ABSTRACT:

Hepatic metabolism is the main determinant in the pharmacokinetics of 5-fluorouracil (5-FU). Its disposition might be affected with liver dysfunction. In the present study, the influence of liver damage induced by bile duct ligation on dihydropyrimidine dehydrogenase (DPD), a rate-limiting enzyme in 5-FU catabolism, CYP2B, and 5-FU pharmacokinetics were compared in male Sprague-Dawley rats. After 3 weeks of the ligation in two different groups of animals for in vitro and pharmacokinetic experiments, significant increases in serum bilirubin level and spleen weight were found in both groups. No significant differences were noted in bilirubin level or spleen weight of the bile duct ligation group between the two experiment groups. In the in vitro experiment, DPD activity and protein levels determined by Western blot analysis in the bile duct ligation group were slightly but significantly greater than those of a sham-operated group, whereas CYP2B activity and protein level were significantly reduced. These findings were supported by mRNA levels of CYP2B and DPD. When 40 mg/kg 5-FU was administered i.v. in the pharmacokinetic experiment, no significant differences in pharmacokinetic parameters were found between the bile duct ligation and sham-operated groups. These results suggested that DPD activity and protein level were maintained and that 5-FU pharmacokinetics was not altered in the presence of liver damage accompanied by a significant reduction in CYP2B activity and protein level, supporting previous clinical studies showing that mild to moderate liver dysfunction does not affect 5-FU disposition.

The elimination of many drugs that undergo hepatic oxidation is impaired in patients with severe liver disease (McLean and Morgan, 1991). Impairment has also been demonstrated in rats with cirrhosis (Mourele et al., 1987; Butner et al., 1993). Previous studies including ours (Murray, 1992; George et al., 1995; Tateishi et al., 1998) suggest that CYP activities and protein level are reduced in the damaged liver and that their reduction is considered to contribute to this impaired elimination of drugs oxidized by the liver, as CYPs play a major role in the oxidative metabolism of a wide variety of endogenous and exogenous substrates (Guengerich, 1992). The influence of liver disease on enzyme activity and drug metabolism is highly variable and difficult to predict (McLean and Morgan, 1991). Therefore, understanding the pharmacokinetics of a drug, especially a cytotoxic agent, in the presence of impaired liver function is of clinical importance.

Although it was introduced over 30 years ago, 5-fluorouracil (5-FU) is still considered to be the most effective agent in the treatment of colorectal cancer (Köhne-Wömpner et al., 1992). It undergoes extensive hepatic metabolism, and in its catabolism, dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme (Naguib et al., 1985). Since hepatic metabolism is the main determinant in the pharmacokinetics of 5-FU (Pinedo and Peters, 1988), its disposition might be affected with liver dysfunction. This study, therefore, was undertaken to compare the influence of chronic liver damage due to bile duct ligation, which is reported to be an experimental model of liver cirrhosis (Kountouras et al., 1984), on DPD and CYP2B and to evaluate the influence on 5-FU pharmacokinetics in the presence of liver dysfunction.

Materials and Methods

Chemicals. [6-3H]5-FU (>99% radiochemical purity, 555 Bq/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Unlabeled 5-FU was a generous gift from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). The radiolabeled drug was diluted with unlabeled 5-FU to give appropriate specific activities. All other chemicals were from Wako Chemicals (Osaka, Japan) and were of analytical grade.

Animals and Surgical Procedure. All procedures were done in accordance with the guiding principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society. Eight-week-old male Sprague-Dawley rats, weighing 250 to 300 g, were purchased from SEAS Co. Ltd. (Saitama, Japan) and were housed in air-conditioned quarters with 12-h light/dark cycles and were given laboratory chow (CE-2; Nippon Clea, Tokyo, Japan) and water ad libitum. The bile duct was ligated in five rats according to the method described by Kountouras et al. (1984). Five sham-operated animals served as controls, and the two groups of rats were used for both in vitro and pharma-
cokinetic experiments 3 weeks after the operation. When rats were sacrificed for in vitro and pharmacokinetic experiments, blood was collected to measure serum total bilirubin level using the Bilirubin BII test (Wako Pure Chemical Industries, Osaka, Japan).

**DPD Activity Assay and Immunoblot Analysis in Liver Cytosols.** After cervical dislocation, the livers were quickly removed, diced, frozen in liquid nitrogen, and stored at −80°C for cytosol preparation. Cytosol was prepared using a method described previously (Tateishi et al., 1996). Protein concentration of the cytosolic fraction was determined according to the method of Lowry et al. (1951). DPD activity was determined by measuring the metabolites of 5-FU formed by reversed-phase HPLC (Tateishi et al., 1996). SDS-polyacrylamide gel electrophoresis was carried out in a 7% acrylamide gel containing 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS using 200-μg aliquots of the cytosolic samples (Lu et al., 1993). Resolved proteins were transferred to nitrocellulose sheets and treated subsequently with primary antibodies (Ogura et al., 1998), goat anti-rabbit IgG-horse radish peroxidase complex, and 4-chloro-1-naphthol plus H2O2. The intensity of the bands corresponding to the protein on each membrane was measured with a densitometer (AE-6920-MF-Densitograph version 2.5; ATTO, Tokyo, Japan).

