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Gini F Fleming · Everett E Vokes · John B Buse
Rosemarie Mick · Jody Dushay · Denise Levitan
M Eileen Dolan

Peripheral blood mononuclear cell dihydropyrimidine dehydrogenase activity in volunteers with and without diabetes mellitus

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Abstract It has been reported that cancer patients with diabetes mellitus receiving a continuous infusion of 5-fluorouracil (5-FU) have more toxicity and higher plasma 5-FU levels than patients without diabetes mellitus. Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of 5-FU. DPD activity in peripheral blood mononuclear cells has been reported to correlate inversely with 5-FU plasma levels in patients. We therefore undertook a study to compare the activity of DPD in peripheral blood mononuclear cells of human subjects with and without diabetes mellitus. The study groups comprised 43 volunteers with and 39 without diabetes mellitus, and peripheral blood mononuclear cell DPD activity was assayed on samples obtained between 8 a.m. and 11 a.m. DPD activity was not decreased in diabetic subjects. There was no relationship between DPD activity and gender, body mass index, or race. There was a modest correlation between DPD activity and age ($r = 0.19$, $P = 0.08$). We conclude that increases in 5-FU-related toxicities in diabetics must be related to factors other than peripheral blood mononuclear cell DPD activity.

G.F. Fleming (✉) · E.E. Vokes · R. Mick · D. Levitan · M.E. Dolan
Section of Hematology/Oncology, University of Chicago Medical Center, 5841 S. Maryland Ave, MC 2115, Chicago, IL 60637-1470, USA
(Fax (312)702-3163)

G.F. Fleming · E.E. Vokes · R. Mick · M.E. Dolan
Cancer Research Center, University of Chicago, Chicago, IL, USA

G.F. Fleming · R. Mick
Committee on Clinical Pharmacology, University of Chicago, Chicago, IL 60637-1470, USA

J.B. Buse[1] · J. Dushay
Section of Endocrinology, Department of Medicine, University of Chicago, Chicago, IL, USA

Present address
[1]University of North Carolina School of Medicine, CB #7170 MacNider (Rm 249), Chapel Hill, NC 27599–7170, USA

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Introduction

5-Fluorouracil (5-FU) is a component of standard chemotherapy for colorectal, breast, and head and neck cancers. Dihydropyrimidine dehydrogenase is the initial enzyme in 5-FU catabolism, accounting for over 80% of drug disposition [4, 11]. Patients who are completely deficient in this enzyme have been reported to have severe and often fatal 5-FU toxicities, as have presumed heterozygotes who have approximately 50% of normal DPD activity [6, 10, 12, 14, 15, 21]. Recently Fleming et al. have reported a highly significant inverse correlation between peripheral blood lymphocyte DPD activity and 5-FU plasma levels in a population of head and neck cancer patients receiving 5-FU as a continuous infusion [7].

Several studies, particularly those using continuous infusion regimens, have demonstrated a correlation between plasma 5-FU levels and toxicity [1, 8, 17, 19, 20]. However, it has been difficult to identify clinical patient characteristics which predict 5-FU clearance or toxicity. Female gender and older age have been reported to be risk factors for toxicity with 5-FU-containing regimens [23], and one group has found women to have a 10% lower median 5-FU clearance than men [16]. The same investigators have reported that the mean DPD activity in women is 15% lower than that in men [5]; others have not confirmed this [14]. Older age has not been associated with decreased 5-FU clearance or DPD activity [5, 14, 16].

Vokes et al. recently noted increased toxicity and higher mean plasma 5-FU levels in diabetic compared with nondiabetic patients with head and neck cancer. In that study, all patients received a combination of cisplatin, 5-day continuous-infusion 5-FU at 800 mg/m^2 per day, leucovorin, and interferon [23]. Of

the 41 patients treated, 7 had type II diabetes mellitus. All initially had a good performance status and normal hepatic and renal function. However, these individuals experienced significantly increased myelosuppression compared to the nondiabetics. All four fatal complications in the study occurred in the diabetic patients. The mean 5-FU concentration was 295 ng/ml in diabetics and 219 ng/ml in nondiabetics ($P = 0.04$). Age, sex, and race were all found not to correlate with plasma 5-FU levels or clearance.

We hypothesized that the difference in plasma 5-FU levels observed between diabetic and nondiabetic patients might be explained by differences in DPD activity.

Material and methods

Non-diabetic volunteers

A group of 39 nondiabetic normal volunteers were recruited from the staff of the University of Chicago Medical Center. To be certain that all were nondiabetic, those with a first-degree relative known to have diabetes mellitus were not enrolled. In addition, a fasting blood glucose was measured at the time the blood sample for DPD activity was obtained. Those with a fasting blood glucose over 105 mg/dl were excluded from analysis.

