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Abnormal Regulation of Methylthioadenosine and Polyamine Metabolism in Methylthioadenosine Phosphorylase-deficient Human Leukemic Cell Lines[1]

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ABSTRACT

Approximately 30 to 40% of human leukemic cell lines are completely deficient in the purine catabolic enzyme 5′-methylthioadenosine phosphorylase. Using two 5′-methylthioadenosine phosphorylase-negative leukemias, we have for the first time been able to measure the synthesis and biological effects of 5′-methylthioadenosine (MTA) in intact mammalian tumor cells. Malignant cells lacking this enzyme, unlike enzyme-positive cells, excreted MTA into the culture medium at a rate of 0.58 to 0.70 nmol/hr/mg protein. The production of the nucleoside was inhibited effectively by nontoxic concentrations of methylglyoxal bis(guanylhydrazone), a putrescine-dependent *S*-adenosylmethionine decarboxylase inhibitor, and also by spermidine and spermine but was enhanced by putrescine. In a reciprocal fashion, MTA at low concentrations progressively increased both the synthesis and concentration of putrescine but suppressed spermine production. The unique alterations in polyamine metabolism induced by elevated MTA levels could offer a selective growth advantage to the 5′-methylthioadenosine phosphorylase-deficient cells and thus may be related to the high frequency of this enzyme deficiency among human leukemic cell lines.

INTRODUCTION

The synthesis of the polyamines spermidine and spermine by mammalian cells yields stoichiometric amounts of MTA[3] (18). However, MTA does not accumulate significantly in mammalian cells (23) but rather is cleaved rapidly into adenine and 5-methylthioribose 1-phosphate by MTAase (17). The latter enzyme has considerable activity in every organ of the rat thus far examined, including the prostate, liver, lung, spleen, kidney, and heart (4, 7).

In 1977, Toohey (31) reported that several malignant murine hematopoietic call lines lacked MTAase. This deficiency rendered the growth of the cells dependent upon an exogenous source of methylthio groups (32, 33). In an effort to extend these observations to humans, we assayed MTAase in 13 established human malignant hematopoietic cell lines; 5 (38%) had undetectable levels of the enzyme and maintained this phenotype with *in vitro* passaging for up to 1 year.[4] However, the MTAase-deficient human leukemic cells, unlike the murine tumors, do not require exogenous methylthio groups for growth.[4]

In principle, as long as spermidine and spermine synthesis persists, MTAase deficiency should lead to an accumulation of MTA, as well as a depletion of 5-methylthioribose 1-phosphate. Since increased levels of polyamines are an established indicator of cell proliferation (5, 11, 28, 30), and thus of neoplastic disease (11, 25, 26), we thought it important to study in detail the regulation of MTA synthesis in intact MTAase-deficient human leukemic cells and the effects of MTA itself on polyamine production. The results demonstrate that MTA synthesis is absolutely dependent on the concomitant production of spermidine and spermine and that even mildly elevated MTA concentrations significantly increase intracellular putrescine levels.

MATERIALS AND METHODS

Cell Lines. Four representative cell lines were studied: CCRF-CEM and MOLT-4, of malignant T-cell origin (6, 15); K562 (12), a leukemia with myeloid and erythroid qualities; and Raji, derived from a patient with African Burkitt's lymphoma (22). When assayed by the method of Pegg and Williams-Ashman (17), the CEM and K562 cell lines contained less than 0.002 nmol/min/mg protein of MTAase activity, compared to 0.958 and 0.864 nmol/min/mg protein in MOLT-4 and Raji, respectively.

Routinely, the cells were grown in suspension culture, in Roswell Park Memorial Institute Medium 1640, supplemented with 10% fetal calf serum, 100 units penicillin per ml, 100 μg streptomycin per ml, and 2 mM glutamine (Flow Laboratories, Rockville, Md.). In the experiments described below, however, heated (56°, 3 hr) horse serum was substituted for fetal calf serum. This modification was necessary because horse serum, unlike fetal calf serum, contained neither MTAase nor diamine oxidase, which converts polyamines to toxic aldehydes (1, 10).

