Tissue folate levels in transgenic mice with tumors and in nontransgenic controls

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Tissues, whole blood, serum, and tumor folate of a tumor-bearing transgenic (TBT) mouse model for human neurofibromatosis were measured to characterize effects of neoplasia on concentration of folate among tissues and tumors. Tissues of 11 TBT mice (killed when tumor burden was estimated to be 1% of body weight to minimize possible effects of cachexia) and eight nontransgenic controls (NTC) of similar age were analyzed for folate. TBT mice had 1.5 ± 0.2 tumors. Tumor mass was 0.35 ± 0.10 g/mouse, and overall tumor folate concentrations were 0.38 ± 0.10 nmol/g. Overall total tumor folate was 0.13 ± 0.04 nmol/mouse. Body weights and erythrocyte and leukocyte counts of TBT and NTC mice were similar. Hematocrits and hemoglobin concentrations were lower in TBT than in NTC mice. Livers and kidneys from TBT mice weighed less than those of NTC mice while spleens weighed more. Both groups had similar folate concentrations in liver and spleen while concentrations in kidney, brain, and serum were lower in TBT than in NTC mice. Folate per liver, kidney, and brain was lower while that per spleen was higher in TBT than in NTC mice. Tumor folate concentrations were lower than those of most mouse nontumor tissues. Folate concentrations of tumors were variable and were ranked as follows: nose ≥ tail ≥ foot ≥ ear. The presence of tumors was associated with reduced folate concentration of some nontumor tissues. Folate concentrations of kidney, brain, and serum decreased as the number and mass of tumors increased.

Keywords: transgenic mice; cancer; folate

Introduction

Folic acid plays a key role in nucleic acid biosynthesis, which is essential for the normal proliferation and function of cells. Anemia is a clinical manifestation of folate deficiency due to abnormal proliferation and function of erythroid cells. Cytologic manifestations of deficiency include megaloblastosis and megalocytosis due to impaired DNA synthesis. Recent reports[1-6] call attention to the possibility that localized folate deficiency may be related to changes in cytology (dysplasia) and may be precursors of cancer. It still remains to be conclusively shown that localized folate deficiency results in cytologic changes that are premalignant and reversible with folate treatment.

The effect of cancer on folate concentrations of various tissues had not been systematically studied. A lack of appropriate animal models is partly responsible for the dearth of experimental evidence that actual depletion of folate occurs in key tissues in association with neoplasia. Suitable animal models in which to test this hypothesis have been difficult to establish due to variability in tumor cell type and growth characteristics. The availability of tumor-bearing transgenic (TBT) mice presented a unique opportunity to compare folate concentration of several tissues (and tumors) with those of corresponding tissues from nontransgenic control (NTC) mice.

The mice selected for this study carry the human T-lymphotropic virus type 1-*tax* (HTLV1-*tax*) gene in their germline under control of its own long terminal repeat (LTR). These transgenic mice are predisposed

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to develop cancer with a well-characterized time of onset, tumor incidence, and tissue involvement.[7] LTR-tax mice develop multiple peripheral nerve sheath tumors with striking similarities to human neurofibromatosis. Tissue folate levels of the TBT mice were compared with those of corresponding tissues from NTC mice. Folate concentrations of tumors from TBT mice were also studied.

Materials and methods

Folate concentrations were measured in liver, spleen, kidney, brain, tumor, whole blood, and serum from 11 TBT and eight NTC mice. Tumors of two TBT mice were barely detectable (because they were young, 67 d old) and so were much too small to be collected. These two mice allowed us to compare tissue folate concentrations of transgenic mice that were essentially tumor free with those of transgenic mice with tumors, thus isolating a tumor effect from a gene effect. When killed, TBT mice were 121 ± 17 d old and weighed 29 ± 2 g, and NTC mice were 126 ± 26 d old and weighed 34 ± 3 g. Mice were housed in a room with a 12 h light-dark cycle, 20° to 23° C ambient temperature, and 50% relative humidity. They had free access to water and to a closed-formula cereal-based diet (Autoclavable Rodent Laboratory Chow #5010, Purina Mills, Inc., St. Louis, MO, USA). By analysis,[8] the diet contained 10.42 nmol folate/kg.

