The site specific transport and metabolism of tacrolimus in rat small intestine

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**Abbreviations:**  HCO-60, polyethoxylated hydrogenated castor oil; P-gp, P-glycoprotein; $P_{\text{app}}$, apparent permeability; AP, apical; BL, basolateral; MDR, multidrug resistance; CYP, Cytochrome P-450; CYP3A, Cytochrome P-450 3A; HPMC, hydroxypropyl methyl cellulose; TEER, transepithelial electrical resistance; DMEM, Dulbecco’s Modified Eagle Medium; NEAA, non-essential amino acids; EDTA, ethylenediaminetetraacetic acid; FkmAb, FK506 monoclonal antibody; FK-POD, FK506-conjugated peroxidase
ABSTRACT

The objective of this study was to evaluate the absorption of tacrolimus by means of simultaneous perfusion of intestinal lumen and blood vessels in rats. In our previous report (Tamura et al., 2002), the permeability of tacrolimus was found to be higher in the jejunum than in the ileum or colon, suggesting the site-dependent absorption after oral administration. However, in this paper, simultaneous perfusion technique revealed that the extent of absorption into blood vessels was similar in the jejunum and the ileum regardless of the site difference in permeability as the absorption rate. In addition to the multidrug resistance (MDR)-associated protein-mediated efflux, Cytochrome P-450 (CYP) mediated metabolism could be a possible mechanism to explain the inconsistencies in the site dependency of tacrolimus absorption. Two enzyme inhibitors, ketoconazole and midazolam, were co-perfused in rat intestinal lumen with tacrolimus to specify the effect of P-gp and CYP. In the jejunum, both inhibitors significantly enhanced the absorbed amount of tacrolimus, while the permeability was not affected. It was suggested that both inhibitors mainly suppress CYP-mediated metabolism in the upper region of the intestine. In contrast, in the ileum, ketocollazol significantly enhanced both the absorbed amount and the permeability of tacrolimus. However, midazolam failed to enhance the absorption of tacrolimus, indicating the dominant role of P-gp mediated efflux in the lower region. From these findings, it is concluded that the site-dependent differences in P-gp and/or CYP activity could be the prime cause of large intra- and inter-individual variability in the oral absorption of tacrolimus.

Tacrolimus is a 23-member macrolide lactone (molecular weight = 803.5 Da) with potent
immunosuppressive activity that has been shown to be effective in the prophylaxis of organ rejection following liver, heart, kidney and small bowel transplantation (Starzl et al., 1989; Todo et al., 1990). It is commercially available as capsules for oral administration and as an injectable for intravenous infusion. Because of its low solubility in water (4-12 µg/mL), intravenous dosage forms of tacrolimus contain surfactants and the oral dosage form is available only as a solid dispersion formulation containing hydroxy propyl methyl cellulose (HPMC). The pharmacokinetics of tacrolimus in normal volunteers and in liver and kidney transplant patients have been reported previously (Jusko et al., 1995; Venkatramanan et al., 1995). Tacrolimus is primarily eliminated from the body by hepatic metabolism and less than 1% of the dose is recovered in urine. The oral bioavailability of tacrolimus is low and exhibits large intra- and inter-individual variability, ranging from 4% to 89% (mean of around 25%) in liver and kidney transplant recipients and in patients with renal impairment (Venkatramanan et al., 1995). The correlation of dose to blood concentration is low as a result of a large variability in pharmacokinetic parameters among patients. Because of this variability and the narrow therapeutic index of tacrolimus, post-treatment monitoring of blood levels is an integral part of patient care to maintain drug levels within the therapeutic range in order to optimize therapy and reduce undesirable toxicity effects (Armstrong and Oellerich, 2001).

Several factors have been suggested as possible determinants of the low and variable oral bioavailability of tacrolimus. These include a low solubility in the intestine, extensive metabolism by CYP3A in both liver and gut, P-gp-mediated drug efflux and the influences of intake of food and concomitant medications. Several studies on the absorption of tacrolimus in animal models have been reported (Jain et al., 1990; Fukukawa et al., 1992; Kagayama et al., 1993; Swaminathan et al., 1993; Mekki et al., 1994). In studies with oral absorption of tacrolimus in rats, the possibility of an absorption window in the upper small intestine has been suggested.
However, the mechanism of tacrolimus absorption, its intrinsic permeability to the intestinal membrane in animals and humans and the underlying cause of its highly variable bioavailability have not been fully addressed.