In short-term experiments, the effects of various compounds on growth were monitored by determining the incorporation of L-[4, 5-^{3}H]leucine (5 Ci/mmol) and [*methyl*-^{3}H]thymidine (2 Ci/mmol) into acid-precipitable material as described previously (2).

Measurement of MTA in Cells and Medium. Suspension of cells in horse serum-containing medium was centrifuged at 700 × *g*. After the removal of the supernatants, the cellular pellets were washed twice in cold phosphate-buffered saline (0.15 M NaCl/0.01 M phosphate buffer, pH 7.4). Then the medium and the packed cells were separately agitated with ice-cold PCA to a final concentration of 0.4 M, followed by the immediate addition of 1.25 to 10 nmol 5′-chloroadensine as an internal standard. 5′-Chloroadenosine had been synthesized in

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[3] The abbreviations used are: MTA, 5′-methylthioadenosine; MTAase, 5′-methylthioadenosine phosphorylase; PCA, perchloric acid; HPLC, high-performance liquid chromatography; MGBG, methylglyoxal bis(guanylhydrazone).

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this laboratory according to published methods (3). The PCA-insoluble material was removed by centrifugation, and the supernatant was neutralized to pH 7 to 8 with 5 M KOH. The additional precipitated salt was removed, and the soluble material was passaged through a boronate affinity column (Affigel 601; Bio-Rad Laboratories, Richmond, Calif.), as described by Uziel *et al.* (34), to concentrate *cis*-diols, including ribonucleosides. The resulting MTA-enriched preparations were lyophilized, resuspended in 200 μl water, and analyzed by reverse-phase HPLC on a C_{18}-μBondapak column (Waters Associates, Milford, Mass.), and eluted with 8% acetonitrile/0.01 M potassium phosphate (pH 6.0) at ambient temperature and a flow rate of 1.5 ml/min. Under these conditions, 5′-chloroadenosine and MTA eluted at 12.8 and 17.6 min, respectively, and were well separated from other UV-absorbing materials. The areas under the respective peaks of UV absorbance at 254 nm, corresponding to MTA and 5′-chloroadenosine, were compared with authentic standards to determine the percentage of recovery and MTA concentration.

Measurement of Polyamine Concentrations and Synthesis. The concentrations of putrescine, spermidine, and spermine in PCA extracts of the various cell lines were determined by the method of Seiler (27). To measure polyamine synthesis, 5 × 10^5 cells were cultured for 6 hr at 37° with 5 μCi L-[3-^{3}H]-ornithine (22.5 Ci/mmol; New England Nuclear, Boston, Mass.). The cells were pelleted, washed twice in phosphate-buffered saline, and treated with PCA, after which the polyamines were dansylated, extracted into toluene, and separated by the method of Seiler (27). Aliquots of the purified polyamines were examined for relative fluorescence and radioactivity. The S.E. of the determinations of polyamine concentrations was less than 5%, while that of polyamine synthesis was less than 6% (n = 6 for each determination).

Thin-Layer Chromatography. MTA isolated from the medium was cochromatographed with authentic standard in 9 different chromatographic systems, derived from 3 kinds of thin-layer plates (polyethylenimine cellulose, cellulose, and silica gel) and 3 developing solutions [Solution A, 1 M ammonium acetate; Solution B, 50% methanol, and Solution C, acetronitrile/28% ammonia/0.1 M ammonium acetate/butanol (10/10/20/60, by volume)].

RESULTS

Dependence on Polyamine Synthesis of MTA Production by Cell Lines. HPLC was an accurate and reproducible method for the determination of MTA concentrations in the medium of leukemic cell cultures and could detect as little as 0.02 nmol nucleoside (the S.D. was 1.9% of the mean in 10 replicate assays). Chart 1 shows a typical HPLC tracing of the medium from K562 leukemic cells. The Peak II thus isolated was clearly identified as MTA by its UV-absorbance spectrum and migration on thin-layer chromatography plates in 9 different chromatographic systems. Furthermore, the purified MTA from K562 cultures was converted to adenine by cytoplasmic extracts only of MTAase-positive cells, *i.e.*, MOLT-4 and Raji.

