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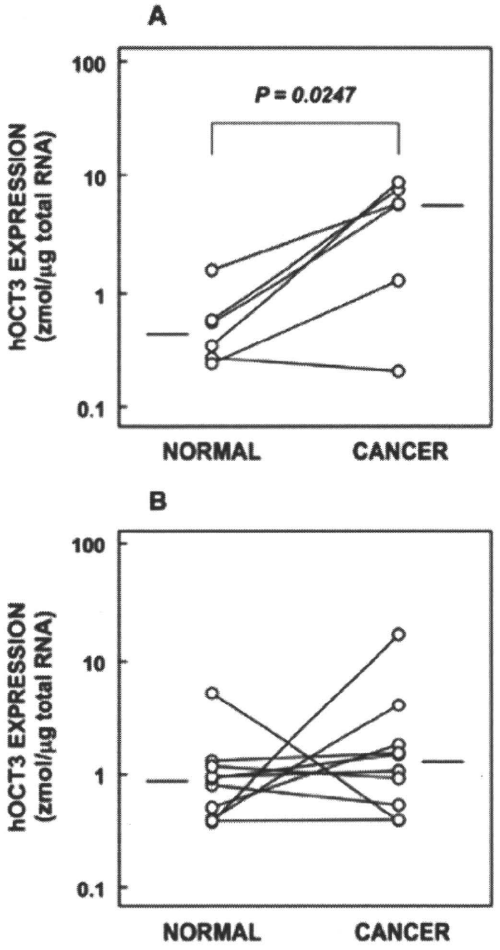

FIG. 1. hOCT3 mRNA expression in normal or cancerous tissues derived from Japanese patients. The expression of hOCT3 mRNA in colon (A) or rectum (B) was detected by real-time PCR. The numbers of patients with colon and rectum cancer were 6 and 10, respectively. The horizontal bars represent the median of hOCT3 mRNA expression.

and compared the levels of OCT3 mRNA in colorectal cancer and normal colorectum and among colorectal cancer-derived cell lines. In addition, the cytotoxicity and platinum accumulation in cultured cells caused by the treatment with oxaliplatin or cisplatin were examined.

Materials and Methods

Cell Culture. Human colorectal cancer-derived cell lines, T84 (CCL-248; American Type Culture Collection, Manassas, VA), SW480 (CCL-228; American Type Culture Collection), HCT116 (91091005; European Collection of Cell Cultures, Wiltshire, UK), HT29 (HTB-38; American Type Culture Collection), SW837 (JCRB9115; Health Science Research Resources Bank, Osaka, Japan), and Lovo (JCRB9083; Health Science Research Resources Bank) were used. Human embryonic kidney (HEK)293 cells (CRL-1573; American Type Culture Collection) were used as a host for gene transfection (Yonezawa et al., 2006). Cell lines were cultured in an atmosphere of 5% CO_2-95% air at 37°C. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) was used for SW480, HT29, HCT116, Lovo, and HEK293. DMEM containing 10% FBS and 1% nonessential amino acid (Invitrogen) was used for SW837, and a 1:1 mixture of DMEM and nutrient mixture Ham's F12 medium (Sigma-Aldrich) with 10% FBS was used for T84. T84, HT29, and Lovo cells were seeded onto 24-well plates or 96-well plates at a density of 4.0×10^5 cells/ml. SW480 and HCT116 cells were seeded at 2.0×10^5 cells/ml, and SW837 cells were seeded at 5.0×10^5 cells/ml. Seventy-two hours after the seeding, the cells were used for the experiments.

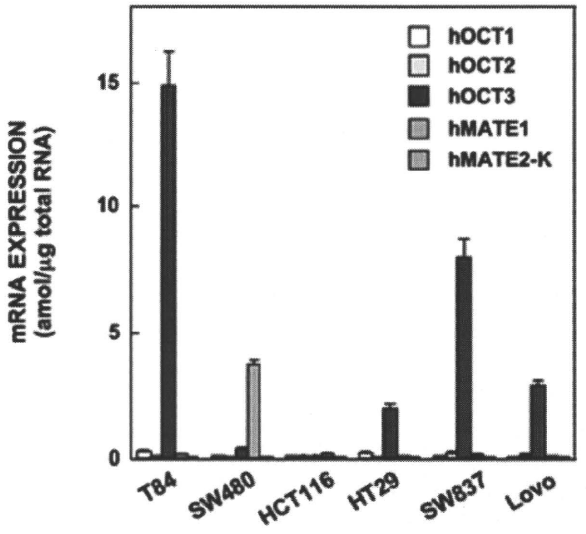

FIG. 2. mRNA levels of organic cation transporters in colorectal cancer-derived cell lines. Total RNA from the cell lines was reverse-transcribed, and the OCT1, hOCT2, hOCT3, hMATE1, or hMATE2-K expression levels were measured by using real-time PCR. Each column represents the mean ± S.E. for three wells. The plot of real-time PCR results for hOCT3 and glyceraldehyde-3-phosphate dehydrogenase is found in the supplemental data.

Transfection. For a transient expression system, pCMV6-XL4 plasmid vector DNA (OriGene Technologies, Rockville, MD) containing hOCT3 cDNA was purified using an EndoFree Plasmid Mega Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions (Yonezawa et al., 2006). The day before transfection, HEK293 or SW480 cells were seeded onto poly-D-lysine-coated and noncoated 24-well plates, respectively. The cells were transfected with 800 ng of plasmid DNA per well in a combination of empty vector and hOCT3 cDNA using 2 μl of Lipofectamine 2000 (Invitrogen) per well according to the manufacturer's instructions. The amount of hOCT3 cDNA was 800 ng except in the experiment examining the transporter cDNA dependence. Forty-eight hours after the transfection, the cells were used for the experiments.

Uptake Experiment. Cellular uptake of [^{3}H]1-methyl-4-phenylpyridium acetate (MPP) (2.7 TBq/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) was measured with monolayer cultures grown on 24-well plates. The composition of the incubation buffer was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM HEPES (pH 7.4 adjusted with NaOH). As previously reported, experiments on the uptake were performed (Urakami et al., 2004).

For measurement of the cellular accumulation of oxaliplatin or cisplatin, cells seeded on 24-well plates were incubated with DMEM containing 10% FBS and oxaliplatin (Wako Pure Chemical Industries, Osaka, Japan) or cisplatin (Sigma-Aldrich) for 2 min or 1 h. After this incubation, the monolayers were rapidly washed twice with ice-cold incubation buffer containing 3% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) and then washed three times with ice-cold incubation buffer. The cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined using inductively coupled plasma-mass spectrometry (ICP-MS) by the Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan). The protein content of the cell monolayers solubilized in 0.5 N NaOH was determined with a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA).

Cytotoxicity Assay. The cytotoxicity of oxaliplatin was measured with cells seeded on 24-well plates for the lactate dehydrogenase (LDH) assay and on 96-well plates for the caspase 3/7 assay. Cells were incubated with medium containing oxaliplatin for 6 h for the LDH assay. After removal of the medium, a drug-free medium was added to the wells. After incubation for 24 h, the medium was collected, and the LDH activity in it was measured using a LDH Cytotoxicity Detection Kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. LDH release (percent) was calculated as described previously (Yonezawa et al., 2006). For the caspase assay, cells were incubated

with medium containing oxaliplatin for 8 h. After the incubation, caspase 3/7 activity was determined by using a Caspase-Glo 3/7 Assay (Promega, Madison, WI), according to the manufacturer's instructions. Caspase activity (fold increase) represents (caspase 3/7 activity in oxaliplatin-treated cells)/(caspase 3/7 activity in cells without oxaliplatin).

Isolation of Total RNA and Real-Time PCR. Total RNA was isolated from each cell line on 24-well plates using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and the concentrations of total RNA were measured by spectrophotometry. Total RNA was reverse-transcribed with random hexamers using Superscript II reverse transcriptase (Invitrogen), followed by digestion with RNase H (Invitrogen). For the detection of the expression of hOCT3 mRNA in cancerous or normal colon and rectum, the same batch of cDNA samples as used by Terada et al. (2005) was subjected to real-time PCR. Detailed information about the patients was given in the report of Terada et al. (2005). The conditions and primer-probe sets for real-time PCR were described previously (Motohashi et al., 2002; Masuda et al., 2006). The glyceraldehyde-3-phosphate dehydrogenase mRNA level was used as an internal control. This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee.

Cancer Profiling Array. The cDNA cancer profiling array, Cancer Profiling Array I (Clontech, Mountain View, CA) was used. It includes normalized cDNAs from cancer and corresponding normal tissues from individual patients, amplified using SMART technology. Preparation of the cDNA probe for hOCT3, hybridization to the array, and signal detection on X-ray film were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Ltd., Basel, Switzerland) according to the manufacturer's instructions. The relative intensity of each dot was determined densitometrically using ImageJ 1.38x (National Institutes of Health, Bethesda, MD).

Statistical Analysis. Data are expressed as means ± S.E. Data were analyzed statistically using the paired Student's *t* test. Probability values of less than 0.05 were considered statistically significant.

Results

Expression of hOCT3 mRNA in Normal and Cancerous Colorectal Tissues. The expression of hOCT3 mRNA in colon ($n = 6$) or rectal ($n = 10$) tissue derived from Japanese patients was measured by real-time PCR. Figure 1 shows the difference in the expression between normal and cancerous colorectum. In cancerous colon tissue, the level of hOCT3 mRNA was significantly higher than that in normal tissue, and the mean increase in individuals was 9.7-fold (Fig. 1A). The median values of hOCT3 in normal and cancerous colon tissue were 0.44 (range 0.24–1.59) and 5.59 (range 0.20–8.62) zmol/μg of total RNA, respectively ($P = 0.0247$, by the paired Student's *t* test). hOCT3 mRNA expression in rectum tended to increase in cancerous tissue, but the difference was not significant (Fig. 1B). The median values of hOCT3 in normal and cancerous rectal tissue were 0.87 (range 0.39–5.17) and 1.26 (range 0.41–17.1) zmol/μg of total RNA, respectively ($P = 0.363$, by the paired Student's *t* test).

mRNA Expression of Organic Cation Transporters in Colorectal Cancer-Derived Cell Lines. We examined the expression of hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K mRNA in colorectal cancer-derived cell lines, T84, SW480, HCT116, HT29, SW837, and Lovo, by real-time PCR. The hOCT3 transcript was found in all of these cells except HCT116 and was strongly detected in T84 and SW837 (Fig. 2). hMATE1 mRNA was only expressed in SW480. However, the mRNA expression of hOCT1, hOCT2, hMATE1, and hMATE2-K in these cells was almost negligible.

[^{3}H]MPP Uptake by Colorectal Cancer-Derived Cell Lines. To check the functional activity of hOCT3 in cultured cells, we measured the cellular uptake of its typical substrate, [^{3}H]MPP. The accumulation of [^{3}H]MPP was greater in T84 cells, SW480 cells expressing hOCT3, and HEK293 cells expressing hOCT3 than in SW480 cells, SW480 cells transfected with vector cDNA, and HEK293 cells transfected with vector cDNA (Fig. 3A). In addition, we examined [^{3}H]MPP uptake in other colorectal cancer-derived cell lines, HCT116, HT29, SW837, and Lovo. SW837 showed the highest level of activity to transport [^{3}H]MPP among these six cell lines (Fig. 3B). The transport activity of the cells was confirmed, and then these cells and expression systems were used in subsequent experiments on the cytotoxicity and the cellular transport of platinum agents.

