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Report

Methylthioadenosine phosphorylase in human breast cancer

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Abstract

Methylthioadenosine (MTA) phosphorylase activity was measured in 47 biopsies from primary breast cancers (n = 34) and metastatic tumors (n = 13). Most specimens were also evaluated by DNA flow cytometry and determination of estrogen and progesterone receptor contents. Median MTA phosphorylase activity was 317 pmol/mg protein/min (range 50–1312 pmol/mg protein/min), but great variations were observed. Samples from four individuals had very low MTA phosphorylase activity (≤70 pmol/mg protein/min). No correlation with aneuploidy, receptor status, or the presence of metastases in the lymph nodes could be demonstrated. However, MTA phosphorylase activity showed a significant (p = 0.009) negative correlation with the fraction of cells in the S-phase of the cell cycle.

Introduction

Methylthioadenosine (MTA) is formed in stoichiometric amounts from S-adenosylmethionine during biosynthesis of the polyamines spermidine and spermine by dividing mammalian cells [1]. MTA exerts a negative feedback inhibition on certain steps in polyamine synthesis. In normal cells this effect is relieved by metabolic degradation of MTA to adenine and 5-methylthioribose 1-phosphate. This reaction is catalyzed by the ubiquitous enzyme MTA phosphorylase [1, 2]. These metabolic relations are depicted in Fig. 1.

MTA phosphorylase has considerable activity in normal tissues tested so far [2], including human prostate, placenta, muscle, and erythrocytes [3–6]. In 1977, Toohey reported that MTA phosphorylase was lacking in four murine tumor cell lines [7]. Since this pioneering work, lack of MTA phosphorylase has been demonstrated in 23% of human malignant cell lines [8], and in human leukemic cells in vivo [9].

The mechanism and possible implications of MTA phosphorylase deficiency are subjects of considerable interest. MTA phosphorylase is encoded in a fragile region of chromosome 9, and deletions or translocations affecting this region may be responsible for loss of enzyme activity [10].

Lack of MTA phosphorylase may be an adaptive process which probably has a considerable impact on polyamine synthesis and transmethylation reactions as well as metabolism of adenine nucleotides and methionine [10]. This in turn may affect the growth potential of the tumor cell. Finally, exploitation of MTA phosphorylase deficiency in cancer chemotherapy has been suggested [8].

Several prognostic factors have been established in human breast cancer. These include tumor size, lymph node metastasis [11, 12], steroid receptor status [11, 13], and also ploidy and S-phase fraction as determined by flow cytometry [13–15]. It is conceivable that MTA phosphorylase may also be related to the growth potential of malignant tumors

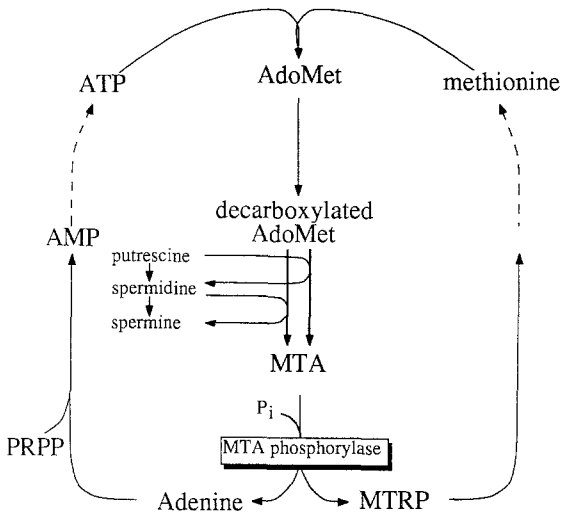

Fig. 1. Diagram of the metabolism of MTA, polyamines, and related compounds. AdoMet, S-adenosylmethionine; MTA, methylthioadenosine; MTRP, 5'-methylthioribose-1-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate.

in humans. This enzyme activity and its possible prognostic impact has not been evaluated in breast cancer or other human solid cancers. Therefore, we determined the enzyme activity in breast cancer biopsies and correlated this activity to several known prognostic factors, as listed above.

Patients and methods

Patient characteristics and collection of tissue specimens

Tumor samples were obtained from 47 patients, median age 64 years (range 35–89), who either underwent modified radical mastectomy with axillary node dissection for a primary breast cancer (34 patients), or were biopsied from local or regional metastatic tumors (13 patients). Axillary nodes were dissected out, the tissue specimens were cleared in cedar wood oil [16], and the per cent positive nodes was recorded. MTA phosphorylase activity was determined in tumor samples from 47 patients, receptor status in 46 samples, and DNA histogram in 30 samples. Patient characteristics recorded are summarized in Table 1.