Only the MTAase-negative cells accumulated the nucleoside in the culture medium. When K562 cells in log-phase growth were washed and resuspended in Roswell Park Memorial Institute Medium 1640 supplemented with 10% horse serum, they began to produce MTA within 1 hr (Chart 2*A*). The production of the nucleoside was affected by cell density; the lower the cell density, the more rapid was MTA production within the range tested (Chart 2*B*). CEM and K562 at a density of 5 × 10^5 cells/ml excreted MTA into the medium at rates of 0.58 and 0.70 nmol/hr/mg protein, respectively, during the first 6 hr. In stationary-phase cultures, the concentration of MTA in the medium reached as much as 1 to 5 μM.

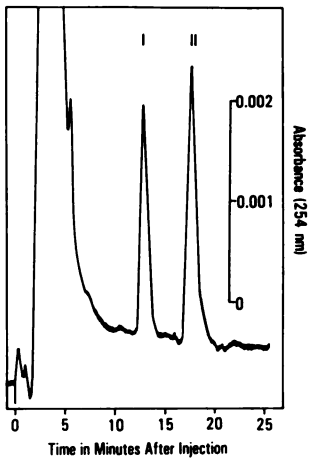

Chart 1. HPLC pattern of MTA in the medium of K562 culture. *I*, 5′-Chloroadenosine (added as an internal standard); *II*, MTA.

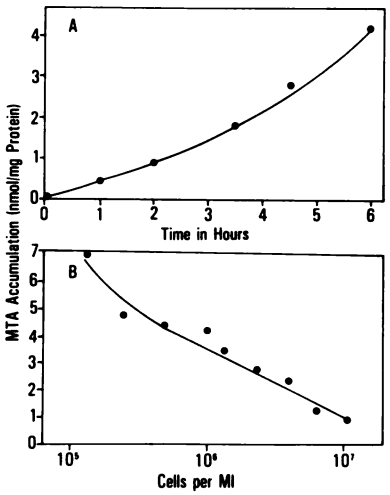

Chart 2. Time-dependent accumulation of MTA and the effect of the cell density. *A*, K562 cells were cultured at a cell density of 5 × 10^5/ml, and MTA accumulated in the medium was measured at various time points; *B*, cultures at various cell densities incubated for 6 hr.

Washed cell pellets contained ≤1% of the total MTA produced by the MTAase-negative cell lines. Because of the small volumes of the washed cells, it was impossible to ascertain the exact intracellular concentration of MTA. However, the levels were estimated to be at least equal to and probably greater than MTA concentrations in the medium.

The synthesis of MTA by K562 cells and the incorporation of [^{3}H]ornithine into spermidine were inhibited dose dependently by MGBG, which blocks the putrescine-dependent decarboxylation of *S*-adenosylmethionine (36) (Chart 3). During the 6-hr period of the experiment, MGBG had no effect on tritiated thymidine or leucine uptake into macromolecular materials. Moreover, even after 72 hr incubation, 1 μM MGBG, which inhibited MTA accumulation by more than 70%, had no effect

on growth. It is therefore probable that inhibition of putrescine-dependent *S*-adenosylmethionine decarboxylase is responsible for the inhibition of MTA production by MGBG.

In horse serum-containing medium, which as mentioned previously lacks diamine oxidase, 10 μM spermidine and spermine each inhibited MTA production by approximately 40%, while 10 μM putrescine increased synthesis by 20% (Table 1). These concentations of polyamines had no effect on radioactive leucine or thymidine uptake during the same period.

Effects of MTA on Polyamine Metabolism. As little as 3 μM MTA added in the medium markedly increased the incorporation of [3-^{3}H]ornithine into putrescine by MTAase-negative K562 cells over 6 hr (Chart 4*B*). On the contrary, at concentrations higher than 10 μM, MTA decreased radioactivity incorporated into spermine. The increase in ornithine incorporation into putrescine was not due to a lack of putrescine metabolism, since total radioactivity incorporated into polyamines increased in parallel. Moreover, in these short-term experiments, MTA also elevated putrescine concentrations in a dose-dependent manner with no measurable effect on spermidine or spermine levels (Chart 4*A*).