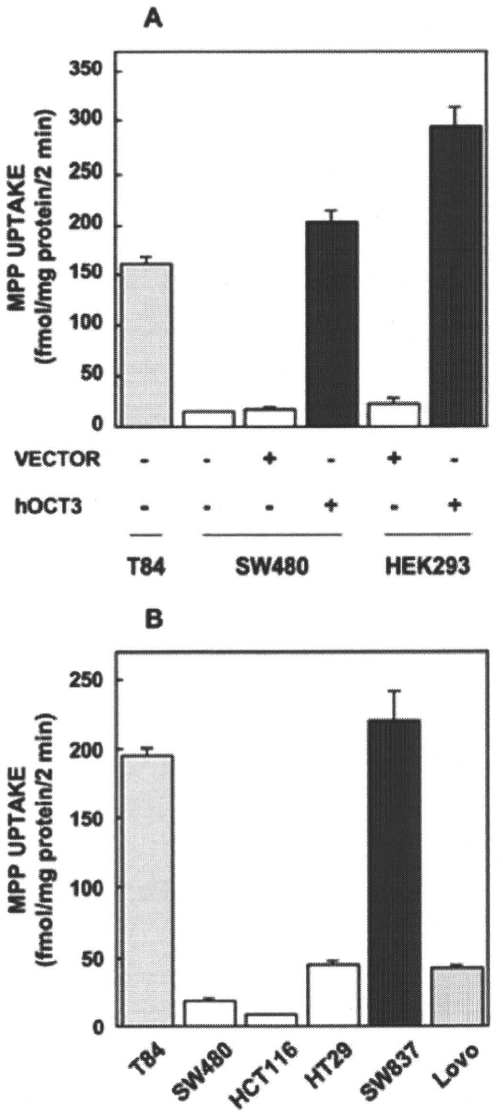

FIG. 3. Uptake of [^{3}H]MPP by colorectal cancer-derived cell lines or HEK293. After preincubation, native T84 or SW480 and SW480 cells or HEK293 cells transiently transfected with empty vector (vector) or hOCT3 cDNA (A), or T84, SW480, HCT116, HT29, SW837, or Lovo cells (B) were incubated with 13.7 nM [^{3}H]MPP for 2 min at 37°C. Each column represents the mean ± S.E. for three wells.

hOCT3 Expression and Oxaliplatin-Induced Cytotoxicity. We examined the effect of hOCT3 expression in a colon cancer-derived cell line, SW480. When SW480 cells transfected with 800 ng of empty vector or hOCT3 were treated with 500 μM oxaliplatin for 6 h and subsequently cultured in normal medium for 24 h, the release of LDH into the culture medium was increased by the expression of hOCT3 (Fig. 4A). In addition, we measured the amount of LDH released by treatment with 500 μM oxaliplatin in other colorectal cancer-derived cell lines. The amount of LDH released was greatest in

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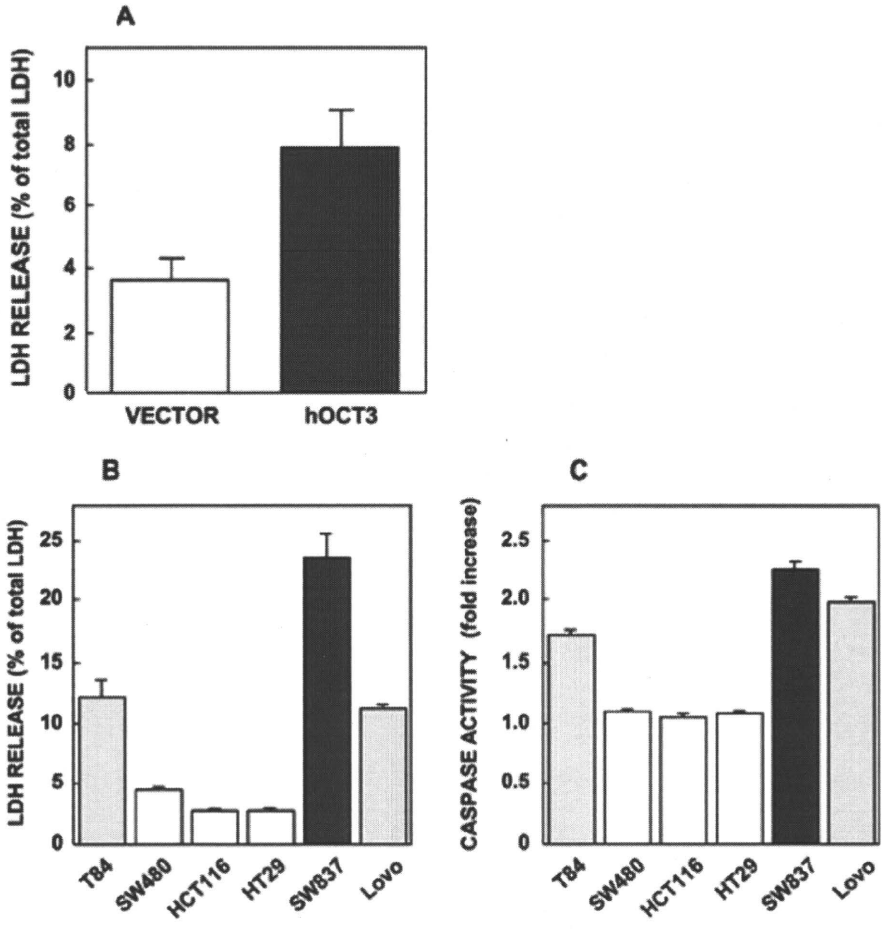

FIG. 4. Role of hOCT3 expression in oxaliplatin-induced cytotoxicity. SW480 cells transiently expressing hOCT3 or empty vector (A) or T84, SW480, HCT116, HT29, SW837, or Lovo cells (B) were treated with 500 μM oxaliplatin in the culture medium for 6 h. Then the cells were incubated in the normal culture medium for 24 h. The amount of LDH released into the medium was measured. C, T84, SW480, HCT116, HT29, SW837, or Lovo cells were treated with 50 μM oxaliplatin in the culture medium for 8 h, and then caspase 3/7 activity was measured. Each column represents the mean ± S.E. for three wells.

SW837 and was also large in T84 and Lovo cells (Fig. 4B). SW480, HCT116, and HT29 cells showed little release of LDH with oxaliplatin treatment.

In addition, caspase 3/7 activity induced by treatment with 50 μM oxaliplatin was examined in these cell lines. The most potent activation of caspase 3/7 was in SW837 but T84 and Lovo cells also showed strong caspase 3/7 activity (Fig. 4C). The results of caspase activity were consistent with those of LDH release.

Transport of Oxaliplatin. We examined the accumulation of oxaliplatin with the increase of hOCT3 cDNA on transfection of SW480 cells (Fig. 5A), because almost no hOCT3 mRNA was found in SW480 cells (Fig. 2). When SW480 cells transfected with 50 to 800 ng of hOCT3 cDNA per well were treated with 1000 μM oxaliplatin for 1 h, the level of platinum accumulated in the cells was increased, depending on the amount of hOCT3 cDNA transfected (Fig. 5A). Based on these results, we determined the platinum accumulation in T84 cells, SW480 cells, and SW480 cells transfected with 800 ng of hOCT3 cDNA. When treated with 100, 500, or 1000 μM oxaliplatin for 1 h, T84 cells and SW480 cells expressing hOCT3 transported oxaliplatin extensively in a concentration-dependent manner compared with SW480 cells or SW480 cells transfected with empty vector (Fig. 5B). Moreover, we examined the amount of platinum accumulated after the treatment with oxaliplatin in other colorectal cancer-derived cell lines, HCT116, HT29, SW837, and Lovo. Platinum was most abundant in SW837 cells at all three concentrations when the cells were incubated with the culture medium containing oxaliplatin for 2 min (Table 1). The same tendency was observed when they were treated for 1 h (Table 2). In HT29 and Lovo cells, the amount of platinum accumulated was approximately half of that in SW837 cells, and the levels in SW480 and HCT116 cells were low compared with those in other cultured cells.

Relation among hOCT3 mRNA Expression, LDH Release, and Platinum Accumulation. When cultured cells were treated with 500 μM oxaliplatin, the release of LDH was increased by the hOCT3 mRNA expression (Fig. 6A). The accumulation of platinum in the cells after the incubation with 500 μM oxaliplatin was also dependent on hOCT3 mRNA expression (Fig. 6B). By combining the data from Fig. 6, A and B, the release of LDH was also comparable with the accumulation of platinum (Fig. 6C). On the other hand, when cells were treated with 500 μM cisplatin, the accumulation of platinum was independent of hOCT3 mRNA expression (Fig. 6D).

Cancer Profiling Array. We examined the differences in hOCT3 expression between normal and cancerous tissues derived from Caucasians using dot blotting, and the density of each dot was quantified using ImageJ 1.38x (Fig. 7). In the colon, the level of hOCT3 was significantly higher in cancerous tissues (Fig. 7A). This result was consistent with that in Fig. 1A. A significant increase of hOCT3 expression was also observed in the rectum and stomach (Fig. 7, B and C). Inversely, a significant decrease of hOCT3 expression in cancerous tissue was detected in the uterus, breast, ovary, and lung (Fig. 7, D–G). In the kidney, there was no significant difference in hOCT3 mRNA expression between normal and cancerous tissue (Fig. 7H).

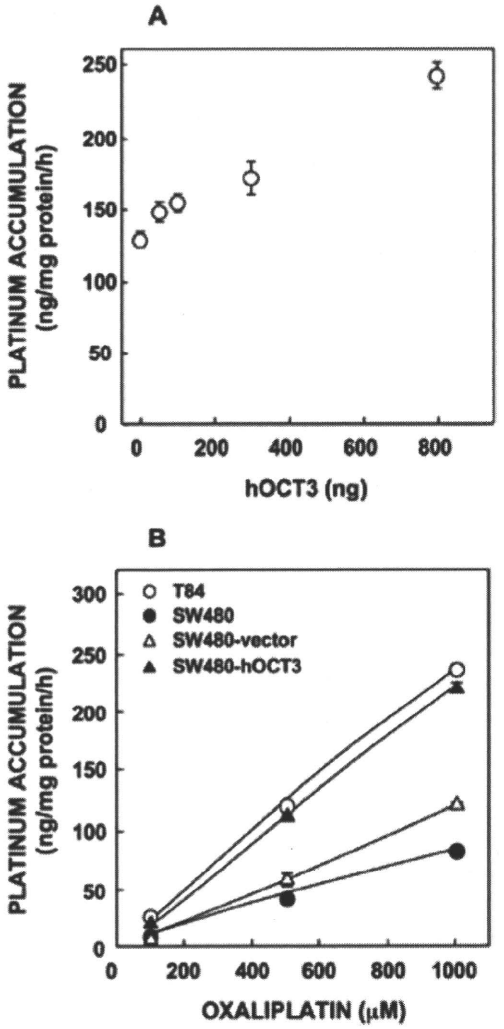

FIG. 5. Uptake of oxaliplatin by colorectal cancer-derived cell lines. A, SW480 cells were transfected with an amount of hOCT3 cDNA and vector plasmid added to 800 ng using 2 μl of Lipofectamine 2000. The cells were exposed to 1000 μM oxaliplatin in the culture medium for 1 h. B, T84 cells, SW480 cells, or SW480 cells transiently expressing empty vector or hOCT3 were treated with culture medium containing 100, 500, or 1000 μM oxaliplatin for 1 h. After being washed, these cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each point represents the mean ± S.E. of four wells.

