ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of 5-fluorouracil (Ura), one of the most widely used anticancer drugs. Previous studies from our laboratory demonstrated the clinical importance of DPD in cancer patients (G. D. Heggie, J-P. Sommadossi, D. S. Cross, W. J. Huster, and R. B. Diasio. Cancer Res., 47: 2203-2206, 1987; B. E. Harris, R. Song, S-J. Soong, and R. B. Diasio. Cancer Res., 50: 197-201, 1990), particularly in those with DPD deficiency who experience severe FUra toxicity (including death) following FUra treatment (R. B. Diasio, T. L. Beavers, and J. T. Carpenter. J. Clin. Invest., 81: 18-51, 1988; B. E. Harris, J. T. Carpenter, and R. B. Diasio. Cancer (Phila.), 68: 499-501, 1991). We now suggest that measurement of DPD activity may be useful in routine screening of cancer patients prior to FUra treatment. In this paper, we describe the following serial studies: (a) we developed a sensitive, accurate, and precise DPD assay and a storage method to stabilize DPD activity, permitting large scale DPD screening in cancer patients; (b) we demonstrated a normal distribution (Gaussian distribution) of human DPD activity from peripheral blood mononuclear cells (PBMDPD) in a population study. Baselines for PBMDPD with fresh and frozen samples were 0.425 ± 0.124 (SD) and 0.189 ± 0.064 nmol/min/mg protein, respectively. The 95% and 99% distribution ranges for both fresh and frozen samples were also determined, providing criteria for detection of DPD-deficient patients; (c) we identified nine new patients with profound or partial DPD deficiency; (d) we determined a baseline for human liver DPD activity, which was shown to be 0.360 ± 0.182 nmol/min/mg protein (frozen samples); (e) we did a preliminary evaluation of liver DPD activity from deficient patients. Low liver DPD activity in two deficient patients correlated with low PBMDPD activity. Using a polyclonal antibody raised against human liver DPD in our laboratory (Z. Lu, R. Zhang, and R. B. Diasio. J. Biol. Chem., 267: 17102-17109, 1992), Western blot analysis demonstrated decreased DPD protein in the liver cytosol from DPD-deficient patients compared to normal subjects. These results may be useful in improving the effectiveness and/or lessening the toxicity of FUra chemotherapy.

INTRODUCTION

DPD (EC 1.3.1.2) is the initial and rate-limiting enzyme in FUra catabolism (1-4). Although it has been thought that the cytotoxic effects of FUra are directly mediated by the anabolic pathway (1-4), studies from our laboratory have demonstrated the importance of the catabolic pathway with more than 80% of administered FUra being catabolized by DPD (5). It has also been shown that the anticancer efficacy of FUra is related to DPD activity in an experimental animal tumor model (6). We (7) and others (8, 9) have demonstrated that DPD activity is correlated with FUra pharmacokinetics, suggesting an important role in the regulation of FUra metabolism and thus determining the amount of FUra available for anabolism. DPD activity in humans (7, 10) and experimental animals (11-14) follows a circadian pattern, which is inversely correlated with FUra plasma level in cancer patients treated with FUra by continuous infusion (7). Additional studies with competitive DPD inhibitors (15-18) have also shown the importance of DPD in FUra chemotherapy.

More importantly, the role of DPD in FUra chemotherapy has been shown in cancer patients with confirmed (19-21) or suspected (22, 23) DPD deficiency. These patients experienced severe FUra toxicity (including death) following FUra treatment. A clinical pharmacological study of one of these deficient patients (19) demonstrated minimal catabolism of FUra with a 10-fold longer FUra half-life compared to patients with normal FUra activity. Familial studies (19-21) have demonstrated that this pharmacogenetic syndrome follows an autosomal recessive pattern of inheritance.

Although extensive studies in DPD biochemistry have been conducted with experimental animals and crude human tissue preparations (24-27), little has been known about human DPD until recently. In our laboratory, human liver DPD has recently been purified and characterized with preparation of a polyclonal antibody to DPD (28, 29), permitting further investigation of DPD in cancer patients.

