Decreased Dihydropyrimidine Dehydrogenase Activity in a Population of Patients with Breast Cancer: Implication for 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 (5-FU), one of the most widely used chemotherapeutic agents in the treatment of breast cancer. The objective of this study was to determine the population characteristics of DPD activity in patients with breast cancer as well as the frequency of DPD deficiency in this population. DPD activity in peripheral blood mononuclear cells (PBM-DPD) was determined in 360 patients with breast cancer, with the mean PBM-DPD (0.26 ± 0.01 nmol/min/mg protein) being significantly lower than that observed in female controls (0.44 ± 0.02 nmol/min/mg protein; $P < 0.01$). ANOVA analysis examining the significance of differences in DPD activity among various groups indicated that only disease difference (breast cancer *versus* normal subjects) was significant after adjustments for race and age. In the present study, 21 (5.8%) patients were considered to be DPD deficient, indicating that this pharmacogenetic syndrome may be more common than anticipated (no DPD-deficient individual was found in the controls). Significantly lower DPD activity in patients with breast cancer may predispose to 5-FU-associated toxicity. These results provide further rationale for individualizing the 5-FU dose, thus reducing the risk of toxicity and/or improving therapeutic efficacy in patients with breast cancer.

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INTRODUCTION

5-FU[3] is one of the most widely used anticancer drugs (1, 2), ranking in the top three anticancer drugs prescribed in the United States (3). It is used frequently in the treatment of common malignancies such as cancers of the breast, colon, and head and neck (1–4). In general, 5-FU-associated toxicity occurs in the gastrointestinal mucosa and bone marrow and, less frequently, in the neurological system, presenting as cerebellar ataxia and somnolence (4, 5). Like many other antineoplastic agents, 5-FU has a relatively narrow therapeutic index, such that toxicity is likely to increase as the dose is escalated. The biochemical mechanism of 5-FU-associated toxicities is thought to be related to its anabolic pathway, in particular, inhibition of thymidylate synthase and incorporation into RNA and DNA (1, 2). However, the role of 5-FU catabolism in toxicity and/or therapeutic responses has not been appreciated until relatively recently. It has been demonstrated that more than 85% of 5-FU administered to patients with cancer is degraded through the catabolic pathway (6), which has an important role in regulating the availability of 5-FU for anabolism (1).

DPD is the initial, rate-limiting enzyme in 5-FU catabolism (1, 2). The importance of DPD activity in 5-FU catabolism has been studied extensively (1, 2). Coadministration of thymidine with 5-FU (leading to release of thymine that competes with 5-FU for DPD) has been shown to induce unexpected life-threatening toxicity (7, 8). Our pharmacokinetic studies of patients receiving 5-FU by continuous infusion demonstrated that plasma 5-FU levels had a circadian variation that varied inversely with circadian variation in DPD activity in PBM cells, suggesting that the plasma 5-FU levels were regulated by DPD (9). Fleming *et al.* (10) have also shown the relationship between DPD levels and 5-FU pharmacokinetics in patients receiving 5-FU by continuous infusion. In other studies, we (6, 11, 12) and others (13) have further demonstrated that the metabolism and pharmacokinetics of 5-FU are correlated with DPD activity. The importance of DPD in 5-FU pharmacokinetics and toxicity may be best shown in patients with DPD deficiency, a recently defined pharmacogenetic syndrome (14–21). Following 5-FU-based chemotherapy, these patients developed profound toxicity, with some eventually dying. More importantly, in our earlier studies (15–18), 10 of the 11 patients were women with either breast or colorectal cancer. In view of this striking sex difference in patient studies from our laboratory (15–18) and others (14, 19), as well as the frequent use of 5-FU in patients with breast cancer, it is important to determine the population

[3] The abbreviations used are: 5-FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; PBM-DPD, DPD activity in peripheral blood mononuclear cells; HPLC, high-performance liquid chromatography.

characteristics of DPD activity in patients with breast cancer using a large-scale prospective study design.

It is believed that the liver is the major site for 5-FU catabolism (22); however, the risk of invasive procedure to measure liver DPD activity is not justified. Previous studies suggested that PBM-DPD can be used as a marker for liver-DPD activity (18, 23, 24). Population characteristics of PBM-DPD have been described in several recent studies (10, 18, 25–29) in normal volunteer and cancer patients from overseas; however, there is limited information available in patients with breast cancer in the United States. The present study was undertaken to characterize DPD activity in patients with breast cancer as well as the frequency of DPD deficiency in this population.

MATERIALS AND METHODS

Chemicals

5-FU, BSA, NADPH, and Histopaque were purchased from the Sigma Chemical Co. (St. Louis, MO). [6-^{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 (30) to be greater than 99%. All other solvents and reagents were purchased in the highest grade available.