Discussion

Oxaliplatin has a much more potent anticolorectal cancer effect than cisplatin (Grem et al., 1993; de Gramont et al., 2000). However, the molecular mechanism(s) that cause the difference in the effect has not been made clear. We previously determined that oxaliplatin but not cisplatin was transported by OCT3 (Yonezawa et al., 2006; Yokoo et al., 2007). Therefore, we hypothesized that the level of hOCT3 in cancerous tissues contributed to the superior anticancer effect of oxaliplatin. In the present study, the level of hOCT3 mRNA was significantly higher in cancerous colon than in normal colon tissues derived from Japanese patients (Fig. 1A). In addition, this tendency was reproduced in Caucasians using a cancer profiling array (Fig. 7, A and B). These findings indicated that the level of hOCT3 mRNA was heightened by colorectal cancerous transformation independent of ethnicity.

In human colorectal cancer-derived cell lines, hOCT3 mRNA expression was correlated with the release of LDH and accumulation of platinum induced by the treatment with oxaliplatin (Fig. 6, A and B). These results suggested that hOCT3 expression is a candidate marker for the efficacy of oxaliplatin treatment. Moreover, the release of LDH and accumulation of platinum caused by the incubation with cisplatin was independent of the hOCT3 mRNA level (Fig. 6D). This result was consistent with the report that cisplatin was not transported by hOCT3 (Yonezawa et al., 2006). Therefore, hOCT3 expression is suggested to be closely associated with the anticancer activity of oxaliplatin but not that of cisplatin.

Cisplatin plays an essential role in chemotherapy against solid tumors of the prostate, bladder, lung, testis, liver, and brain (Ho et al., 2003). However, the effect of cisplatin on colorectal cancer is weak. Loehrer et al. (1988) and Grem et al. (1993) reported rates of response of colorectal cancer to cisplatin-based chemotherapy of 22 and 19%, respectively. On the other hand, for oxaliplatin-based chemotherapy, de Gramont et al. (2000) reported that the response rate was 50%. The differences in molecular mechanisms whereby cisplatin has a weak effect but oxaliplatin has a strong effect on colorectal cancer have been unclear. The anticancer activity and resistance to platinum agents have been considered to be related to the DNA repair pathway, nucleotide excision repair, base excision repair, mismatch repair, and double-strand break repair, or the substrate specificity of copper transporters, CTR1, ATP7A, and ATP7B (Kelland, 2007). However, recently, we and others reported the contribution of organic cation transporters in the cellular transport of platinum agents (Ciarimboli et al., 2005; Yonezawa et al., 2005, 2006; Zhang et al., 2006; Yokoo et al., 2007; Kitada et al., 2008). Zhang et al. (2006) reported that the effect of oxaliplatin against colon cancer was related to the expression of hOCT1 and hOCT2. Kitada et al. (2008) reported that the levels of ATP7A and hOCT1 mRNA affect the sensitivity to oxaliplatin. However, we reported that oxaliplatin was transported by both human and

TABLE 1

Platinum accumulation in colorectal cancer-derived cell lines (2 min)

Colorectal cancer-derived cell lines were treated with medium containing 100, 500, or 1000 μM oxaliplatin for 2 min. After being washed, these cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each value represents the mean ± S.E. for four wells.

Cell Lines	Platinum Accumulation		
	100 μM	500 μM	1000 μM
	ng/mg protein/2 min		
T84	0.82 ± 0.03	5.12 ± 0.06	9.77 ± 0.46
SW480	0.48 ± 0.01	2.27 ± 0.02	4.19 ± 0.04
HCT116	0.46 ± 0.03	2.72 ± 0.08	5.23 ± 0.26
HT29	0.64 ± 0.01	3.55 ± 0.05	7.85 ± 0.19
SW837	1.07 ± 0.02	5.77 ± 0.10	14.3 ± 1.13
Lovo	0.61 ± 0.01	3.59 ± 0.07	7.27 ± 0.10

TABLE 2

Platinum accumulation in colorectal cancer-derived cell lines (1 h)

Colorectal cancer-derived cell lines were treated with medium containing 100, 500, or 1000 μM oxaliplatin for 1 h. After being washed, these cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each value represents the mean ± S.E. for four wells.

Cell Lines	Platinum Accumulation		
	100 μM	500 μM	1000 μM
	ng/mg protein/hr		
T84	14.0 ± 0.3	70.5 ± 1.2	160.2 ± 3.9
SW480	4.9 ± 0.1	25.0 ± 0.5	64.0 ± 1.4
HCT116	6.0 ± 0.2	32.3 ± 0.6	79.2 ± 2.8
HT29	8.7 ± 0.1	47.8 ± 0.1	123 ± 2.6
SW837	16.7 ± 0.4	91.6 ± 2.3	222 ± 3.8
Lovo	9.6 ± 0.0	52.4 ± 1.2	126 ± 2.1

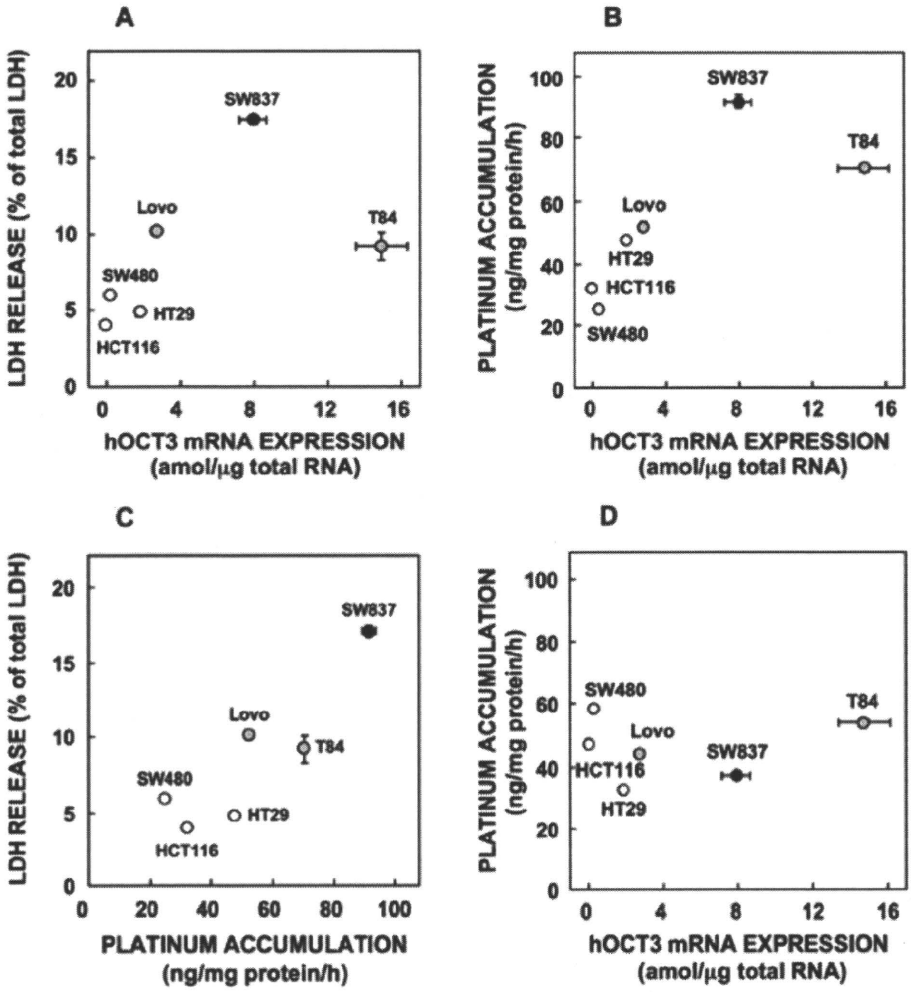

FIG. 6. Relation among hOCT3 mRNA expression, LDH release, and platinum accumulation. The data on mRNA expression are from Fig. 2, and the data on the release of LDH and accumulation of platinum are from Fig. 4B and Table 2, respectively. A, hOCT3 mRNA expression versus LDH release on treatment with 500 μM oxaliplatin. B, hOCT3 mRNA expression versus platinum accumulation on treatment with 500 μM oxaliplatin. C, platinum accumulation versus LDH release on treatment with 500 μM oxaliplatin. D, hOCT3 mRNA expression versus platinum accumulation on treatment with 500 μM cisplatin.

rat OCT2 and OCT3, but not by OCT1 (Yonezawa et al., 2006; Yokoo et al., 2007). In the present study, only hOCT3 mRNA was found in the six cell lines derived from colorectal cancers, and the cytotoxicity of oxaliplatin was associated with the expression level. Based on these findings and the present results, at least in colorectal cancer, OCT3 is thought to be important for sensitivity to oxaliplatin.

In the six colorectal cancer-derived cell lines, hOCT3 mRNA levels were markedly higher than hOCT1, hOCT2, hMATE1, or hMATE2-K mRNA levels (Fig. 2). Previously, we reported that oxaliplatin was also transported by hOCT2 (Yonezawa et al., 2006). However, in these cell lines, the expression of hOCT2 mRNA was little detected by real-time PCR (Fig. 2). Therefore, the contribution of hOCT2 to the anticancer effect of oxaliplatin was suggested to be small. The transport activity of hOCT3 in these cells was confirmed by using [^{3}H]MPP, a typical substrate of OCT3 (Fig. 3). Okuda et al. (2000) reported that the cytotoxicity of cisplatin differed at low and high doses, that is, 30 and 1000 μM cisplatin induced apoptosis and necrosis, respectively. In the present study, we used two indexes of cytotoxicity, LDH release and caspase 3/7 activity, as indicators of necrosis and apoptosis, respectively. Both LDH release and caspase activity showed a similar tendency; that is, values were high in cell lines expressing high levels of hOCT3 mRNA (Figs. 2 and 4, B and C). From these results, the hOCT3-mediated cellular accumulation of oxaliplatin might be a trigger for the subsequent cytotoxic effects.

Although the cytotoxicity of oxaliplatin in T84 cells was lower than expected, given the expression level of hOCT3 (Fig. 6A), the LDH release in these cells correlated quite well with the platinum accumulation (Fig. 6C). These results suggest some mechanisms including an unknown oxaliplatin efflux transporter to reduce the intracellular platinum concentration in T84 cells compared with that in SW837 cells.

We had reported that the nephrotoxicity caused by treatment with platinum agents was closely associated with their renal accumulation, which is determined by the substrate specificity of the OCT and MATE families (Yonezawa et al., 2005, 2006; Yokoo et al., 2007). There had also been a report that the uptake of imatinib, a tyrosine kinase inhibitor effective in the treatment of chronic myeloid leukemia, was mediated by hOCT1 (Thomas et al., 2004). Recently, two groups showed that hOCT1 was a determinant of outcome in imatinib-treated chronic myeloid leukemia (White et al., 2007; Wang et al., 2008). Patients with a high level of hOCT1 had a greater probability of achieving a cytogenetic response and superior progression-free and overall survival (Wang et al., 2008). These reports showed the participation of hOCT1 in the clinical effects of imatinib. Therefore, the results of this study, that the cytotoxicity of oxaliplatin depended on hOCT3 expression, may be expanded to include effectiveness in clinical cases.

