Dihydropyrimidine Dehydrogenase Activity in Hepatocellular Carcinoma: Implication in 5-Fluorouracil-based Chemotherapy[1]

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) is the initial, rate-limiting enzyme in the catabolism of 5-fluorouracil, one of the most widely used cancer chemotherapeutic agents. Previous studies have demonstrated the clinical importance of determination of DPD in cancer patients, suggesting that the efficacy and toxicity of 5-fluorouracil may directly relate to the DPD activity in both tumor and host tissues. In the present study, DPD activity was determined in 50 pairs of tumor and uninvolved liver specimens in Chinese cancer patients with hepatocellular carcinoma. Mean enzyme activity in uninvolved liver tissues (0.45 ± 0.02 nmol/min/mg protein) was significantly higher than that in tumor specimens (0.34 ± 0.03 nmol/min/mg protein). Statistical analysis revealed no significant differences in DPD activity of tumor and uninvolved liver specimens among different age and gender groups. Compared to previously reported tumor studies, hepatomas were found to have relatively high DPD activity. Since high levels of DPD would be expected to metabolize 5-fluorouracil, these findings may provide an explanation for the relative 5-fluorouracil resistance of hepatoma and may have implications for designing a new therapeutic strategy such as modulation of 5-fluorouracil chemotherapy by DPD inhibitors.

INTRODUCTION

5-FU[3] remains one of the most widely used chemotherapeutic agents in the treatment of common malignancies such as cancers of the breast, colon, and head and neck. 5-FU administrated to cancer patients is metabolized by both anabolic and catabolic pathways (1, 2). Previous studies demonstrated that anticancer effects and toxicity of 5-FU are directly related to the anabolism of 5-FU to its nucleotides that can then exert effects through inhibition of thymidylate synthase activity or incorporation into RNA and/or DNA (1, 2). However, the role of catabolism in 5-FU toxicity and/or therapeutic response has not been appreciated until relative recently. It has been demonstrated that more than 85% of 5-FU administered in cancer patients is degraded through the catabolic pathway (3) and thus has an important role in regulating the availability of 5-FU for anabolism (2).

DPD is the initial and rate-limiting enzyme in 5-FU catabolism (2). The importance of DPD activity in 5-FU catabolism has been well documented. DPD activity has been shown to follow a circadian pattern with levels varying 3-fold over a 24-h period (4, 5). In patients receiving continuous 5-FU infusion, DPD levels have been shown to vary inversely with 5-FU levels in the plasma (6). The circadian variation in DPD activity has been exploited in the design of a time-modified regimen of 5-FU delivery in hopes of improving the effectiveness of 5-FU in the treatment of colorectal cancer (7). The critical role of DPD in 5-FU chemotherapy has been further demonstrated in several recent studies of a new pharmacogenetic disorder associated with DPD deficiency, which has been shown to predispose to 5-FU-associated toxicity (8–14). One of the mechanisms responsible for severe 5-FU-induced toxicity in DPD-deficient patients has been shown to be alteration of 5-FU pharmacokinetics (9). Family studies have been conducted in several of the cancer patients with DPD deficiency, suggesting an autosomal recessive pattern of inheritance (9–12, 14).

Our laboratory (6) and others (15–17) have demonstrated that DPD activity is correlated with 5-FU pharmacokinetics. Population characteristics of DPD activity in peripheral blood mononuclear cells have been described in several recent studies (12, 16–18). Liver is believed to be the major site of pyrimidine metabolism and the major location of DPD (19, 20). The population distribution and characteristics of liver DPD activity in a population study in the United States has recently been determined (21). Although extensive biochemical and molecular studies have been carried out (22–25), the basis for differences

Received 7/18/96; revised 11/20/96; accepted 12/2/96.
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[1] Supported by USPHS/NIH Grant CA-64214 and U. S. Army Grant DAMD17-94-J-4115.
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[3] The abbreviations used are: 5-FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; HPLC, high-performance liquid chromatography.

in 5-FU responsiveness among different tumors remains unclear. Previous *in vitro* studies have demonstrated that the DPD activity in experimental tumor cell lines is related to the sensitivity to 5-FU (20, 26), suggesting that DPD activity in tumors may be related to the responsiveness to 5-FU. A recent study in patients with head and neck cancers suggested that DPD activity might play an important role in 5-FU resistance (27). It is well documented that hepatoma is relatively resistant to 5-FU (28). In the present study, DPD activity was determined in paired samples of nodular hepatoma and uninvolved liver tissues from 50 Chinese patients with primary hepatocellular carcinoma.