Thus, in a previous report, we have used a single-pass intestinal perfusion technique in rats to evaluate the intrinsic permeability of tacrolimus as well as to identify the factors that contribute to its low and variable oral absorption (Tamura et al., 2002). We have successfully demonstrated that tacrolimus is an intrinsically high permeability drug, but shows significant site dependency in intestinal permeability. The permeability was found to be highest at jejunum followed by ileum and colon. Perfusion with verapamil, a potent inhibitor of P-gp, did not affect the tacrolimus permeability in the jejunum while ileal and colonic permeabilities were markedly enhanced. These results indicate that the differences in P-gp function among these three sites cause the site-dependent permeability of tacrolimus.

It has been known that tacrolimus is also a substrate of CYP3A and is extensively metabolized in the intestinal epithelium (Benet et al., 1999). Differences in CYP3A activity in the different intestinal regions, therefore, could also be a determinant for overall absorption of tacrolimus. In the present study, we used the simultaneous perfusion techniques of intestinal lumen and blood vessels in rats to address the roles of P-gp and CYP on the overall absorption of tacrolimus. These studies in rat are expected to provide a better understanding of the factors that control intestinal absorption of tacrolimus in humans and allow the design of improved drug delivery strategies in order to reduce large intra- and inter-subject variability in absorption following oral administration.

**METHODS**

**Materials**

Tacrolimus was provided by the Fujisawa Pharmaceutical Co., Ltd.(Osaka, Japan).
Polyoxyethylated hydrogenated caster oil 60 (HCO-60) was supplied by NIKKO Chemicals Co., Ltd. (Tokyo, Japan). The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco’s modified Eagle medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS), L-glutamate, trypsin (0.25%)-EDTA (1mM) and antibiotic-antimycotic mixture (10000 U/mL penicillin G, 10000 µg/mL streptomycin sulfate and 25 µg/mL amphotericin B in 0.85% saline) were purchased from Gibco Laboratories (Lenexa, KS, USA). All other chemicals were of analytical grade and commercially available.

**Preparation of Perfusion Solution**

The perfusion solution for intestinal lumen was similar to that previously used (Tamura et al., 2002). Tacrolimus (25 µg/mL) was dissolved in a citric acid-dibasic sodium phosphate buffer containing 1% (w/v) HCO-60 to reduce adhesion of tacrolimus to the lining of the tube. The pH was adjusted to 6.5 by using NaOH or HCl. FITC Dextran (0.01%) was added to the perfusion solution to measure the net water flux in intestinal perfusion studies, resulting from water absorption or exsorption in the intestinal segment. When investigating the effect of enzyme inhibitors, ketoconazole or midazolam was added to the perfusion solution. As the vascular perfusate, Krebus-phosphate bicarbonate buffer (KHBB, pH = 7.4) that contains bovine serum albumin (BSA, 3%) and D-glucose (10mM) was used (Taki et al. 1995) The perfusion solution was maintained at 37°C by water bath.

**Simultaneous Perfusion Study**

All experiments were performed on male Charles River rats, 200-300 g, and age 60 to 80 days. The rats were fasted overnight, 12 to 18 hr before each experiment. A small intestinal loop (jejunum or ileum, about 10 cm) was isolated from rats and its blood vessels were perfused according to the method of Yamashita et al. (1994). Briefly, the superior mesenteric artery and the portal vein were cannulated with polyethylene tubes (i.d. 0.5 mm) for vascular perfusion. At both
ends of the intestinal loop, polyethylene tubes (i.d. 3 mm) were placed, through which tacrolimus was introduced and withdrawn. The cannulated intestinal segment was isolated from other portions and suspended in a serosal bath containing 100 mL of Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4) warmed to 37°C with a water jacket as illustrated in Fig 1.

Perfusion of the blood vessels was started just after the intestinal segment was isolated and continued throughout the experiment. KHBB containing D-glucose (10 mM) and BSA (3%) was used as the vascular perfusate at a rate of 2.5 mL/min and changed from 1.0 to 4.0 mL/min so that the blood flow rate dependency of drug absorption could be studied. Then the intestinal lumen was perfused with the perfusion solution containing tacrolimus at a perfusion flow rate of 0.2 mL/min. The perfusion solution leaving the intestinal lumen was taken at 10-min intervals and the vascular outflow was collected at 5-min intervals for 60 minutes.