OCT3 is widely distributed in many tissues (Kekuda et al., 1998), but its function has been examined mainly in the brain (Wu et al.,

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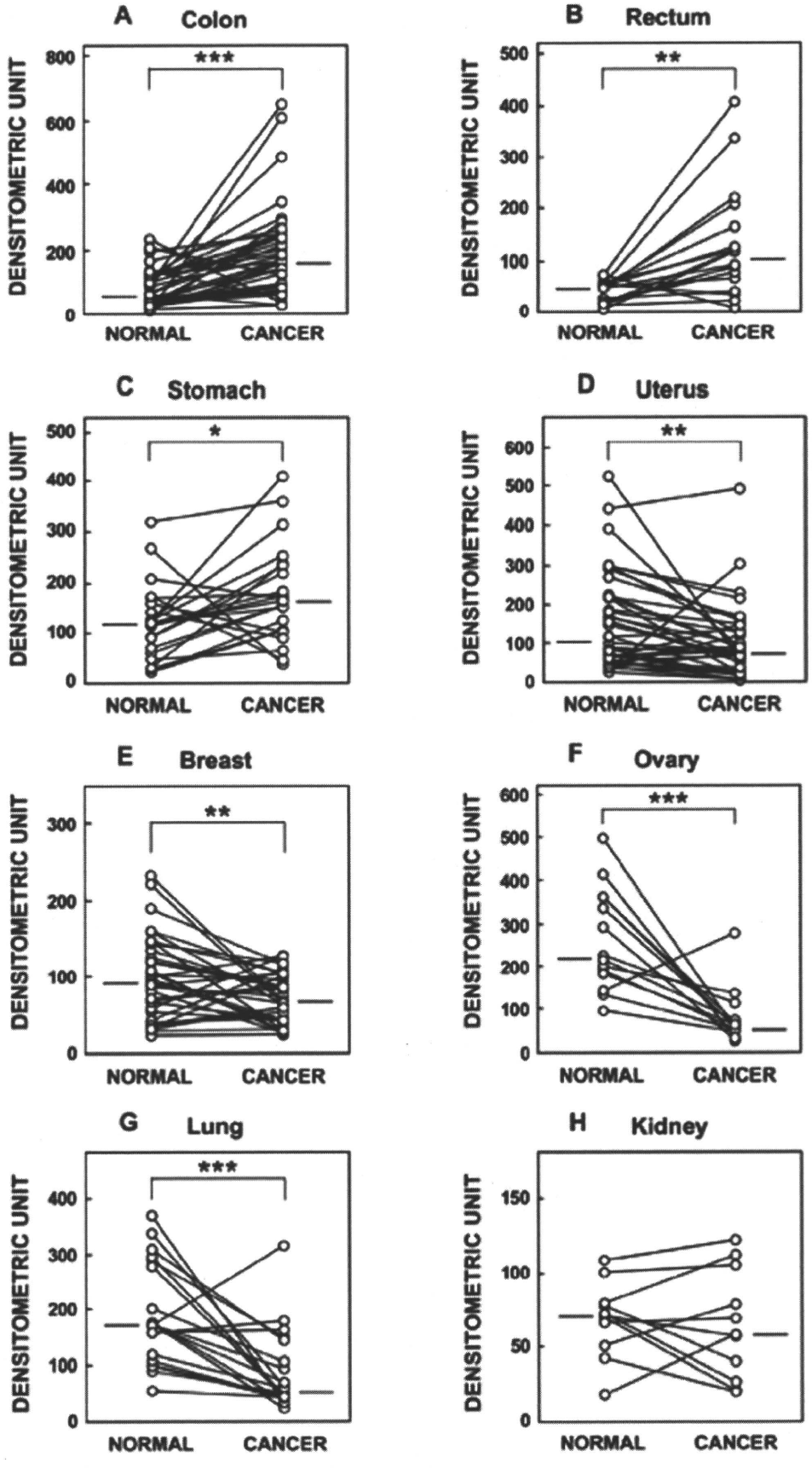

FIG. 7. The differences in hOCT3 expression between normal and cancerous tissues. The differences in hOCT3 expression between normal and cancerous tissue were examined by dot blotting. The density of each dot was quantified using ImageJ 1.38x. Figures represent the colon (A, n = 39), rectum (B, n = 18), stomach (C, n = 23), uterus (D, n = 42), breast (E, n = 35), ovary (F, n = 14), lung (G, n = 20), and kidney (H, n = 11). The bars represent median values.

1998; Gasser et al., 2006). The results of this study suggested a new role for OCT3, as a determinant of the sensitivity of treatment with oxaliplatin against colorectal cancer. At present, oxaliplatin is used for colorectal cancer as a key drug of FOLFOX regimens (de Gramont et al., 2000). Other combinations including oxaliplatin for colorectal cancer or other cancers have been used in clinical trials (Goldberg et al., 2004; Zhu et al., 2006). The level of hOCT3 in cancerous tissue was significantly higher in colon, rectum, and stomach (Fig. 7, A–C). Conversely, the level was significantly lower in uterus, breast, ovary, and lung (Fig. 7, D–G). These changes in hOCT3 expression might contribute to the sensitivity and selectivity of oxaliplatin-based chemotherapy. Recently, there were several reports that oxaliplatin was effective against gastric cancer in phase II trials (Lordick et al., 2005; Park et al., 2006; Kim et al., 2008). Considering the present results, there is a possibility that the increase of hOCT3 expression in cancerous tissue affects the results of clinical trials. Therefore, taking a positive attitude to use of oxaliplatin-based chemotherapy for other cancers that express high levels of hOCT3 compared with normal tissue may lead to good clinical results.

In the present study, we clearly found selective induction of hOCT3 mRNA expression in colon cancer and colorectal cancer-derived cell lines. The cytotoxicity and accumulation of platinum caused by the treatment with oxaliplatin but not cisplatin depended on the expression of hOCT3 mRNA. In conclusion, the uptake of oxaliplatin into the cancer cells via hOCT3 was suggested to be an important mechanism for its cytotoxicity, and the expression of hOCT3 in cancers may became a marker for including oxaliplatin in cancer chemotherapy.

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Drug Metab. Pharmacokinet. 23 (6): 469–475 (2008).

Regular Article

Effect of Intestinal and Hepatic First-pass Extraction on the Pharmacokinetics of Everolimus in Rats

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: The aim of this study was to quantitatively evaluate the effects of intestinal and hepatic extraction on the pharmacokinetics of everolimus in rats. Everolimus was administered intravenously, intraportally or intraintestinally in order to separately evaluate the intestinal and hepatic first-pass extraction. Cyclosporine or tacrolimus was administered into rat intestines, and after 10 min everolimus pharmacokinetics were evaluated. The blood concentrations of everolimus were measured by the high-performance liquid chromatography with tandem mass spectrometry. Total body clearance of everolimus was constant in the dosage from 0.2 to 1.0 mg/kg. The bioavailability after intraportal and intraintestinal administration were 48.0% and 21.2%, respectively. Concomitantly administered cyclosporine (5 mg/kg) , but not tacrolimus (1 mg/kg), significantly decreased the total body clearance of everolimus compared with the control, and also increased the bioavailabilty of everolimus after intraintestinal administration 1.75-fold. Cyclosporine significantly increased the area under the blood concentration-time curve of everolimus after the intraintestinal constant infusion 3-fold, and increased that after the intraportal constant infusion only 1.35-fold. In conclusion, the intestine as well as liver contributes to the first-pass extraction for everolimus in rats. Intestinally administered cyclosporine inhibited the intestinal extraction of everolimus more than its hepatic extraction.

Keywords: everolimus; first-pass effect; pharmacokinetics; cyclosporine; tacrolimus; interaction

Introduction

Everolimus is an immunosuppressive macrolide bearing a stable 2-hydroxyethyl chain substitution at position 40 on the sirolimus (rapamycin) structure. Both everolimus and sirolimus inhibit the mammalian target of rapamycin (mTOR) and suppress the activation of lymphocytes and cell proliferation.[1] Everolimus was developed in an attempt to improve the oral bioavailability of sirolimus,[2] and is used generally with a calcineurin inhibitor cyclosporine for renal or heart transplantations.[3–6] When everolimus is used together, synergistic pharmacological interactions are expected to potentiate the immunosuppressive effects of cyclosporine,[7] and the dose of cyclosporine can be reduced and various adverse effects, such as nephrotoxicity or neurotoxicity, caused by cyclosporine can be substantially decreased.[5,6]

Everolimus is absorbed rapidly, but has variable pharmacokinetics,[3,8] which is probably explained by different activities of drug efflux pump P-glycoprotein and of metabolism by the cytochrome P450 (CYP) 3A subfamily, as well as cyclosporine or tacrolimus.[9–11] Since higher blood concentrations of everolimus are related to the incidence of adverse effects such as hypertriglycemia, hypercholesterolemia and thrombocytopenia, blood concentration monitoring is required.[12] Therefore, pharmacokinetic interactions between everolimus and other drugs that inhibit the transport by P-glycoprotein and/or metabolism by CYP3A subfamily should be carefully considered to achieve safe therapies. It was reported that the blood concentration of everolimus was elevated with combination cyclosporine treatment in patients and rats,[2,7,13] probably due to inhibition of hepatic and/or intestinal metabolism of everolimus by cyclosporine. However, the quantitative contribution of intestinal and hepatic first-pass extraction of everolimus to its phar-

Received; February 29, 2008, Accepted; July 17, 2008
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This work was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the 21st Century COE Program 'Knowledge Information Infrastructure for Genome Science'.

macokinetics have not been well understood, and the effect of another concomitantly administered calcineurin inhibitor, tacrolimus, on everolimus pharmacokinetics have not been sufficiently enough evaluated.

In this study, we administered everolimus by different routes namely, intraintestinally, intraportally and intravenously, and separately evaluated the effect of intestinal and hepatic extraction on the pharmacokinetics of everolimus in rats. Next, we examined the effects of cyclosporine and tacrolimus on the first-pass extraction of everolimus. Although it should be noted that there are some peculiarities of drug metabolism in rats when bioavailability or drug-drug interactions are extrapolated to humans, the findings presented here could be helpful to understand the bioavailability of everolimus and interaction mechanisms of calcineurin inhibitors in humans, because injectable formulation of everolimus is not on the market and its bioavailability in humans is unknown.

Materials and Methods

Materials: Everolimus was a gift from Novartis Pharma AG (Basel, Switzerland) and it was in microemulsion and injection formulations. Tacrolimus injection solution (Prograf injection, 5 mg/mL) was obtained from Astellas Pharma Inc. (Tokyo, Japan). Cyclosporine (Sandimmun injection, 50 mg/mL) was obtained from Novartis Pharma KK (Tokyo, Japan). 32-Desmethoxyrapamycin was obtained from Wyeth (Madison, NJ). All other chemicals used were of the highest purity available.

