Human Tumor Growth in Nude Mice Is Associated with Decreased Plasma Cysteine and Homocysteine

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Abstract. *The methionine cycle and its metabolites homocysteine and cysteine serve several important functions in cellular metabolism. Abnormalities in metabolism of the methionine cycle have been associated with cancer. We determined plasma levels of methionine, homocysteine and cysteine in nude mice implanted with human cancer cell lines (MDA-MB-435 breast, PC-3 prostate, HT29 colon, BX-PC3 pancreas) over a prolonged period of tumor growth. The data were compared with correspondins values in nontumor-bearing controls. Nude mice were injected s.c. in the right flank with 10^6 cancer cells. Tumor growth was measured over time. Methionine was measured in plasma by HPLC. Cysteine and homocysteine were measured in plasma by recombinant enzyme assays and spectrophotometry to measure products. The concentrations of cysteine and homocysteine in plasma decreased significantly as a result of progression of breast, prostate and the pancreas tumor types implanted in the nude mice at least over a two-month period. Data for the colon tumors were nonsignificant for both cysteine and homocysteine. In the case of methionine, the decrease was significant only due to progression of the breast tumors, grown over a long time period, as compared to the mice without tumors control. The results suggest that sulphur amino acids may be plasma or serum biomarkers for cancer progression.*

Abnormalities of methionine metabolism have long been associated with cancer (1-3). Deficiencies of the main dietary sources of methyl groups, methionine and choline, lead to the formation of liver cancer in rodents (4, 5). The transulfuration pathway links the methionine cycle to cysteine. Cysteine is associated with reduced risk of breast cancer in women when it is at high levels in their plasma (6). Homocysteine is the precursor of both methionine and cysteine and therefore is a critical bridge between the methionine and transulfuration pathways. The present study has evaluated the plasma levels of methionine, homocysteine and cysteine in human tumor-bearing mice, including tumors of the prostate, breast, pancreas and colon, over a time period up to 10 months. The results suggest the potential of measurement of these amino acids as biomarkers of cancer progression.

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Materials and Methods

Animal model. Six-week-old, male and female, immunocompromised NCR nude mice (nu/nu) weighing approximately 25 g were housed in a barrier room with HEPA filtration and fed Purina rodent chow *ad libitum* (Newco Distributor, Rancho Cucamonga, CA, USA). Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23) under assurance number A3873-01.

Cell culture. The MDA-MB43 human breast cancer, PC-3 human prostate cancer, HT 29 human colon cancer and Bx-PC 3 human pancreatic cancer cell lines were genetically engineered to express green fluorescent protein (GFP) or red fluorescent protein (RFP) using a retrovirus expression vector according to the methods previously reported by our group (7, 8). The cells were grown in monolayer in RPMI-1640 supplemented with 5%-10% fetal bovine serum and incubated in a humidified chamber at 37˚C with 95% air and 5% CO_2.

Tumor implantation. Nude mice were injected s.c. in the right flank with 1×10^6 cancer cells grown in culture. Cells were first harvested by trypsinization and washed three times with cold serum-free medium and then injected in a total volume of 50 μl within 40 min of harvesting.

Quantitative analysis of tumor growth. Periodically, the tumor-bearing mice were examined by whole-body fluorescence imaging. Whole-body images were acquired and analyzed by using a FluorVivo small animal imaging system (Indec Systems, Inc., Capitola, CA, USA). The size of each individual tumor was evaluated based on the total number of pixels in fluorescent areas.

Table I. *Circulating amino acids in mice with different human tumors.*

Conditions of nude mice	L-Cysteine μM	Homocysteine μM	Methionine μM
Controls (n=15)	235±14.2	7.5±0.6	39.2±1.91
Prostate cancer (n=3)	68.8±0.58	2.2±0.15	39.2±0.60
P-value	0.002	0.003	0.994
Breast cancer (n=3)	153.5±35.3	4.5±0.15	38.5±3.70
P-value	0.031	0.041	0.877
Colon cancer (n=5)	248±25.9	8.6±0.5	N/D
P-value	0.700	0.047	0.877

Data are presented as mean±SEM compared to control values; $P<0.05$ is significant. The age of mice was 3 ½ months and tumors grew for 2 months.