The perfusion samples were frozen immediately after collection and stored in a freezer at –20°C until analyzed. After the last sample was taken, the length was measured by placing a piece of string along the intestine and measuring the string with a ruler. Drug concentration in the serosal bath fluid was also measured. The rate of drug disappearance from intestinal lumen and drug appearance into the vascular outflow was calculated from the drug concentration in each sample.

**Caco-2 Transport Study**

Caco-2 monolayers were obtained from the American Type Culture Collection (Rockville, MD) at passage 17. Caco-2 monolayers were grown in DMEM supplemented with 10% FBS, 1% L-gultamine, 1% NEAA, and 5% antibiotic-amimycotic solution at 37°C in humidified air-5% CO₂ atmosphere. The culture medium (1.5 mL in the insert and 2.6 mL in the well) was first replaced after 72 hr and every 48 hr thereafter. The transport studies were conducted with monolayers between 15 to 20 days in culture. All transport experiments were performed for 1 hr
at 37°C. Prior to the experiments, the culture medium was replaced with transport medium and cell monolayers were subsequently equilibrated for 30 minutes at 37°C before the transport study. Transport medium was composed of 136.89 mM NaCl, 5.36 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.41 mM MgSO₄·7H₂O, 19.45 mM glucose, 1.26 mM CaCl₂, 0.49 mM MgCl₂·6H₂O, 4.17 mM NaHCO₃ and 10 mM Hepes, and the pH adjusted to 6.5 for apical (AP) solution and to 7.5 for basolateral (BL) solution.

In AP-to-BL transport studies, transport medium (1.5 mL) containing tacrolimus (25 µg/mL) with or without enzyme inhibitor (ketoconazole or midazolam) (300 µM) was applied to the AP side. Then, the 6-well cell culture plate was gently swirled to ensure complete mixing. Samples (100 µL) were taken carefully from the BL side and replaced with the same volume of fresh transport medium. In BL-to-AP transport studies, transport medium (2.6 mL) containing tacrolimus (25 µg/mL) was applied to the BL side. In this case, ketoconazole or midazolam (300 µM) was applied to the AP side. Then, the 6-well cell culture plate was gently swirled to ensure complete mixing. Samples (100 µL) were then withdrawn carefully from the AP side and replaced with the same volume of fresh transport medium. The AP-to-BL and BL-to-AP permeability of tacrolimus was calculated from its flux rate into the opposite side of the initial application of tacrolimus. Caco-2 monolayers were checked for confluency by measuring the transepithelial electrical resistance (TEER) before and after the transport study.

**HPLC Assay**

Tacrolimus concentration in intestinal perfusion solution was determined by HPLC: pump (Model 510), a WISP automatic sampler (Model 712), and a UV detector (Model 481) from Waters (Milford, MA, USA) and a TSK-gel 120 column (5µm, 25cm x 4.6 mm i.d. Tosho Co. Ltd., Tokyo, Japan) were used. After addition of 0.02 mL of ethanol, 1.0 mL of pH7 phosphate buffer and 4.0 mL of ethyl acetate to 0.2 mL samples of the intestinal perfusion solution, tacrolimus was
extracted into the ethyl acetate phase. Then 3 mL of the ethyl acetate phase was taken into a clean tube and evaporated to dryness at 40°C under a stream of nitrogen gas. The resulting residue was redissolved by the addition of 100 µL of 80% methanol solution. Then 20 µL of sample solution was injected into HPLC. Mixture water and methanol and acetonitrile (40:30:30, v/v) were used as a mobile phase with a flow rate of 1.0 mL/min. The wavelength of the detector was 210 nm. The calibration curve of tacrolimus was prepared in each assay in a concentration range from 0 to 50 µg/mL. The correlation coefficient was always more than 0.99, and a detection limit of quantification was 0.5 µg/mL.

**EIA Assay**

According to the method of Kobayashi et al. (1991), tacrolimus was measured by competitive enzyme immunoassay with mouse anti-FK506 monoclonal antibody (FKmAb) and FK506-conjugated peroxidase (FK-POD). Tacrolimus in vascular perfusion solution, serosal bath fluid and Caco-2 transport medium was extracted with ethyl acetate and evaporated. The residue was dissolved in FK-POD solution and was added to a microtiter plate well, previously coated with FKmAb, to determine competitive binding of tacrolimus and FK-POD with FKmAb. POD activity was measured using o-phenylenediamine and hydrogen peroxide as cosubstrates. The reaction was stopped by addition of H₂SO₄, and the optical density was measured by a microplate reader (CS-9300PC, Shimadzu, Kyoto, Japan). Tacrolimus content was determined by comparison with a standard curve. The calibration curve of tacrolimus was prepared in each assay in a concentration range from 0 to 40 ng/mL. The correlation coefficient was always more than 0.99, and a detection limit of quantification was 0.1 ng/mL.

