Methylthioadenosine Phosphorylase cDNA Transfection Alters Sensitivity to Depletion of Purine and Methionine in A549 Lung Cancer Cells

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ABSTRACT

Methylthioadenosine phosphorylase (MTAP), an enzyme involved in purine and methionine metabolism, is present in all normal tissues but is frequently deficient in a variety of cancers. It has been suggested that this metabolic difference between normal and cancer cells may be exploited to selectively treat MTAP-negative cancers by inhibiting de novo purine synthesis and by depleting l-methionine. However, these therapeutic strategies have only been tested in naturally occurring MTAP-positive and -negative cell lines, which might have additional genetic alterations that affect chemotherapeutic sensitivity. Therefore, it is of importance to examine the feasibility of enzyme-selective treatment using paired cell lines that have an identical genotype except for MTAP status. MTAP-negative A549 lung cancer cells were transfected with eukaryotic expression vectors encoding MTAP cDNA in sense and antisense orientations. The resultant stable transfectomas were treated with inhibitors of de novo purine synthesis such as methotrexate, 5,10-dideazatetrahydrofolate, and l-alanosine and by methionine depletion. The A549 cells transfected with an antisense construct (antisense transfectoma) expressed no MTAP protein and were more sensitive to both purine and methionine depletion than were cells expressing MTAP protein (sense transfectoma). Methylthioadenosine phosphorylase was able to completely rescue the sense transfectedoma but not the antisense transfectoma from growth inhibition by depletion of purine and methionine. These results prove that MTAP deficiency contributes directly to the sensitivity of cancer cells to purine or methionine depletion. Inhibition of de novo purine synthesis, combined with methionine depletion in the presence of methylthioadenosine, is a highly selective treatment for MTAP-negative cancers.

INTRODUCTION

MTAP1, an enzyme involved in purine and methionine metabolism (Fig. 1), is present in all normal tissues but is frequently deficient in many kinds of cancer cell lines and primary tumors such as leukemias, brain tumors, non-small cell lung cancers, and pancreatic cancers (1-5). The gene for the enzyme resides on chromosome 9p21 in close proximity to the tumor suppressor genes CDKN2A and CDKN2B, and these genes are frequently codeleted (6, 7). MTAP-negative cancer cells cannot use MeSAdo, a substrate of MTAP, to produce adenine and methionine under conditions in which purines and methionine are readily to the sensitivity of cancer cells to purine or methionine depletion. Inhibition of de novo purine synthesis, combined with methionine depletion in the presence of methylthioadenosine, is a highly selective treatment for MTAP-negative cancers.

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3 The abbreviations used are: MTAP, methylthioadenosine phosphorylase; MeSAdo, methylthioadenosine; DDATHF, 5,10-dideazatetrahydrofolate; MTX, methotrexate; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

chchemosensitivity of cancer cells has never been evaluated in syngeneic human cancer cell lines with different MTAP status.

In this report, we generated MTAP transfectomas from the MTAP-negative human lung cancer cell line A549, because the sensitivity to de novo purine synthesis inhibitors and methionine depletion in this cell line has been studied in a previous report (4). We then determined the precise role of MTAP in the chemosensitivity of cancer cells by using these transfectomas. The results of the present study prove that MTAP-negative cancers can be treated selectively and efficiently by the combination of de novo purine synthesis inhibitors and methionine starvation.

MATERIALS AND METHODS

Cell Lines. Lung cancer cell lines A549 and A427 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% dialyzed horse serum (Life Technologies), which lacks MTAP activity (9).

Chemicals. DDATHF was kindly provided by Lilly Research Laboratories (Indianapolis, IN). DDATHF was dissolved in 0.1 N NaOH and adjusted to pH 7.4 with PBS (10). l-Alanosine was generously provided by the Drug Synthesis and Chemistry Branch (National Cancer Institute). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Transfection with MTAP Expression Vectors. To construct MTAP expression vectors, the human MTAP cDNA (11) was inserted into a EcoRI site of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA). The orientation of the insert was determined by digesting plasmid DNA with HindIII. Plasmid DNA containing MTAP cDNA in either sense or antisense orientation (designated sense or antisense MTAP expression vector, respectively) was prepared in a large quantity by using the Qiagen plasmid kit (Qiagen-tip; Qiagen, Inc., Chatsworth, CA) according to the supplier’s instructions. Twenty μg of either sense or antisense MTAP expression vector was used to transfect A549 cells grown at 80% confluency in a 75-cm2 tissue culture flask using the calcium phosphate transfection system (Life Technologies).

