]
% of metabolized	23.0 ± 2.5	−0.7 ± 6.1[a]

Note: Results are mean ± S.E. of four separate experiments.
[a] Significantly different from respective value without miconazole, $P < .05$ according to non-paired *t*-test.

DISCUSSION

The bioavailability of orally administered tacrolimus is poor and variable, ranging from 4 to 89% (with mean of about 25%) in kidney and liver transplant recipients (1). This may be attributed to several factors including poor and variable absorption, and first pass metabolism in the intestine and liver. However, the contributions of metabolism in each organ to first pass metabolism are unknown. In this study, we clarified the contribution of the intestine as well as the liver to the first pass effect of tacrolimus in rats. In addition, the first pass effect in the intestine may be more related to metabolism by CYP3A than to drug exsorption by P-glycoprotein.

The pharmacokinetics of tacrolimus after intraportal administration were linear at a dose of 1.0 mg/kg or with a blood concentration of 100 ng/ml in rats. That is, the F_{portal} value was dose-independent and about 40% (Table 2). The hepatic extraction ratio of tacrolimus administered at a dose of 1.0 mg/kg was 60.2%, which was calculated from $1\text{-}F_{portal}$. The extraction of tacrolimus in the intestine could be also attributed to metabolism because it was absorbed almost completely (99.8%). To evaluate the intestinal extraction ratio of tacrolimus, we assumed that intestinal metabolism does not contribute to its systemic clearance after intravenous administration. This assumption was supported by a previous report that intestinal metabolism is negligible for systemic clearance (11). With this assumption, we calculated the intestinal extraction ratio at a dose of 1.0 mg/kg from $1\text{-}F_{intestinal}/F_{portal}$. Thus, the intestinal extraction ratio was estimated as 34.2%. Therefore, about 34% of the absorbed tacrolimus was considered to be metabolized in the intestine and about 60% of the tacrolimus reaching the portal circulation was metabolized in the liver after intestinal administration in rats.

Recently, it was suggested that CYP3A is expressed in the intestine as well as liver (12), and that the intestinal metabolism contributes largely to the oral bioavailability of tacrolimus and other CYP3A substrates in clinical studies (6,13–15). By using the everted sac technique, we determined whether the extraction of tacrolimus in the intestine is caused by CYP3A. In this study, 23% of the tacrolimus applied disappeared during 60-min incubation in the absence of miconazole. On the other hand, tacrolimus did not disappear during 60-min incubation in the presence of miconazole, a specific inhibitor of the CYP3A subfamily (Table 3). This observation suggested that tacrolimus is metabolized by CYP3A in the intestine.

Recently, it was suggested that the exsorption by P-glycoprotein, as well as metabolism by CYP3A4 in the intestine, contribute to the first pass effects of some drugs including tacrolimus and cyclosporine, which are substrates of these proteins (6,12). In this study, however, exsorption by P-glycoprotein may not affect the first pass effect of tacrolimus directly because tacrolimus was absorbed almost completely (99.8%) after intraintestinal administration. In addition, tacrolimus was absorbed rapidly, and the blood tacrolimus concentration reached the maximum from 15 to 30 minutes after administration (Fig. 1). Therefore, we suppose that P-glycoprotein contributes little to the extraction of tacrolimus in the intestine. On the other hand, it was suggested that P-glycoprotein may cause a shift in drug localization to the distal small intestine which contains lower amounts of CYP3A4, and that P-glycoprotein may prolong the duration of absorption (16). Thus, P-glycoprotein may affect the extraction of drugs in the intestine. In the everted sac experiment, the amount of remaining tacrolimus in the tissue in the presence of miconazole was significantly greater than that in absence of miconazole (Table 3). The reason for this phenomenon is not clear, but it may be due to the inhibitory effect of miconazole on drug efflux by P-glycoprotein.

In conclusion, we showed that absorbed tacrolimus is moderately metabolized in the intestine as well as in the liver as one step of the first pass effect. The present findings provide useful information for further studies of drug interactions with CYP3A in the intestine as well as of the mechanism of poor and variable bioavailability of tacrolimus in human.

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