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SHORT COMMUNICATION

Dietary Folate and Folylpolyglutamate Synthetase Activity in Normal and Neoplastic Murine Tissues and Human Tumor Xenografts

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ABSTRACT. The importance of polyglutamation for the activation of natural folates and classical antifolates and recent evidence for the role of dietary folate as a biochemical modulator of antifolate efficacy led us to investigate the influence of changes in dietary folate on folylpolyglutamate synthetase (FPGS) activity. Activities were measured using lometrexol (6*R*-5,10-dideazatetrahydrofolic acid) as a substrate for FPGS with extracts of murine tissues, murine tumors, and human tumor xenografts from mice on standard diet or low folate diet. Tissues and tumors from mice on standard diet exhibited a 6-fold range of FPGS activity. Kidney had the lowest activity (36 pmol/hr · mg protein), followed by the human xenograft PANC-1 pancreatic carcinoma (46 pmol/hr · mg protein), liver (109 pmol/hr · mg protein), murine C3H mammary tumor (112 pmol/hr · mg protein), and the human xenograft MX-1 mammary carcinoma (224 pmol/hr · mg protein). In response to restricted dietary folate, four out of five tissues had significantly increased (25–50%) FPGS activity. Only the tumor with highest FPGS activity under standard diet conditions (MX-1 mammary) did not respond to low folate diet. The results indicate that changes in dietary folate intake can modulate FPGS activity significantly *in vivo* and suggest that the tissue distribution and toxicities of classical antifolates requiring polyglutamation for activation and cellular retention will be influenced significantly by folate status of the host. BIOCHEM PHARMACOL 52;9:1477–1479, 1996.

KEY WORDS. folylpolyglutamate synthetase; dietary folate

Increased interest in understanding the role of dietary folate in modulating folate metabolism is reflected in recent *in vivo* studies on the effect of restricted dietary folate on cellular biochemistry involved in the maintenance of folate homeostasis. For example, Schmitz *et al.* [1] demonstrated significant changes in plasma and tissue folate pools in response to dietary folate restriction. Specifically, reduced folate pools in tumors and livers decreased 5-fold, from 175 to 34 pmol folate/mg protein, and 2-fold, from 522 to 268 pmol folate/mg protein, respectively. Similarly, Ward and Nixon [2] determined total tissue folate concentrations in rats fed folate-sufficient or deficient diets. They reported a 3-fold decrease for liver and a 2-fold decrease in kidney folate pools in response to low dietary folate. Gates *et al.* [3] reported that changes in dietary folate affect both the isotype and concentration of folate receptors expressed in normal and neoplastic murine tissues. Additionally, clinical experiences with inhibitors of folate-dependent enzymes such as 5-fluorouracil [4] or lometrexol [5] demonstrated that supplemental folate significantly modulated their efficacy or toxicity.

Polyglutamation of folates and "classical" antifolates by FPGS† is essential for their intracellular retention [6]. In addition, polyglutamation increases the affinity of several antifolates to their target enzyme, creating more potent, tight-binding inhibitors [7, 8]. Although the effect of folate concentration on FPGS activity and mRNA has been studied *in vitro* with murine and human cell lines [9–11], the effect of changes in dietary folate on FPGS activity *in vivo* has not been explored. Lometrexol, an excellent substrate for FPGS [12], was selected for study because dietary folate supplementation has been shown to modulate the clinical toxicity of this agent in cancer patients [5]. Therefore, we determined FPGS activity using lometrexol in normal tissues involved in folate homeostasis and in neoplastic murine tissues and human tumor xenografts in mice maintained on standard and low folate diets.

MATERIALS AND METHODS

Materials

[^{14}C]-L-Glutamate (261.6 mCi/mmol) was purchased from Dupont-NEN (Boston, MA). Lometrexol (6R-5,10-dideazatetrahydrofolate) was synthesized in the laboratory of

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† *Abbreviations*: FPGS, folylpolyglutamate synthetase; and DTT, dithiothreitol.

C. Shih at Lilly Research Laboratories (Indianapolis, IN). Sep-Pak Plus C18 cartridges were purchased from Waters (Milford, MA).

Murine Diets

Mice were placed on one of the following diets prior to tissue sampling: (a) standard diet, Purina Chow No. 5001 containing ≥5.9 ppm folate; or (b) low folate diet, Purina Chow No. 5831C-2 containing 0.032 ppm folate.

Normal and Neoplastic Murine Tissues and Human Tumor Xenografts

C3H mammary carcinoma was maintained in C3H mice from the Taconic Corp. (Germantown, NY). Charles River Laboratories (Wilmington, MA) supplied CD1 Nu/Nu mice, in which the human tumor xenografts PANC-1 pancreatic and MX-1 mammary were maintained. Tumors were originally obtained from Jackson Laboratories, Bar Harbor, ME (C3H mammary) and the National Cancer Institute, Bethesda, MD (MX-1 mammary). PANC-1 pancreatic carcinoma was developed at Lilly Research Laboratories from a cell line purchased from the American Type Culture Collection (Rockville, MD) [13]. Mice were placed on respective diets 2 weeks prior to tumor implantation and remained on that diet throughout the study. Tumors were implanted by trocar in the left axillary region 21 days prior to excision for PANC-1 pancreatic and MX-1 mammary carcinomas and 10 days for C3H mammary carcinoma. Kidney and liver were excised from C3H mice maintained on standard diet or low folate diet for a minimum of 3 weeks.