**Analysis of Absorption Processes of Tacrolimus**

On the basis of drug disappearance from the intestinal lumen and its appearance into the vascular outflow at the steady-state condition in the simultaneous perfusion study, the absorption
processes of tacrolimus across rat intestinal epithelium were divided into three steps. The first step was the uptake into the intestinal epithelium and was represented by the parameter of uptake amount. The uptake amount was calculated from the difference in the tacrolimus concentration in the luminal perfusate before and after the single-pass perfusion and was expressed as uptake rate per unit length of intestine (µg/min/cm). Apparent permeability (Papp, cm/sec) of tacrolimus was also calculated from the difference in the concentration in the luminal perfusate before and after the perfusion, as described in a previous report (Tamura et al., 2002). The second process was the absorption into the blood and was represented by the parameter of absorbed amount. Absorbed amount was calculated from the recovered amount of tacrolimus into the vascular outflow sample in simultaneous perfusion study and was expressed as absorption rate per unit length of intestine (µg/min/cm). The third process was the enzymatic degradation in the intestinal epithelial cells and was represented as the metabolized amount. The metabolized amount was estimated as the difference between the uptake and the absorbed amount. The ratios of absorbed and metabolized amount of tacrolimus to the uptake amount were also defined in order to consider the enzyme activity in the intestinal epithelium.

RESULTS

Validation of Experimental Method to Evaluate The Absorption of Tacrolimus

Apparent permeability (Papp) of tacrolimus in rat jejunum and ileum was measured with or
without vascular perfusion by means of the luminal perfusion of the intestinal tract in order to check the effect of vascular perfusion on the intestinal permeability. Papp of tacrolimus measured in both conditions in jejunum and ileum are shown in Table 1. Since no significant differences were observed in Papp in both conditions, it was confirmed that vascular perfusion caused no effect on the intestinal permeability of tacrolimus during the experimental period. This result provides the validity of our method to estimate tacrolimus absorption across the intestinal membrane.

Yamashita et al. (1994) have reported that the absorption of high permeability drug shows vascular flow rate-dependency in simultaneous perfusion study. Since tacrolimus is classified as a high permeability drug (Tamura et al., 2002), the effect of vascular perfusion rate on the absorption of tacrolimus should be investigated to define the appropriate perfusion rate. Uptake and absorbed amounts of tacrolimus at 1 mL/min, 2.5 mL/min and 4 mL/min of vascular flow rates are shown in Figure 2. There were no significant differences in the uptake amount among the three flow rates, whereas the absorbed amount at 1 mL/min of vascular flow rate was significantly lower than other rates, suggesting that the absorption of tacrolimus was limited by the vascular flow rate in this condition. In the following study, vascular perfusion rate was fixed to 2.5 mL/min.

In most experiments, tacrolimus was not detected in the serosal bath fluid at the end of experiment and, if the significant amount of tacrolimus was detected, the experiment was regarded as unsuccessful due to the technical problem and whole data were omitted. Therefore, in this paper, it is reasonable to consider that the difference between uptake and absorbed amount of tacrolimus represents the amount metabolized in the enterocytes during the absorption.
Site Dependency of Tacrolimus Absorption in Rat Intestine

Figure 3 shows uptake and absorbed amounts of tacrolimus in rat jejunum and ileum in simultaneous perfusion study. Uptake of tacrolimus in the jejunum was approximately two times greater than that in the ileum. This also means that Papp of tacrolimus is two times higher in the jejunum than in the ileum. However, no significant differences in the absorbed amount (into vascular perfusate) were observed in either site. The ratio of absorption/uptake was thus greater in the ileum than in the jejunum. These findings indicate that, although tacrolimus shows clear site dependency in terms of permeability, the amount absorbed into the portal vein should be the same in upper and lower part of the small intestine. Since the difference between the uptake and absorbed amount was regarded as the amount degraded in the intestinal epithelium, Figure 3 shows that about 50% of the uptake amount of tacrolimus degraded in the jejunum during the absorption, but only 20% in the ileum. It has been reported that tacrolimus is a good substrate of CYP3A and extensively metabolized in the intestine as well as in the liver (Benet et al., 1999). In the following study, therefore, we have tried to clarify the site-dependent differences in the activity of CYP as well as P-gp in rat small intestine by using two kinds of enzyme inhibitors.

