Variations in 5-Fluorouracil Concentrations of Colorectal Tissues as Compared with Dihydropyrimidinase Dehydrogenase (DPD) Enzyme Activities and DPD Messenger RNA Levels

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) is the initial key enzyme in 5-fluorouracil (5-FU) catabolism. We measured DPD activities represented as DPD protein levels (units/mg protein) and the associated mRNA levels in tumorous and normal tissues from 40 colorectal cancer patients, and we studied the relation to 5-FU concentrations in the same samples after treatment with doxifluridine, a prodrug of 5-FU. DPD mRNA levels were also measured in biopsy samples before treatment for comparison with those in surgical samples. 5-FU concentrations in tumors were higher than those in normal tissues (P < 0.05) and were inversely associated with DPD protein levels (r = −0.463; P < 0.05). DPD activities in tumorous and normal tissues showed a significant correlation (r = 0.527; P < 0.01). DPD protein levels correlated with their mRNA levels detected by semiquantitative reverse transcription-PCR in tumor tissues (r = 0.740; P < 0.01). DPD mRNA levels in tumor biopsy specimens correlated with those in surgical specimens (r = 0.366; P < 0.05). These results suggest DPD activities in tumors to be predictive of 5-FU levels in colorectal cancer tissues and are reflected by DPD mRNA levels as measured by reverse transcription-PCR.

INTRODUCTION

5-FU3 is one of the most popular and effective anticancer drugs for several neoplasms including colorectal cancer. The efficacy of 5-FU chemotherapy is based on inhibition of DNA synthesis and RNA functions in tumors (1). Oral administration of 5-FU drugs is readily acceptable to patients, even long term, and 5-FU effectively inhibits DNA synthesis, even at low doses. However, 5-FU concentrations in tumors must be maintained at efficacious levels during treatment.

In humans, >80% of 5-FU-based drugs administered are degraded to inactive metabolites by DPD, the first and rate-limiting enzyme in the catabolism of fluoropyrimidines (2). The activity of this enzyme limits the efficacy of 5-FU, whereas 5-FU itself is considered to be among the most effective of anticancer agents. Severe, occasionally fatal side effects of 5-FU chemotherapy have been seen in some patients with enzyme deficiency, and the genetic basis for this deficiency has been identified (3–5).

The distribution of DPD enzyme activities varies among organs (6). In 1985, Naguib et al. (7) reported levels of DPD activities to be variable in several neoplastic human tissues, whereas the highest levels were found in the liver and lymphocytes. More recently, several studies (8, 9) have reported a relationship between DPD activities in tumors and the antitumor effect of 5-FU. 5-FU is not only metabolized in the liver but also in tumors and normal tissues. Therefore, it is possible that the DPD activity in the tumor itself is the main determinant of the actual 5-FU dose as well as the efficacy of treatment. Several studies (9, 10) found a good correlation between DPD enzyme activities and DPD mRNA levels in gastrointestinal cancers. Therefore, quantification of mRNA is a reliable and acceptable means of predicting DPD enzyme activities.

This is more feasible than estimation of enzyme activities, especially in small biopsy specimens. Several methods such as semiquantitative RT-PCR have been established that, in contrast to Northern blot hybridization, allow the quantification of the low expression level of DPD mRNA in tumor tissues (11). Forty colorectal cancer patients were analyzed to study the relationship between the DPD expression and actual 5-FU concentrations in the same sample after treatment with 5′-DFUR, a prodrug of 5-FU. In addition, mRNA levels in biopsy and surgical specimens were quantitated to gain insight into the influence of treatment on the DPD mRNA expression level.

MATERIALS AND METHODS

Study Characteristics. Forty advanced colorectal cancer patients who were <75 years of age and without any serious complications participated in this study. All patients gave informed consent for preoperative 5′-DFUR treatment as neoadjuvant chemotherapy. There were 24 males and 16 females, and the mean age of the group was 65 years (range, 46–75 years). Patients were treated with 5′-DFUR (800–1200 mg/day) over a period of 14 days and during a preoperative period of 6–12 h. The average dose administered to each patient was 16.3 g of 5′-DFUR.
**Tissue Sample Collection.** Before the administration of 5'-DFUR, two additional biopsy specimens, one from the tumor and the other from normal surrounding tissues, were collected by colorectal endoscopy for histological diagnosis of the cancers and examination of the surgical margin. Biopsy specimens were immediately submerged in Isojen (phenol/guanidine isothiocyanate) and stored at -80°C until mRNA extraction. One hundred mg each from the normal and tumorous portions of the specimens were collected intraoperatively, immediately frozen in liquid nitrogen, and then stored at -80°C until use. We collected the tissues in either the morning (11 a.m.–12 a.m.) or afternoon (3 p.m.–4 p.m.).