Transgenic mice

Mice were originally derived via microinjection of the LTR-*tax* gene construct into fertilized eggs from superovulated CD1 females crossed with C57BL/6 × DBA2 F1 males.[9] Mice used in the present experiment were derived from two of these original founder lines (6-2 and 8-4), which are maintained as breeding colonies at the University of California, Davis.

Tail biopsies (~1 cm) were taken at 10 days of age and digested with proteinase K. DNA was extracted, digested with *Bg*III restriction enzymes, electrophoresed in 0.8% agarose, transferred to nylon membranes,[10] and hybridized with ^{32}P-labeled HTLV-I *tax* DNA[9] labeled via the random primer procedure.[11] Membranes were washed and exposed to X-ray film for 24 h. The presence or absence of a band on the X-ray film corresponding to HTLV-I *tax* DNA was used to classify mice as TBT or NTC, respectively.

Collection of tissues

Mice were anesthetized with diethyl ether and bled by cardiac puncture. Blood was transferred into tubes without anticoagulant. An aliquot was drawn into microhematocrit tubes for hematocrit determination. Another aliquot (0.1 ml) was transferred into heparinized glass tubes containing 0.9 ml 50 mmol/L sodium phosphate buffer (pH 6.1) with 11.35 mmol/L ascorbate (hereafter referred to as buffer), mixed, and stored at −10° C. Another aliquot (40 μl) was transferred into 20 ml of Isoton II for leukocyte counts; 200 μl of this mix were transferred into another 20 ml Isoton II for erythrocyte counts. Leukocytes (after treatment with 6 drops of Zapoglobin II) and erythrocytes were counted electronically. (The electronic counter [Model ZBI], Isoton II, and Zapoglobin were from Coulter Electronics, Hialeah, FL, USA). Remaining whole blood was allowed to clot for 30 min at room temperature and cooled on ice; serum was separated by centrifugation. Serum was transferred into glass vials and stored at −10° C. Liver, spleen, kidneys, brain, and tumors were promptly excised, freeze clamped in liquid nitrogen, weighed, and stored at −10° C.

Folate analysis

Aliquots (~0.5 g or the entire organ if less than 1 g) of frozen livers, spleens, kidneys, brains, and tumors were homogenized (Polytron, Brinkman Instruments, Westbury, NY, USA) in 9 volumes of buffer, autoclaved for 10 min at 121° C, and cooled in an ice water bath. Cooled homogenates were centrifuged for 15 min at 2000*g*, and clear supernatant extracts were transferred to plastic vials and stored at −10° C.

Frozen serum was thawed, diluted with 6 volumes of buffer, autoclaved at 121° C for 10 min, cooled, and centrifuged at 10,000*g* for 15 min to deproteinize the serum. Deproteinized serum was transferred to plastic vials and stored at −10° C. Frozen diluted whole blood was thawed, autoclaved for 10 min at 121° C, cooled, and centrifuged at 10,000*g* for 15 min. The supernatant extract of whole blood was transferred to plastic vials and stored at −10° C.

Frozen supernatant extracts (except serum) were thawed, and 150 μl aliquots were transferred to tubes containing 2.79 ml buffer plus 60 μl of chicken pancreas conjugase and mixed. The mixture was incubated at 37° C for 6 h to allow folacin polyglutamates to be converted to diglutamates[12] and stored at −10° C until analyzed for folate. Four different aliquots of the conjugase-treated extract of each tissue and three different aliquots of each deproteinized serum or whole blood extract containing 0.23 to 4.5 pmol folate were analyzed for folate with *L. casei* (ATCC No. 7469) using the medium of Waters and Mollin.[13] Folate concentrations were calculated per gram of tissue or ml of whole blood or liter of serum. Erythrocyte folate concentrations were calculated as: (whole blood folate concentration − serum concentration (1 − hematocrit))/hematocrit. Concentrations were multiplied by tissue mass to obtain folate per total tissue. Results are expressed as means ± SEM.