Given the frequent use of FUra in cancer chemotherapy, the importance of DPD in FUra pharmacokinetics, and the clinical significance of DPD deficiency, we suggest the potential value of DPD screening in cancer patients prior to and during FUra treatment. In order to provide the basis for large scale DPD screening in cancer patients, the following studies were undertaken: (a) development of a sensitive, accurate, precise, and clinically useful DPD assay; (b) establishment of a baseline for DPD activity and criteria for diagnosis of partial and profound DPD deficiency; (c) identification of additional patients with DPD deficiency; (d) determination of liver DPD activity in the general population and in cancer patients with DPD deficiency initially identified by PBMDPD assay. In addition, using a polyclonal antibody raised from purified human liver DPD (28), we also conducted Western blot analyses to determine if DPD protein in the liver cytosol from DPD-deficient patients was decreased compared to that of normal subjects. The serial studies reported in this paper demonstrate the potential value of measuring DPD activity. We believe that determination of DPD activity in cancer patients prior to and/or during FUra treatment may be beneficial in improving the clinical effectiveness of FUra.

MATERIALS AND METHODS

Chemicals and Radiochemicals

FUra, bovine serum albumin, NADPH, and Histopaque were purchased from the Sigma Chemical Co. (St. Louis, MO). [3H]FUra (25 Ci/mmole) was obtained from New England Nuclear Corp. (Boston, MA). The purity of
unlabeled and labeled FUra was confirmed by HPLC (30) to be greater than 99%. Acrylamide and prelabeled major weight markers were purchased from Bio-Rad (Richmond, CA). Alkaline phosphatase-labeled goat anti-rabbit antibody, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt were obtained from Southern Biotechnolog (Birmingham, AL). All other solvents and reagents were purchased in the highest grade available.

The major buffer (buffer A) used in both the enzyme preparation and DPD assay contained 35 mM potassium phosphate (pH 7.4), 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol. Since NADPH, the critical cofactor in the enzyme reaction, is light sensitive and unstable with long-term storage, it was freshly prepared.

**Determination of PBM-DPD Activity in a Normal Population and Patients**

**Healthy Volunteers.** One hundred twenty-four healthy volunteers participated in this study. This normal population consisted of faculty, staff, and students from the University and its affiliated units. Among them, DPD activity was also determined with frozen samples from 109 subjects, along with another 14 frozen samples from healthy volunteers. The characteristics of this population are listed in Table 1. Informed consent was obtained from each healthy volunteer, using an institutionally approved protocol.

**Cancer Patients.** In collaboration with more than 20 medical institutions, 25 cancer patients who were treated with FUra (in combination with other agents) and who had experienced moderate to severe drug-related toxicity were obtained from each institution. Consent was obtained from each patient.

**Blood Collection and Isolation of PBM Cells.** Because of a known circadian pattern of DPD activity (7), blood samples were collected between 8 a.m. and 10 a.m. Blood samples (25–50 ml) were drawn from a peripheral vein into a 60-ml syringe containing 5 ml heparin (1000 units/ml). After collection, the syringe was inverted carefully to mix heparin and blood. The blood sample was then loaded onto a centrifuge tube containing 15 ml Histopaque. After centrifugation at 500 × g for 30 min at 25°C, the PBM cell fraction (located between the plasma fraction and Histopaque) was carefully removed and washed 3 times with phosphate-buffered saline. Contaminating RBC were centrifuged at 14,000 X g for 15 min at 4°C, the supernatant was removed by sonication (3 times for 10 s with a 30-s interval between sonication). After collection, the enzyme reaction, is light sensitive and unstable with long-term storage, it was freshly prepared.

**Reverse-Phase HPLC Analysis.** Separation of FUra and its catabolites was performed by reverse-phase HPLC using a method we have described in detail previously (30).

**Table 1 PBM-DPD activity in healthy volunteers (with fresh samples and frozen samples)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fresh sample</th>
<th>Frozen sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean activity (nmol/min/mg)</td>
<td>0.425</td>
<td>0.189</td>
</tr>
<tr>
<td>SD</td>
<td>0.124</td>
<td>0.064</td>
</tr>
<tr>
<td>SE</td>
<td>0.011</td>
<td>0.006</td>
</tr>
<tr>
<td>Distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% range</td>
<td>0.182-0.668</td>
<td>0.064-0.314</td>
</tr>
<tr>
<td>99% range</td>
<td>0.105-0.745</td>
<td>0.024-0.354</td>
</tr>
<tr>
<td>Characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>124</td>
<td>123</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>56 (45.2)*</td>
<td>55 (44.7)*</td>
</tr>
<tr>
<td>Females</td>
<td>68 (54.8)</td>
<td>68 (55.3)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>77 (62.1)</td>
<td>74 (60.2)</td>
</tr>
<tr>
<td>Non-Caucasian</td>
<td>47 (37.9)</td>
<td>49 (39.8)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>45 (36.3)</td>
<td>39 (31.7)</td>
</tr>
<tr>
<td>30</td>
<td>39 (31.5)</td>
<td>42 (34.2)</td>
</tr>
<tr>
<td>40</td>
<td>25 (20.2)</td>
<td>25 (20.3)</td>
</tr>
<tr>
<td>50</td>
<td>15 (12.1)</td>
<td>17 (13.8)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