Diabetic volunteers

A group of 43 diabetic volunteers were recruited from the University of Chicago Hospitals' Endocrinology Clinic. Because the original patients of Vokes et al. [23] in whom elevated 5-FU plasma levels were noted all had type II diabetes, only patients with type II diabetes mellitus were enrolled. Information was prospectively collected on whether patients used insulin or sulfonylurea drugs for diabetic control. A blood glucose (nonfasting) and a hemoglobin A_1C were measured at the time the blood sample for DPD activity was obtained. All diabetic and nondiabetic volunteers signed written informed consent in accordance with federal and institutional guidelines.

Collection of peripheral blood mononuclear cells

Because of a reported circadian variation of up to threefold in DPD activity [9], all blood samples from volunteers were obtained between 8 a.m. and 11 a.m. Approximately 20–30 ml of whole blood was drawn from a peripheral vein into heparinized tubes which were inverted several times. The blood was then mixed 1:1 with RPMI-1640 cell culture medium, layered 2:1 onto 15 ml of Histopaque 1077, and centrifuged at 400 *g* for 30 min at 25°C. The mononuclear cell layer was carefully removed, washed twice with phosphate-buffered saline, resuspended in 0.5 ml 35 m*M* sodium phosphate buffer (pH 7.5) and frozen at − 70°C until further analysis.

Chemicals

Dihydrofluorouracil was kindly provided by Dr. Gerard Milano (Centre Antoine-Lacassagne, Nice, France). [2-^{14}C]5-Fluorouracil (56 mCi/mmol) was purchased from Moravek Biochemicals (Brea, Calif.) and partially dried to remove ethanol. [2-^{14}C]5-Fluorouracil was diluted to 2.9 nmoles/10 µl. Histopaque 1077 and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Assay for DPD activity

Minor modifications to a previously published method were made [9, 22]. Frozen cells were thawed in an ice bath and lysed by sonication (five times for 10 s with a 30-s interval). Samples were then centrifuged for 30 min at 20 000 *g* at 4°C, and the supernatant was aspirated and kept on ice. Protein concentration was determined using the method of Bradford [2].

DPD activity for each sample was determined in triplicate. The reaction mixtures consisted of 40, 50 and 60 µg protein in 35 m*M* sodium phosphate buffer, pH 7.4, 250 µ*M* NADPH, 2.5 m*M* $MgCl_2$, and 17 µ*M* ^{14}C-5-FU (55 mCi/mmol) in a total volume of 125 µl. A stock solution of NADPH was prepared immediately before it was added to the reaction mixture. The reaction mixture was incubated at 37°C for 30 min, and terminated by the addition of 125 µl ice-cold ethanol. Samples were stored at − 20°C for up to 18 h, vortexed, and then centrifuged for 5 min at 250 *g* to precipitate the protein. Prior to high performance liquid chromatography (HPLC) analysis, samples were dried under nitrogen and resuspended in mobile phase.

Reaction products were eluted from a Beckman ODS 5-µm C_{18} column (Beckman instruments, San Ramon, Calif.) with an ODS-GU precolumn (Applied Biosystems, Foster City, Calif.) with 0.005 *M* tetrabutylammonium hydrogen sulfate and 0.0015 *M* potassium phosphate, pH 8.0, at 1 ml/min at room temperature. Radioactivity was monitored using a radioactivity flow detector (Radiomatic Instruments, Tampa, Fl.) with a Flo-Scint II/mobile phase ratio of 4.5:1 (Packard, Downers Grove, Ill.). The retention times for [^{14}C]dihydrofluorouracil and [^{14}C]fluorouracil were 4 and 9 min, respectively. The identity of the reaction product, [^{14}C]dihydrofluorouracil, was confirmed by comparison of retention time with that of authentic standard. Interassay coefficients of variation were 1% and 14% on two separate subjects (samples divided and run in separate assays). To ensure that blood glucose concentration did not affect DPD activity, whole blood from two normal volunteers was incubated with 100 µl 20% glucose solution (final concentration 300 mg/dl) or 100 µl water (control) for 30 min prior to isolation of lymphocytes. DPD activity remained unchanged.

Statistical Analysis

In the study by Fleming et al. [7] correlating DPD activity with 5-FU clearance for head and neck cancer patients, a 35% lower plasma 5-FU clearance corresponded approximately with a 50% lower DPD activity in peripheral blood mononuclear cells. A 25% reduction in 5-FU plasma clearance, as reported for diabetic patients by Vokes et al. [23] would therefore be expected to be associated with a 36% lower DPD activity. Given a preliminary estimation of DPD activity of 0.6 ± 0.2 nmol min per mg protein (mean ± SD), a sample size of 38 diabetic and 38 nondiabetic patients was projected to give 90% power to detect a 25% difference in mean DPD activity; a 20% difference could be detected with 73% power. Comparisons of demographic characteristics between normal and diabetic subjects were performed by the Chi-squared test for categorical data or Student's *t*-test for continuous data. Pearson's correlation coefficient was employed to assess linear correlation between natural log-transformed DPD and age or body mass index (BMI) (weight/height^2; BMI is used as a measure of degree of overweight) [3]. Analysis of covariance was employed to assess the effect of diabetes on DPD activity while adjusting for age as a covariate. All statistical tests were two-sided.