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

Patients

Three hundred sixty patients with breast cancer seen in the outpatient oncology clinics of the University of Alabama at Birmingham Comprehensive Cancer Center during a 4-year study period were enrolled in the present study. All patients had been diagnosed with breast cancer by pathological confirmation. The study included patients at various stages of diagnosis, treatment, and follow up, with fewer than 50% of the patients receiving 5-FU at the time of this study. All patients gave informed consent to participate in this study. The protocol used in this study was approved by the Institutional Review Board of University of Alabama at Birmingham. Clinical data were collected by the Oncology Clinics and kept blind to the laboratory personnel conducting the DPD assay.

Determination of PBM-DPD Activity in Breast Cancer Patients

Blood Collection and Isolation of PBM Cells. Blood samples (25 ml) were drawn between 8:00 and 10:00 am (central time), from a peripheral vein into heparinized tubes and then loaded onto a centrifuge tube containing 15 ml of Histopaque. After centrifugation at 500 × *g* for 30 min at 25°C, the PBM cell fraction was carefully removed and washed three times with PBS. Contaminating RBCs were hypotonically lysed. The resulting PBM cells were used in the subsequent enzyme assay.

Preparation of PBM Cytosol. Fresh PBM cells were suspended in buffer A, placed in an ice bath, and then lysed by sonication (three times for 10 s each with 30-s intervals between sonications). After centrifugation at 14,000 × *g* for 15 min at 4°C, the supernatant was removed and used in the subsequent enzyme assay. Using the method of Bradford (31), the amount of protein in the sample was determined before enzyme assay to add the appropriate amount of protein into the reaction mixture.

Enzyme Assay. DPD activity was determined by a radioassay, measuring the catabolites of 5-FU formed by reversed-phase HPLC (18, 20). The reaction mixture contained 200 μM NADPH, 20 μM [6-^{3}H]5-FU (25 Ci/mmol), buffer A, and enzyme solution (250 μg total protein) in a final volume of 1 ml. The mixture was incubated at 37°C, and 175 μl of the reaction sample was taken out at various times (5, 10, 20, 30, and 60 min) and mixed with the same volume of ice-cold ethanol to stop the reaction. The mixture was then kept in a freezer (−20°C) for 30 min and subsequently filtered through a 0.2-μm Acro filter (Gelman Sciences, Ann Arbor, MI) before HPLC analysis.

Reversed-Phase HPLC Analysis. Separation of 5-FU and its catabolites was performed by a reversed-phase HPLC method described previously (30).

Calculation of DPD Activity. After HPLC analysis, the formation of 5-FU catabolites at each time point was quantitated. The data were plotted using products formed (y) *versus* time (x) to calculate the slope of the reaction (products formed/min) by linear regression analysis. The enzyme activity was calculated by dividing the slope by the amount of protein added and 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 in PBM cells and SD or SE were calculated for different groups divided by age and race. The differences in DPD activity among the different groups were analyzed by the Student t test or ANOVA as appropriate (18, 23). To determine the distribution pattern in the population of breast cancer patients, probability testing was used (18, 23).

RESULTS

PBM-DPD Activity in the Breast Cancer Patient Population. Using freshly prepared PBM cell samples, DPD activities of 360 patients with breast cancer were determined. The population characteristics of this study are summarized in Table 1. The mean PBM-DPD activity in patients with breast cancer was 0.265 nmol/min/mg protein, with highest and lowest values being 0.571 and 0.013 nmol/min/mg protein, respectively. The distribution of PBM-DPD activity in this population is shown in Fig. 1. Statistical analysis by probability testing indicated that human PBM-DPD activity follows a normal distribution.

PBM-DPD Activity in Breast Cancer Patients Grouped by Age and Race. The mean PBM-DPD activities in each group by age are shown in Table 1. Analysis of different age groups indicated that PBM-DPD activities in breast cancer patients age 40 and above were slightly higher than that observed in patients less than 40 (Table 1). However, these differences were not statistically significant. The mean PBM-DPD activities in each group by race also are shown in Table 1. Statistical analysis indicated that there was no significant dif-

Table 1 PBM-DPD in breast cancer patients

	DPD activity (nmol/min/mg protein)					
	Patients with breast cancer				Controls[a]	
	n	Mean ± SD	Highest	Lowest	*n*	Mean ± SD
Total	360	0.265 ± 0.109[b]	0.571	0.013	68	0.443 ± 0.128
African American	48	0.243 ± 0.093[b]			28	0.460 ± 0.116
Caucasian	312	0.270 ± 0.110[b]			40	0.431 ± 0.126
Age (yr)						
20					22	0.402 ± 0.117
30	35	0.230 ± 0.092[b]			22	0.481 ± 0.131
40	108	0.265 ± 0.106[b]			17	0.482 ± 0.136
50	113	0.274 ± 0.106[b]			7	0.365 ± 0.108
60	61	0.282 ± 0.115				
70	43	0.265 ± 0.118				

[a] Data from a previous study in our laboratory using a similar study design (18).
[b] $P < 0.01$, compared with corresponding controls.