Table II. *Circulating amino acids in mice with pancreatic cancer.*

Conditions of nude mice	L-Cysteine μM	Homocysteine μM	Methionine μM
Controls (n=4)	183±15.4	4.9±0.47	41±6.99
Pancreatic cancer (n=3)	82.5±17.5	2.4±0.05	27.3±1.71
P-value	0.00007	0.004	0.274

Data are presented as mean±SEM compared to control values; $P<0.05$ is significant. The age of mice was 6 months and tumors grew for 4 ½ months.

Table III. *Circulating amino acids in mice with breast cancer over a long time period.*

Conditions of nude mice	L-Cysteine μM	Homocysteine μM	Methionine μM
Control (n=8)	210.7±15.17	3.2±0.28	59.8±5.68
Breast cancer (n=3)	159.2±20.7	3.3±0.93	20.7±1.29
P-value	0.093	0.895	0.004

Data are presented as mean±SEM compared to control values; $P<0.05$ is significant. The age of mice was 12 months and tumors grew for 10 months.

Blood samples. Blood samples were collected and plasma fractions prepared after the animals were sacrificed at different time points after cancer cell implantation.

Enzymatic cysteine assay. *In vitro* total cysteine in plasma was assayed by an L-cysteine kit (A/C Diagnostics, San Diego, CA, USA). The method is based on that of Han *et al*. (9). The assay was carried out in a 96-well plate. The protocol comprised the following three steps involving two enzymes and four reagents: (i) 10 μl of plasma samples and assay buffer containing L-dithiothreitol (DTT), adenosine and recombinant *S*-adenosylhomocysteine hydrolase (rSAHH) were incubated at 37˚C for 30 minutes. (ii) The assay buffer containing recombinant methioninase (rMETase) (20 μl of 0.375 mg/ml) was then added and incubated at 37˚C for 5 minutes using an IEMS incubater/shaker (Thermolab Systems, Waltham, MA, USA). (iii) The enzymatic reaction was stopped with the addition of 25 μl *N,N*-dibutyl phenylene diamine (DBPDA) in 3 M HCl followed by the addition of 15 μl of the oxidizing agent potassium ferricyanide. The mixture was incubated at room temperature for 5 minutes. The cysteine was measured at 675 nm using a Tecan-Sunrise Reader (Tecan US, Durham, NC, USA).

Table IV. *Tumor growth.*

Tumor type	Tumor size in pixels (mean±SEM)		
	Initial	Final	n
Breast	369±35	1970±352	5
Prostate	727±119.5	19172±4347	3
Colon	1303±139.5	6786±669	5
Pancreas*	991±189.7	44957±5765	3
Breast**	395±37	2625±463	3

*4 ½ Months after implantation; **10 months after implantation. The remaining tumors were two months after implantation.

Table V. *Body weight changes in tumor-bearing mice.*

Tumor type	Weight (g) (mean±SEM)		n
Control	24.6±1.0	29.1±0.9	15
Breast	22.7±0.8	26.2±1.1	5
Prostate	27.8±2.1	31.0±1.3	3
Colon	22.5±0.9	25.0±2.3	5
Pancreas	26.9±0.1	31.3±1.2	5

Tumors are two months after implantation except for the pancreatic tumors which had grown for 4 ½ months in 6-month-old mice.

Enzymatic homocysteine assay. Homocysteine was measured by an L-homocysteine kit (A/C Diagnostics). The assay procedure is based on that of Tan *et al*. (10) and was as follows: 5 μl of sample or calibrator and control were placed into one well of a 96-well plate or in a tube (Thermowell™ tube), followed by addition of 165 μl of 10 mM phosphate buffer with 1 mM dithiothreitol (DTT) (reducing reagent). The mixture was incubated at room temperature (24-28˚C) for 60 minutes, or at 37 ˚C for 30 minutes, then 30 μl of 0.05/ml recombinant homocysteinase was added and incubated at room temperature (24-28˚C) for 5 minutes. Subsequently, 50 μl *N,N*-dibutyl phenylene diamine (DBPDA) (12.5 mM) in 1.5 N H_2SO_4 was added and the mixture was allowed to stand at room temperature for 5 minutes. A volume of 30 μl of 5 mM $K_3Fe(CN)_6$ in 10 mM sodium phosphate buffer, pH 7.6, was added and the mixture was incubated at room temperature for 10 minutes. If the assay was carried in 96-well plates, the solution was transferred to Thermowell™ tubes, and fluorescence was read at emission 710 nm/excitation 660 nm. The homocysteine (HCY) value was determined directly by the A/C Diagnostics Fluorescence Reader. Calibrators, controls and samples were run in duplicate.