MATERIALS AND METHODS

Chemicals and Radiochemical

5-FU, used as the substrate for determination of DPD activity, was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]5-FU (25 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The purity of unlabeled and labeled 5-FU was confirmed by HPLC (29) to be more than 99%. All other solvents and reagents were purchased in the highest grade available. The major buffer (buffer A) used in the enzyme assay contained 35 mM potassium phosphate (pH 7.4), 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol.

Patients and Samples

Subjects. Fifty cancer patients, hospitalized in the Cancer Hospital, Sun Yat-sen University of Medical Sciences in Guangzhou, China, were enrolled in the present study. All patients were diagnosed with primary hepatocellular carcinoma that was relatively small and surgically removable. All patients gave informed consent to participate in this study. The protocol used in this study was approved by the University Institutional Review Board of Sun Yat-sen University of Medical Sciences.

Sampling. The hepatocellular carcinoma and the adjacent uninvolved liver tissue samples (about 10 g) were removed during the operation. Each sample was divided into two parts: one for pathological evaluation and another for DPD analysis. Samples for pathology were fixed in formalin solution and kept until analysis. The samples for DPD analysis were frozen immediately and stored in liquid nitrogen until transported in dry ice to our laboratory. These samples were then stored at −70°C until DPD analysis. The permission to import biological samples was obtained from the U.S. Center for Disease Control. The mean storage time for these samples was 13 months, ranging from 9 to 16 months. No impact of storage time on DPD activity was found.

Determination of DPD Activity

Sample Treatment. The slowly thawed tumor and liver tissues were washed with ice-cold physiological saline (0.9% NaCl), weighed, minced, and homogenized in 4 volumes of buffer A. The resulting homogenate was centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant was removed and used in the subsequent analysis as enzyme solution. Prior to enzyme assay, the amount of protein in each sample was determined according to the method of Bradford (30) to add the appropriate amount of protein to the enzyme reaction.

Enzyme Assay. DPD activity was determined by a radioassay (12, 21), measuring the 5-FU catabolites by HPLC. The reaction mixture in buffer A contained 20 μM [^{3}H]-5-FU, 200 μM NADPH, and enzyme solution in a final volume of 2 ml. The mixture was incubated at 37°C, and 350 μl of the reaction sample were taken out at various times (5, 10, 15, and 20 min) and then mixed with the same volume of ice-cold ethanol to stop the reaction. The mixture was then kept in a freezer (−20°C) for at least 30 min and subsequently centrifuged and filtered through a 0.2-μm Acro filter (Gelman Sciences, Ann Arbor, MI) prior to HPLC analysis.

Reversed-Phase HPLC Analysis. Separation of 5-FU and its catabolites was performed with a reversed-phase HPLC method that we have described in detail previously (29). The enzyme activity was expressed as nmol/min/mg protein. For samples with extremely low enzyme activity, at least two separate assays were performed to verify the results.

Statistical Analysis

Mean DPD activity and SD or SE in tumor and uninvolved liver tissues were calculated for different groups by age and gender. The differences in DPD activity among the different groups were analyzed using Wilcoxon's signed rank test or ANOVA as appropriate (21). To determine the correlation between enzyme activities in tumor and uninvolved liver tissue, the correlation coefficient test was conducted.

RESULTS

Pathological Confirmation of Uninvolved Liver and Hepatocellular Carcinoma Specimens. Pathological evaluation for each uninvolved liver and hepatocellular carcinoma sample was performed under a microscope in the Department of Pathology, Sun Yat-sen University of Medical Sciences. Forty-seven of 50 uninvolved liver samples were confirmed to be normal liver tissue. Three other samples were shown to be hepatic cirrhosis. However, the DPD activity in three samples with cirrhosis was not statistically different from that in uninvolved liver specimens (data not shown). All of the tumor samples were shown to be primary hepatocellular carcinoma including well differentiated, moderately differentiated, and poorly differentiated based on WHO standard classification criteria.