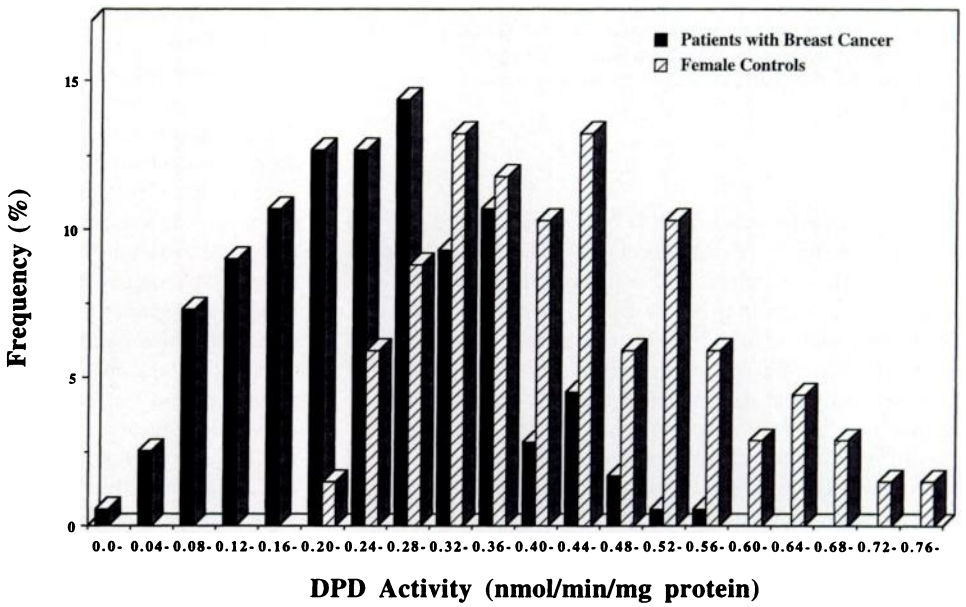

Fig. 1 Population distribution of DPD activity in 360 patients with breast cancer, compared with the general population. Statistical analysis demonstrated that DPD activities follow a normal distribution.

ference of PBM-DPD activity between Caucasians and African Americans, although the mean PBM-DPD activity in African Americans was slightly lower than that in Caucasians.

PBM-DPD Activity in Breast Cancer Patients Compared with Healthy Volunteers. As illustrated in Fig. 1, although the distribution pattern of PBM-DPD activity in patients with breast cancer was essentially the same as seen in the general female population (18), the distribution and mean activity were shifted to the left, indicating that mean PBM-DPD activity in the breast cancer patient population was significantly lower than that observed in female controls ($P < 0.01$; Table 1). Further examination of the effect of disease, race, and age on PBM-DPD activity by ANOVA analysis revealed that only disease difference (cancer *versus* normal subjects) was statistically significant after adjustments for race and age.

Frequency of DPD Deficiency in Patients with Breast Cancer. Twenty-one (5.8%) patients were classified as DPD deficiency (their PBM-DPD activities were less than 0.1 nmol/min/mg protein). In a previous study with 68 controls, no DPD-deficient individual was found.

DISCUSSION

DPD has 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 (1–6). However, the clinical value of determination of DPD activity has not been widely appreciated until recently. This is due to limited knowledge of population characteristics of DPD activity in different populations, including the general population and different cancer patient populations, the frequency of DPD deficiency, as well as the relationship between DPD activity and 5-FU-associated toxicity. In the last decade, our laboratory and others have been conducting investigations on DPD, including pharmacokinetics and pharmacodynamics of 5-FU in relation to DPD (1, 2, 6, 9–13, 27), biochemical and

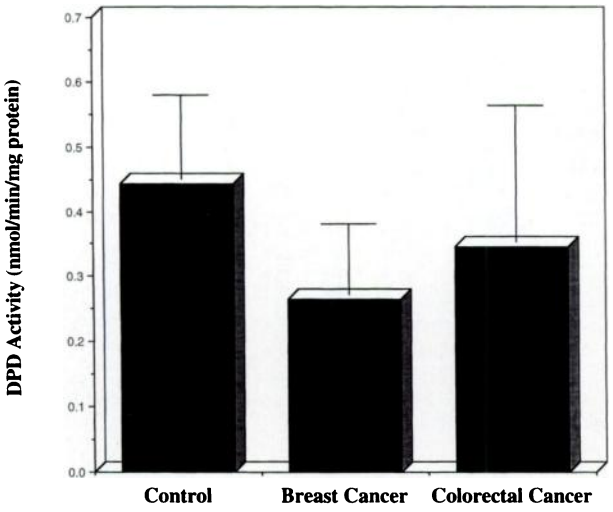

Fig. 2 Comparison of DPD activity in normal volunteers, patients with breast cancer, and patients with colorectal cancer. Statistical analysis demonstrated that there are significant differences: breast cancer *versus* female control, $P < 0.01$; breast cancer *versus* colorectal cancer, $P < 0.05$. *Bars*, SE.