Tumor number and mass/mouse were correlated with nontumor tissue folate concentrations in TBT mice by linear regression and differences between means of TBT and NTC mice were evaluated by Student *t* test.[14]

Results

Hematologic parameters, tissue weights, folate concentrations, and folate per tissue are summarized in *Table 1*. Hematocrits and hemoglobin concentrations were lower in TBT than in NTC mice. Erythrocyte and leukocyte counts were similar in both groups. All hematologic values were within normal physiological ranges.[15]

Mean concentrations of liver folate of both groups of mice were similar. However, mean liver mass of TBT mice was 82% of NTC mice. Therefore, TBT mice had 79% as much folate in liver as did NTC mice. Mean spleen folate concentrations of both groups were also similar, but spleens of TBT mice weighed 2.2 times more and, therefore, had almost 2.3 times more total folate than did spleens of NTC mice. Kidneys of TBT mice weighed 75% as much and had 66% as much folate/g mass as those of NTC mice. Therefore, kidneys of TBT mice had only 50% as much total folate as NTC mice. Brain weights were similar for both groups of mice. Mean folate concentration of brain of TBT mouse brain was only 52% that of NTC mouse brain. Therefore, brain total folate of TBT mice was only 51% that of NTC mice. Mean serum folate concentration of TBT mice was 77% that of NTC mice. Whole blood and erythrocyte folate concentra-

Table 1 Hematologic values and tissue folate of nontransgenic (control) and transgenic mice

	Control	Transgenic	*P* value
Hematology			
Hematocrit (%)	47.1 ± 1.3	40.8 ± 2.3	0.05
Hgb mg/dL	14.9 ± 0.3	13.2 ± 0.4	0.01
RBC × 10^9 mL	5.78 ± 0.27	5.51 ± 0.33	0.57
WBC × 10^6 mL	4.18 ± 0.43	5.12 ± 0.65	0.31
Liver folate			
Weight (g)	2.10 ± 0.13	1.72 ± 0.12	0.05
nmol/g	21.52 ± 0.52	20.96 ± 0.91	0.63
nmol/liver	45.63 ± 3.62	36.20 ± 3.01	0.06
Spleen folate			
Weight (g)	0.131 ± 0.013	0.293 ± 0.057	0.03
nmol/g	2.879 ± 0.152	2.956 ± 0.170	0.78
nmol/spleen	0.376 ± 0.020	0.865 ± 0.050	0.0001
Kidney folate			
Weight (g)	0.652 ± 0.059	0.487 ± 0.049	0.04
nmol/g	8.176 ± 0.535	5.390 ± 0.838	0.02
nmol/kidney	5.186 ± 0.365	2.621 ± 0.492	0.001
Brain folate			
Weight (g)	0.500 ± 0.008	0.481 ± 0.012	0.26
nmol/g	0.949 ± 0.023	0.498 ± 0.113	0.004
nmol/brain	0.476 ± 0.018	0.242 ± 0.057	0.004
Blood folate			
Serum: nmol/L	98.96 ± 5.89	75.80 ± 8.81	0.06
RBC: nmol/ml	1.044 ± 0.054	1.278 ± 0.143	0.21

Note: Values are means ± SEM for $n = 8$ (control) and $n = 11$ (transgenic) mice, respectively. *P* values are for Students *t* test.

tions were similar for both groups of mice. Mean folate concentration of tissues from the two TBT mice that were essentially tumor free were 20.14 ± 2.87, 3.136 ± 0.106, 8.965 ± 0.478, 1.005 ± 0.081, 87.89 ± 14.67 and 1.121 ± 0.905 nmol/g liver, spleen, kidney, brain, /L serum, and /ml red blood cells (RBC), respectively. These values were not different from the corresponding values of NTC mice; thus, alterations in tissue folate levels seen in TBT mice were associated with the presence of tumors. Prominent features of TBT mice included splenomegaly, decreased liver and kidney weights, and kidney folate concentrations only two-thirds and brain folate concentrations only half those of NTC mice.