The tumor specimens were divided into four parts. The portions used for determination of enzyme and receptor activities were immediately put into liquid nitrogen and stored at −70° C until use. The tumor specimens for flow cytometry were placed in 0.9% NaCl at 4° C for up to 2 hours, whereas the parts to be examined microscopically were fixed in formalin, embedded in paraffin, and stained in haematoxylin-eosin.

Microscopic examination revealed that all patients had infiltrating carcinoma, except for one patient who had Paget's disease of the nipple.

Materials

MTA, adenine, and RNAase (1 mg/ml in distilled water) were obtained from Sigma Chemical Co., St. Louis, Mo, USA, and pepsin from Rikshospitalets Apotek, Oslo, Norway. Packing materials for HPLC (Partisil SCX, 10 μm, and ODS Hypersil, 3 μm) were obtained from HPLC Technology, Cheshire, U.K., and Shandon Southern Products Ltd, Cheshire, U.K., respectively. Pelliguard LC 18, 40 μm, was from Supelco, Bellafonte, PA, USA. The columns (Partisil SCX, 0.46 × 25 cm; OSD Hypersil, 0.46 × 10 cm) were packed in stainless steel tubes, using a column packer from Shandon Southern, Ltd.

Table 1. Clinical material and parameters recorded.

Parameter	
Age of patients in years median (range)	64 (35–89)
Premenopausal	7 patients
Postmenopausal	40 patients
Primary tumors	34 patients
Stage I	3 patients
Stage II	15 patients
Stage III	12 patients
Stage IV	4 patients
Local recurrence/regional metastatic tumor	13 patients
MTAase measurements	47 specimens
Receptor measurements	46 specimens
DNA histograms	30 specimens

Determination of MTA phosphorylase activity

Frozen tissues were pulverized in a pre-cooled mortar, and then immediately homogenized in 10 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM KH_2PO_4, using an Ultra-Turrax homogenizer.

Tissue extract (9.5% v/v), KH_2PO_4 (50 mM) in 50 mM Tris-HCl buffer, pH 7.5, was preincubated for 5 minutes at 37 °C in a total volume of 500 μl in 1.5 ml polypropylene tubes. The reaction was started by addition of MTA (150 μM). After 2, 5, 10, and 20 minutes of incubation at 37 °C, the reaction was terminated by mixing 50 μl aliquots with an equal volume of ice-cold 0.8 N perchloric acid. The acidic mixture was centrifuged at 9000 g for 5 minutes.

The reaction product, adenine, was separated from MTA by HPLC. Samples (25–75 μl) of the perchloric acid extract were injected into a Partisil SCX column which had been equilibrated with 60 mM ammonium formate, pH 3.5, containing 1% 1-propanol. The column was eluted isocratically with the same mobile phase, at a flow rate of 1.5 ml/min. The absorbance of the effluent was recorded at 254 nm. The identities of MTA and adenine were determined by retention times in this system, and were confirmed by rechromatography on an ODS Hypersil column eluted as described [17].

The chromatographic system consisted of a Perkin-Elmer ISS 100 autosampler for HPLC, a Spectra-Physics SP 8700 solvent delivery system, a Beckman model 160 ultra-violet detector, and a Hewlett-Packard HP 3390 A integrator.

The amount of product formed was linear with respect to time of incubation and concentration of tissue extract. The MTA phosphorylase activity is given as pmol adenine formed per mg of protein per min.

Flow cytometry

The tumor samples were minced with scissors in cold saline, and disintegrated with a whirlmixer to further loosen cell adhesions, in order to obtain a maximal yield of single cells in dispersion. The resulting suspension was syringed through a 0.8 mm gauge, and then centrifuged at 1500 rpm for 10 minutes. The cell pellet was resuspended in 1 ml saline, and fixed in cold (−30 °C) 96% ethanol. After fixation, the cells were washed twice in saline, incubated with 3–4 ml pepsin (0.5% in 0.2% HCl) at 37 °C for 30 minutes, washed once in saline, and treated with RNAase (1 mg/ml). Then the sample was filtered through a 50 μm mesh. For specific staining of DNA, a solution of ethidium bromide (7.14 mg/ml) plus mithramycin (7.14 mg/ml) in Tris buffer, pH 7.5, was added [18].

For the DNA analysis, a Phywe ICP 22 Pulse Cytophotometer (Ortho Diagnostic Systems) was used. Ten to seventy thousand nuclei were routinely collected for each analysis. The coefficient of variation of both the diploid and aneuploid G1-peak ranged from 1–5%. Human lymphocytes were used as a diploid standard by adding them to the tumor sample as an external control. The DNA index was calculated from the ratio between the G1-peak of the tumor cells and the G1-peak of the lymphocytes. The DNA index of the diploid tumor cells was 2.0 ± 0.1. Tumor cells were considered to be aneuploid if a separate G1-peak with DNA index outside this range was present. Tumor cells with DNA index equal to $4.0c \pm 0.2$ and the presence of a distinct S- and G2 + M-phase were considered tetraploid. The number of doublets was estimated by fluorescence microscopy showing that doublets were negligible.