Population Distribution of DPD Activities in Uninvolved Liver and Hepatocellular Carcinoma Specimens. DPD activity of 50 pairs of uninvolved liver specimens and tumor tissues were quantified in a Chinese population of cancer patients with hepatocellular carcinoma. The population characteristics of this study are summarized in Table 1. Distribution of DPD activities in both uninvolved liver and tumor tissues is shown in Fig. 1. DPD activity in hepatocellular carcinoma was significantly lower than that in uninvolved liver specimens (Wilcoxon's signed rank test, $P < 0.01$). Of note, a small proportion of uninvolved liver and hepatocellular carcinoma specimens had very high DPD activity (Fig. 1). Further statistical analyses showed that the mean DPD activities in uninvolved liver specimens and hepatocellular carcinoma among groups by gender and age had no significant difference. However, in each subgroup, the mean hepatocellular carcinoma DPD

Table 1 DPD activity in uninvolved liver specimens

		DPD activity (nmol/min/mg protein)					
		Uninvolved liver tissue			Hepatocellular carcinoma tissue		
Group	*n*	Mean ± SE	Highest	Lowest	Mean ± SE	Highest	Lowest
Total	50	0.45 ± 0.02	0.85	0.19	0.34 ± 0.03	1.45	0.02
Men	43	0.45 ± 0.02	0.85	0.19	0.35 ± 0.03	1.45	0.07
Women	7	0.38 ± 0.02	0.45	0.28	0.31 ± 0.08	0.70	0.02
Age (yr)							
30–	8	0.47 ± 0.06	0.73	0.32	0.22 ± 0.03	0.34	0.11
40–	24	0.44 ± 0.03	0.78	0.19	0.38 ± 0.06	1.45	0.02
50–	9	0.43 ± 0.06	0.85	0.19	0.34 ± 0.05	0.61	0.15
60–	9	0.44 ± 0.06	0.80	0.22	0.36 ± 0.06	0.63	0.06

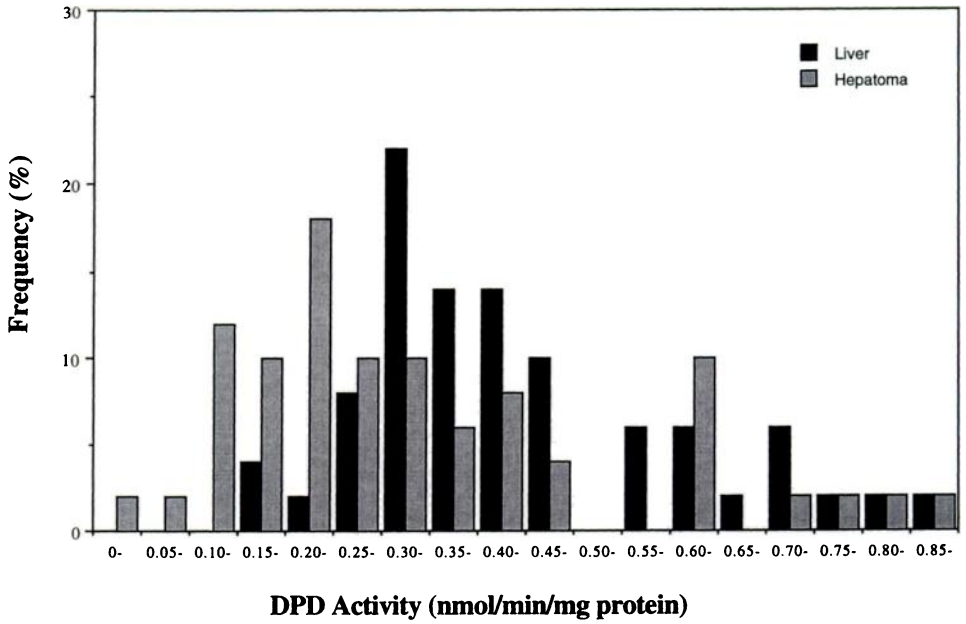

Fig. 1 Population distribution of DPD activity in uninvolved liver specimens and hepatocellular carcinoma.

activities were consistently significantly lower ($P < 0.01$) than the mean uninvolved liver specimens DPD activities (Table 1 and Fig. 2). In addition, statistical analysis indicated that there was no correlation between disease stages (based on WHO classification) and DPD activity in uninvolved liver or tumor tissues.

Correlation between Uninvolved Liver DPD Activity and Hepatocellular Carcinoma DPD Activity. The correlation analysis of DPD activity between uninvolved liver and hepatocellular carcinoma tissues showed no significant linear correlation ($r = 0.196$, $P > 0.05$; data not shown).