**RNA Preparation and Northern Blot Analysis.** The total RNA fraction was extracted from homogenized liver using the acid guanidine thiocyanate-phenol-chloroform method (Gisin et al., 1974), and poly(A)+ RNA was purified from the pooled total RNA using oligo(dT)-Latex (Takara Shuzo Co., Ltd., Tokyo, Japan) (Kimura et al., 1998). The integrity of the RNA was checked by agarose gel electrophoresis. Glyoxal-treated RNA was separated by electrophoresis and transferred to a nitrocellulose membrane. This membrane was hybridized with radiolabeled rat liver DPD cDNA (Kimura et al., 1998), rat CYP2B cDNA (Kimura et al., 1998), or human β-actin cDNA (Nakajima et al., 1985). The membrane was then washed and exposed to scientific imaging film (Fuji Film Co., Tokyo, Japan).

**Determination of 5-FU Pharmacokinetics.** Both sham-operated (n = 5) and bile duct-ligated (n = 5) rats received a single i.v. injection of 40 mg/kg 5-FU containing 20 μCi/ml [6-3H]5-FU (80 μCi/kg) in the tail vein around 10 AM. Blood (300 μl) was collected by the tail vein on the reverse side at 5, 15, 30, 45, 60, and 90 min after injection of 5-FU. Blood samples were mixed with a minimal amount of heparin centrifuged; separated plasma was stored at −30°C until assay. The livers and spleens were harvested and weighed after the rats were decapitated. After 100 μl of plasma was mixed with the same volume of 5% perchloric acid and centrifuged, supernatant was removed, filtered (Ultrafree-MC, 0.45 μm; Millipore Corp., Bedford, MA) and injected into the HPLC system used for DPD activity assay (Tateishi et al., 1996). Plasma 5-FU concentration was estimated in terms of the dpm count of a 5-FU fraction. The area under the plasma 5-FU concentration-time curve was determined by the trapezoidal rule. The values were extrapolated to infinity by dividing the last measured plasma concentration by the slope of the terminal elimination phase (β) obtained by least-squares linear regression analysis. Elimination half-life (T1/2) was calculated as ln2/β. The total clearance (CL) and the volume of distribution (Vd) were calculated as dose/area under the plasma 5-FU concentration-time curve and Vd = CL/β, respectively.

**Statistical Analysis.** Data are presented as mean ± S.D. The one-way ANOVA was used for comparisons between the ligation and sham-operated groups.

**Results**

The body and organ weights and serum total bilirubin level of rats in the in vitro experiment were reported in a previous study (Tateishi et al., 1998). After 3 weeks of bile duct ligation, the spleen weight (2.10 ± 0.80 g and 0.84 ± 0.07 g for the bile duct-ligated and sham-operated groups, respectively) and the plasma bilirubin level (240.7 ± 69.5 μmol/liter and 3.3 ± 0.4 μmol/liter for the bile duct-ligated and sham-operated groups, respectively) of the bile duct-ligated group were increased significantly. Body weight in the ligation group was decreased significantly in comparison with that in the sham-operated group (289.6 ± 47.4 g versus 378.6 ± 18.3 g for the bile duct-ligated and sham-operated groups, respectively).

**Figure 1.** Effect of 3-week bile duct ligation on the activities of DPD activity (A) and pentoxyresorufin O-deethylase (B), a probe for CYP2B. The body and organ weights and serum total bilirubin level of rats
drug-metabolizing enzymes such as cytochrome P-450s. This has because of the reduced activities and expressions of microsomal impaired in the presence of liver damage (McLean and Morgan, 1991) of any drug that undergoes oxidative metabolism in the liver is especially antitumor agents such as 5-FU. In general, the elimination essential to prescribing drugs for patients with reduced liver function, the effect of liver damage on enzyme activity and pharmacokinetics is activity affects the efficacy and toxicity of 5-FU. Fully understanding 1988). These studies and case reports have suggested that the DPD lethal side effects with 5-FU (Tuchman et al., 1985; Diasio et al., 1994). However, a deficiency of this enzyme activity induces the antitumor effect of 5-FU (Baccanari et al., 1993; Cao et al., 1995; Tateishi et al., 1998). The present study showed that the clearances of 5-FU metabolic pathways in the damaged liver. Since 5-FU clearance was close to hepatic blood flow in a rat (Malik et al., 1976; Groszmann et al., 1982), 5-FU may be a drug of high hepatic drug extraction ratio, and its pharmacokinetics remained unaltered in the presence of liver damage induced by bile duct ligation. Since 5-FU clearance was close to hepatic blood flow in a rat (Malik et al., 1976; Groszmann et al., 1982), 5-FU may be a drug of high hepatic drug extraction ratio, and its extraction ratio by the liver is high enough that i.v. administration is not as sensitive as other routes for detecting changes in hepatic activity (Daneshmend and Roberts, 1984). Although the reasons for the increase in DPD level are not clear, the effects of liver damage on different enzymes seems to vary. For example, pharmacokinetics of many drugs metabolized by hepatic glucuronidation are reported to be unaffected in patients with liver cirrhosis, and glucuronidation is considered to be relatively stable in patients with liver disease (McLean and Morgan, 1991). DPD, like glucuronidase, might be an enzyme that remains stable in the presence of liver disease. In addi-