Cell cycle distribution was estimated according to a modification of the rectangular method of Baisch et al. [19]. The number of cells in the mid S-phase was counted, and the number of cells located between the G1- and G2 + M-peaks, which represents the S-phase, could then be calculated. This was done by dividing the number of cells in the mid s-phase by the corresponding channel interval in the histogram, and multiplying this with the difference in channel number of the G1- and G2 + M-peaks. The remaining part of the G1-peak represented the G1-phase, and the remaining part of the G2 + M-peak represented the G2 + M-phase.

By counting the cells in mid S-phase and in this way calculating the number of cells in the S-phase, there will be a minor or no overlap with S- and

G2 + M-phase of diploid tumors with aneuploid tumors with DNA indices equal to or higher than 3.0. The reason is that the mid S-phase of the aneuploid population will be located largely or totally outside the S- and G2 + M-phase of the diploid population. In our material only 5 patients had a DNA index between 2.0 and 3.0. The rest of the patients with aneuploid tumors had a DNA index of 3.5 or higher.

Determination of estrogen and progesterone receptor activities

These assays, based on adsorption of free steroids by dextran-coated charcoal, have been described previously [20].

Determination of protein

Protein was determined by the method of Bradford [21] using a kit from Bio-Rad, or by the method of Lowry *et al.* [22]. Bovine serum albumin was used as standard.

Statistical analysis

Statistical analyses were done using the Wilcoxon Rank Sum Test and the Spearman Rank Correlation Test.

Results

Characterization of tumors by flow cytometry and steroid receptor status

DNA histograms were obtained from 30 out of 47 tumors samples. The distribution between diploid and aneuploid tumors were 43% and 57%, respectively. The S-phase fraction could be estimated in 28 cases, and was for the diploid tumors 6.7% and for the aneuploid tumors 16.5%. These values are in accordance with data published previously [23–27].

Evaluation of receptor status showed that 17.4% were estrogen receptor negative, and 37% were progesterone receptor negative (<10 pmol/g).

MTA phosphorylase activity

The median value for MTA phosphorylase activity in these tumor samples was 317 pmol/mg protein/min. However, a wide distribution (range 50–1312) was observed (Fig. 2). No tumor sample was MTA phosphorylase negative. There was no significant difference in MTA phosphorylase activity between primary and metastatic tumors ($P>0.1$), and there was no relationship between the enzyme activity and age of the patient (data not shown).

The median value for MTA phosphorylase activity was 288 pmol/mg protein/min for diploid tumors and 317 pmol/mg protein/min for aneuploid tumors. This difference was not statistically significant. There was no significant correlation between MTA phosphorylase activity and the estrogen or progesterone receptor level, and no correlation was found between enzyme activity and T category or the nodal status (data not shown).

The MTA phosphorylase activity showed a statistically significant negative correlation ($n = 28$, $p = 0.009$, Spearman rank test) with % S-phase of the cell cycle (Fig. 3).

Discussion

Most tumor specimens tested for MTA phosphorylase activity in the present work were evaluated by flow cytometry and determination of estrogen and progesterone receptors as well as tumor size and axillary node status. Based on the flow cytometric parameters and receptor status, the tumors investigated showed properties typical for breast cancer, as reported by others [11, 13, 23–27]. These parameters may serve as useful prognostic markers.

The search for markers for cell proliferation has stimulated numerous studies to investigate the relationship between cell proliferation and polyamines and enzymes involved in polyamine biosynthesis. Cancer cells contain higher levels of

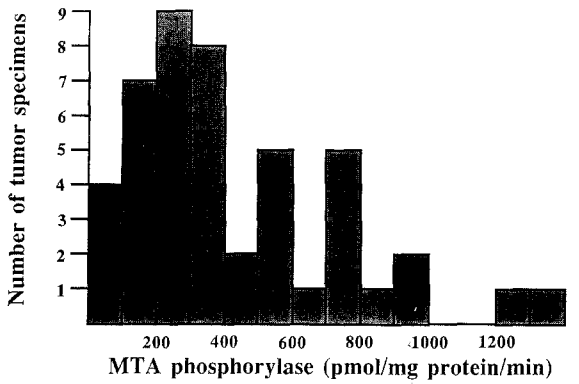

Fig. 2. The distribution of MTA-phosphorylase activity in biopsies from breast cancer.