The primary antibody used in the study was the purified rabbit polyclonal antibody generated against human liver DPD (28). A 7% SDS-PAGE was performed using the freshly prepared liver supernatant centrifuged at 100,000 X g. SDS-PAGE was carried out in a 1.0-mm-thick 7% (w/v) polyacrylamide gel containing 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. Samples were mixed with an equal volume of sample buffer (0.0625 M Tris-HCl, pH 6.8–10% glycerol-0.2% SDS (w/v)-80 mM 2-mercaptoethanol) and then boiled for 5 min. Electrophoresis was performed at a constant current of 30 mA for 45 min at 25°C. The proteins were transferred from the gel to a nitrocellulose filter following the method described previously by Towbin et al. (32). Following incubation overnight at 4°C with the primary antibody (diluted 1:2000) in a 120 mM borate-saline solution containing 1% (w/v) bovine serum albumin (pH 8.5), the nitrocellulose filter was washed with borate-saline containing 0.5% (v/v) Tween 20 and then incubated with a secondary, alkaline phosphatase-labeled goat anti-rabbit antibody. The location of immunoreactive proteins on the nitrocellulose filter was detected in a 0.1 M sodium carbonate buffer (100 ml), pH 9.5, containing 30 mg nitro blue tetrazolium (added as a 1-mL solution dissolved in 70% dimethylformamide) and 15 mg 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (added as a 1-mL solution dissolved in 100% dimethyformamide).

**Statistical Analysis**

The differences among the groups by sex, age, and race were analyzed by the Student t test or analysis of variance as appropriate. To determine the distribution pattern in the general population, probability testing was used to test the hypothesis that the sample population distribution of DPD activity follows a normal distribution (Gaussian distribution). Following confirmation
of a normal distribution, additional statistical parameters were calculated, including mean, SD, SE, and the 95% and 99% distribution ranges. In determining the correlation of DPD activity between fresh samples and frozen samples, Pearson correlation coefficients were calculated.

RESULTS

Optimal Assay Conditions and Evaluation of the PBM-DPD Assay. In the present study, we optimized the assay conditions for quantitation of PBM-DPD activity, particularly in samples with decreased PBM cells commonly seen in cancer patients with leukopenia. In brief, the conditions included: substrate (FUra) concentration at 20 μM; enzyme reaction maintained at pH 7.4 and 37°C; incubation time between 5 and 60 min; and a protein level of PBM cytosol between 25 and 100 μg/100 μl reaction mixture. Substrate and product inhibition were observed with PBM-DPD. Both protein (enzyme) level and incubation time were shown to be critical factors in determining DPD activity.

Statistical analysis further confirmed that the above method was sensitive (lowest detectable products being 0.005 nmol), accurate, and precise (the inter- and intraday coefficients of variations being less than 5%). Evaluation of the stability of DPD with frozen PBM cells indicated that decreased DPD activity (~50%) occurred in the freezing process, but DPD activity was stable afterwards for at least 1 month (data not shown). The correlation coefficient between fresh sample and frozen sample was 0.858 with statistical significance (Fig. 1; P < 0.0001).

Population Distribution of PBM-DPD Activity. With freshly prepared samples, PBM-DPD activities in 124 healthy volunteers were shown to follow a normal or Gaussian distribution (Fig. 2). Further cross-analysis revealed that there was no significant difference among groups by sex, race, or age (Table 2). Using a normal distribution model, several statistical parameters were calculated, including mean, SD, SE, and 95% and 99% distribution ranges (Table 1) for the population. The 95% or 99% distribution range (i.e., theoretically 95% or 99% persons in population have a DPD activity in the corresponding range) was subsequently used in the identification of DPD deficiency (see below).

With frozen samples, PBM-DPD activities in 123 healthy volunteers (among them were 109 paired samples from the above study with fresh samples) were also shown to follow a normal distribution (data not shown) with no differences in sex, age, or race. Using a normal distribution model, several statistical parameters for the population were also calculated, including mean, SD, SE, and 95% and 99% distribution ranges (Table 1). As with the fresh samples above, the 95% or 99% distribution range was used for identification of DPD deficiency with frozen samples.