Cloning of Stable Transfectomas. After transfection with MTAP expression vectors, cells were selected in DMEM containing G418 sulfate (Geneticin; Life Technologies) at 800 μg/ml. Following incubation for 2 weeks in the presence of G418, resistant cells were collected and subjected to cloning by limiting dilution. Individual clones were screened for the presence of MTAP transcript and protein by RT-PCR and Western blot analysis, respectively, as described below. The growth rate of each transfectoma was determined by counting the viable cell number with the erythrosin B dye exclusion method after seeding 1 X 105 cells in six-well tissue culture plates (Falcon 3046; Becton Dickinson, Lincoln Park, NJ).

RT-PCR Analysis. For detection of the MTAP transcript in the transfectomas, total RNA was isolated with RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX) and transcribed to cDNA using a Superscript preamplification system (Life Technologies) followed by PCR amplification of a partial sequence of the MTAP gene as well as the GAPDH gene as a control. The PCR amplification was carried out in a 25-μl reaction mixture containing 1 μg of transcribed cDNA, 1X PCR buffer [10 mm Tris-HCl (pH 8.0), 50 mm KCl, 1.5 mm MgCl2, and 0.001% gelatin], 400 μM of each deoxynucleoside triphosphate, 50 ng each of sense and antisense primers, and 2.5 units of Taq polymerase (Stratagene, La Jolla, CA). Thirty cycles were performed with the programmable cyclic reactor (GeneAmp PCR system 9600; Perkin Elmer, Norwalk, CT). Each cycle consists of denaturation at 94°C for 1 min, annealing
at 55°C for 1 min, and extension at 72°C for 1 min. The PCR product was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Primers used for amplification of the MTAP and GAPDH genes are: MTAP sense primer, 5'-CCCAAAAACGAGAGGAGTGCTCTTTATAT-3'; MTAP antisense primer, 5'-ACCTGACGGTCCGCCTTCTTCTC-3'; GAPDH sense primer, 5'-AAGAAGATGCGCTAGCTGCAAGCCCAT-3'; and GAPDH antisense primer, 5'-CTCTATGTTCAACCCATGACGAAACATG-3'.

Western Blot Analysis. MTAP protein was detected by Western blotting as described (3, 4), with a modification as follows. Crude cell extracts were prepared from transfectedomas, separated in 12% polyacrylamide gels containing 0.1% SDS, and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). Blots were incubated with a rabbit anti-MTAP antiserum diluted to 1:200 for 1 h, followed by detection of a 32-kilodalton trimeric subunit of MTAP protein with a Western-Light chemiluminescent detection system (Tropix, Inc., Bedford, MA).

Consumption of MeSAdo by Stable Transfectomas. MeSAdo in medium is metabolized by MTAP-positive cells but not by negative cells. Therefore, the disappearance of MeSAdo from culture medium is an indicator of the presence of active MTAP in the cells. To determine the functionality of MTAP protein expressed in transfectedomas, sense and antisense transfectedomas were incubated for 4 days in the presence of 16 μM MeSAdo in methionine-free DMEM or in DMEM supplemented with 0.3 μM DDATHF. MeSAdo was extracted with ice-cold 0.5 M perchloric acid from aliquots of medium collected every 12 h and quantitated by ion pair reverse-phase high-performance liquid chromatography as described (12).

In Vitro Treatment of Transfectomas with Inhibitors of de Novo Purine Synthesis and with Methionine Depletion. Transfectomas were cultured at an initial cell density of 0.5 × 10^6 cells/ml in DMEM containing MTX, L-alanosine, or DDATHF at varying concentrations in 96-well plates. The medium containing MTX was supplemented with 16 μM thymidine to abolish the antipyrimidine action of MTX. Similarly, the sensitivity of transfectedomas to methionine depletion was determined by culturing cells in methionine-free DMEM. To determine the effect of the combination of de novo purine synthesis inhibitors and methionine depletion, transfectedomas were incubated with MTX, L-alanosine, or DDATHF in methionine-free medium. MeSAdo was added to the medium at 16 μM to assess its rescue effect on the growth inhibition by de novo purine synthesis inhibitors or by methionine depletion. The viability of the cells was measured spectrophotometrically with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylenetetrazolium bromide (13) after a 3-day incubation.
Depletion. The sense and antisense transfectomas characterized transfectoma has enzyme activity, and that transfection of expression cells; 2, A549 cells; 3, sense transfectoma; 4, antisense transfectoma.

The subunit of MTAP protein was detected by chemiluminescence as described in "Materials and Methods." Lanes: 1, A427 cells; 2, A549 cells; 3, sense transfectoma; 4, antisense transfectoma.

