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SHORT COMMUNICATIONS

Proliferation-dependency of folylpolyglutamyl synthetase activity in maturating luminal epithelial cells of mouse small intestine

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Isolation and fractionation of luminal epithelial cells from mouse small intestine have allowed us to examine specific transport and enzymic processes associated with folate homeostasis during maturation of this tissue compartment [1]. Information has also been obtained [1,2] on the cellular pharmacokinetics of folate and nucleoside analogs in the crypt-cell fraction, the site of their limiting toxicity in this rodent. We now report on studies of folylpolyglutamyl synthetase (FPGS) in this same maturating cellular system. This enzymic activity has importance, since it provides folate polyglutamates as co-factors for thymidylate and purine biosynthesis and is a determinant of 4-amino-folate cytotoxicity[3].

Materials and methods

The isolation of epithelial cells from mouse intestine as four individual fractions has been described in detail in our earlier report [1]. Measurement of the activity and cellular content of dihydrofolate reductase [4], and of the activities of folylpolyglutamyl hydrolase [5], thymidine kinase [1], and alkaline phosphatase [1] have been described. The method of HPLC analysis of [^{3}H]aminopterin ([3′,5′-9-^{3}H] aminopterin, sp. act. 10–15 Ci-mol, purchased from Moravek Biochemicals, City of Industry, CA) and aminopterin polyglutamates has also been described [6]. Aminopterin polyglutamates (AM + Gl, +G2, +G3, and +G4) were provided by Dr. J. R. Piper, Southern Research Institute, Birmingham, AL.

A saturating concentration (100 μM) of aminopterin (provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) was used as substrate for determination of FPGS activity according to the method of McGuire *et al.* [3]. Mouse epithelial cell fractions were suspended in ice-cold (0°) 0.5 M Tris–HCl (pH 8.85) with 0.2 M 2-mercaptoethanol and homogenized. Aliquots of supernatant fluid after centrifugation were assayed 1 day after storage at −70°. The specific activity of the L-[2,3-^{3}H]glutamate (New England Nuclear, Waltham, MA) used in the assay was 12–14 × 10^6 dpm/μmol. The FPGS activity for a particular extract was determined by assaying aliquots in duplicate for 1 hr (400 μg protein) and 2 hr (200 μg protein) which provided linearity with respect to time and enzyme amount [3]. The activity of each sample was corrected for activity obtained in the absence of aminopterin. For determining the effects of endogenous folates on FPGS activity, cell-free extract was treated with and without added 2.5% dextran-treated charcoal [7], centrifuged, and assayed.

Results and discussion

The properties of the epithelial cell fractions used during the current studies are summarized in Table 1. The total yield of intact cells in the form of four distinct fractions was approximately 16 × 10^7 cells per individual small intestine with the majority derived within fractions II and III. Fraction I contained primarily mature columnar cells, whereas fraction IV contained primarily immature cells that were less dense and spherical in appearance. The ratios of each cell type in fractions II and III were intermediate between these two extremes. The level of thymidine kinase activity, a marker for proliferative potential [1], was lowest in fraction I, increased in fractions II and III, and was highest in fraction IV cells. The converse was seen for alkaline phosphatase, a marker for intestinal epithelial cell maturation[1].

Results on the relative levels of FPGS activity in cell-free extracts from various fractions of intestinal epithelium are given in Table 2. We employed aminopterin as a substrate in these assays because it is more rapidly converted to polyglutamates in this tissue than most other folate compounds [8]. It can be seen that specific enzyme activity was lowest in fraction I and fraction II cells, was increased 3-fold in fraction III and 4-fold in fraction IV cells. Charcoal treatment of these extracts to remove endogenous folates did not alter the level of enzyme activity obtained (data not shown). Also, no measurable hydrolysis of methotrexate + G1 by folylpolyglutamyl hydrolase was detected under these same conditions which is probably due to the fact that FPGS activity was measured at a pH (pH 8.4) that prevents measurable [5] hydrolysis of this polyglutamate by this endopeptidase under these reaction conditions. To eliminate the possibility that lower levels of