Tumors were found at four sites in TBT mice: ear, nose, foot, and tail. TBT mice had 1.5 ± 0.2 tumors. Mass and folate content of tumors are presented in *Table 2*. Overall mean tumor mass was 0.35 ± 0.10 g/mouse, and overall tumor folate concentration was 0.38 ± 0.10 nmol/g. Based on average mass, tumors at various sites were ranked as follows: foot ≥ ear ≥ nose ≥ tail. Average mass of foot tumors was 4.7 times greater than those of tail tumors. On the basis of mean folate concentration, tumors from various sites were ranked as follows: nose ≥ tail ≥ foot ≥ ear. Nose tumors had 5.5 times more folate per unit mass as ear tumors. On the basis of folate per total tumor, tumors from various sites were ranked as follows: foot ≥ nose ≥ ear ≥ tail. Overall total tumor folate was 0.13 ± 0.04 nmol/mouse. Differences in folate concentrations among tumors from various sites were not significant.

Among TBC mice, tumor mass was negatively correlated with folate concentrations of brain ($r = -0.54$), kidney ($r = -0.61$), and serum ($r = -0.44$). Tumor mass was also negatively correlated with RBC counts ($r = -0.72$), hematocrit ($r = -0.76$), and hemoglobin concentration ($r = -0.72$). Tumor mass was positively correlated with white blood cell (WBC) counts ($r = 0.71$). Tumor mass was not correlated with body weight or with the concentrations of folate in liver and spleen. All of the correlations between tumor mass and tissue folate concentrations and hematological parameters of TBT mice are consistent with the differences between control and TBT mice shown in *Table 1*.

Discussion

Transgenic technology is a powerful tool in which genetic elements can be manipulated to generate experi-

Table 2 Weight and folate concentrations of tumors from various sites of transgenic mice

Site	Mouse no.	Tumor weight (g)	nmol Folate/g Tumor	nmol Folate/Tumor
Ear	1	0.277	0.1382	0.0383
	2	0.238	0.1402	0.0394
	3	0.235	0.0670	0.0158
	7	0.474	0.1728	0.0820
	Mean ± SEM	0.306 ± 0.057	0.129 ± 0.022	0.044 ± 0.014
Nose	6	0.530	0.3851	0.2041
	13	0.028	1.0376	0.0326
	Mean ± SEM	0.279 ± 0.251	0.711 ± 0.326	0.118 ± 0.086
Foot	3	0.726	0.1486	0.1078
	4	0.228	0.7762	0.1769
	8	0.099	0.3298	0.3657
	Mean ± SEM	0.351 ± 0.191	0.4182 ± 0.186	0.217 ± 0.077
Tail	1	0.045	0.1101	0.0050
	3	0.056	0.3101	0.0174
	5	0.090	1.0254	0.0922
	7	0.108	0.2358	0.0254
	Mean ± SEM	0.075 ± 0.015	0.420 ± 0.206	0.035 ± 0.020

mental animals predisposed to neoplasia. Transgenic animals may be particularly useful for studies in nutrition and cancer because the tumors that develop in the HTLV-1 *tax* system are highly reproducible in both time of onset and tissue type and develop at multiple sites simultaneously, involving ears, nose, limbs, and tail. Since folate is essential for cell division, we proceeded with the hypothesis that it may be utilized at a higher rate in neoplastic cells compared with normal cells and that higher rates of utilization might be manifested as tissue depletion. If increased utilization is associated with depletion, it is still not clear if increased utilization is coincidental, or if it has a causal role in carcinogenesis.

Prominent findings in this study included lower brain and kidney folate concentrations, splenomegaly, and decreased liver and kidney weights in TBT compared with NTC mice. The 48% reduction in brain folate in tumor-bearing animals is a significant finding because some investigators have indicated that brain folate is conserved even with inadequate intake.[16,17] However, these investigators used nutritionally inadequate diets. More recent investigations using nutritionally adequate diets (except for folate) have shown that brain folate can be depleted.[18,19] The effect of folate depletion on brain function is unknown, since cell division is not a prominent feature of a mature brain. (We have noticed in other studies in our laboratory that folate-depleted mice spill more food than nondepleted controls.)

The low tumor folate concentrations found in the present study may be associated with a greater requirement for a rapidly growing tissue, or it may reflect altered folate metabolism in this tissue. A greater folate requirement by tumor tissue might enhance the effectiveness of antifolate chemotherapy. An altered folate metabolism, where influx of folate into tumors is diminished, might reduce the effectiveness of antifolate chemotherapy. Because the mouse model system yields tumors with predictable kinetics, it may provide the opportunity to study folate metabolism and antifolate chemotherapy in vivo.