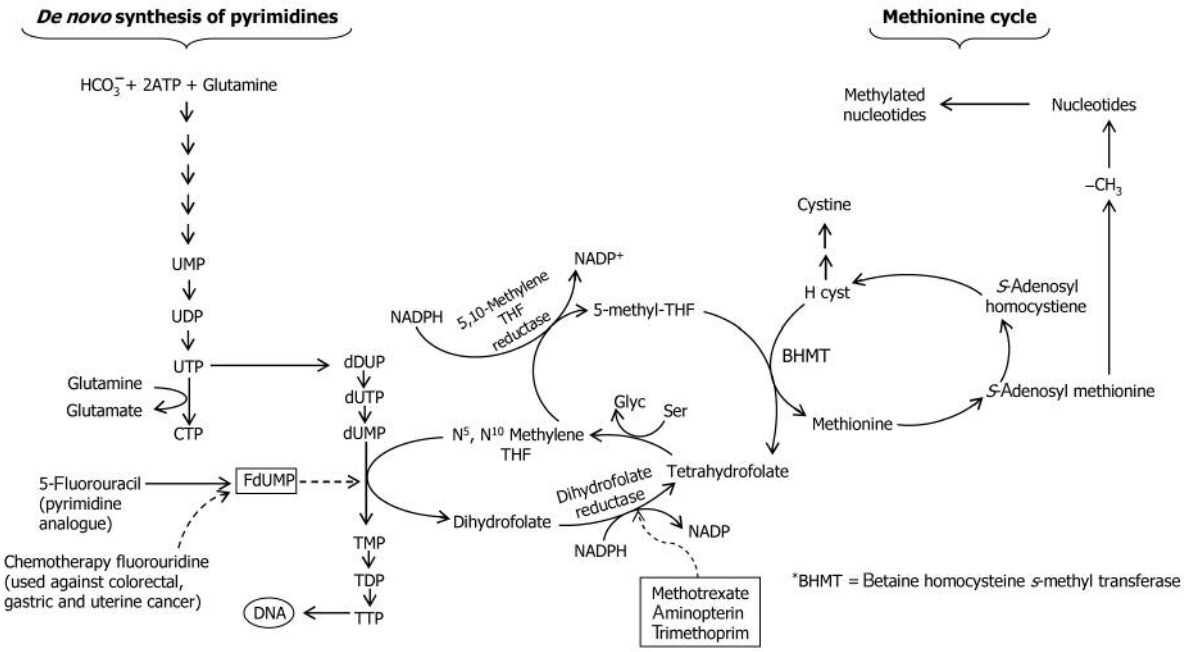

Figure 1. *The methionine cycle (15, 16).*

HPLC assay for methionine. The methionine concentration in plasma was measured by precolumn derivatization, followed by high-performance liquid chromatography separation (11). Briefly, 10 μl plasma sample or MET standard were precipitated with 30 μl acetonitrile, followed by centrifugation at 10,000 rpm for 1 minute. Aliquots of 10 μl supernatant were mixed with 5 μl of a fluoraldehyde derivative reagent, *o*-phthaldiadehyde, for 1 min. at room temperature, followed by the addition of 150 μl 0.1 M sodium acetate (pH 7.0). The reaction mixture (20 μl) was loaded on a reversed-phase Supelcosil LC-18 DB column (25×4.8 cm, particle size 5 μl; Supelco, Bellefonte, PA, USA). The amino acid derivatives were separated using a gradient elution of 40-60% solution B (methanol) in solution A [tetrahydrofuran/methanol/0.1 M sodium acetate (pH 7.2); 5/95/900] at a flow rate of 1 ml/min. A fluorescence spectrophotometer was used for detection with excitation at 350 nm and emission at 450 nm (Hitachi L-6200 A Intelligent Pump Model; Hitachi Ltd., Tokyo, Japan). The plasma MET was identified by the retention time of a MET standard solution and the concentration was measured according to a MET standard curve.