Animals: Male Wistar/ST rats weighing 220 to 260 g (8 week old) were used for in the vivo study. Before the experiment, rats were fasted overnight, but given free access to water. Animals were anesthetized with sodium pentobarbital (1 mg/kg i.p.). Supplemental doses of pentobarbital were administered as required. Body temperature was maintained with appropriate heating lamps. The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

Pharmacokinetic studies in rats: In each experiment, the femoral artery was cannulated with a polyethylene tube (PE-50; BD Biosciences, San Jose, CA) filled with heparinized saline (50 U/mL) for blood sampling. In the experiments for intravenous administration, the femoral vein was cannulated, and everolimus was administered from the femoral vein. In separate experiments for intraportal administration, portal vein was cannulated with a polyethylene tube (PE-10) with a 26 G needle, and everolimus was administered intraportally for 60 min using an infusion pump at a rate of 2.2 mL/hr. In separate experiments for intraintestinal administration of everolimus, the abdominal cavity was opened via a midline incision, and the upper site of the duodenum was exposed to administer everolimus. To examine effect of intraintestinal administration of 5 mg/kg cyclosporine or 1 mg/kg tacrolimus, the abdominal cavity was opened via a midline incision, and the upper site of the duodenum was exposed to administer each drug. Everolimus was administered intravenously or intraintestinally at 10 min after intraintestinal administration of cyclosporine, tacrolimus or saline (control). Blood samples were collected from the femoral artery at 5, 15, 30, 60, 120, 180 and 240 min after the start of the administration of everolimus. Samples were placed into EDTA anticoagulant tubes.

To separately evaluate the influence of calcineurin inhibitors on the intestinal and hepatic first-pass effects of everolimus, cyclosporine (5 mg/kg) and tacrolimus (1 mg/kg) was intraintestinally administered 10 min before everolimus administration, and everolimus were administered intraportally or intraintestinally for 60 min using an infusion pump at a rate of 2.2 mL/hr. Blood samples were collected from the femoral artery at 5, 15, 30 and 60 min after the beginning of everolimus administration. Samples ware placed into EDTA anticoagulant tubes.

Analytical methods: Whole blood samples (150 μL) were transferred to 13 mL glass tubes and spiked with the internal standard (10 μL of 300 ng/mL of 32-desmethoxyrapamycin in blood). The analytical method for everolimus from the whole blood samples was performed by a previous method for sirolimus using high performance liquid chromatography with tandem mass spectrometry (LC/MS/MS).[14] Mass analysis was performed using a triple quadrupole mass spectrometer (API4000, Applied Biosystems Japan Ltd., Tokyo, Japan) equipped with an electrospray ionization interface and operated in the positive ion mode. The ion transitions monitored were the mass-to-change ratio (m/z) 975.6→908.6 for everolimus and m/z 901.5→834.7 for the internal standard. The lower limit of quantification for everolimus was 0.5 ng/mL.

Pharmacokinetic analysis: The pharmacokinetic parameters of everolimus, the area under the blood concentration-time curve from time zero to infinity (AUC), total body clearance (CL), volume of distribution at steady-state (Vd_{ss}) and half-life ($T_{1/2}$) were calculated with the software WinNonlin version 4.0.1 (Pharsight Co. Mountain View, CA) using the two-compartment model for the dose-dependent study and non-compartment model for other studies. Maximum concentration (C_{max}) and time of maximum concentration (T_{max}) were obtained from concentration-time curve of everolimus. The bioavailability after intraintestinal (F) or intraportal administration (F_h, hepatic availability) was calculated using the dose-normalized AUC after intraintestinal or intraportal administration divided by the dose-normalized AUC after intravenous administration, respectively. The apparent intestinal availability was obtained by dividing F by F_h. The area under the blood concentration-time curve

for 60 min (AUC_{0-60}) values of everolimus after intraportal and intraintestinal infusion were calculated by the trapezoidal method.

Statistical analysis: Values are expressed as means ± standard error of the mean (SE) for n experiments, except for that T_{max} is shown as median (min-max). The dose dependence of CL and Vd_{ss} was examined using the linear regression. Comparison of mean blood concentrations among three groups was performed by the repeated measures ANOVA with the post-hoc Dunnett test. The statistical significance of mean pharmacokinetic parameters among three groups was performed using Dunnett test following ANOVA. The statistical analysis for the distribution of T_{max} was performed using Kruskal-Wallis test. Difference was considered significant at $p<0.05$.

Results

Dose dependency of everolimus pharmacokinetics: We first examined the dose proportionality of everolimus after intravenous administration (0.2, 0.5 and 1 mg/kg). The everolimus concentration profile showed a two-phase decline (**Fig. 1**). The CL did not correlate with the dose ($P=0.096$), while Vd_{ss} significantly correlated with the dose from 0.2 to 1.0 mg/kg ($P<0.05$, **Table 1**).

First-pass extraction of everolimus by the intestine and liver: To clarify the contribution of the intestine and liver to the first-pass effect of everolimus, 0.5 mg/kg of everolimus was administered intraintestinally, intraportally and intravenously (**Fig. 2**). The bioavailability after intraportal and intraintestinal administration were 48.0% and 21.2%, respectively, assuming that the pharmacokinetics of everolimus was linear in this condition (**Table 2**). The values of intestinal and hepatic availability were calculated as 44.2% and 48.0%, respectively.

Effect of calcineurin inhibitors on the pharmacokinetics of everolimus: The pharmacokinetics of everolimus after intravenous and intraintestinal administration with or without the pre-administration of cyclosporine or tacrolimus was evaluated. After the intravenous administration (0.2 mg/kg), the everolimus concentration was not significantly elevated with each calcineurin inhibitor (**Fig. 3A**). The AUC and CL values after the intravenous administration with cyclosporine were significantly changed compared with those in the control, and those with tacrolimus were slightly changed (**Table 3**). When everolimus was administered intrain-

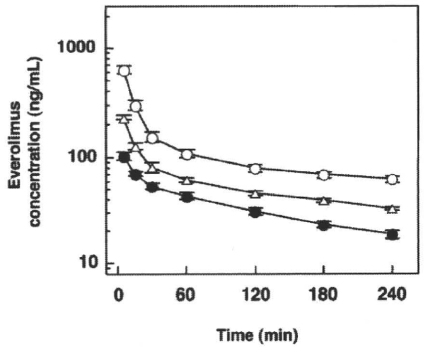

Fig. 1. Time-concentration profiles of everolimus after intravenous administration
Blood concentrations of everolimus at a dose of 1 mg/kg (open circles; n=5), 0.5 mg/kg (open triangles; n=5) and 0.2 mg/kg (closed circles; n=6) were plotted. Each line shows a simulation curve fitted to the two-compartment model. Each point shows the mean ± SE.

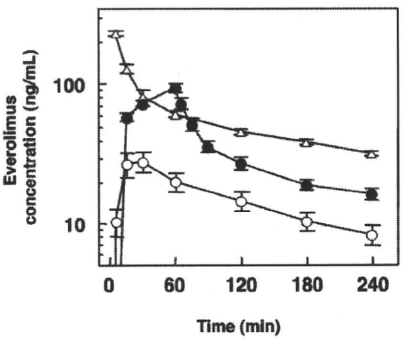

Fig. 2. Time-concentration profiles of everolimus after intravenous, intraportal and intraintestinal administration
Blood concentration of everolimus following intraintestinal (open circles; n=5), intraportal (closed circles; n=6) or intravenous (open triangles; n=3) administration at a dose of 0.5 mg/kg were plotted. Each point shows the mean ± SE.

Table 1. Total body clearance (CL) and volume of distribution at steady-state (Vd_{ss}) after intravenous administration at a dose of 0.2, 0.5 or 1 mg/kg by the two-compartment model

Dose (mg/kg)	CL (L/hr/kg)	Vd_{ss}(L/kg)
0.2 (n=6)	0.96±0.06	3.29±0.31
0.5 (n=5)	1.25±0.04	5.55±0.63
1.0 (n=5)	1.19±0.11	6.03±1.12

Each point shows the mean ± SE.

Table 2. Area under the blood concentration-time curve (AUC) and bioavailability following intravenous, intraportal or intraintestinal administration of everolimus in rats

Administration Route	Dose (mg/kg)	AUC (mg·hr/L)	Bioavailability (%)
Intravenous (n=3)	0.5	0.433±0.027	100
Intraportal (n=6)	0.5	0.208±0.016	48.0
Intraintestinal (n=5)	0.5	0.092±0.015	21.2

Each value shows the mean ± SE.

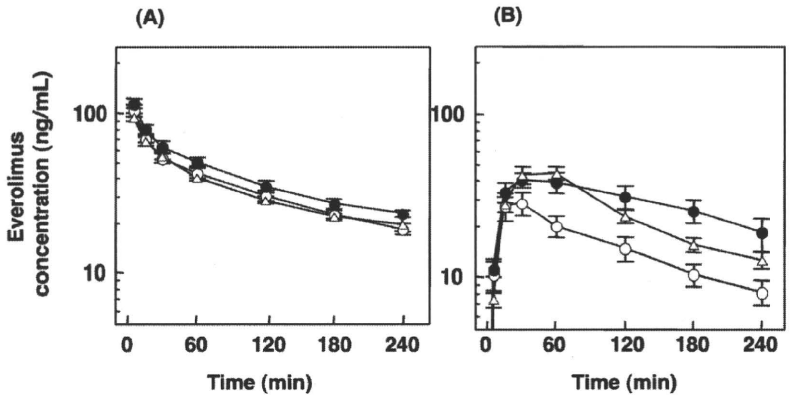

Fig. 3. Effect of calcineurin inhibitors on blood concentration of everolimus in rats
Blood concentrations of everolimus following intravenous (0.2 mg/kg, panel A) or intraintestinal (0.5 mg/kg, panel B) administration were plotted. Saline (open circles; control, n=6 for panel A, n=5 for panel B), 1 mg/kg of tacrolimus (open triangles; n=5 for panel A, n=4 for panel B) or 5 mg/kg of cyclosporine (closed circles; n=5 for panel A, n=5 for panel B) were intraintestinally administered 10 min before everolimus administration. Each point shows the mean±SE.

Table 3. Pharmacokinetic parameters of everolimus after intravenous and intraintestinal administration with or without administration of cyclosporine and tacrolimus by the non-compartment model

Parameter	Control	Cyclosporine	Tacrolimus
Intravenous administration (0.2 mg/kg)			
n	6	5	5
AUC (mg·hr/L)	0.217±0.016	0.263±0.013*	0.226±0.010
CL (L/hr/kg)	0.945±0.068	0.768±0.036*	0.892±0.038
Vd_{ss} (L/kg)	3.47±0.38	2.86±0.24	3.55±0.24
$T_{1/2}$(hr)	3.65±0.24	3.70±0.14	3.97±0.17
Intraintestinal administration (0.5 mg/kg)			
n	5	5	4
AUC (mg·hr/L)	0.092±0.015	0.194±0.045	0.137±0.012
T_{max} (min)	30 (15–30)	60 (30–60)	60 (30–60)
C_{max} (ng/mL)	28.9±2.7	41.7±4.6	44.5±5.1
F (%)	16.9	29.5	24.3

Each value shows the mean±SE. *$p<0.05$ compared with the control.

testinally (0.5 mg/kg), blood concentrations of everolimus tended to elevate by pre-administration of both cyclosporine and tacrolimus (**Fig. 3B**). The AUC value with cyclosporine was doubled, but did not reach statistical significance (**Table 3**). The F value of everolimus was calculated as 16.9% in the control, and increased to 29.5% and 24.3%, respectively, with pre-administration of cyclosporine and tacrolimus (**Table 3**).