New Cases of DPD Deficiency. Using the above methods, we collaborated with medical oncologists within our university and at more than 20 medical institutions and have been able to identify 9 new deficient patients who experienced moderate to severe FUra toxicities including mucositis, granulocytopenia, neuropathy, and death. The characteristics, therapy, major toxicity, and DPD activity (expressed as a percentage of mean DPD activity in population) are shown in Tables 3 and 4. Three profoundly deficient patients, listed in Table 3, experienced Grade V toxicity (death). DPD activity in this group was less than 10% of the mean DPD activity in the general population and below the lower limit of the 99% distribution range. Six partially deficient patients listed in Table 4 experienced Grade II–III toxicity. DPD activity was less than 30% of the mean DPD activity in the general population and below the lower limit of the 95% distribution range.
We have been able to conduct familial studies with four of the patients (patients 1 and 2 in Table 3; patients 5 and 7 in Table 4) and confirmed an autosomal recessive pattern of inheritance (data not shown).

Liver DPD Activity in "Normal" Donors. Using frozen liver samples, DPD activities of 59 "normal" donor livers collected over the preceding 24 months were determined and shown to follow a normal distribution (data not shown) with no differences in age and race, although the DPD activity of females was slightly higher than that of males. The means and SD as well as the SE of liver DPD activity in each group by sex, age, and race are shown in Table 5.

Liver DPD Activity and DPD Protein in Deficient Patients. After their deaths, liver DPD activities of two patients initially identified as being DPD deficient by PBM-DPD assay were determined. Interestingly, liver DPD activities in the two patients correlated with their PBM-DPD activity. Patient 6, whose PBM-DPD activity was 17% of the mean PBM-DPD activity of the normal population, had a liver DPD of 38.9% of the mean liver DPD activity of the normal population. Patient 2 had activities of 6% for PBM-DPD and 14.7% for liver DPD. These results suggest a relationship between PBM-DPD and liver DPD activity. Using affinity-purified polyclonal antibody against human DPD, Western blot analysis revealed that DPD protein was significantly decreased in liver cytosol from the two patients compared with normal subjects. As illustrated in Fig. 3, with an equal amount of protein loaded onto the gel, the DPD protein band for patient 6 had a lower density compared to the normal subjects; an even lower density was seen with patient 2. These results suggest a relationship between DPD activity and DPD protein in the liver.

DISCUSSION

Recent studies have shown the important role that DPD has in FURA catabolism with regulation of the availability of FURA for anabolism, potentially determining the resultant anticancer efficacy and/or toxicity of FURA. However, the clinical value of determination of DPD activity has not been widely appreciated. This is due to limited knowledge of "normal" DPD activity in the general population and the relationship between PBM-DPD and liver DPD, as well as the unavailability of a sensitive and reproducible assay. The major purpose of the present study was initially to develop and evaluate an assay that can be used in DPD screening and subsequently to determine the distribution of DPD activity (in both PBM-DPD and liver DPD) in the general population. Results from the present study suggest that measurement of DPD activity could be performed routinely in cancer patients prior to FURA treatment.

The initial study optimized the assay conditions for determining DPD activity. In subsequent studies evaluating storage conditions, we demonstrated that the freezing process affected PBM-DPD initially but no significant change in enzyme activity was observed during storage (at least for 1 month). This method has been demonstrated to be sensitive, accurate, and precise. More importantly, DPD activity in fresh and frozen samples was strongly correlated, permitting utilization of frozen samples in large scale screening in the general population and in routine testing in the clinic.

Prior to a large scale study of DPD activity in patients, one needs to clearly determine the population distribution of DPD activity and establish criteria for identification of deficient patients. Furthermore, it is necessary to determine whether there are any differences in DPD activity for different groups by sex, age, or race. In a study of cancer patients, Milano et al. (33) reported a possible influence of sex on FURA clearance and suggested that it may be related to variations in DPD activity. However, a more recent study from the same laboratory (23) demonstrated no significant sex difference in DPD activity. It is worth noting that these results were obtained from a relatively small sample of the cancer patient population consisting of 66 patients with only 10 females being studied. In the present population study with 124 subjects (45% males and 55% females), statistical analysis demonstrated no significant sex difference in DPD activity. It is necessary to determine whether there are any differences in DPD activity for different groups.
Given the wide use of FUra in the clinic and the presence of severe toxicity (including death) in patients with DPD deficiency, it would be desirable to determine DPD activity in cancer patients prior to administration of FUra. Clinical investigations in the present study further support this suggestion. In addition to three cases reported previously from this laboratory (19–21), we identified nine new patients with profound or partial DPD deficiency in the present study, strongly suggesting that DPD deficiency is more frequent than previously thought. Using the established baseline for DPD activity, we suggest that the lower limit of the 95% distribution range be used as a criterion for identification of DPD deficiency.

