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Methylthioadenosine Phosphorylase Deficiency in Human Leukemias and Solid Tumors[1]

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ABSTRACT

5′-Methylthioadenosine (MTA) is a naturally occurring nucleoside which is degraded by MTA phosphorylase (MTAase) to adenine and methylthioribose-1-phosphate in all normal mammalian cells. These products of the phosphorylytic cleavage of MTA are recycled to the nucleotide pool and methionine, respectively. Thus, supplemental MTA could theoretically be utilized by MTAase-containing cells as a source of methionine and adenine. In fact, *in vitro* experiments have shown that MTAase-containing cells proliferate normally in methionine-free medium if MTA is added to the cultures (M. K. Riscoe and A. J. Ferro, J. Biol. Chem., *259:* 5465–5471, 1984). In contrast, MTAase-deficient malignant cell lines do not proliferate under these conditions. In light of these observations and the recent demonstration (N. Kamatani *et al.*, Blood, *60:* 1387–1391, 1982) that a proportion of acute lymphoblastic leukemias lack MTAase, we wished to determine if this enzyme deficiency occurs in a variety of human neoplasms. Accordingly, malignant cells from eight patients with acute nonlymphocytic leukemia and ten patients with various solid tumors were assayed for MTAase activity. Samples from one of the eight acute nonlymphocytic leukemia patients and three of the 10 solid tumor patients (one with melanoma, one with squamous cell lung cancer, and one with adenocarcinoma of the rectum) had undetectable MTAase activity. In contrast, erythrocytes, neutrophils, and monocytes isolated from normal subjects and from patients with immunodeficiency syndromes or cancer all contained enzyme activity. In addition, the methods of preservation, storage, and cell disruption did not affect MTAase activity. These observations confirm and extend the findings of Kamatani *et al.* (Blood, *60:* 1387–1391, 1982) by demonstrating that MTAase deficiency occurs in a variety of human malignancies including acute nonlymphocytic leukemia and solid tumors. This metabolic difference between normal and malignant cells may be therapeutically exploitable.

INTRODUCTION

MTA[3] is a naturally occurring nucleoside synthesized stoichiometrically during biosynthesis of the polyamines spermidine and spermine (1, 2). Increased synthesis and cellular levels of the polyamines are associated with the initiation of cell growth and with rapidly proliferating tissues (3, 4). MTA, however, does not accumulate in cells in amounts commensurate with the concentration of polyamines. This observation led to the discovery of the phosphate-dependent breakdown of MTA by a specific nucleoside phosphorylase designated MTAase (5). The products of the phosphorylytic cleavage of MTA are MTR-1-P and adenine (5). Both of these products are recycled: the adenine to nucleic acids via the purine salvage pathway and MTR-1-P to methionine via α-methylthioketobutyrate (1, 2).

MTAase is abundant in normal mammalian tissues and in cell lines derived from normal cells (1, 2). In 1977, Toohey (6) reported that some malignant murine hematopoietic cell lines were devoid of MTAase activity. Subsequently, Kamatani *et al.* (7) found the same enzyme deficiency in 7 of 31 human malignant cell lines including leukemias, melanomas, and breast cancers. None of 16 cell lines of benign origin lacked the enzyme.

MTAase deficiency is not confined to mammalian cells in culture. Recently, malignant cells from 20 patients with leukemia and 9 with solid tumors were assayed for MTAase (8). Leukemic cells from one individual with pre-T-cell ALL and one with common ALL were MTAase deficient. In contrast, normal cells from the same patients, including peripheral blood lymphocytes, thymic lymphocytes, and bone marrow cells, contained the enzyme (8).

In order to determine if MTAase deficiency occurs in many types of human malignancy, we have examined tissue samples from patients with various forms of neoplasia. We show that the enzyme deficiency occurs in acute nonlymphocytic leukemia and a variety of solid tumors.

MATERIALS AND METHODS

Tissue Samples. Leukemia cell samples were obtained in heparinized syringes from the blood or aspirated bone marrow of informed and consenting patients at initial presentation or during relapse. The samples were layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged at 400 × *g* for 25 min, washed, and resuspended in tissue culture medium to obtain a single-cell suspension of low-density cells depleted of erythrocytes and mature neutrophils (9). The cells were then frozen in RPMI-1640 medium supplemented with 10% fetal bovine serum and 7.5% DMSO at 10^7 cells/ml and cryopreserved in liquid nitrogen. When the samples were thawed for analysis, a cytospin preparation of an aliquot of the low-density cells was made to determine the purity of the leukemic cell population (uniformly 98% blasts or greater) and cell viability (by trypan blue dye exclusion).