Over a 48-hr period, MTA progressively increased putrescine levels in K562 cells and decreased spermine concentrations (Chart 5). The effect of MTA on spermidine concentrations was biphasic, with an initial increase at 10 to 100 μM MTA followed by a return to basal levels with increasing concentrations of the nucleoside. It is apparent from these results that among the MTA-induced changes in polyamine concentrations and synthesis in the leukemic cells, the increase in putrescine synthesis began earliest, was the most notable, and required the lowest concentrations of nucleoside. Similar results were obtained when the MTAase-negative leukemic cell line CEM was studied.

There was no detectable breakdown of MTA by enzyme-negative cells grown in the horse serum-containing medium. Thus, the effect of the nucleoside on polyamine metabolism was not attributable to a metabolic by-product, nor was the MTA at concentrations less than 5 μM toxic to the enzyme-negative cells.

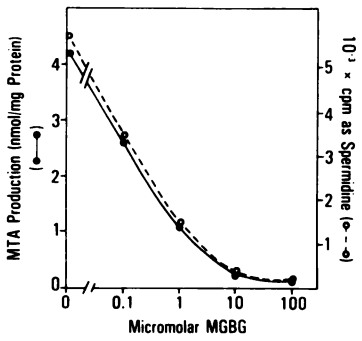

Chart 3. Effect of MGBG on MTA production and spermidine synthesis. K562 cells were cultured with or without MGBG at a cell density of 5 × 10^5/ml for 6 hr. ●, MTA accumulated in the medium; ○, [^{3}H]ornithine incorporated into spermidine.

Table 1

Effect of polyamines on MTA production

K562 cells were cultured for 6 hr at a density of 5 × 10^5/ml, and MTA production was measured.

Compound	MTA production (nmol/6 hr/mg protein)
None	4.90 ± 0.11[a]
Putrescine (10 μM)	5.80 ± 0.08
Spermidine (10 μM)	2.78 ± 0.23
Spermine (10 μM)	2.73 ± 0.14

[a] Mean ± S.D. obtained from 3 replicate cultures.

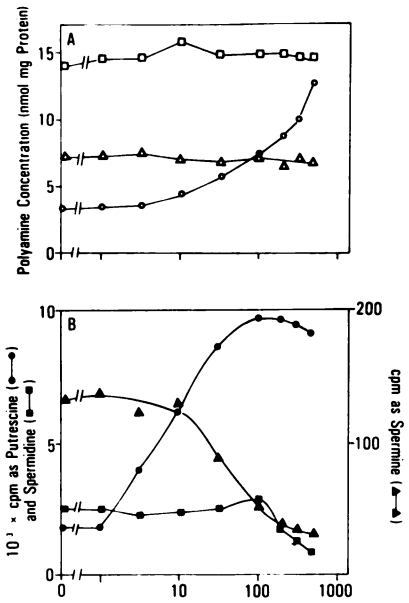

Chart 4. Effect of MTA on polyamine concentrations and on the incorporation of [^{3}H]ornithine into polyamines in short-term experiments. K562 cells (5× 10^5/ml) were cultured with [^{3}H]ornithine and various concentrations of MTA. After 6 hr, polyamine concentrations (*A*) and incorporation of radioactivity into polyamines (*B*) were measured. ○, ●, putrescine; □, ■, spermidine; △, ▲, spermine. Each *point* shows the mean of duplicate determinations. The pattern of each curve was reproduced in at least 2 different experiments.

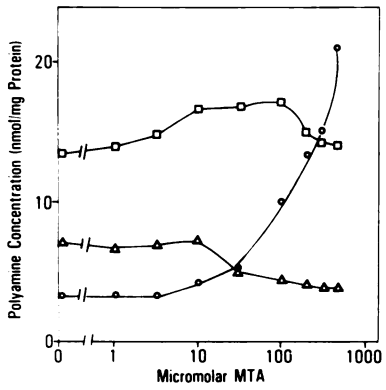

Chart 5. Effect of MTA on polyamine concentrations in long-term experiments. K562 cell cultures were started at an initial cell density of 1.25 × 10^5/ml with various concentrations of MTA added to the medium. After 48 hr, the concentrations of polyamines were measured. ○, putrescine; □, spermidine; △, spermine. Each *point* shows the mean of duplicate determinations. The pattern of each curve was almost the same in at least 2 different experiments.