Effects of Ketoconazole and Midazolam on Tacrolimus Absorption

In order to determine whether the P-gp mediated efflux or the CYP mediated metabolism is responsible for the observed site-dependent absorption of tacrolimus, the effect of enzyme inhibitors on the absorption of tacrolimus was investigated. Ketoconazole and midazolam were used as enzyme inhibitors. Ketoconazole is a potent inhibitor of CYP3A (Ki of ~ 1 µM) and a moderately potent inhibitor of P-gp (K of ~ 120 µM) (Benet et al., 1999). Midazolam is a typical substrate of CYP3A (Km of ~ 1 µM) (Obach et al., 2002), and was reported not appear to be a P-gp substrate (Kim et al., 1999).
Since Caco-2 cells are known to express P-gp on their apical (brush-border) membrane, first, we determined the effects of ketoconazole and midazolam on the transport of tacrolimus across Caco-2 monolayers in order to clarify the effect of both inhibitors on P-gp mediated efflux. Figure 4 shows apical to basal (AP-BL) and basal to apical (BL-AP) transport of tacrolimus across Caco-2 monolayers with or without enzyme inhibitors. Without enzyme inhibitors, BL-AP permeability of tacrolimus was about 5-fold higher than that of AP-BL direction. Ketoconazole markedly decreased BL-AP transport of tacrolimus and increased AP-BL, showing that ketoconazole interacts with P-gp to inhibit the efflux of tacrolimus effectively. In the case of midazolam, neither AP-BL nor BL-AP permeability of tacrolimus was affected. These findings clearly indicated that ketoconazole, but not midazolam, inhibit the efflux of tacrolimus mediated by P-gp.

Simultaneous perfusion studies were performed in the jejunum and the ileum in the presence of ketoconazole or midazolam (300 µM). Figure 5 illustrates a mass-balance of tacrolimus across rat enterocyte, that is, the uptake, absorbed and metabolized amounts. The width of the arrow expresses approximately the amount of tacrolimus transported or metabolized in each process. Apparent permeability was also calculated based on the uptake amount of tacrolimus during the perfusion.

Under the control condition (without inhibitors), uptake amount, or the permeability, of tacrolimus was 2-times greater in the jejunum than in the ileum as was shown in Fig. 3. In the jejunum, however, more than 50% of tacrolimus was metabolized in the intestinal cells, thus the absorbed amount was almost the same level as that observed in the ileum. In the ileum, only 20% of uptake amount suffered metabolism. Ketoconazole, a potent inhibitor for both P-gp and CYP, enhanced the absorbed amount of tacrolimus in both sites, but the mechanism of increased absorption might be site-dependent. In the jejunum, metabolized percentages were dramatically reduced by adding ketoconazole, while the uptake did not change. In contrast, the main cause for
increased absorption in the ileum was the enhanced uptake at the apical membrane. In the ileum, the metabolized amount did not change although the metabolized percentages were reduced to 10% due to the increase in uptake amount. The results with ketoconazole infer that the rate-limiting or rate-determining process of tacrolimus absorption is different between the jejunum and the ileum. In the jejunum, the upper part of the small intestine, tacrolimus absorption is limited by the metabolism in the intestinal cells and efflux (by P-gp) does not play a major role to control its absorption. In contrast, P-gp dominantly limits the absorption of tacrolimus by lowering the permeability at the apical membrane in the ileum. These ideas were well confirmed by the experiment with midazolam, an inhibitor of CYP with no significant effect on P-gp mediated efflux (Fig. 4). In the jejunum, midazolam also increased the absorption of tacrolimus by suppressing the metabolism in the cells. However, midazolam failed to enhance the absorption of tacrolimus in the ileum since it has no inhibitory effect on P-gp.