Statistical analysis. Statistical analyses were carried out using Microsoft Excel (Microsoft) and Statview (SAS, Cary, NC, USA) software. Differences among groups were assessed using ANOVAs and two-tailed Student's *t*-tests. $P<0.05$ was considered to be statistically significant. Data are reported as mean ± standard error (SE).

Results

The plasma levels of L-cysteine, homocysteine and methionine in age-matched controls and tumor-bearing mice were determined (Table I).

L-cysteine. The level in age-matched (3 ½ months) non-tumor-bearing controls was 235 μM; in the mice with prostate cancer, 68.8 μM (p=0.002); in the mice with breast cancer, 154 μM (p=0.031); in the mice with colon cancer, 248 μM (p=0.700); in the mice with pancreatic cancer, 83 μM, while that in the age-matched (6 months) controls was 183 μM (p=0.00007). In the mice with breast cancer grown over 10 months, the level was 159 μM and that of the age-matched controls was 210.7 μM (p=0.093).

Homocysteine. The level in age-matched controls (3 ½ months) was 7.53 μM; in the mice with prostate cancer, 2.2 μM (p=0.003); in the mice with breast cancer, 4.5 μM (p=0.041); in the mice with colon cancer, 8.6 μM (p=0.470); in the mice with pancreatic cancer, 2.4 μM, while that in the age-matched (6 months) controls was 4.9 μM (p=0.004). In the mice with breast cancer grown over 10 months, the level was 3.3 μM and while that in the age-matched controls was 3.2 μM (p=0.895).

Methionine. The level in age-matched controls was 39 μM. In the mice with prostate cancer, the level was 39 μM (p=0.994); in the mice with breast cancer, 38.5 μM (p=0.877); in the mice with pancreatic cancer, 27 μM and in the age-matched controls, 41 μM (p=0.274). Methionine was not determined in mice with colon cancer. In the mice with breast cancer grown over 10 months, the level was 21 μM while that in the age-matched controls was 60 μM (p=0.004).

Discussion

For all the cancer types tested, there was extensive tumor growth (Table IV) suggesting a possible inverse correlation between tumor size and circulating levels of cysteine and homocysteine. The data suggest the possibility of extensive use of cysteine and homocysteine by the prostate, breast and pancreatic tumors, at least over two months of tumor growth. Future experiments should attempt to discern the fate and possible role of these amino acids in tumor growth and progression. The colon tumors grew extensively without depleting cysteine and homocysteine. Future experiments will determine whether different tumor types have differential requirements for homocysteine and cysteine.

Although extensive studies have demonstrated the methionine dependency of tumors (1-3), the levels of methionine were not depleted in the tumor-bearing mice, except in the animals with breast cancer grown over a 10-month period. Further studies are needed to determine the relationship of plasma methionine levels with tumor growth.

The animals with the long-term growing breast tumors (Table III) have a different pattern of plasma amino acids from the animals with the shorter term-growing breast tumors (Table I). It is possible over the long term that the breast tumors use more methionine than cysteine or homocysteine. These tumors may be candidates for methioninase therapy (12-14).

In all cases, the body weights of the tumor-bearing mice were stable or increased (Table V). Therefore the observed changes in circulating amino acid levels cannot be explained by body weight loss (cachexia).

In addition to information on the mechanism of tumor growth and progression, further pursuit of studies described in this report may lead to new biomarkers for cancer, such as circulating amino acids. Since folate and vitamin B_6 are central to the methionine and transulfuration cycle, respectively (Figure 1), the plasma measurements of tetrahydrofolate and pyridoxal-6-phosphate should also be considered in future experiments.

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