Solid tumor samples were obtained during diagnostic or therapeutic surgical procedures. The specimens were either analyzed fresh or placed in medium/7.5% DMSO and stored in liquid nitrogen. At the time of analysis, specimens were cut into small pieces with a scalpel and disrupted by either repeated freeze-thawing or digitonin treatment (see below) followed by processing in a Dounce homogenizer.

Erythrocytes and neutrophils were obtained by dextran sedimentation (10). To isolate monocytes, a mononuclear leukocyte suspension was obtained by centrifugation over Ficoll-Hypaque (9) followed by adherence to serum-coated dishes (11). The nonadherent cells (lymphocytes) were then poured off, and the adherent cells (monocytes) were lifted off with a rubber policeman.

Cell Disruption. In early studies, cell extracts were prepared by freeze-thawing 5 times in liquid nitrogen, removing cellular debris by centrifugation, and assaying the supernatant immediately for enzyme activity. To determine if cryopreservation, storage, or the preparative freeze-

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[3] The abbreviations used are: MTA, methylthioadenosine; MTAase, methylthioadenosine phosphorylase; MTR-1-P, methylthioribose-1-phosphate; ALL, acute lymphoblastic leukemia; DMSO, dimethyl sulfoxide; ANLL, acute nonlymphocytic leukemia; LDH, lactate dehydrogenase; PMN, polymorphonuclear leukocytes.

Table 1 *MTA phosphorylase activity in acute nonlymphocytic leukemia cells*

Patient	FAB subtype	Cell viability (%)	MTAase (pmol/min/mg)	LDH (nmol/min/mg)	Previous treatment[a]
G. H.	M4	20	51.2	42	DAT
H. C.	M4	78	50.0	49	DAT
V. C.	M2	56	48.7	26	None
T. G.	M1	39	48.2	26	DAT
M. G.	M5	33	42.3	41	None
M. B.	M2	79	38.1	48	None
D. P.	M2	54	31.7	36	None
J. S.	M1	42	0[b]	40	TAD twice; high-dose ara-C

[a] D, daunomycin; A, 1-β-D-arabinofuranosylcytosine; T, 6-thioguanine; ara-C, 1-β-D-arabinofuranosylcytosine.
[b] No detectable activity on four determinations.

thawing technique affects the stability of MTA phosphorylase, two different cell disruption methods were used, and samples were tested fresh or after varying periods of frozen storage. In these experiments, MTAase activity was compared in cell extracts prepared by the freeze-thaw method *versus* digitonin disruption (12). When it became evident that the two techniques gave comparable results, digitonin disruption was adopted as the routine because of its greater convenience.

Enzyme Assays. MTAase activity was determined by measuring the conversion of $^{14}CH_3$-labeled MTA to $^{14}CH_3$-labeled MTR-1-P by ion-exchange chromatography under reducing conditions (2 mM dithiothreitol) as previously described (13). The lowest detectable level of MTAase by this method is 0.3 pmol/min, and values are reproducible within 10% on repeated testing of enzyme-containing samples (13). All samples without detectable MTAase activity were tested at least 3 times. In addition, most samples were assayed for LDH by standard techniques (14) to test for nonspecific enzyme degradation which could result from a decrease in cell viability or some consequence of cell processing. Protein concentration was determined by the method of Lowry *et al.* (15).

RESULTS

Leukemia Samples. Specimens of blood or bone marrow from 8 patients with ANLL in relapse were analyzed for MTAase activity. These samples had been frozen for up to 18 mo. The viability of the cells, as determined by trypan blue dye exclusion, varied from 20% to 79%. Of the 8 specimens, one (J. S.) was completely devoid of MTAase activity (Table 1). MTAase activity in the other 7 specimens ranged from 31.7 to 51.2 pmol/min/mg of protein. It is unlikely that the lack of enzyme activity in Sample J. S. was due to low cell viability, since 3 other specimens (G. H., T. G., and M. G) had lower viabilities but "normal" enzyme activities. In addition, we have found that MTAase activity is very stable when cell-free extracts have been stored for periods in excess of 2 yr.[4] Moreover, we assayed each of these samples for LDH activity, an enzyme completely unrelated to MTAase, and found that the specimen which lacked MTAase activity (J. S.) contained LDH activity within the same range exhibited by those samples which contained MTAase.