**DISCUSSION**

In general, anticancer drugs are toxic not only to cancer cells, but also to normal cells. If there is a metabolic difference between normal and cancer cells, one could exploit this difference to develop selective chemotherapy. MTAP deficiency is one of a few clear-cut metabolic differences between normal and cancer cells. Since its discovery in murine leukemias (14), many human cancer cell lines and primary tumors have also been found to be deficient in this enzyme (1–5). It was previously demonstrated that the growth of MTAP-negative cancer cell lines can be selectively inhibited by de novo purine synthesis inhibitors MTX, d-alanosine, and DDATHF. As expected, the rescue effect of MeSAdo was not observed in the antisense transfectoma. The rescue effect for the sense transfectoma was complete in L-alanosine treatment but not in treatment with MTX or DDATHF at the concentration that causes significant growth inhibition in the antisense transfectoma. As shown in Fig. 4, when the sense transfectoma was treated similarly in methionine-free medium, MeSAdo completely rescued the cells from growth inhibition by all three inhibitors tested. The other sense transfectoma expressing a lesser amount of MTAP protein was not studied further.

**Table 1** Growth of sense and antisense transfectomas in methionine-free medium supplemented with or without MeSAdo

<table>
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<tr>
<th>Transfectoma</th>
<th>Growth (% of control)</th>
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<tr>
<td></td>
<td>Without MeSAdo</td>
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<tr>
<td>Sense transfectoma</td>
<td>68 ± 9.0</td>
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<tr>
<td>Antisense transfectoma</td>
<td>48 ± 6.7</td>
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*a Each value is the mean ± SD of three determinations.

cell-doubling times were 21.0, 22.6, and 22.1 h, respectively, for parental cells, the sense transfectoma, and the antisense transfectoma.

These results indicate that MTAP protein expressed in the sense transfectoma has enzyme activity, and that transfection of expression vectors caused no changes in the morphology or proliferation rate of the transfectomas.

**In Vitro Sensitivity of Transfectomas to Purine and Methionine Depletion.** The sense and antisense transfectomas characterized above were used to elucidate the role of MTAP in cell growth inhibition by methionine depletion and by inhibitors of de novo purine synthesis. The two transfectomas demonstrated different sensitivities to methionine depletion (Table 1). The growth of the antisense transfectoma was inhibited to 47.5% of control by methionine depletion, whereas the sense transfectoma was less sensitive to methionine depletion (67.8% of control). The growth inhibition of the sense transfectoma by methionine depletion was completely prevented by the addition of 16 μM MeSAdo to the growth medium. In contrast, MeSAdo was not able to restore the growth of the antisense transfectoma cultured in the absence of methionine.

These transfectomas were also incubated with de novo purine synthesis inhibitors MTX, d-alanosine, and DDATHF, the sites of metabolic inhibition of which are shown in Fig. 1. Instead of the commonly used IC50, we determined and used the IC25 in the present experiments, because the synergistic or additive effects of purine and methionine depletion may easily be observed. In methionine-containing medium, the IC25 in the sense transfectoma was achieved at concentrations of 0.77 μM for MTX, 9.1 μM for d-alanosine, and 0.60 μM for DDATHF. In the antisense transfectoma, 3.5-, 7.5-, and 4.0-fold lower concentrations of these inhibitors, respectively, exerted equivalent growth-inhibitory effects (Table 2). Moreover, the IC25 in methionine-free medium for d-alanosine and for DDATHF was greater than 12-fold lower in the antisense transfectoma than that in the sense transfectoma, whereas the IC25 in methionine-free medium for MTX was 6.2-fold lower in the antisense transfectoma than that in the sense transfectoma. It is noteworthy that the IC25 for these three inhibitors decreased substantially (20–87%), when both transfectomas were cultured in methionine-free medium. Particularly, the IC25 for d-alanosine decreased by 73 and 87%, respectively, in the sense and antisense transfectomas. These results indicate that methionine depletion enhances growth inhibition by inhibitors of de novo purine synthesis.