Effect of calcineurin inhibitors on the intestinal and hepatic first-pass extraction of everolimus: To further examine the effect of calcineurin inhibitors on the intestinal and hepatic extraction of everolimus, everolimus (0.5 mg/kg) was administered intraportally or intraintestinally at a rate of 2.2 mL/hr for 60 min after calcineurin inhibitors were administered intraintestinally. After the intraportal administration, blood concentrations of everolimus in the cyclosporine and tacrolimus groups were not significantly elevated compared with that of the control (**Fig. 4A**). The AUC_{0-60} values in the cyclosporine and tacrolimus groups were not significantly increased after intraportal infusion (**Table 4**). On one hand, blood concentrations of everolimus after the intraintestinal infusion were significantly elevated in the cyclosporine group compared with control (**Fig. 4B**), and the AUC_{0-60} value in the cyclosporine group was significantly increased by about 3-fold (**Table 4**). Blood concentrations of everolimus and the AUC_{0-60} value in the tacrolimus group were not significantly changed compared with the control (**Fig. 4B, Table 4**).

Discussion

Everolimus is eliminated from the body by the metabolism of CYP3A subfamily,[10] and CYP3A4 as well as CYP3A5 are known to be expressed in human liver and intestine.[15] In adult male rats, CYP3A2, CYP3A9, CYP3A18, CYP3A1/23 and CYP3A62 are expressed in livers, and CYP3A62 as well as CYP3A9 and CYP3A18 are detected in the intestinal tract.[16] Cao *et al.*[17] suggested that a rat model could not be used to predict drug metabolism or oral bioavailability in humans, since the two species exhibit distinct expression levels and patterns for metabolizing enzymes in the intestine. On one hand, the bioavailability of tacrolimus and cyclosporine, typical substrates of CYP3A subfamily, in humans are poor and varies from 4% to 89% (mean approximately 25%) and from <5% to 89% (average approximately 30%, classical formulation), respectively,[18,19] and the reported bioavailability in rats are similar to the mean values in humans.[20,21] Everolimus has been on the market only as an

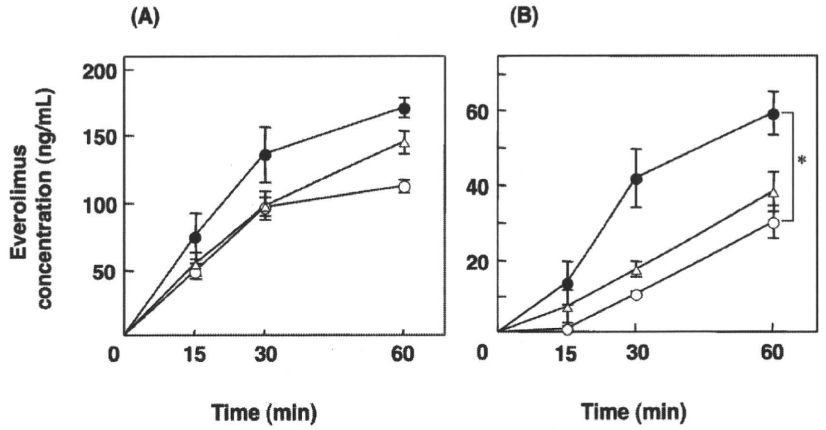

Fig. 4. Time-concentration profile of everolimus after intraportal infusion (panel A) and intraintestinal infusion (panel B) at a dose of 0.5 mg/kg
Saline (open circles; control, n = 5 for panel A, n = 3 for panel B), 1 mg/kg of tacrolimus (open triangles; n = 5 for panel A, n = 4 for panel B) or 5 mg/kg of cyclosporine (closed circles; n = 3 for panel A, n = 4 for panel B) were intraintestinally administered 10 min before everolimus administration. Each point shows the mean ± SE. *$p < 0.05$ compared with control.

Table 4. Area under the blood concentration-time curve for 60 min (AUC_{0-60}, mg·hr/L) values of everolimus after intraportal and intraintestinal infusion with or without administration of cyclosporine and tacrolimus

	Control	Cyclosporine	Tacrolimus
Intraportal	0.084 ± 0.004	0.113 ± 0.014	0.087 ± 0.007
	(n = 5)	(n = 3)	(n = 5)
Intraintestinal	0.012 ± 0.001	0.034 ± 0.005*	0.018 ± 0.002
	(n = 3)	(n = 4)	(n = 4)

Each value shows the mean ± SE. * $p < 0.05$ compared with the control.

oral formulation, and there is no information about its bioavailability in humans. Therefore, we investigated the effect of intestinal and hepatic first-pass extraction on the pharmacokinetics of everolimus in rats to understand the bioavailability in humans, although the species difference should be taken into consideration.

To evaluate the pharmacokinetic parameters of everolimus, we first examined its dose proportionality. The CL was constant, but Vd_{ss} significantly correlated with the dose from 0.2 to 1.0 mg/kg (**Table 1**). Since everolimus was reported to exhibit moderate non-linear binding to red blood cells,[22] the CL as well as Vd_{ss} may increase as the blood concentrations increased. In stable renal transplant patients, dose-normalized AUC values were not significantly different for doses in the range 2.5–25 mg but were higher at the 0.75 mg dose.[3] Considering that the blood concentration profile after intravenous administration well fitted to the two-compartment model at each dose, everolimus pharmacokinetics can be regarded as linear in a relatively small concentration range. Since the everolimus concentration was between 10 and 100 ng/mL after intravenous administration of 0.2 mg/kg, we carried out the following experiments in this concentration range, assuming the pharmacokinetic linearity.

To quantitatively evaluate the intestinal and hepatic first-pass extraction, everolimus was administered intravenously, intraportally and intraintestinally at a dose of 0.5 mg/kg (**Fig. 2**). As a result, the intestinal and hepatic availability were 44.2% and 48.0%, respectively, showing both intestine and liver function to be absoption barriers to everolimus. These results were consistent with a previous report that 50% of everolimus was metabolized in the intestinal mucosa in an *in situ* rat jejunum administration study at a dosage of 0.5 mg/kg.[2] Hashimoto *et al.*[20] reported that the tacrolimus availability in the small intestine and liver were 65.7% and 38.8%, respectively. Therefore, everolimus was considered to have more difficulty in permeating the small intestine mucosa than tacrolimus in rats.

Since the therapeutic range of cyclosporine and tacrolimus early after liver transplantation is approximately 600–1,000 ng/mL at 2 hr post dose and 10 to 20 ng/mL at the trough point, respectively,[23,24] we set each dose at 5 mg/kg of cyclosporine and 1 mg/kg of tacrolimus following previous reports.[20,21] Actually, in the present study, the blood concentrations of cyclosporine and tacrolimus after 240 min were 1,458 ± 183 ng/mL and 3.8 ± 0.9 ng/mL (mean ± SE, n = 4–5) with a fluorescence polarization immunoassay method and a microparticle enzyme immunoassay method, respectively. From our results, cyclosporine significantly decreased the CL, and increased the F of everolimus after intraintestinal administration, while tacrolimus showed a less potent effect than cyclosporine. It was reported that the everolimus concentration was elevated in combination with cyclosporine in rats, in which the oral dose of cyclosporine

was 2.5 mg/kg and that of everolimus was 0.6 mg/kg.[2] Therefore, the same tendency to elevate the everolimus concentration was observed with cyclosporine in our experiments. Kovarik et al. reported that the C_{max} and AUC of everolimus was significantly elevated by 84% and 168%, respectively, in co-administration of a microemulsion formulation of cyclosporine, to healthy subjects.[13] On the other hand, concomitantly administered tacrolimus had no significant effects on the concentrations of everolimus in renal transplant patients.[25] Cyclosporine and tacrolimus competitively inhibited the CYP3A-mediated nifedipine oxidation activity with the inhibition constants of 0.36 and 1.42 μM in human liver microsomes.[26] In addition, tacrolimus stimulates the P-glycoprotein-ATPase activity with an affinity in the 100 nM range, and cyclosporine acts as a potent competitive inhibitor of verapamil-stimulated P-glycoprotein-ATPase activity with an affinity constant in the 20–25 nM range.[27] Taking these findings into consideration, lower blood concentrations of tacrolimus in the clinical situation compared with its inhibition or affinity constants for CYP3A or P-glycoprotein might have little influence on everolimus pharmacokinetics, while the blood concentrations of cyclosporine correspond to its inhibition or affinity constants. On one hand, pharmacodynamic interactions remain to be clarified in a future study because both everolimus and tacrolimus bind to FK506 binding protein (FKBP12), but the complex of everolimus and FKBP12 has no immunosuppressant effects.[1,28]

The F of everolimus was increased 1.75-fold with the concomitant administration of cyclosporine, while its CL was decreased to about 80% of the control (**Table 3**). These results showed that cyclosporine inhibited the first-pass extraction in the intestine in addition to the inhibition of hepatic extraction. To clarify the effects of calcineurin inhibitors on the intestinal and hepatic extraction of everolimus, we administered everolimus via constant intestinal or intraportal infusion for 60 min with or without calcineurin inhibitors. As a result, cyclosporine interacted with everolimus in the intestine more than in the liver, and tacrolimus did not show a significant effect (**Fig. 4**, **Table 4**). Therefore, intestinal first-pass metabolism may play an important role when considering the interaction of everolimus with CYP3A or P-glycoprotein-mediated inhibitors after oral administration. The difference between the interaction potency in the liver and intestine might be related to in part the difference in the concentration of inhibitory drugs in interaction sites, namely higher concentrations in enterocytes than in hepatocytes after oral administration. Thummel et al.[29] suggested that enzymes of the gut wall might represent an important and highly sensitive site of metabolically-based interactions for orally administed drugs, because of their unique anatomical location.

In conclusion, everolimus was extracted by the intestine to a similar extent as in the liver in rats. In addition, intraintestinally administered cyclosporine inhibited the first-pass extraction of everolimus by the intestine, rather than that by the liver in rats. It should be clarified in humans if the pharmacokinetic interaction between cyclosporine and everolimus is more evident in the intestinal first-pass extraction compared with the hepatic first-pass extraction after both drugs are orally administered.

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Drug Metab. Pharmacokinet. 23 (5): 313–317 (2008).

Regular Article

Required Transient Dose Escalation of Tacrolimus in Living-Donor Liver Transplant Recipients with High Concentrations of a Minor Metabolite M-II in Bile

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: The profiles of tacrolimus metabolites in the whole blood and bile were examined in two living-donor liver transplant patients, who transiently required higher doses of tacrolimus. Even when the 16 mg/day or oral 10 mg/day and intravenous infusion of 0.5 mg/day of tacrolimus were administered, its trough level in each patient did not reach over 15 ng/mL. By use of liquid chromatography-tandem mass spectrometry/mass spectrometry methods, a minor metabolite M-II was found to be a major metabolite both in blood and bile in these cases. However, a primary metabolite M-I was confirmed as the majority in the bile of other 8 control cases. Each graft liver and native intestine carried *CYP3A5*1/*3* or **3/*3* and **1/*3* or **1/*3*, respectively. Therefore, the *CYP3A5* genotype could not explain the present phenomena. After removing the bile drainage tube to allow the bile flow into intestine, the required doses of tacrolimus were decreased to around 20% compared to each maximum dosage. In conclusion, a minor metabolite M-II was first found in the human bile, suggesting that the appearance of M-II in bile could associate with the extensive metabolism of tacrolimus and/or the requirement of larger oral dosage.