Table 1. Characteristics of isolated intestinal epithelial cells from mouse small intestine

Fraction	Total no. of cells/intestine (+10^7)	% as Columnar cells	% of Maximum Thymidine kinase	% of Maximum Alkaline phosphatase
I	2.18 ± 0.5*	90–94	5	100
II	4.51 ± 0.8	76–82	18	97
III	5.76 ± 0.8	34–42	39	42
IV	2.98 ± 0.7	4–7	100	22

Epithelial cells were stripped from everted small intestine in a stepwise manner as described previously [1]. Cell counting and morphologic classification were done microscopically. Other experimental details are given in the text. Values represent an average of three determinations each of which was done on separate days.

* Average ± SE.

Table 2. Folylpolyglutamyl synthetase activity in cell-free extracts prepared from various fractions of mouse epithelial cells

Expt.	Incubation time[1]* (hr)	Folylpolyglutamyl synthetase activity (pmol/hr/mg) I	II	III	IV
A	1	134	103	317	502
	2	135	86	335	361
B	1	94	76	225	429
	2	105	132	284	440
Average		119 ± 18	99 ± 18	290 ± 28	408 ± 27

A total of four separate determinations were made on extracts prepared following two individual preparations on separate days. Experimental details are provided in the text. Averages of four determinations done in two separate experiments. ± standard error are given.

* For 1-hr and 2-hr determinations, respectively, 400 and 200 μg of intracellular protein were incubated with substrate.

FPGS activity in fractions I, II, and III were related to the presence of an endogenous inhibitor, we determined that enzyme activity was additive after mixing cell-free extract from each of the epithelial cell fractions with a similarly prepared extract from H35 hepatoma cells (data not shown).

From these results, we concluded that FPGS activity is a proliferation-dependent enzyme activity that decreases during maturation of this epithelial cell compartment. Additional evidence was provided in the following manner. Mice were administered 12 mg/kg [^{3}H]aminopterin s.c. 4 hr later the animals were killed and the intestinal epithelial cells were stripped [1] from small intestine as four fractions. Due to the pharmacokinetic clearance that occurs in this organ following administration of drug [4] and the wash out that resulted during the isolation and fractionation of these cells [1], we anticipated that all of the [^{3}H]aminopterin and its polyglutamates in these cells would be bound to dihydrofolate reductase. Data in Table 3 show that this was actually the case. The content of this enzyme varied approximately 4-fold among these cellular fractions. Levels were lowest in fraction I cells and increased progressively to a maximum in fraction IV cells. Total drug ([^{3}H]aminopterin and its polyglutamates) found in these cellular fractions was equivalent to the levels of dihydrofolate reductase measured in each case. Total [^{3}H]aminopterin as polyglutamate was found to vary 10-fold between fraction I and fraction IV. This was due to the variation in dihydrofolate reductase content observed among these fractions and to the difference in the relative amount of polyglutamylation of this folate analog seen among these fractions. The relative amount of [^{3}H]aminopterin polyglutamates in fraction IV cells was 63% of total drug, which progressively decreased in fractions II and III and represented only 19% of total drug in fraction I cells.

After correction for that contributed by contaminating columnar epithelial cells in fraction I, we found that the differential in activity of dihydrofolate reductase and FPGS between this fraction and fraction IV was approximately 5-fold. In contrast, levels of folylpolyglutamyl hydrolase activity [5] and folate compound transport inward [1] derived from our earlier studies were unchanged between these two fractions. We also point out that levels of dihydrofolate reductase and FPGS activities found in fraction I cells are probably even lower since they could reflect some contamination [1], as well, by stromal and blood cell elements.