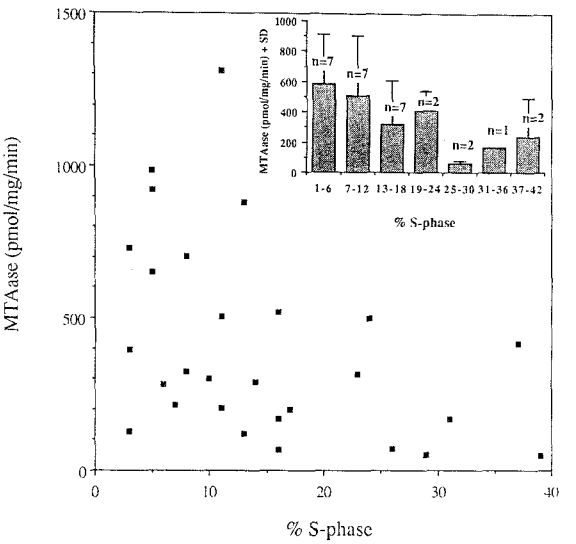

Fig. 3. The relation between % S-phase and MTA-phosphorylase activity in the tumor specimens.

polyamines than normal cells [10, 28–31]. Measurements of polyamines have been performed in human cancer [32–34], including breast cancer, and polyamine content has been related to the tumor size and nodal status [33–34].

There is a close relation between metabolism of polyamines and MTA. Furthermore, consistent reports have been published on the lack of MTA phosphorylase in some cancer cells [7–9], including a cell line derived from human breast cancer [8], and in human leukemia cells *in vivo* [9]. In spite of this, MTA phosphorylase has not been determined in human breast cancer *in vivo*.

We investigated MTA phosphorylase activity in 47 samples from human breast cancer. The enzyme activity varied over a wide range from 50 to 1312 pmol/mg protein/min. No tumor was MTA phosphorylase negative. This contrasts to the distribution of MTA phosphorylase *in vivo* in human leukemias, which were either MTA phosphorylase positive or negative. Leukemic cells with intermediate levels of enzyme were not found [9]. The varying enzyme activities in breast cancer may reflect the heterogeneity displayed in breast cancer cells both within a particular tumor and between tumors from different patients [15]. In addition, the presence of non-malignant cells in the tumor may be a source to MTA phosphorylase activity. It is conceivable that the tumor cells in some specimens are lacking MTA phosphorylase and that the low enzyme activity in these specimens stems from non-malignant cells.

The mechanism behind the MTA phosphorylase deficiency in some malignant cells is a subject of current interest. We found no relation between aneuploidy and MTA phosphorylase activity. This supports the view [10] that lack of MTA phosphorylase is not associated with gross karyotypic abnormality. Furthermore, no patient included in the present study had received chemotherapy against cancer, and the low enzyme activity in our material is therefore not induced by anticancer agents.

We found no relationship between MTA phosphorylase activity and some parameters which are associated with the aggressiveness of the tumor: nodal metastases, large tumor size, or aneuploidy. Similarly, Kingsnorth et al. [34] reported that no relationship exists between polyamine levels in primary breast cancer and tumor size or nodal status [34], but they could demonstrate a correlation with the estrogen receptor status [34]. We found no relation between the estrogen and progesterone receptor activities and MTA phosphorylase activity.

It is noteworthy that a significant negative correlation exists between MTA phosphorylase activity and S-phase fraction. This may be explained either by low MTA phosphorylase activity in rapidly proliferating cells measured as a high

S-phase fraction, or by a low S-phase fraction of cell populations rich in MTA phosphorylase.

Differentiation between these two possibilities and evaluation of the biological implications are difficult since data on the possible role of MTA and MTA phosphorylase in cell proliferation are not conclusive. In isolated cells, MTA phosphorylase peaks at late G_1, but shows a transient decrease during S-phase [35]. However, loss of MTA phosphorylase activity in a mutant murine lymphoid cell line did not alter growth rate, cloning efficiency, or tumor forming ability compared to the MTA phosphorylase containing parent cells [36]. Lack of MTA phosphorylase probably causes elevated levels of cellular MTA. Low concentrations of MTA may support growth of cells in culture [37, 38] whereas high concentrations of MTA have a cytostatic effect [10, 37–41]. One may speculate whether low levels or lack of MTA phosphorylase in breast cancer cells and other malignant cells may be an adaptive mechanism opposing the effect of a continuous stimulus favoring uncontrolled growth.

In conclusion, the present evaluation of MTA phosphorylase as a biochemical marker in breast cancer did not reveal any relationship between the enzyme activity in tumor biopsies and some established clinical parameters. However, the enzyme activity varied over a wide range and showed a negative correlation with the fraction of cells in the S-phase of the cell cycle. The biological and clinical implications of this finding remain to be settled.

Acknowledgements

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