in the pharmacokinetic experiment are shown in Table 1. No significant differences were found between the in vitro and pharmacokinetic experiments in terms of the body and spleen weights and serum total bilirubin level. There were no significant differences in the pharmacokinetic parameters obtained from the plasma 5-FU concentration-time profiles of the ligation and sham-operated groups (Fig. 4 and Table 2).

Discussion

We studied the effect of 3-week bile duct ligation on the expression and activity of DPD in hepatic cytosol and on the pharmacokinetics of 5-FU. In the in vitro experiment, DPD activity and protein level were not affected or were slightly increased in damaged liver showing decreased CYP2B activity and protein level (Tateishi et al., 1998), and these findings were supported by mRNA expression levels. The similar change in DPD may be expected from the pharmacokinetic experiment because similar serum bilirubin levels and organ weights were obtained. Although DPD activity was slightly increased in the ligation group, no significant difference was found between the two groups in 5-FU pharmacokinetics.

5-FU undergoes extensive hepatic metabolism, and DPD is considered the initial and rate-limiting enzyme in 5-FU metabolism (Naguib et al., 1985). Coadministration of DPD inhibitors is intended to enhance the antitumor effect of 5-FU (Baccanari et al., 1993; Cao et al., 1994). However, a deficiency of this enzyme activity induces lethal side effects with 5-FU (Tuchman et al., 1985; Diasio et al., 1988). These studies and case reports have suggested that the DPD activity affects the efficacy and toxicity of 5-FU. Fully understanding the effect of liver damage on enzyme activity and pharmacokinetics is essential to prescribing drugs for patients with reduced liver function, especially antitumor agents such as 5-FU. In general, the elimination of any drug that undergoes oxidative metabolism in the liver is impaired in the presence of liver damage (McLean and Morgan, 1991) because of the reduced activities and expressions of microsomal drug-metabolizing enzymes such as cytochrome P-450s. This has been shown in previous studies including ours (Murray, 1992; George et al., 1995; Tateishi et al., 1998). The present study showed that the activity and protein level of DPD was slightly increased and that 5-FU pharmacokinetics remained unaltered in the presence of liver damage induced by bile duct ligation. Since 5-FU clearance was close to hepatic blood flow in a rat (Malik et al., 1976; Groszmann et al., 1982), 5-FU may be a drug of high hepatic drug extraction ratio, and its extraction ratio by the liver is high enough that i.v. administration is not as sensitive as other routes for detecting changes in hepatic activity (Daneshmend and Roberts, 1984). Although the reasons for the increase in DPD level are not clear, the effects of liver damage on different enzymes seems to vary. For example, pharmacokinetics of many drugs metabolized by hepatic glucuronidation are reported to be unaffected in patients with liver cirrhosis, and glucuronidation is considered to be relatively stable in patients with liver disease (McLean and Morgan, 1991). DPD, like glucuronidase, might be an enzyme that remains stable in the presence of liver disease. In addi-
tion, DPD activity has been shown to be reduced in differentiating and regenerating rat liver cells and in hepatomas (Queener et al., 1971). Degeneration of the normal lobular architecture, observed in liver cirrhosis, might affect DPD activity and protein expression.

There are some clinical studies on 5-FU pharmacokinetics with liver dysfunction or liver metastases (Kawata et al., 1987; Nowakowska-Dulawa, 1990; Fleming et al., 1992). In agreement with the findings of the current study, mild to moderate liver dysfunction has been reported not to affect 5-FU disposition or pharmacodynamics, and no dose reduction is recommended for patients with these conditions (Fleming et al., 1992). Although the present study showed 5-FU clearance relatively maintained even in the presence of liver damage accompanied by the increased plasma bilirubin and reduced CYP2B, Aguiar et al. (1987) reported that bone marrow toxicity induced by 5-FU was greater in rats with thioacetamide-induced liver cirrhosis compared with those without it because of reduced elimination of 5-FU. The influence of severe liver dysfunction on 5-FU pharmacokinetics and toxicity remains to be studied in humans.

Acknowledgment. We thank Dr. Yoshiaki Fujii-Kuriyama (Tohoku University) for providing the rat CYP2B cDNA.

References