The ability to predict tissue nutrient levels is important because current epidemiological evidence relating diet to disease risk is limited to correlations among nutrient intake, blood nutrient levels (which are assumed to reflect tissue levels), and the incidence of cancer. The lower serum folate in TBT compared with NTC mice is consistent with several reports in the literature, showing that serum (or plasma) folate levels are lower in cancer patients than in controls.[2,20-22] Furthermore, because all mice in the present experiment were fed the same diet, the changes seen in tissue folate levels are disease and not diet related.

Weight loss, tissue wasting, and cachexia are common in patients with neoplasia. To avoid potential complications associated with cachexia and tissue wasting in the present study, TBT and NTC mice were of similar age and weight and were killed before wasting (weight loss) became apparent and the tumor burden was light (~1% of body weight).

The variability of folate concentrations in tumors was unexpected. One explanation is that more connective tissue was present in some tumors. However, it is also possible that a different percentage of neutrophils was present in various tumors. Additional studies are needed to examine these possibilities. It may be important to quantitate tissue folate levels of primary tumors and those of metastatic lesions in humans.

Folate concentrations of specific organs (brain, kidney, and serum) but not others (spleen and liver) were lower in NTC than in TBT mice. These differences appear to be related to tumor burden because of the correlations (negative for some and unrelated to others) between tumor burden and folate concentrations of tissues in TBT mice. Tumor burden was negatively correlated with folate concentrations of brain, kidney, and serum and unrelated to those of spleen and liver. The relationship of tumor burden to hematologic values and tissue folate concentrations may be important because tumor burden appears to have organ-specific effects on folate metabolism that may manifest as hematologic alterations. The reduced serum folate concentrations observed in the TBT mice may be relevant to reductions in serum (or plasma) folate seen in human cancer patients.

Tissue and tumor folate concentrations reported by other investigators are as follows. Gailani et al.[20] reported folate concentration declined in a cancer patient receiving a folate-deficient diet: liver (from 10.2 to 0.680 nmol/g), tumors (from 0.45 to 0.057 nmol/g), blood (from 0.159 to 0.045 nmol/ml), and serum (from 7.9 to 2.3 pmol/ml). Folate depletion of tumor was approximately parallel to that of liver and blood. Patients with esophageal dysplasia or cancer had lower erythrocyte (0.548 vs. 0.696 nmol/ml) and plasma folates (9.81 vs. 12.01 pmol/ml)[21] than controls with normal cytology. Heimburger et al.[2] compared plasma and erythrocyte folate levels of smokers without metaplasia to those of similar individuals with metaplasia (14.3 vs. 11.1 pmol/ml plasma and 0.628 vs. 0.569 nmol/ml erythrocyte), respectively, and concluded that compromised folate status of smokers may facilitate the initiation of lung carcinoma. Lower mean folate levels in smokers with metaplasia may indicate that the levels of this vitamin play a role in squamous metaplasia and atypia. However the dietary intake of folate was not evaluated by Heimburger et al.[2] Rosen and Nichol[23] reported liver and Walker carcinosarcoma 256 concentrations of 35.86 and 0.838 nmol/g, respectively. The growth of these tumors is restricted by folate deficiency but refractory to amethopterin treatment. Zeigler[22] reported plasma and erythrocyte folate levels in oral contraceptive users with cervical dysplasia (8.2 pmol/ml and 0.365 nmol/ml) and in nonusers without dysplasia (11.83 pmol/ml and 0.428 nmol/ml). The concentrations of folate in tissues and tumors found in the present experiment are similar to those reported above.

The genotypically similar control mice used in the present experiment allowed evaluation of subtle changes in tissue folate concentrations associated with

the presence of cancer. Additional studies of the LTR-*tax* transgenic mouse model of cancer with controlled dietary folate intake[24] would permit the effects of dietary folate and tumor burden on folate metabolism to be evaluated.

Finally, the relationship between cell enlargement (megalocytosis) and folate deficiency remains an issue of considerable interest. However, since the megaloblastic changes in human cancer have been primarily described in epithelial cells, it will be necessary to study this issue in a transgenic model with epithelial rather than mesenchymal neoplasms.

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