**DISCUSSION**

Intestinal single-pass perfusion experiment has often been used to determine the permeability of drugs to the intestinal membrane. Since the obtained permeability of various drugs correlate with their oral absorption in human (Amidon et al. 1988), absorbability of drugs can be predicted from this in situ experiment. By using the single-pass perfusion technique in rat, we have already reported (1) tacrolimus has intrinsically high permeability in the intestinal membrane, (2) the permeability is higher in the jejunum than in the ileum or colon, and (3) P-gp mediated efflux may contribute to this site-dependent permeability of tacrolimus (Tamura et al., 2002). However, since tacrolimus is known to be a substrate of CYP3A and extensively
metabolized in the intestine as well as in the liver, information obtained from the luminal perfusion study may not give enough information to consider the overall absorption of tacrolimus into the blood.

In this study, we have successfully characterized the overall processes of tacrolimus absorption across rat jejunum and ileum by using a simultaneous perfusion technique. The same type of study was performed to evaluate intestinal absorption of a peptide drug, metokephamid, in our previous report (Taki et al., 1995), where the degradation and the permeation of the peptide in the intestinal membrane was calculated. In this report, tacrolimus absorption was divided into three processes: uptake, metabolism and absorption (Fig. 5). Furthermore, the study with enzyme inhibitors clearly demonstrated the role of P-gp and CYP in regulating the absorption of tacrolimus. Simultaneous perfusion technique and the mass-balance analyses on the drug movement are quite useful to consider the absorption of drugs that are chemically or enzymatically unstable in the intestine.

The results in Fig. 5 clearly demonstrate the differences in P-gp and CYP functions in the jejunum and the ileum. It is obvious that tacrolimus absorption was regulated mainly by CYP-mediated metabolism in the jejunum, but by P-gp mediated efflux in the ileum. The existence of P-gp on the brush-border membrane of rat intestinal mucosa has already been demonstrated (Hsing et al., 1992). Regional differences in the functional expression of P-gp were investigated in rat and human intestine, indicating the higher activity of P-gp in the ileum and the colon than in the jejunum. The difference in P-gp expression between the upper and the lower intestine in human was approximately 2-fold (Fojo et al., 1987; Makhey et al, 1998). Trezise et al. (1992) have also reported that the expression of multidrug resistance to MDR1 mRNA varies in rat intestine, with moderate expression in the duodenum and the jejunum, maximal expression in the ileum, and then a decrease in expression through the proximal and distal colon. Thus,
regional differences in P-gp mediated efflux could be one of the possible mechanistic explanations for the site-dependent absorption of tacrolimus. The reason why the uptake amount of tacrolimus in the jejunum was not affected by ketoconazole is not clear at present. One plausible explanation is that the high permeability of tacrolimus and the low P-gp expression in the upper intestine lead to the saturation of P-gp which might mask the effect of ketoconazole in the jejunum. This hypothesis can also explain the fact that the permeability in the ileum in the presence of ketoconazole was similar to that observed in the jejunum.

CYP3A is the major subfamily of CYP enzymes leading to the oxidative biotransformation in both humans and rats, and is abundant in rat and human enterocytes as well as in the hepatocytes (Kolars et al., 1992). Some reports have shown that CYP3A is a major CYP enzyme for the metabolism of some drugs in rat intestinal microsomes (Zhang et al., 1998; Higashikawa et al., 1999). CYP3A has also been identified as being responsible for the O-demethylation of tacrolimus in the human and rat liver (Sattler et al., 1992; Vincent et al., 1992; Shiraga et al., 1993). In addition, enterocytes CYP3A was suggested to be involved in the first pass metabolism of tacrolimus (Lampen et al., 1996; Floren et al., 1997). Hashimoto et al. (1998) have evaluated the contribution of intestine to the first pass effect of tacrolimus in rats, indicating about 34% of the absorbed tacrolimus was metabolized in rat intestine. This first-pass effect in the intestine should be related to the metabolism by CYP3A since the blood concentration of tacrolimus was increased by the co-administration of fluconazole, which is known to inhibit CYP enzymes in humans and animals (Manez et al., 1994). Therefore, differences in CYP3A activity in different regions of the intestine could also account for the site-dependent absorption of tacrolimus. Several reports have shown that CYP3A activity is higher in the jejunum compared to that in the ileum or colon (Paine et al., 1997). Also it was reported that metabolite formation of tacrolimus in the gastrointestinal tract of pig varied depending on the regions where the highest concentration
of metabolites was found in the duodenum, followed by the jejunum, ileum and colon (Lampen et al., 1996). This site-dependent metabolism in pig gastrointestinal tract is coincides with the distribution of CYP3A in human intestine (Kolars et al. 1994). Shimomura et al. (2002) have investigated the metabolism of tacrolimus in the rat intestine using in vitro everted sac experiments. They have clearly demonstrated the regional differences in tacrolimus metabolism in the small intestine where the metabolic activity in the jejunum was higher than that in the ileum. These results are in agreement with our findings reported here.