Solid Tumor Samples. The results of MTAase (and LDH) assays on samples from 10 patients with solid tumors are given in Table 2. Of the 10 samples examined, 7 contained MTAase activity while 3 (C. A., D. C., and M. P.) were devoid of activity. The samples from Patients C. A. (malignant melanoma) and M. P. (rectal adenocarcinoma) contained LDH activity within the range found in the MTAase-containing specimens. This finding was particularly important for the sample from Patient M. P., since his tumor had been irradiated before surgery. The sample from Patient D. C. had relatively low LDH activity, and it is therefore less certain that the deficiency of MTAase in his cancer cells was specific.

[4] A. J. Ferro and J. H. Fitchen, unpublished observation.

Table 2 *MTA phosphorylase activity in solid tumor samples*

Patient	Diagnosis	MTAase (pmol/min/mg)	LDH (nmol/mg/min)	Previous treatment
W. C.	Renal cell carcinoma	487	74	None
M. G.	Melanoma	37	109	None
S. O.	Melanoma	30	55	DTIC,[a] cis-Plat
A. B.	Large cell lymphoma	37	100	None
R. M.	Brain metastases (adenocarcinoma)	556	ND	None
D. P.	Cervical cancer	1068	74	None
D. H. C.	Brain metastases (melanoma)	238	139	None
C. A.	Melanoma	0[b]	65	None
D. C.	Squamous lung carcinoma	0[b]	20	None
M. P.	Rectal adenocarcinoma	0[b]	67	5000 rads

[a] DTIC, dacarbazine; cis-Plat, *cis*-diamminedichloroplatinum; ND, not determined.
[b] No detectable activity on at least three determinations.

Table 3 *MTA phosphorylase activity in human blood cells*

Subject	Cell type	Condition	MTAase activity (pmol/min/mg protein)
F. O.	RBCs	Normal	236
T. Y.	RBCs	Normal	175
A. V.	RBCs	Normal	187
B. E.	RBCs	Immunodeficiency	187
L. A.	RBCs	Immunodeficiency	175
C. L.	RBCs	Immunodeficiency	186
M. O.	RBCs	Lung cancer	185
M. K.	RBCs	Melanoma	220
R. N.	RBCs	Breast cancer	172
K. D.	RBCs	Esophageal cancer	182
J. H.	RBCs	Colon cancer	182
F. O.	Monocytes	Normal	170
T. Y.	Monocytes	Normal	152
A. V.	Monocytes	Normal	145
B. E.	Monocytes	Immunodeficiency	146
L. A.	Monocytes	Immunodeficiency	158
C. L.	Monocytes	Immunodeficiency	137
F. O.	PMNs	Normal	44
T. Y.	PMNs	Normal	27
A. V.	PMNs	Normal	72
B. E.	PMNs	Immunodeficiency	76
L. A.	PMNs	Immunodeficiency	23
C. L.	PMNs	Immunodeficiency	44

It is interesting to note that 2 of the tumor samples had extremely high MTAase levels. The specimens from R. M. (brain metastasis from adenocarcinoma of the gall bladder) and D. P. (cervical carcinoma) had MTAase activity 3- to 5-fold higher than that noted in normal cells (see Table 3).

MTA Phosphorylase Activity in Normal Human Blood Cells. Erythrocytes (RBCs), neutrophils (PMNs), and monocytes were isolated from normal subjects and from patients with immunodeficiency syndromes or various cancers. All cells examined contained enzyme activity, and there was no significant difference between normal individuals and those with either cancer or immunodeficiency within the same cell type (Table 3). Lower activity was uniformly found in PMNs compared to RBCs or monocytes.

Effect of Storage and Freeze-Thawing on MTA Phosphorylase Activity. To determine if cryopreservation, storage, or the method of cell disruption affects MTA phosphorylase activity, we compared 2 different cell disruption techniques and tested samples fresh or after varying periods of frozen storage. The

results indicate that MTAase (and LDH) activities are comparable when cell extracts are prepared by freeze-thawing or digitonin disruption, and that enzyme activity does not decline significantly during prolonged storage (Table 4).