Results

Subject characteristics are shown in Table 1. No normal subjects had a fasting blood glucose over 105 mg/dl. The diabetic and nondiabetic groups were well matched for gender, but the diabetic subjects were significantly older ($P < 0.0001$), more obese ($P = 0.0002$), and less likely to be Caucasian ($P < 0.0001$). Overall DPD activity ranged from 0.2 to 1.21 nmol/min per mg. The distribution of DPD activity was not Gaussian. A natural log transformation of DPD produced a normal distribution (Kolmogorov-Smirnov test, $P > 0.10$). All subsequent analyses utilized natural log-transformed DPD activity. We found no significant differences in DPD activity by gender or race. There was no correlation with BMI and only a modest positive correlation with age ($r = 0.19$, $P = 0.08$). There was no difference in DPD activity between diabetic and nondiabetic subjects ($P = 0.12$ unadjusted, $P = 0.92$ adjusted for age, Table 2). Population distributions of peripheral blood mononuclear cell DPD activity in diabetic and nondiabetic volunteers are shown in Fig. 1. The mean $\pm$ SD of 0.56 ± 0.21 nmol/min per mg for nondiabetic volunteers was very similar to the estimate used for our power calculations. This is higher than the mean reported for frozen cells in some other laboratories [5, 14], which is probably accounted for by some minor differences in technique. Ranges relative to the mean are similar. The protein assay methodology was cross-checked with standards prepared by an outside laboratory. Within the diabetic population, there was no correlation between DPD activity and years since diagnosis of diabetes, peripheral blood glucose, hemoglobin A_1C, and no differences by race or method of diabetic control (insulin or sulfonylurea). There was no clustering of females at the low end of the population distribution. Of the five subjects with DPD activities below 0.286 (antilog of the lower 5th percentile of distribution for natural log of DPD), two were female and four were diabetics. No unifying factors, such as use of certain medications or comorbid conditions including renal insufficiency, could be identified among these diabetic patients.

Table 2 Comparison of DPD activities in subgroups

	N	*DPD activity* (*nmol*/min/mg) Mean	Median (range)	2-sided *P*-values[a]
Diabetics	43	0.67	0.63 (0.20–1.21)	
Non-Diabetics	39	0.56	0.54 (0.22–1.14)	0.12
Males	30	0.65	0.62 (0.21–1.16)	
Females	52	0.60	0.56 (0.20–1.21)	0.70
Caucasian	42	0.57	0.56 (0.21–1.15)	
Other	40	0.67	0.62 (0.20–1.21)	0.19
Total	82	0.62	0.57 (0.20–1.21)	

[a]Student's *t*-test on ln(DPD)

Discussion

A number of recent reports have clearly associated partial or complete deficiency of DPD activity, usually as measured in peripheral blood mononuclear cells, with severe toxicities after 5-FU administration. One group [7] has reported a correlation between 5-FU clearance and DPD activity in patients with head and neck cancer. It has been suggested that DPD activity be measured prior to administration of 5-FU both in order to screen for DPD deficiency and to adjust 5-FU dosage in nondeficient patients to increase efficacy and decrease toxicity. The value of such strategies depends on the percentage of 5-FU toxicity that can be accounted for by variability in DPD activity. Clearly not all cases of severe 5-FU toxicities are associated with partial or complete DPD deficiency. Lu et al. [14] analyzed peripheral blood mononuclear cells for DPD activity in 25 patients who had experienced moderate to severe (grade II to V) drug-related toxicity after receiving 5-FU in combination with other agents. Only nine patients, or about one-third, were identified as totally or partially DPD deficient. Increased age has been associated with increased 5-FU toxicity [18] but not with decreased DPD activity [5, 14].