In contrast, Zhang et al. (1996) have reported that only low amounts of CYP3A enzyme were found in rat enterocytes. Although several subfamilies of CYP, including 1A1, 2B1, 2B2, 2A1, 3A1 and 3A2, have been detected in rat enterocytes by the enzyme activity measurements, some authors were unable to detect the message for CYP3A2 and inferred that intestinal microsomes contain only CYP3A1 (Kolars et al., 1992; Zhang et al., 1996). In the human intestine, CYP3A4 has been identified as a major enzyme responsible for tacrolimus metabolism. Although Lampen et al (2002) have demonstrated the formation of demethylated tacrolimus in rat small intestinal microsomes, the enzymes which contributed to this metabolism have not been specified yet. Further studies are necessary to clarify the subfamily of CYP enzymes, which contribute to the intestinal first-pass metabolism of tacrolimus in rat.

The cooperative effects of CYP and P-gp as a major barrier to oral drug delivery have been the subject of several reports (Hebert, 1997; Benet et al., 1999; Kim et al., 1999). It was suggested that continued passive absorption and active P-gp efflux of the drug would subject the drug to repeated exposure to CYP3A at concentrations much lower than required for saturation effects (Benet et al., 1999). Therefore, even very low levels of intestinal CYP3A may impact drug metabolism substantially, provided it is coupled with a highly active intestinal P-gp function.

Through the study reported here, we propose a new interpretation on the compensative
collaboration of CYP enzymes and P-gp as a barrier for tacrolimus absorption. Shown in Figure 6
is an image of the intestinal barrier for which CYP and P-gp are responsible. CYP-mediated
metabolism works dominantly in the upper portion, then P-gp mediated efflux becomes more
important in the lower. In total, barrier function might be constant throughout the intestine. This
idea corresponds to our results where the absorbed amounts of tacrolimus into the portal vein
were not significantly different in the jejunum and the ileum in simultaneous perfusion study.
However, since tacrolimus is a drug with high permeability to the intestinal membrane and the low
oral dose, its absorption is considered to complete quickly in the upper intestine after oral
administration. In fact, Tmax of tacrolimus is reported to be only 2 hours (Bekersky et al., 1998). In
such a case, the role of P-gp as an absorption barrier in the lower intestine becomes insignificant.
Wacher et al (2002) have reported that the absorption of sirolimus, a P-gp substrate, was not
influenced by oral co-administration of P-gp inhibitor. If the similar study is performed with
tacrolimus, the same result will be obtained. On the other hand, Chiou et al (2000) have reported
that the absorption of slowly and/or incompletely absorbed compounds are affected by P-gp
because these compounds keep being absorbed in the lower intestine. Therefore, the
compensative roles of CYP and P-gp in the intestine are profoundly related to the permeability,
solubility and the dose of drugs. For the therapy with tacrolimus, our findings are further important
to develop a modified release product, which expects the absorption in the lower intestine. If
tacrolimus is administered as a modified release product, both CYP and P-gp would impact the
rate and extent of absorption and be the factors of intra- and inter-subject variability.

Although CYP and P-gp act cooperatively as a barrier against the absorption of tacrolimus in
this study, their essential role is a defense against the invasion of various ingested toxic chemicals,
including drugs. The overall disposition profiles of CYP and P-gp in the intestine, therefore, should
be important for functioning as a detoxication system.
CONCLUSIONS

In conclusion, by using a simultaneous perfusion technique, we have successfully demonstrated the role of CYP enzymes and P-gp as a barrier for tacrolimus absorption in rat small intestine. Tacrolimus absorption was found to be regulated mainly by CYP-mediated metabolism in the jejunum, but by P-gp mediated efflux in the ileum. These results support the hypothesis that CYP-mediated metabolism and P-gp-mediated efflux in the intestine cause a low oral bioavailability of drugs that are CYP and/or P-gp substrates. Although the contribution of other mechanisms limiting the intestinal absorption of tacrolimus could not be ruled out, differences in P-gp and CYP function in each intestinal site could be a main cause of the large variability in its absorption. The present findings will be useful in improving the absorption of tacrolimus and reduce intra- and inter-individual variability by providing better knowledge of intestinal absorption of tacrolimus.
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Schematic diagram of simultaneous perfusion study. (Simultaneous perfusion of intestinal lumen and blood vessel)