molecular characterization of DPD from human tissues (32) and from various species of experimental animals (33), and clinical characterization of patients with a newly defined pharmacogenetic syndrome characterized by DPD deficiency (18, 20, 21). The present study was undertaken to determine the distribution pattern of PBM-DPD activity in patients with breast cancer, the potential difference of PBM-DPD activities among groups by age and race, as well as the frequency of DPD deficiency.

To evaluate the distribution pattern of DPD activity and determine whether a genetic polymorphism for DPD exists, several studies have now been undertaken in healthy volunteer and cancer patient populations (18, 23, 25–28, 34). Most of the previous studies used small populations without balance in the number of subjects in each subgroup by sex, age, and race. In a recent study with 124 subjects (45% males and 55% females), we demonstrated a normal distribution of PBM-DPD activity with an approximate 4-fold difference in enzyme activity (18). In the general population, the variation in DPD activity was not related to sex, age, or race (18, 23). In a study of cancer patients, Milano *et al.* (26) reported a possible influence of sex on 5-FU clearance and suggested that it may be related to variations in DPD activity. However, more recent reports from the same laboratory showed no significant sex difference in DPD activity (25, 29).

Although the liver is thought to be the major site of 5-FU metabolism (22), the population distribution of liver DPD activity was not known until recently. In a previous study (22), a 288-fold variation in human liver DPD activity was observed, presumably due to variation in the quality of liver tissue preparation. Recently, we demonstrated in freshly processed human liver that DPD activity had no significant difference by race or age, whereas mean liver DPD activity of females was observed to be slightly higher than that of males (23). Given the abundance of DPD activity in the liver, it is necessary to establish the relationship between DPD activity in liver and PBM cells to permit the use of PBM-DPD as a pharmacologically relevant marker of total body DPD activity. In our recent study, we demonstrated that cancer patients identified as DPD deficient by PBM-DPD assay had both decreased DPD activity and decreased DPD protein in the liver (18), indicating the usefulness of PBM-DPD assay in quantitating DPD activity and identification of DPD deficiency.

In the present study, the PBM-DPD activity was determined in 360 patients with breast cancer patients, representing the largest population study of DPD activity in cancer patients thus far. Results from the present study demonstrated that PBM-DPD activity in patients with breast cancer generally follows a normal distribution, as seen in the general population (18). The mean PBM-DPD activity in patients with breast cancer was, however, significantly lower than that in female controls (18). The difference remained after cross analysis by age and race.

Preliminary analysis of a recently initiated study of colon rectal cancer patients also suggested that mean PBM-DPD activity in breast cancer patients was lower than that observed in patients with colorectal cancer ($P < 0.05$; Fig. 2), although the number of colorectal cancer patients is still relatively small ($n = 22$). Because lower DPD activity is likely to be associated with decreased catabolism of 5-FU, patients with breast cancer may have a higher risk to develop 5-FU-associated toxicity after treatment with a standard dose of 5-FU and, therefore, may need to have a reduction of the 5-FU dose. Initial analysis of the clinical data suggested that the frequency of 5-FU-associated toxicity was greater in patients with PBM-DPD activity lower than 0.10 nmol/min/mg protein than those with higher DPD activity (ongoing prospective study; data not shown). The mechanisms responsible for the decreased DPD activity in patients with breast cancer are not clear at this time but may be related to disease status, nutritional effect, hormone levels, and concurrent treatments. Because limited information is available on DPD activity in other cancer patient populations, additional studies should be undertaken to clarify whether the depression of DPD activity in breast cancer patients is unique.

Among 360 patients with breast cancer enrolled in the current study, 4 patients had profoundly decreased PBM-DPD activity (<0.05 nmol/min/mg protein), whereas 17 patients had PBM-DPD activity between 0.05 and 0.10 nmol/min/mg protein, suggestive of partial DPD deficiency. As suggested earlier (18), we have now used the lower limit of 99% distribution range in the general population (PBM-DPD activity less than 0.1 nmol/min/mg protein) as a criterion for identification of DPD deficiency. The frequency of DPD deficiency in patients with breast cancer in the present study was found to be 5.8%, which is higher than that observed in other populations reported previously (25, 26, 28, 29). The relationship between PBM-DPD activity and 5-FU-associated toxicity and the value of PBM-DPD activity as a marker for individualizing 5-FU dosage remain to be determined.

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