Fig. 3 demonstrates the rescue effect of MeSAdo on the growth inhibition of the sense and antisense transfectomas by de novo purine synthesis inhibitors in methionine-containing medium. MeSAdo rescued the sense transfectoma from the toxicity of MTX, d-alanosine, and DDATHF. As expected, the rescue effect of MeSAdo was not observed in the antisense transfectoma. The rescue effect for the sense transfectoma was complete in d-alanosine treatment but not in treatment with MTX or DDATHF at the concentration that causes significant growth inhibition in the antisense transfectoma. As shown in Fig. 4, when the sense transfectoma was treated similarly in methionine-free medium, MeSAdo completely rescued the cells from growth inhibition by all three inhibitors tested. The other sense transfectoma expressing a lesser amount of MTAP protein was not studied to see whether the increased drug sensitivity depends on the expressed level of MTAP protein.
synthesis inhibitors or by methionine depletion in the presence of MeSAβo (1, 4, 15). However, MTAP-positive and -negative human cancer cell lines studied previously were not established from the same tumor sample and had differences in their genetic backgrounds, in addition to that in MTAP status. Such differences might alter sensitivity to purine or methionine depletion, independently of MTAP. To prove the role of MTAP in the chemosensitivity of cancer cells, we established a transfection model by transfecting parental MTAP-negative A549 lung cancer cells with MTAP sense and antisense expression vectors. The MTAP protein expressed in the sense transfectoma was functional, whereas no MTAP protein was detected by Western blotting in the antisense transfectoma.

The antisense transfectoma cells were more sensitive to inhibition of de novo purine synthesis and methionine depletion than the sense transfectoma cells. This occurs because adenine and methionine are synthesized endogenously from MeSAβo in the enzyme-positive cells, but not in the enzyme-negative cells, when these nutrients are depleted. Although both therapeutic strategies have been shown to selectively kill MTAP-negative cancer cells in separate experiments (4), they have never been studied in combination. Therefore, we tested the combination therapy of purine and methionine starvation in the MTAP transfectomas and also tested three inhibitors of de novo purine synthesis that act at separate sites in purine metabolism (Fig. 1). Our results indicate that methionine depletion enhances cytotoxic effects of de novo purine synthesis inhibitors on MTAP-negative cells without affecting the rescue effect of MeSAβo on MTAP-positive cells (Table 2 and Fig. 4). However, the efficacy of MeSAβo in rescuing the sense transfectoma cells from growth inhibition varied depending on the inhibitor used. MeSAβo completely restored the growth-inhibitory effect of L-alanosine but not toxicity of DDATHF or MTX at higher concentrations (Figs. 3). The contrasting rescue effects of MeSAβo may be attributable to the mode of action of the three inhibitors in purine metabolism. L-alanosine condenses with aminoimidazole carboxylate ribonucleotide to inhibit adenylosuccinate synthetase (16) and selectively blocks the synthesis of AMP, whereas DDATHF or MTX combined with thymidine blocks the synthesis of IMP, which is the source of both AMP and GMP (Fig. 1). Because MeSAβo is converted to adenine, which is the source of AMP, MTAP-positive cells can replete AMP more easily than GMP in the presence of MeSAβo. This explains why MeSAβo was able to completely rescue the sense transfectoma from the growth inhibition by L-alanosine but not by DDATHF or MTX and thymidine at higher concentrations.

In methionine-free medium supplemented with MeSAβo, MTX was
not as selectively growth inhibitory to MTAP-negative cells as either L-alanosine or DDATHF (Table 2). In addition to the inhibition of purine and pyrimidine synthesis, MTX also blocks other folate-dependent reactions such as the regeneration of methionine from homocysteine and pyrimidine synthesis, MDC also blocks other folate-dependent reactions such as the regeneration of methionine from homocysteine and pyrimidine synthesis, MDC also blocks other folate-dependent reactions such as the regeneration of methionine from homocysteine and pyrimidine synthesis, MDC also blocks other folate-dependent reactions such as the regeneration of methionine from homocysteine and pyrimidine synthesis, MDC also blocks other folate-dependent reactions such as the regeneration of methionine from homocysteine and pyrimidine synthesis.

Chemosensitivity in A549 Cells Transfected with MTAP cDNA

Fig. 3. Sensitivity of the transfec toms to inhibitors of de novo purine synthesis and growth rescue effects of MeSAdo in methionine-containing medium. Cells were treated with MTX (A), L-alanosine (B), and DDATHF (C) at the indicated concentrations at an initial cell density of 0.5 × 10⁶ cells/ml (200 μl/well) in methionine-containing medium with or without MeSAdo. Sixteen μM thymidine was added in medium with MTX to circumvent the inhibitory effect on thymidylate synthetase. The viability of the cultured cells was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide after a 3-day incubation. Each point is the mean of three determinations. The SD was less than 15% of the mean. △, sense transfec toma; ■, sense transfec toma in the presence of MeSAdo; Δ, antisense transfec toma; □, antisense transfec toma in the presence of MeSAdo.

REFERENCES


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CHEMOSENSITIVITY IN A549 CELLS TRANSFECTED WITH MTAP CDNA

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