DISCUSSION

The common occurrence of complete MTAase deficiency among human leukemic cell lines enabled us to examine for the first time the regulation of MTA synthesis by intact tumor cells and the effects of elevated MTA concentrations on polyamine metabolism. The MTAase-negative leukemic cell lines were completely unable to metabolize the nucleoside and indeed excreted large quantities of MTA into the culture medium

during periods of rapid growth. The concentration of the nucleoside in MTAase-negative leukemic cell cultures reached levels as high as 1 to 5 μM.

The production of MTA and the incorporation of [^{3}H]ornithine into spermidine were progressively inhibited by the *S*-adenosylmethionine decarboxylase inhibitor MGBG in almost the identical dose-dependent manner. These results strongly support experiments with partially purified enzymes, which have shown that MTA is a product of the synthesis of spermidine and spermine (18). The other polyamine-independent route of MTA production, which has been reported in the literature (29), contributes little, if at all, to MTA synthesis in viable human leukemic cell lines.

The determination of MTA excretion into the medium of growing cells is an accurate and independent measurement of spermine and spermidine production. Moreover, following MTA levels avoids the pitfalls associated with isotopic methods for measuring polyamine synthesis, which do not control for changes in endogenous pool sizes and alternative pathways of precursor metabolism. By this means, the rate of spermidine-spermine synthesis per cell was shown to be inversely related to cell density as early as 6 hr after resuspension in fresh medium. These results suggest that the synthesis of MTA, and hence of spermidine and spermine, is sensitive to the concentration of excreted metabolites which could include the various polyamines, as well as MTA itself (14, 20). Indeed, we were also able to demonstrate that exogenous putrescine at concentrations as low as 10 μM increased spermidine-spermine synthesis, whereas the latter 2 polyamines at similar concentrations significantly depressed their own production.

Inasmuch as MTA production was closely related to spermidine and spermine synthesis in the leukemic cells, so elevated concentrations of MTA induced marked alterations in polyamine metabolism. As low as 3 μM MTA increased the incorporation of radioactive ornithine into putrescine. As noted previously, this concentration of MTA could be achieved in unsupplemented medium during the routine maintenance of MTAase-negative cell lines. Furthermore, although MTA was excreted by the cells, it is probable that the concentration of the nucleoside under dynamic conditions was higher intracellularly.

The exact mechanism by which MTA increased putrescine synthesis remains uncertain. MTA is a known inhibitor of spermidine and spermine synthetases (9, 16), and indeed 32 μM or greater MTA decreased the synthesis and concentration of spermine in the MTAase-deficient leukemic cells. The lack of inhibition of spermidine synthesis by low concentrations of MTA remains unexplained. Similar effects have been noted for a described inhibitor of polyamine biosynthesis, *S*-adenosyl-(±)-2-methylmethionine, which inhibited spermine but not spermidine synthesis in concanavalin A-transformed lymphocytes (35). It is possible that the increased synthesis of putrescine induced by MTA resulted from a feedback mechanism sensitive to transient decreases in spermine and/or spermidine concentrations, which are rapidly corrected. Alternatively, MTA may have a direct effect on putrescine production.

The accelerated rate of putrescine synthesis induced by low concentrations of MTA might have a beneficial effect on the growth of the MTAase-deficient human leukemic cell lines. Both *in vivo* and in tissue culture, the onset of cell proliferation is preceded by an increase in putrescine synthesis (11, 13, 30). There is circumstantial evidence that such elevated putrescine levels have a growth-promoting effect on a variety of mammalian cells (8, 19, 21). Indeed, some investigators have postulated that an important aspect of the growth of malignant cells may be an altered polyamine-sensitive "restriction point" at the G_1-S boundary of the cell cycle (24).

There are 2 explanations for the common occurrence of MTAase deficiency among the human leukemic cell lines: (*a*) the normal cells from which the tumors arose may have been enzyme deficient; (*b*) the loss of the enzyme may have developed during the growth of the malignant cells *in vivo* or *in vitro*. If correct, the latter possibility requires that MTAase deficiency provide a selective advantage for the malignant cells. Our results suggest that MTA at low concentrations uniquely elevates intracellular putrescine concentrations. This change could provide a continuous pressure for the selection of MTAase-negative human leukemic cell lines.

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