**Enzyme Activities of DPD Represented as Protein Levels in Surgical Specimens.** The amounts of DPD protein in both tumor and normal tissues were determined by a sandwich ELISA consisting of two monoclonal antibodies specific to human DPD, according to a previous investigation (6). Tissue samples were homogenized in a 10-fold volume of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl₂, and 50 μM potassium phosphate, and the homogenates were centrifuged at 10,000 x g for 15 min. The protein concentration of the supernatant was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). An ELISA plate coated with anti-DPD monoclonal antibody 4B9 was incubated with serially diluted DPD standards and test samples, and the peroxidase activity was assessed by electrophoresis on 1.2% denaturing formamide method according to a method reported previously (12). The column temperature was 35°C. The mean 5-FU concentration was 0.411 ± 0.206 (μg/g) in tumors than in normal colorectal tissues, and the difference was statistically significant (P < 0.05; Fig. 1). The β-actin antisense primer (5'-CTACCTGCTTGCCCGTTGAGC-3'), the 5'-sense primer (5'-AGGATTCAGACAGCTTTAAGC-3'), and the β-actin antisense primer (5'-CCCCGACCCAG-GCAC-3'), all of which were designed according to a method described previously (15, 16). PCR was carried out in a final volume of 50 μl containing 5 μl of cDNA template, 1.25 units of Taq Gold (Perkin-Elmer), 10× PCR Gold Buffer (the composition of which is a company secret), 25 mM MgCl₂, and 2 mM deoxynucleotide triphosphates and specific primers (30 pmol of DPD and 1.5 pmol of β-actin) using a thermal cycler (TP-3000; Takara, Tokyo, Japan). Initial heating at 95°C for 12 min was followed by 30 PCR cycles (95°C for 1 min, 60°C for 2 min, and 72°C for 2 min). Amplification was terminated with a 7-min extension step at 72°C. Amplified DNA fragments were separated on an ethidium bromide-stained 2.5% agarose gel. A picture of the gel was scanned (EPSON scanner 9500), and the density of each band was quantitated by NIH image software (Macintosh 1.59/PPC). Relative rates for DPD mRNA levels were calculated as DPD/β-actin ratios.

**Measurement of 5-FU Concentrations in Tissues.** The 5-FU concentrations in tumors and normal tissues of the colon after treatment with 5'-DFUR were determined by high-performance liquid chromatography as described previously (17). High-performance liquid chromatography was carried out using a Scherisorb 5 ODS 2 column (25-cm × 0.46-cm inside diameter, packed with 5-μm particles) and 0.05 M phosphate buffer as the mobile phase (pH 3), with UV detection at 280 nm. The column temperature was 35°C.

**Statistical Analysis.** Comparisons of DPD protein levels and 5-FU concentrations were performed by Pearson’s correlation, and the relative risk was calculated as an odds ratio using the SPSS software package (version 9.0J). The statistical significance limit was P < 0.05. The relationships between DPD mRNA and protein levels were analyzed by Pearson’s correlation. Pearson’s correlation was also used to assess the relationship between DPD mRNA levels before and after chemotherapy.

**RESULTS**

**5-FU Concentrations in Tumorous and Normal Tissues.** 5-FU concentrations were assessed in 40 tumors and normal surrounding tissues from the surgical specimen. Fig. 1 shows the range of 5-FU concentrations. Distributions of 5-FU concentrations in tumors varied within a 10-fold range, and the distributions in normal tissues varied within a 4-fold range. There were small interindividual differences in 5-FU concentrations in normal tissues, compared with the tumor tissues, as shown in Fig. 1. The mean 5-FU concentration was 0.411 ± 0.381 (μg/g) in the tumorous portions of the specimens and 0.180 ± 0.206 (μg/g) in the normal portions. 5-FU concentrations were higher in tumors than in normal colorectal tissues, and the difference was statistically significant (P < 0.05; Fig. 1).

**Comparison of DPD Protein Levels in Tumors and Normal Tissues.** DPD protein levels were measured in all tumorous and normal portions of the surgical specimens. DPD protein levels varied within a 20-fold range from 8.3 to 190.1 in tumors and within a 10-fold range from 14.5 to 99.8 in normal tissues.
tissues. The mean DPD protein levels were 53.39 ± 32.54 (units/mg) in the tumorous portions of specimens and 37.74 ± 16.49 (units/mg) in the normal portions. Fig. 2 shows the association of DPD protein levels in tumorous and normal tissues. At the individual level, DPD protein levels in the tumorous and normal tissues correlated well (r = 0.527; P < 0.01).

Fig. 3 shows the DPD protein levels and corresponding 5-FU concentrations in tumors. DPD protein levels were inversely associated with 5-FU concentrations in tumors (Pearson’s correlation, r = −0.463; P < 0.05; Fig. 3A). In contrast, no such correlation was found in normal tissues (r = −0.24; P = 0.135; Fig. 3B). Thus, high DPD activities correlated with low 5-FU concentration only in tumors.

We studied the relationship between protein levels and mRNA expression using semiquantitative RT-PCR in tumor tissues after chemotherapy. DPD protein levels and corresponding DPD mRNA levels in tumors are summarized in Fig. 4. DPD protein and mRNA expression levels correlated well in tumor tissues (r = 0.740; P < 0.01; Fig. 4A). In contrast, DPD mRNA levels in normal tissues did not correlate with DPD protein levels (r = 0.054; P = 0.734; Fig. 4B). We confirmed that DPD mRNA levels in tumors are predictive of enzyme activities.