We chose a group of subjects, diabetics, who had been reported to have both increased 5-FU related toxicities and decreased 5-FU clearance, and compared the DPD activity in their peripheral blood mononuclear cells to that of nondiabetics. Although four of five subjects with very low DPD activity were diabetic, this difference was not significant. Our results showed that DPD activity was not lower in the diabetics; indeed, it tended to be higher. This difference disappeared when DPD activity was adjusted for age. Since two previous reports had noted no difference in DPD activity with respect to age, and one had reported no difference with

Table 1 Characteristics of study groups

	All	Non-diabetic	Diabetic	2-sided *P*-values
N	82	39	43	
% Males	37	31	42	0.30
% Caucasian	51	80	26	< 0.0001
Mean age (range) (years)	47 (22–81)	31 (22–51)	63 (27–81)	< 0.0001
Mean BMI (range)	28 (19–69)	26 (19–49)	31 (23–69)	0.0002

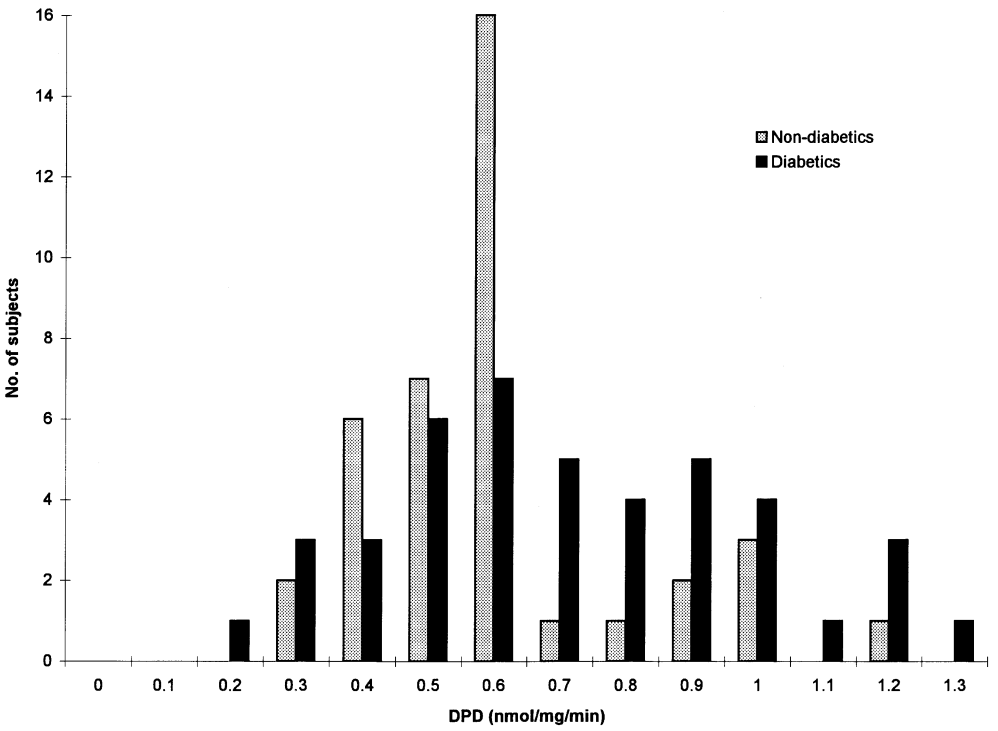

Fig. 1 Population distribution of peripheral blood mononuclear cell activity broken down by diabetic status

respect to race, we did not attempt to match the groups for these variables. Because of the strong correlation of age with diabetes in this study, either variable could account for the small increase in DPD activity in the diabetic group. However, age was found to have greater statistical significance. Although our study did not have adequate power to detect the 15% difference in DPD activity reported by Etienne et al. [5], we found no evidence of decreased activity in women.

There are several possible explanations for the fact that DPD activity, as measured in peripheral blood mononuclear cells, is not decreased in diabetics. The findings of Vokes et al. [23], in only seven diabetic patients with head and neck cancer, may relate to some unidentified covarying factor not present in our population of diabetic volunteers. It may also be that diabetics do, in fact, have more 5-FU-related toxicities, but that the finding of higher plasma levels and decreased clearance was due to chance. Although some investigators have been able to correlate 5-FU plasma levels with toxicity, there is considerable overlap in plasma 5-FU levels between the groups of patients who do and do not experience toxicity [17]. 5-FU must be activated in target tissues, and toxicities may depend on many factors other than plasma drug levels, such as thymidine kinase activity, thymidylate synthase activity, and folate pools in target tissue [24]. Target tissues in diabetics might be more sensitive because of underlying decreased reserve which is not identified in routine screening tests. Finally, increased plasma 5-FU levels may, in fact, occur in diabetic patients, but may be accounted for by factors other than peripheral blood mononuclear cell DPD activity. It is possible that DPD activity in the liver, where the bulk of 5-FU clearance is believed to occur, does not always correlate with DPD activity in peripheral blood mononuclear cells. Diabetes mellitus is well known to be associated with alterations in hepatic drug metabolism in experimental animals although results of studies in humans have been variable [13]. Changes in drug detoxification in the liver related to alterations in insulin-regulated metabolism may not be reflected in peripheral blood cells.

In summary, increased toxicity of 5-FU in diabetics is not related to decreased DPD activity in peripheral blood mononuclear cells. Other explanations should be investigated.

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