Keywords: FK506, metabolism, pharmacokinetics, prograf

Introduction

Tacrolimus is a 23-member macrolide lactone with potent immunosuppressive properties, and has been used clinically for the prevention of rejection in organ transplantations, including living-donor liver transplantation (LDLT).[1,2] Tacrolimus is extensively metabolized in the liver by the cytochrome P450 (CYP) 3A subfamily, with little excretion of the unchanged drug in the urine, bile, or feces, and biliary excretion is thought to be the major route of elimination of tacrolimus metabolites.[3] The chemical structure of 3 major metabolites (M-I, M-II, M-III, **Fig. 1**) was determined *in vitro* with liver microsomes, however, only M-I among the three metabolites was detected in the blood of liver transplant recipients.[4,5]

Tacrolimus is a drug with a narrow therapeutic range (from 5 to 20 ng/mL), and shows large variability in bioavailability after oral administration.[6–8] Even when the blood concentration of tacrolimus is kept in the therapeutic range, patients may experience rejection episodes or adverse effects. Therefore, the pharmacological efficacy of tacrolimus is highly variable, and it is difficult to optimize the dosage and target range.[9]

In the present case report, we have retrospectively examined the whole blood and bile concentrations of tacrolimus and its three main metabolites in LDLT recipients whose blood tacrolimus concentration had been difficult to control.

Received; December 4, 2007, Accepted; February 24, 2008
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This work was supported by a Grant-in-Aid from the Japan Health Sciences Foundation "Research on Health Sciences focusing on Drug Innovation", by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, and by the 21st Century COE program 'Knowledge Information Infrastructure for Genome Science'. M. S. is supported as a Research Fellow by the 21st Century COE program 'Knowledge Information Infrastructure for Genome Science'.

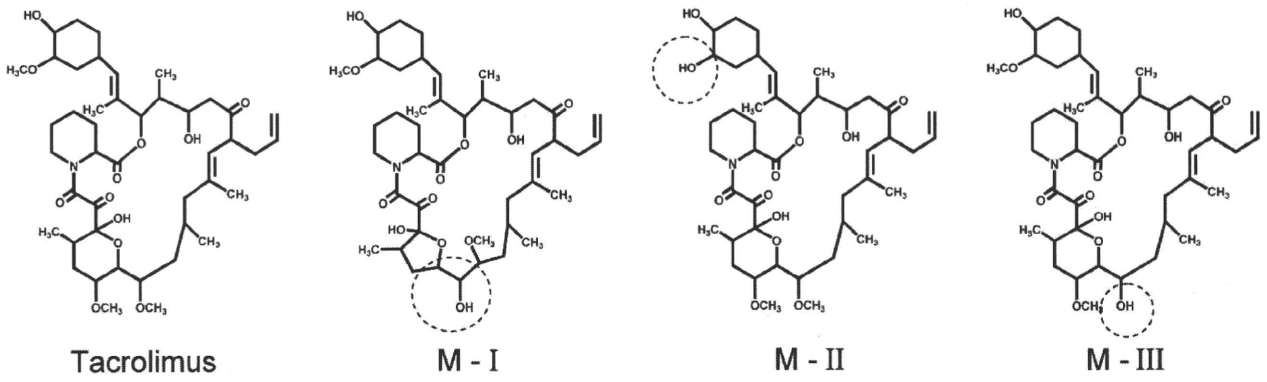

Fig. 1. Chemical structures of tacrolimus and its three metabolites.
Dotted circles indicated the positions of biotransformation.

Materials and Methods

Two LDLT patients whose tacrolimus doses were raised transiently and eight patients whose blood tacrolimus concentrations were controllable enrolled in this study after providing written informed consent. All patients had received liver transplantation at Kyoto University Hospital. The genetic analysis was performed in accordance with the Declaration of Helsinki and its amendments, and approved by Kyoto University Graduate School and Faculty of Medicine, Ethics Committee.

The blood sampling was performed once a day in the morning before the next administration of tacrolimus. The daily dose of tacrolimus was adjusted by the blood concentration of tacrolimus determined by using a semi-automated microparticle enzyme immunoassay (MEIA) (IMx®, Abbott Japan Co., Ltd., Tokyo, Japan). The excess amounts of blood samples were subjected to analyze the metabolite profile. Bile was collected from the bile drainage tube after measurement of the bile flow rate. The whole blood samples and bile samples (150 μL) were transferred to 13 mm × 100 mm conic tubes and spiked with the internal standard (25 μL, 100 ng/mL of rapamycin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.)). Then, 600 μL of water and 2 mL of extraction solution (methyl-t-butyl ether/cyclohexane, 1:3 w/v) were added to the tubes. Each tube was capped securely and mixed on a horizontal shaker for 30 min, and centrifuged at 3000 rpm for 10 min. The organic layer was transferred to a clean tube, and evaporated with an Automatic Environmental Speed Vac® System (Savant Instruments, Inc., Farmingdale, NY, U.S.A.). Each tube was reconstituted with 100 μL of mobile phase and vortexed for 1 min. The concentrations of unchanged tacrolimus and its metabolites (M-I, M-II and M-III) were quantified by liquid chromatography-tandem mass spectrometry/mass spectrometry (LC-MS/MS). A 20-μL aliquot of each sample was injected into the LC-MS/MS system. Briefly, the system comprised two pumps, an analytical column (Inertsil-ODS3, 150 × 2.1 mm i.d., GL Sciences, Inc., Tokyo, Japan), and a MS/MS detector (API3000System, Applied Biosystems, CA, U.S.A.). The mobile phase consisted of a multiple gradient of solvent A (methanol/1 mM ammonium acetate) and solvent B (1 mM ammonium acetate). The flow rate was set at 250 μL/min, and the eluent was introduced directly into the electrospray ion source of the mass spectrometer. Selected reaction monitoring transitions monitored in the positive ion mode were m/z 821→m/z 768 for tacrolimus, m/z 807→m/z 772 for M-I, m/z 807→m/z 754 for M-II, m/z 807→m/z 754 for M-III, and m/z 931→m/z 864 for rapamycin (internal standard). Tacrolimus, all metabolites, and rapamycin were detected as ammonium adducts ions ($m + NH_4$). Peak areas were linear from 0.5 to 50 ng/mL for tacrolimus and 0.5 to 20 ng/mL for metabolites.

Genomic DNA from graft liver was isolated with a MagNAPure LC DNA isolation kit (Roche, Mannheim,

Table 1. Patients' background

Case number	I	II
Sex	Female	Male
Age (y)	45	60
Blood type	O	O
Body weight (kg)	55.8	58.8
Primary disease	HCV-LC, HCC	HCV-LC, HCC
CYP3A5 genotype	*1/*3	*1/*3
Donor		
Relation	Husband	Son
Age (y)	49	21
Blood type	O	B
CYP3A5 genotype	*1/*3	*3/*3
ABO blood group match	Identical	Incompatible
Graft lobe	Right	Right
GRWR (%)	1.59	1.37

HCV; hepatitis virus C, LC; liver cirrhosis, HCC; hepatocellular carcinoma, GRWR; graft-to-recipient body weight ratio.

Germany).[10] Genotyping of the *CYP3A5* gene was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.[11,12]

Case Reports

Patients' backgrounds are shown in **Table 1**. Immunosuppression by tacrolimus was introduced with an oral administration in case I, and with an oral administration and intravenous infusion in case II. The dosage was modified mainly on the basis of the daily trough levels of tacrolimus. A small amount of prednisolone was administered for post-surgical inflammation or additional immunosuppression in both cases. During the observation period, biochemical parameters of liver function remained near the normal level in both cases (**Figs. 2A** and **3A**). By a genetic polymorphism analysis, the genotype of *CYP3A5* in the graft liver was determined as *1/*3 in case I and *3/*3 in case II. Therefore, the patients in cases I and II were classified as a CYP3A5 expressor and as CYP3A5 defective in the grafted liver, respectively. The genotype of *CYP3A5* in patients was *1/*3 in both cases, so CYP3A5 was expressed in the native intestine.

Case I: Signs of mild acute rejection were observed in the biopsy specimen, and mycophenolate mofetil (MMF) was administered from the 9th postoperative day (**Fig. 2**). This patient was discharged from our hospital on the 22nd postoperative day, and was readmitted on the 34th postoperative day with suspicion of chronic rejection. On the 44th postoperative day, the required dose of tacrolimus was raised (14 mg/day) in comparison with the mean dosage of the first 21 days post-surgery (6.5 mg/day). On the 52nd postoperative day, the bile drainage tube was removed to allow the bile to flow into

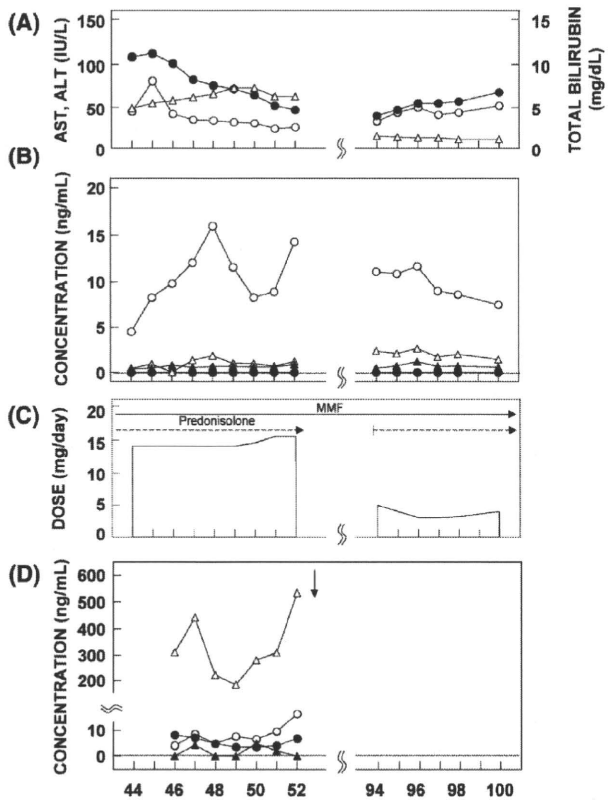

Fig. 2. Monitoring of concentrations of tacrolimus and its metabolites in whole blood and bile in case I
(A) AST (*open circle*), ALT (*closed circle*) and total bilirubin (*open triangle*) values were determined. (B) Trough tacrolimus (*open circle*), M-I (*closed circle*), M-II (*open triangle*) and M-III (*closed triangle*) levels were quantified by the LC-MS/MS method. (C) The daily oral doses of tacrolimus were documented. Arrows indicate the administration of other drugs. (D) Tacrolimus (*open circle*), M-I (*closed circle*), M-II (*open triangle*) and M-III (*closed triangle*) levels in bile were quantified by the LC-MS/MS method. The arrow indicates the time of the bile drainage tube removed.