Figure 2. Effects of vascular perfusion rate on uptake (○) and absorbed (●) amount of tacrolimus in simultaneous perfusion study. The uptake amount was calculated from the difference in the tacrolimus concentration in the luminal perfusate before and after the perfusion. Absorbed amount was calculated from the recovered amount of tacrolimus into the vascular outflow sample. *, Difference was significant (P<0.05) compared with other vascular perfusion rate by Tukey's test. Each point represents the mean ± sd of three experiments.

Figure 3. Site-dependent differences in tacrolimus absorption in rat intestine. Uptake and absorbed amount of tacrolimus in rat jejunum and ileum were obtained by simultaneous perfusion study. Absorbed ratio means the percent of absorbed amount to the uptake amount of tacrolimus. Results are expressed as the mean ± sd of three experiments.

Figure 4. Apical to basal and basal to apical transport of tacrolimus across Caco-2 monolayers in the presence or absence of enzyme inhibitors. Tacrolimus (25 μg/mL) was added to the apical or basal solution, then the samples were taken from the opposite side to calculate the permeability. Enzyme inhibitor, ketoconazole or midazolam (300 μM), was applied to the apical side in all experiments. AP-BL: apical to basal transport, BL-AP: basal to apical transport. Results are expressed as the mean ± sd of four experiments. *, Difference was significant (P<0.05) compared with the control by Student's t-test. n.s., Difference was not significant.
Figure 5. Overall absorption processes of tacrolimus in rat jejunum and ileum evaluated by the simultaneous perfusion study in the presence or absence of enzyme inhibitors. In the figure, the width of the arrow expresses approximately the extent of tacrolimus amount transported or metabolized in each process. Intestinal epithelial cells in the upper row correspond to the jejunum and those in the lower row to the ileum. Metabolized amount of tacrolimus in the cells is calculated as the difference between the uptake and the absorbed amount. Results are expressed as the mean ± sd of three experiments.

\[ a \] absorbed ratio (% of absorbed amount to the uptake amount), \[ b \] metabolized ratio (% of metabolized amount to the uptake amount).

Figure 6. Image of the intestinal barrier for tacrolimus absorption.

---, CYP activity, ---, P-gp activity, -----, Total barrier function for tacrolimus absorption.
Table 1. Effect of vascular perfusion on the apparent permeability of tacrolimus in rats.

<table>
<thead>
<tr>
<th>Intestinal site</th>
<th>Apparent permeability(^a) (10(^{-6})cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without vascular perfusion</td>
</tr>
<tr>
<td></td>
<td>With vascular perfusion</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.352 ± 0.048</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.166 ± 0.048</td>
</tr>
<tr>
<td></td>
<td>0.324 ± 0.029</td>
</tr>
<tr>
<td></td>
<td>0.174 ± 0.011</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± sd of four experiments.

\(^a\) Calculated based on the total tacrolimus concentration in the luminal perfusion solution (25 µg/mL).
Mixed gas
95% O₂
5% CO₂

Vascular perfusion

Pump

2.5mL/min

Intestinal perfusion

0.2mL/min

Vascular perfusate

Serosal bath fluid

Intestinal segment

Intestinal perfusate

Mixed gas
95% O₂
5% CO₂
<table>
<thead>
<tr>
<th></th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ Ketoconazole</td>
</tr>
<tr>
<td>Permeability</td>
<td>0.344±0.019</td>
<td>0.323±0.036</td>
</tr>
<tr>
<td>(10⁻⁴cm/sec)</td>
<td>0.065±0.004</td>
<td>0.061±0.006</td>
</tr>
<tr>
<td>uptake amount</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/min/cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolized</td>
<td>0.034±0.007</td>
<td>0.006±0.001</td>
</tr>
<tr>
<td>amount</td>
<td>(52.2±7.0%)</td>
<td>(19.8±4.3%)</td>
</tr>
<tr>
<td>(g/min/cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorbed</td>
<td>0.031±0.003</td>
<td>0.054±0.009</td>
</tr>
<tr>
<td>amount</td>
<td>(47.8±7.0%)</td>
<td>(88.1±4.8%)</td>
</tr>
<tr>
<td>(g/min/cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>