DISCUSSION

Kamatani *et al.* (8) reported MTAase deficiency in the leukemic cells from 2 of 20 patients with ALL, and Chilcote *et al.* (16) reported undetectable MTAase levels in an additional patient with "lymphomatous" ALL. We have confirmed the occurrence of MTAase deficiency in primary explants of human malignant cells and extended these observations by demonstrating that MTAase deficiency occurs in human malignancies other than ALL. Of 8 samples from acute nonlymphocytic leukemia patients, one was devoid of MTAase activity, and of 10 solid tumors analyzed, 3 (melanoma, squamous lung carcinoma, rectal adenocarcinoma) were found to lack activity of this enzyme. The patient with ANLL had been extensively treated with chemotherapy, and it is therefore possible that MTAase-deficient clones were selected in this patient by previous drug treatment. However, none of the chemotherapeutic agents used in this patient is known to affect MTAase. The patient with rectal adenocarcinoma had received preoperative radiotherapy to the tumor which could account for lowered MTAase activity. However, normal LDH activity was preserved in this sample. The other 2 deficient samples were from patients who had not received anticancer treatment of any kind. Thus, in these latter 2 samples, and probably in the ANLL and rectal adenocarcinoma samples as well, MTAase deficiency appears to be specific. The finding of MTAase deficiency in acute leukemia and a variety of solid tumors, together with the previously demonstrated absence of MTAase in human tumor cell lines of diverse origin, suggests that MTAase deficiency is a phenomenon which occurs in a broad spectrum of human malignancies.

Since many of the samples we tested (including 3 of the 4 that lacked MTAase activity) had been subjected to frozen storage before analysis, it was important to determine if cryopreservation, storage, or the method of cell disruption affects MTAase activity. Results of experiments addressing these issues (Table 4) indicate that MTAase activities are comparable when cell extracts are prepared by freeze-thawing or digitonin disruption, and that enzyme activity does not decline during prolonged frozen storage.

Table 4 *Stability of MTA phosphorylase after storage and freeze-thawing*

Sample	Duration of storage (days)	Cell disruption technique	MTAase (pmol/min/mg)	LDH (nmol/min/mg)
Normal blood lymphocytes	Fresh	Freeze-thaw	64	ND[a]
	Fresh	Digitonin	52	ND
	60	Freeze-thaw	55	ND
	60	Digitonin	49	ND
Normal human serum	Fresh		102	60
	30		98	60
	60		101	58
	120		99	57
Sarcoma	Fresh	Freeze-thaw	36	108
	30	Freeze-thaw	36	108
	60	Freeze-thaw	34	108
	120	Freeze-thaw	34	107
CGL: bone marrow	Fresh	Freeze-thaw	36	ND
	Fresh	Digitonin	34	ND
	60	Freeze-thaw	27	ND
	60	Digitonin	27	ND
CGL-BC: bone marrow	Fresh	Freeze-thaw	15	ND
	Fresh	Digitonin	14	ND
	60	Freeze-thaw	14	ND
	60	Digitonin	14	ND

[a] ND, not determined; CGL, chronic granulocytic leukemia; CGL-BC, chronic granulocytic leukemia in blast crisis.

Our finding of MTAase activity in erythrocytes, monocytes, and neutrophils from normal subjects and patients with immunodeficiency syndromes or cancer corroborates previous reports that all normal human cells contain MTAase activity (1, 2) and that uninvolved tissues in cancer patients are not MTAase deficient (8).

Since the cleavage of MTA is the only source of endogenously synthesized adenine in human cells (17), it has been proposed that a chemotherapeutic strategy involving methotrexate, pyrimidines, and MTA could be used to kill MTAase-deficient malignant cells without harming normal cells (7). This strategy is based on the hypothesis that only MTAase-containing cells can derive their purine requirement from MTA and thereby overcome the methotrexate-mediated block in *de novo* purine synthesis. Indeed, selective inhibition of MTAase-deficient cells using this regimen has been successful *in vitro* (7). Clinical application of such a regimen could be hampered by the fact that human serum contains MTAase (18): enzymatic cleavage of MTA in the serum might therefore generate adenine which could be utilized by MTAase-deficient malignant cells.

An alternative approach, which circumvents the potential problem of MTAase in serum, focuses on the reycling of MTR-1-P to methionine. We have shown that MTAase-containing cells cultured in methionine-free medium can utilize exogenous MTA as a source of methionine, whereas MTAase-deficient cells cannot (18). The presence of MTAase in serum in the culture medium does not alter this differential effect. Thus, a regimen combining methionine deprivation and MTA supplementation could theoretically produce selective killing of MTAase-deficient malignant cells. Experiments designed to test this treatment strategy in experimental animals are currently in progress in our laboratories.

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