The results relating to FPGS activity in this maturing epithelial cell compartment are pertinent to both folate homeostasis and absorption in this organ as well as folate analog pharmacology. Following absorption of folate from the luminal surface of mature epithelial cells, their transport to the basement membrane surface as unconjugated forms is possible because of the low levels of FPGS (Table 2) as well as the high levels of folylpolyglutamyl hydrolase characteristic [5] of these cells. On the other hand, the level of FPGS activity in the proliferative fraction of this cellular

Table 3. Intracellular content of dihydrofolate reductase and of [^{3}H]aminopterin and its polyglutamates from murine intestinal epithelial cells

Fraction	Dihydrofolate reductase (pmol/10^7 cells)	Total drug* (pmol/10^7 cells)	Total as polyglutamates (pmol/10^7 cells)	Parent (%)	+Gl (%)	+G2 (%)	+G3 (%)
I	3.52 ± 0.8†	3.78 ± 0.5	0.76 × 1.3	80.9	15.3	2.7	1.1
II	5.56 ± 1.3	5.08 ± 0.9	1.32 ± 0.2	78.2	17.4	5.4	<1
III	7.53 ± 1.2	7.92 ± 0.9	3.54 ± 0.6	55.2	22.7	20.2	2.0
IV	12.8 ± 2.3	12.4 ± 1.8	7.65 ± 1.3	36.8	35.5	24.6	3.1

Animals were given 12 mg/kg [^{3}H]aminopterin s.c.; 4 hr later, animals were killed, the small intestine was removed and luminal epithelial cells were removed sequentially [1] in fractionation buffer (pH 7.4). Drug was extracted by heating for 15 min at 100° followed by centrifugation. To control for endogenous hydrolytic activity, aminopterin + G1 was added to control cell suspension prior to heat extraction. HPLC analysis of these samples showed than no aminopterin was present in the samples. Shown are averages of two separate experiments done on separate days. Each was replicated two to four times; the average of the two experiments was calculated.

* Recovered following heat extraction as described earlier.

† Average ±SE.

compartment ensures adequate amounts of folates as polyglutamates necessary for optimizing folate-dependent biosynthetic activities during rapid cycling of these cells. Moreover, it is well known [4] that the proliferative fraction of this epithelial cell compartment is the site of limiting toxicity to antifolates in this rodent. Data obtained during the current studies should now allow a more valid estimate of the extent that membrane transport and folylpolyglutamyl synthetase play a role in the accumulation of specific 4-aminopterin polyglutamates in this proliferative tissue compartment. Finally, although there are a number of possible explanations for this variability in FPGS activity among the cellular fractions, it most likely reflects "down-regulation" of FPGS gene expression during maturation in this issue compartment. Further work will be required to confirm this notion.

In summary, folylpolyglutamyl synthetase activity in maturating luminal epithelial cells from mouse small intestine was measured by direct enzymologic assay in cell-free extracts and by monitoring *in vivo* [^{3}H]aminopterin polyglutamate synthesis following administration to mice. Our results revealed that FPGS activity was proliferation-dependent and decreased during maturation of this epithelial cell compartment.

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Interindividual variation in phase II detoxification enzymes in normal human colon mucosa*

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Human colon tissue is in continuous contact with the chemical environment defined by the diet as modified by endogenous gut mucosal metabolism. Some experimental data have given rise to speculation that various compounds or their metabolites play a role in carcinogenesis [1]. If this is true, colon tissue pathways that detoxify xenobiotics are of potential importance in tumor susceptibility. Indeed, an association has been suggested previously between cytochrome P-450-dependent phase I enzyme activity and inducibility and cancer incidence [2]. It has also been suggested that in the process of chemical carcinogenesis some detoxification pathways may be induced, resulting in anti-cancer drug-resistant tumors [3, 4]. A number of studies have reported colon cancer tissue levels of some enzymes to suggest a role in drug resistance [5, 6]. However, there is little data available on normal mucosal phase II detoxification components and, in particular, on the degree of interindividual variability. Such variability would be predicted based on the data on the phase I enzymes and the spectrum of inducers that can influence these pathways [7]. This information will be useful in evaluating levels described in tumors. This paper describes the activities and interindividual variabilities of six detoxification components measured in the normal appearing colon mucosa from fifteen human subjects with colon cancer.

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Materials and methods

Eight women and seven men, mean age 70.1 ± 7.0 years, were studied. All had locally advanced tumors, without any evidence of abdominal or systemic metastasis. Specimens