Preparation of Crude Extract

Mice were euthanized with CO_2. Tissues were excised, weighed, and placed in 2× volume of ice-cold 20 mM HEPES, pH 7.4, containing 10 mM DTT. Livers were perfused *in situ* through the portal vein with 10 mL of ice-cold 20 mM HEPES, pH 7.4, with 0.25 M sucrose prior to excision. Tissues were homogenized using a Tekmar Tissuemizer (Cincinnati, OH), and suspensions were centrifuged at 160,000 *g* for 1 hr at 4°. Protein in the resulting supernatants was determined using the Bio-Rad microassay (Bio-Rad Laboratories, Inc., Melville, NY) utilizing bovine γ-globulin as a standard.

Enzyme Assay

FPGS activity was measured as described by Moran and Colman [14]. Briefly, extract protein was added to 100 mM Tris–HCl containing 20 mM $MgCl_2$, 20 mM KCl, 10 mM ATP, 10 mM DTT, 1 mM [^{14}C]-L-glutamate (4 mCi/mmol) and 50 μM lometrexol, pH 8.4, in a final volume of 0.5 mL at 37°. Lometrexol is an excellent substrate for FPGS with a K_m of 9 μM and a high first order rate constant [12]. Reactions were initiated by the addition of tissue extract containing the following amounts of protein: liver, 1.0 mg; kidney, 1.75 mg; C3H mammary, 1.0 mg; PANC-1 pancreatic, 1.5 mg; MX-1 mammary, 0.5 mg. Reactions were terminated after 2 hr by the addition of ice-cold 10 mM L-glutamate (1.5 mL), pH 7.5. Product was separated from unincorporated [^{14}C]-L-glutamate as previously described by Jansen *et al.* [15]. For each tissue, product formation increased linearly with protein over the range of 0.5 to 2.5 mg. The minimum sensitivity of the assay was 20 pmol product/hr. Initial velocities were determined by measuring product formation during a 5-hr incubation. Product formation, measured at hourly intervals using a fixed protein concentration for each tissue, increased linearly during this period. Specific activity, defined as picomoles of lometrexol-[^{14}C]diglutamate product formed per milligram of protein per hour, remained constant.

Statistical Analysis

Student's *t*-test was utilized to obtain estimates of statistical significance of the data. Any P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

FPGS activity was measured in murine tissue, murine tumor, and human tumor xenografts (Table 1). In standard diet mice, liver had 3-fold higher activity than kidney (P < 0.001). A wide range of FPGS activity was detected in tumors from mice on standard diet. Comparison of activity in normal and neoplastic tissue indicated that only MX-1 mammary had significantly higher activity than liver (P < 0.0002).

The effect of restriction of dietary folate on FPGS activity in liver, kidney, and tumors was assessed utilizing mice on low folate diet. A 50% increase in FPGS activity was detected when liver from low folate diet mice was compared

TABLE 1. Folylpolyglutamate synthetase activity in murine tissues, murine tumor and human tumor xenografts

Tissue	Specific activity (pmol/hr · mg protein)	
	Standard diet	Low folate diet
C3H liver	109 ± 8	164 ± 4*
C3H kidney	36 ± 1	45 ± 1*
C3H mammary	112 ± 9	163 ± 9*
PANC-1 pancreatic	46 ± 1	63 ± 1*
MX-1 mammary	224 ± 13	202 ± 4

Product was measured by incubating protein from liver, kidney or tumors from mice on standard or low folate diet with 1 mM [^{14}C]-L-glutamate and 50 μM lometrexol. Specific activity was determined based on a time course with five hourly poinst; each data point was run in triplicate. Values are the means ± SEM of 3–5 experimental determinations.

* Statistically significant difference from corresponding standard diet value. P values for C3H liver, C3H kidney, C3H mammary, and PANC-1 pancreatic are: P < 0.0002, P < 0.0083, P < 0.0008, and P < 0.0001, respectively.

with liver from standard diet mice (Table 1). In kidney, a significant but smaller increase (25%) in activity resulted in response to low folate diet. The increase in liver FPGS activity is interesting in light of an *in vivo* study which demonstrated that a deficiency in dietary folate caused a 2- to 4-fold increase in hepatic retention of lometrexol [16]. In a similar study using standard diet and low folate diet mice [17], polyglutamates of lometrexol in liver were determined 7 days after an i.v. dose of drug; approximately 15% of lometrexol polyglutamates were in the hepta- and octaglutamate forms in liver of low folate diet mice, whereas liver from standard diet mice contained primarily lometrexol pentaglutamate (95%) and hexaglutamate (5%). The increase in FPGS activity that we observed in livers of low folate diet mice is consistent with accumulation of lometrexol in the liver reported by Habeck *et al.* [17] and observations of delayed and sustained toxicity observed in these animals [18].

In neoplastic tissues, restriction of dietary folate resulted in significant increases in enzyme activity in C3H mammary (46%, $P < 0.0008$) and PANC-1 pancreatic (38%, $P < 0.0001$). In contrast, MX-1 mammary, which had the highest enzyme activity in mice on standard diet, did not exhibit a significant change in FPGS activity when dietary folate was restricted ($P < 0.12$).

Collective *in vivo* observations have shown that dietary folate levels modulate cellular determinants of antifolate efficacy, i.e. folate pools and cellular uptake. Therefore, demonstration of elevated FPGS activity induced with low folate diet *in vivo* is a significant finding. It is apparent that although the extent of cellular influence of dietary folate remains uncertain, a clearer understanding is critical to further development of novel, efficacious antifolate therapeutic agents. Furthermore, the assessment of patient folate status and supplementation of dietary folate in chemotherapy patients may be important for the clinical development of current antifolate therapies.

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