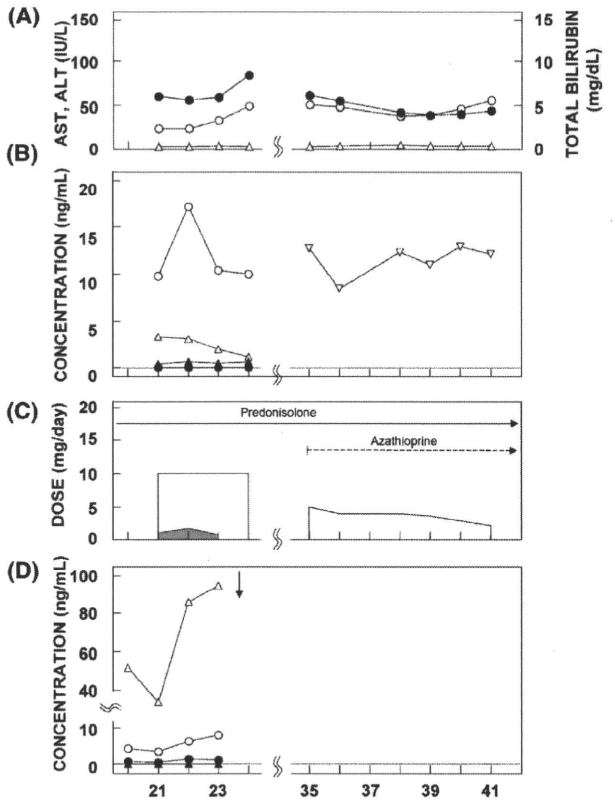

Fig. 3. Monitoring of concentrations of tacrolimus and its metabolites in whole blood and bile in case II
(A) AST (*open circle*), ALT (*closed circle*) and total bilirubin (*open triangle*) values were determined. (B) Trough tacrolimus (*open circle*), M-I (*closed circle*), M-II (*open triangle*) and M-III (*closed triangle*) levels were quantified by the LC-MS/MS method, and tacrolimus concentrations (*inverted open triangle*) were measured by MEIA method. (C) The daily oral and intravenous (*shaded area*) doses of tacrolimus were documented. Arrows indicate the administration of other drugs. (D) Tacrolimus (*open circle*), M-I (*closed circle*), M-II (*open triangle*) and M-III (*closed triangle*) levels in bile were quantified by the LC-MS/MS method. The arrow indicates the time of the bile drainage tube removed.

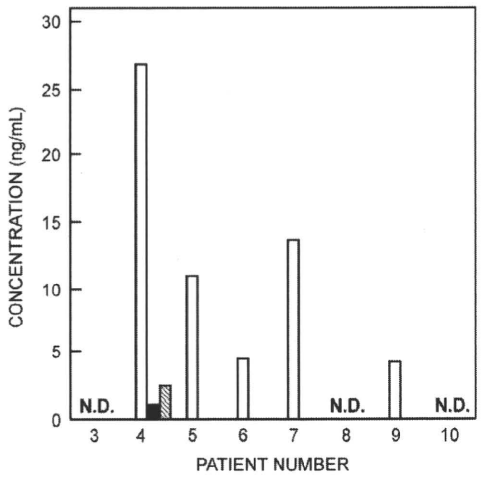

Fig. 4. Concentration of tacrolimus metabolites in the bile of 8 LDLT patients
M-I (*open column*), M-II (*solid column*) and M-III (*hatched column*) levels were quantified by the LC-MS/MS method. N.D.: not detected.

the intestine, and the required dose decreased to 3.7 mg/day, mean dosage, from the 94th to 100th postoperative day.

Case II: From the 30th postoperative day, the dose of prednisolone was decreased, and azathioprine was administered at 50 mg/day (**Fig. 3**). Although the mean dosage of tacrolimus between the 7th and 11th postoperative days was 0.62 mg/day by intravenous infusion, the required dose on the 21st postoperative day was 10 mg/day by oral administration and 0.5 mg/day by intravenous infusion. On the 24th postoperative day, the bile drainage tube was removed to allow the bile to flow into the intestine, and the required dose of tacrolimus decreased to 3.7 mg/day by oral administration only, mean dosage, from the 35th to 41st postoperative day. Because the sample volume was limited, we could not quantify the concentration of unchanged tacrolimus and its metabolite in whole blood from the 35th to 41st postoperative day. Therefore, we used the tacrolimus concentrations measured by MEIA in this period.

Control cases: The main metabolite of tacrolimus in the bile was M-I in five LDLT patients (patients 4, 5, 6, 7 and 9) who were well controlled, and no metabolite was detected in three LDLT patients with cholangitis (patients 3, 8 and 10) (**Fig. 4**). In all eight patients, unchanged tacrolimus was not detected in the bile.

Discussion

In cases I and II, after the required dose of tacrolimus was elevated in order to keep the concentration in the therapeutic range, the main metabolite was M-II in whole blood and bile (**Figs. 2** and **3**). Notably, the concentration of M-II in bile was about 10 times as high as that of unchanged tacrolimus and the other metabolites, and the concentration of M-II in whole blood was higher than that of any other metabolite. On the other hand, the main metabolite of tacrolimus in the bile was M-I in the well controlled patients (**Fig. 4**). These results indicated that the main metabolite under normal conditions was M-I, and that the profile of metabolites would change in the cases involving a remarkable elevation in dose. In addition, because only M-I among the three metabolites was reported to be in the blood,[4,5] this is the first report in which the main metabolite was found to be M-II.

Tacrolimus is extensively metabolized in the liver by CYP3A4 and CYP3A5, and biliary excretion is thought to be the major route of elimination of its metabolites.[3] CYP3A5 shows significant differences in expression caused by a single nucleotide polymorphism. The *CYP3A5*3* allele with a single nucleotide polymorphism in intron 3 causes a splicing error of CYP3A5 mRNA, and results in a defect of protein synthesis.[13,14] We previously reported that the tacrolimus concentration/dose ratio was decreased in patients engrafted with partial liver carrying the *CYP3A5*1/*1* genotype, and that intestinal CYP3A5 played an important role in the first-pass effect of orally administered tacrolimus.[8,11,12] The genotype of *CYP3A5* in native small intestine was *1/*3 in both cases (**Table 1**). It was possible that metabolism by CYP3A5 in the small intestine was responsible for high tacrolimus dose. However, the doses in both cases were elevated transiently (**Figs. 2** and **3**). Therefore, it is difficult to explain the reason for high tacrolimus dose only by intestinal metabolism by CYP3A5.

The main bile metabolite was M-II in this study, but it was reported that both CYP3A4 and 3A5 produced M-I as the main metabolite of tacrolimus.[15] Although M-II and M-III were also produced by CYP3A4 and 3A5, their amounts were 10 to 20-fold lower than that of M-I. In addition, the genotype of *CYP3A5* in the graft liver was *1/*3 in case I and *3/*3 in case II, and the high concentration of M-II is assumed not to be the result of only metabolism mediated by CYP3A5. Further study is necessary to explain the profile of tacrolimus metabolism.

The highest dose in case I was 16 mg/day. That in case II was 10 mg/day by oral administration and 0.5 mg/day by intravenous infusion. By removing the bile drainage tube, the lowest dose was reduced to 3.0 and 2.2 mg/day in cases I and II, respectively. Although it was indicated that the metabolism of tacrolimus had changed transiently in these cases, removing the bile drainage tube could assist the reabsorption of M-II, which had immunosuppressive activity, from the small intestine and enhance the immunosuppressive activity in the case of high M-II concentrations.

In conclusion, the present case report suggested that the transient elevation in the dosage of tacrolimus in the two LDLT patients was associated with the extensive for-

mation of M-II, and this is the first time the main metabolite was found to be M-II. We could decrease the dose of tacrolimus by removing the bile drainage tube. However, further investigation of many cases of LDLT and other transplantations is needed to clarify the relation of metabolites to the pharmacokinetics and pharmacodynamics of tacrolimus, because high M-II concentrations were observed in only two cases. In addition, it may be necessary to study the significance of monitoring the concentration of M-II in blood to the pharmacodynamics of tacrolimus.

Acknowledgments: We thank the Analysis and pharmacokinetics research labs of Astellas Pharma Inc. for the quantification of tacrolimus and its metabolites by LC-MS/MS.

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BIOCHEMICAL PHARMACOLOGY 76 (2008) 894–903

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Transcellular transport of organic cations in double-transfected MDCK cells expressing human organic cation transporters hOCT1/hMATE1 and hOCT2/hMATE1

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ARTICLE INFO

Article history:
Received 23 May 2008
Accepted 1 July 2008

Keywords:
Renal tubular transport
Vectorial transport
OCT1
OCT2
MATE1
Quinidine

ABSTRACT

To clarify the transcellular transport of organic cations via basolateral and apical transporters, we established double-transfected Madin–Darby canine kidney (MDCK) cells expressing both human organic cation transporter hOCT1 and hMATE1 (MDCK-hOCT1/hMATE1), and hOCT2 and hMATE1 (MDCK-hOCT2/hMATE1) as models of human hepatocytes and renal epithelial cells, respectively. Using the specific antibodies, hOCT1 and hMATE1 or hOCT2 and hMATE1 were found to be localized in the basolateral and apical membranes of MDCK-hOCT1/hMATE1 or MDCK-hOCT2/hMATE1 cells, respectively. A representative substrate, [^{14}C]tetraethylammonium, was transported unidirectionally from the basolateral to apical side in these double transfectants. The optimal pH was showed to be 6.5 for the transcellular transport of [^{14}C]tetraethylammonium, when the pH of the incubation medium on the apical side was varied from 5.5 to 8.5. The basolateral-to-apical transport also decreased in the presence of 10 mM 1-methyl-4-phenylpyridinium or 1 mM levofloxacin on the basolateral side of both double transfectants. In MDCK-hOCT2/hMATE1 cell monolayers, but not in MDCK-hOCT1/hMATE1 cell monolayers, the accumulation of [^{14}C]tetraethylammonium was decreased in the presence of 10 mM 1-methyl-4-phenylpyridinium, but significantly increased in the presence of 1 mM levofloxacin. The uptake of [^{14}C]tetraethylammonium, [^{3}H]1-methyl-4-phenylpyridinium, [^{14}C]metformin and [^{3}H]cimetidine, but not of [^{14}C]procainamide and [^{3}H]quinidine, by HEK293 cells was stimulated by expression of the hOCT1, hOCT2 or hMATE1 compared to control cells. However, transcellular transport of [^{14}C]procainamide and [^{3}H]quinidine was clearly observed in both double-transfectants. These cells could be useful for examining the routes by which compounds are eliminated, or predicting transporter-mediated drug interaction.

1. Introduction

Renal tubular secretion of drugs, toxins and endogenous metabolites is one of the most important functions in the kidney. The characteristics of the transport of tetraethylammonium (TEA), a representative substrate of the organic cation transport system, by the basolateral and brush-border membranes revealed that transcellular transport across the renal epithelial cells was mediated by basolateral uptake from blood and subsequent extrusion from the cells into the lumen. The mechanisms of renal secretion of cationic drugs were examined using isolated membrane vesicles from rat kidney

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0006-2952/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2008.07.005