DISCUSSION

DPD plays an important role in 5-FU catabolism with regulation of the availability of 5-FU for anabolism, potentially determining the resultant anticancer efficacy and/or toxicity of 5-FU (1–3, 6, 12). Recent studies with the general population and cancer patients (12, 17, 18) provided a basis in determining population characteristics of DPD activities in Caucasian and African-Americans. However, limited information in other ethnic groups such as Asians is available. The purpose of the present study was to characterize the DPD activity in hepatocellular carcinoma as well as to obtain information on liver DPD activity in Chinese patients with cancer.

Slight differences among different gender and age groups were observed (Table 1). The mean liver DPD activity in the present study is slightly higher than that of the general population in the United States that previously reported (19, 21). However, there was no statistical significance.

The mechanisms for 5-FU resistance have been studied extensively (22–25). Thus far, most of the studies have focused on the anabolic pathway of 5-FU. Very little attention has been paid to the catabolic pathway of 5-FU, especially DPD activity in the target tissues. Naguib *et al.* (20) demonstrated some differences in DPD activity in different tumor samples. Beck *et al.* (26) determined the DPD activity in 19 human cancer cell lines and suggested that DPD activity in tumor cells was related to 5-FU sensitivity. A recent study in the patients with head and neck cancer (27) suggested that

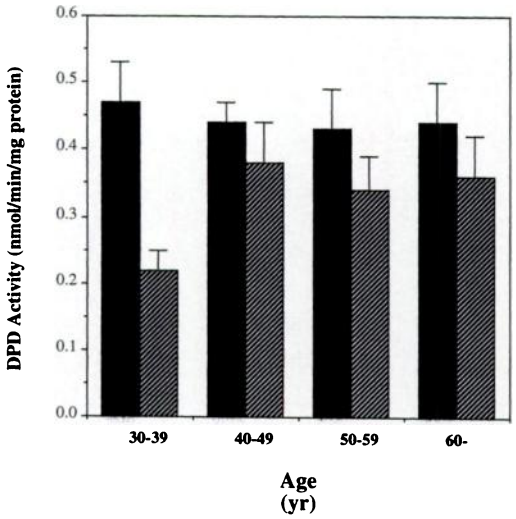

Fig. 2 Population distribution of liver (■) and hepatocellular carcinoma (▨) DPD activity by age.

tumor DPD activity might be a determining factor of 5-FU responsiveness in cancer patients. In the present study, we determined the DPD activity in 50 pairs of uninvolved liver and hepatocellular carcinoma specimens. DPD activity in hepatocellular carcinoma was significantly lower than that in uninvolved liver specimens (Fig. 1 and Table 1). The DPD activities in hepatocellular carcinoma (0.02–1.44 nmol/min/mg protein) appeared to be higher than those of the head and neck tumors (0.01–0.193 nmol/min/mg protein) reported by Etienne *et al.* Recently, in a preliminary study with limited samples, we found that DPD activity in colorectal carcinomas was lower than that in hepatocellular carcinoma (data not shown).

The high DPD activity in hepatocellular carcinoma may explain, at least in part, the resistance of hepatocellular carcinoma to 5-FU chemotherapy reported previously (28). If this is the case, the use of specific DPD inhibitors, especially regional administration of DPD inhibitors, should improve the efficacy and spectrum of 5-FU chemotherapy. Several DPD inhibitors, *e.g.*, 5-benzyloxybenzyluracil (31) and 5-ethynyluracil (32), are under investigation. Recently, Fischel *et al.* (33) reported that enhanced 5-FU cytotoxicity by 5-ethynyluracil occurred only in cell lines with high DPD activity, whereas 5-ethynyluracil did not modify 5-FU cytotoxicity in cell lines with low DPD activity. These results suggest that DPD could be a target for improvement of 5-FU chemotherapy.

ACKNOWLEDGMENTS

We appreciate the participation of our patients in this study. We thank Dr. Ruiwen Zhang for stimulating suggestions and discussion, many surgeons in the Cancer Center of Sun Yat-sen University of Medical Sciences for their help in sample collection, and Jinhua Chui and Hai Liao for their technical assistance in sample preparation.

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Dihydropyrimidine dehydrogenase activity in hepatocellular carcinoma: implication in 5-fluorouracil-based chemotherapy.

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Clin Cancer Res 1997;3:395-399.