Although the liver is thought to be the major site of FUra metabolism (34), it is not practical to directly determine liver DPD activity in patients for diagnosis or research purposes. Since the number of fresh human liver samples available for research purposes is limited, in the present study, we determined the baseline for human liver DPD using frozen liver tissues from “normal” donors. These samples appeared to be free of disease and were rapidly frozen and kept at ~70°C. The DPD activity in frozen liver tissues had been shown previously in our laboratory to be stable for at least 2 years (data not shown). These results are also consistent with a study with rat liver in our laboratory.4 We found no significant difference between fresh liver samples and frozen samples (storage period from 1 week to 6 months). With 59 “normal” liver samples, a baseline for liver DPD activity was determined (Table 5), demonstrating no significant difference in race and age, while mean liver DPD activity of females was slightly higher than that of males.

The present study is the first in which decreased liver DPD activity was demonstrated in cancer patients with DPD deficiency initially identified by PBM-DPD. More interestingly, there was a suggested relationship between liver DPD and PBM-DPD. Considering the similar population distribution pattern of PBM-DPD and liver DPD and the above evidence, we suggest that PBM-DPD could be used as a marker for DPD activity in general. In the two DPD-deficient patients, Western blot analysis demonstrated decreased DPD protein in the liver cytosol compared with normal subjects. Further characterization of the DPD protein from these deficient patients is currently under investigation in our laboratory.

In addition to its obvious importance to DPD-deficient patients, measurement of DPD activity is also potentially important in the therapeutic use of FUra in other cancer patients as well. Theoretically, assay of DPD activity could be used to adjust the FUra dose to be administered prior to or during treatment in order to improve the effectiveness of FUra chemotherapy and/or decrease drug-related toxicity. Further studies are needed to confirm the relationship between DPD activity and FUra therapeutic effectiveness and toxicity in cancer patients, using a prospective pharmacoepidemiological study design.

Another potential use of measurement of DPD activity is related to the use of DPD inhibitors. Several groups have suggested a role for DPD inhibitors in improving FUra effectiveness. Inhibition of DPD activity has been shown to potentiate the effect of FUra both in vitro and in vivo (6, 15–18, 35–37). However, in light of the severe toxicity in patients with DPD deficiency, caution, including DPD determination, should be used prior to administration of these DPD inhibitors in conjunction with FUra chemotherapy.

In summary, the DPD assay described in the present report is a sensitive, accurate, and precise method that can be used in DPD screening in cancer patients. DPD activity in the general population follows a normal distribution with no significant differences in sex, age, or race. Additional DPD-deficient patients have been identified, suggesting that this pharmacogenetic syndrome is not rare. In considering the frequent use of FUra in cancer patients, severe FUra toxicity in DPD-deficient patients, and the potential for measurement of DPD activity in improving FUra chemotherapy, we suggest that the measurement of DPD activity could be routinely conducted in cancer patients prior to or during FUra treatment. Further studies screening the frequency of DPD deficiency in the cancer patient population, prospectively determining the relationship between DPD activity and FUra effectiveness and/or toxicity, and the molecular basis for DPD deficiency are currently under investigation in our laboratory.

ACKNOWLEDGMENTS


REFERENCES

10. Daber, G. C., Harris, B. E., Zhang, R., Willard, E. M., Soong, S.-J., and Diasio, R. B. The role of dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (dThdPase) in the circadian variation of plasma levels of fluorouracil (FUra) and 5-fluordeoxyuridine (FdUrd) following infusion of FUra or FdUrd. Annu. Rev. Chronopharmacol., 7: 227–230, 1990.

Dihydropyrimidinase Dehydrogenase Activity in Human Peripheral Blood Mononuclear Cells and Liver: Population Characteristics, Newly Identified Deficient Patients, and Clinical Implication in 5-Fluorouracil Chemotherapy

Zhihong Lu, Ruiwen Zhang and Robert B. Diasio


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