Finally, we explored whether DPD mRNA levels in biopsy samples are predictive of DPD levels in tumors after chemotherapy. DPD mRNA levels varied markedly among individuals (10-fold). DPD-β-actin mRNA ratios ranged from 0.28 to 2.16 in surgical specimens and varied 7-fold in normal tissues with a range from 0.33 to 1.99. Our results are presented in Fig. 5. At the individual level, DPD mRNA levels in tumor biopsy specimens before treatment often correlated with those found in surgical specimens after chemotherapy, but this was not true for each individual (r = 0.366; P < 0.05). In summary, our investigation of 40 colorectal cancer patients suggested that the DPD mRNA level may predict DPD activities and intratumoral 5-FU concentrations.


discussion
This study demonstrates the relationships among 5-FU concentrations, DPD activities represented as DPD protein levels (units/mg protein), and DPD mRNA levels in tumorous and normal tissues from 40 colorectal cancer patients who were treated preoperatively with 5′-DFUR. We found different individual 5-FU concentrations in tumors after 5-FU treatment, and the 5-FU levels in tumors were modified by the actual DPD activity in the tissues. DPD activities were found to relate to DPD mRNA levels. The latter results indicate that activities can be estimated by quantification of DPD mRNA levels, which can easily be measured even in small tissue samples.

DPD is responsible for degradation of the pyrimidines uracil and thymine and the inactivation of 5-FU (2). DPD activity is highly variable in cancer tissues (18), and this variation in cancer tissues may influence the antitumor efficacy of 5-FU, because the intratumoral drug concentration is one of the most important factors for the determination of antitumor efficacy. We found the intratumoral level of 5-FU concentration to be inversely proportional to DPD activities. Thus, individuals with a low DPD level need less 5-FU to maintain an efficacious 5-FU dose and vice versa. Some investigators (9, 13) proposed that DPD activities in the tumor were related to 5-FU sensitivities in gastric cancer cell lines.

Several factors are known to influence DPD activities. Harris et al. (19) demonstrated that DPD activity follows a circadian variability in rat liver (7-fold) as well as in human blood lymphocytes (2-fold). Because of inherent collection difficulties, no such data exist for other human tissues. Our statistical analysis did not reveal any difference in DPD levels between samples collected in the morning and those collected in the afternoon (data not shown). 5-FU clearance is not only influenced by DPD activity but also by liver function (20). In this study, none of the patients had impaired liver function. Maeda et al. (21) reported that 5-FU levels in gastric cancer tissues had not changed within 4–12 h after 5′-DFUR administration. That was also the case in this study, where the esti-
mated 5-FU concentrations were independent of the time interval (6–12 h) after the last drug administration (data not shown).

In our patients, 5-FU levels were higher in tumors than in surrounding normal tissues, a finding that is in accordance with earlier results (21, 22). This selective antineoplastic effect is mediated by the action of intratumoral thymidine phospholipase enzyme, which converts 5′-DFUR to 5-FU more efficiently in tumors than in normal tissues because of the higher amount of thymidine phospholipase activity in tumorous than in normal portions of tissues (22). The latter is also supported by our observation that 5-FU levels in normal tissues were not modified by 5-FU administration (Fig. 1).

DPD enzyme activities in resected tumors correlated well with mRNA expression levels. Additionally, DPD mRNA levels in tumors before and after 5-FU treatment correlated significantly, although the correlation coefficient was only 0.366. One likely explanation for this might be that DPD expression is also influenced by 5-FU, as are the activities of thymidylate syn-

**Fig. 3** Correlation between DPD protein levels and 5-FU concentrations in tumors ($r = -0.463; P < 0.05; A$) or normal tissues ($r = -0.24; P = 0.135; B$).

**Fig. 4** Correlation between DPD mRNA levels, as determined by semiquantitative RT-PCR, and DPD protein levels, as determined by sandwich ELISA, in tumor tissues ($r = 0.740; P < 0.01; A$) or normal tissues ($r = 0.084; P = 0.734; B$).

**Fig. 5** Correlation between DPD mRNA levels in tumor biopsy specimens versus surgical samples ($r = 0.366; P < 0.05$).
phage, which is inhibited by 5-FU (23). However, there are no reports examining this possibility.

Several studies reported that the efficacy of 5-FU might relate to DPD activity in tumor tissues, but DPD activity or protein levels are difficult to determine in small biopsy samples. DPD mRNA levels and activities correlated well in tumor tissues of surgical specimens, and DPD mRNA levels in tumor surgical specimens after chemotherapy correlated with those found in tumor biopsy specimens. These results may indicate that DPD mRNA levels in biopsy samples may be used to estimate DPD activities. Our findings suggest that DPD mRNA levels, as assessed from biopsy specimens obtained by colonoscopy, may be useful indicators in predicting tumor sensitivity to 5-FU in patients with colorectal cancer.

In summary, our study indicates that DPD activities are predictive for 5-FU concentrations in colorectal cancer tissues. DPD activities can be estimated by quantification of mRNA levels using sensitive techniques such as RT-PCR.

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