Antisense Inhibition of Methylene tetrahydrofolate Reductase Reduces Cancer Cell Survival In vitro and Tumor Growth In vivo

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ABSTRACT

Purpose: Many cancer lines are methionine dependent and decrease proliferation when methionine supply is limited. Methylene tetrahydrofolate reductase (MTHFR) generates the folate derivative for homocysteine remethylation to methionine. We investigated the effect of antisense-mediated inhibition of MTHFR on survival of human cancer cells.

Experimental Design: We examined the in vitro and in vivo anticancer effects of a combination of MTHFR antisense and standard cytotoxic drugs.

Results: Specific antisense against MTHFR (EX5) showed significant inhibitory effects on growth of human colon, lung, breast, prostate, and neuroblastoma tumor cells in vitro compared with that of the control oligonucleotide. Cytotoxic drugs (5-fluorouracil, cisplatin, or paclitaxel) potentiated the effect of EX5. In vivo, antisense alone or in combination with cytotoxic drugs inhibited the growth of human colon and lung carcinoma xenografts. In comparison with control oligonucleotide, treatment with EX5 inhibited growth of colon tumors and lung tumors by 60% and 45%, respectively. EX5 with 5-fluorouracil decreased growth of colon tumors by an additional 30% compared with EX5 alone, and EX5 with cisplatin decreased growth of lung tumors by an additional 40% compared with cisplatin alone. Growth inhibition by EX5 was associated with decreased amounts of MTHFR protein and with increased amounts of an apoptosis marker.

Conclusions: Our results confirm that MTHFR inhibition decreases tumor growth and suggest that inhibition of MTHFR by antisense or small molecules may be a novel anticancer approach.
MATERIALS AND METHODS

Cell Lines. Human colon (SW-620), lung (A-549), breast (MCF-7), and prostate (PC-3) carcinoma and human neuroblastoma (SK-NFi) tumorigenic cell lines were purchased from American Type Culture Collection (Rockville, MD) and cultured according to instructions of the supplier. SW-620 and SK-NFi lines were grown in MEM, MCF-7 in RPMI 1640, and PC-3 and A-549 in F-12K medium. Cells were maintained in 75-cm² flasks or 10-cm² Petri dishes in a humidified 37°C incubator in 5% CO₂.

Antisense Oligonucleotides. The 20-mer desalted phosphorothioate oligonucleotides were purchased from the University of Calgary (University Core DNA Services, Calgary, Alberta, Canada). All CpG dinucleotides were methylated for in vivo experiments because unmethylated CpG motifs are potential immune stimulators. The EX5 MTHFR specific antisense (EX5; sequence 5′-AGC TGC CGA AGG GAG TGG TA-3′) targets nucleotides 796 to 815 in exon 5 of the human MTHFR mRNA. Selection of EX5 was based on the results of our previous work. The control scrambled antisense oligodeoxynucleotide (CTS; sequence 5′-TGT TGA CAA AAG GAG TGG TG-3′) has the same base composition in random order. The control 6-bp mismatched antisense oligodeoxynucleotide (CT6; sequence 5′-GTG GAC TAG GAC AGC-3′) was used only for in vitro experiments. These antisense oligodeoxynucleotide sequences did not show any homology to known human genes in a BLAST search of the National Center for Biotechnology Information.

Transfection with Oligonucleotides. Cells in the exponential growth phase were seeded at 50,000 per well in triplicate in six-well tissue culture plates and incubated overnight in complete medium. Transfection mix contained serum-free OPTI-MEM medium and Lipofectin at 10 μg/mL/200 nmol/L of antisense. Lipofectin was allowed to complex with antisense oligodeoxynucleotide for 30 minutes before use. Complete medium was washed away from each well with OPTI-MEM and then overlaid with transfection mix for 5 hours. Transfection mix was then replaced with complete medium alone for incubation at 37°C overnight. The transfection was repeated the next day and cells were counted 24 hours after the second round of transfection, using the Cell Counting Kit-8 (Dojindo, Gaithersburg, MD). Each experiment was done twice, in triplicate.

In vitro Treatment with Cytotoxic Drugs and Antisense. Cytotoxic drugs were selected based on the known sensitivity of cancer cell lines. Cisplatin and 5-fluorouracil (5-FU) were kindly donated by Mayne Pharma (Canada) Inc. (Montreal, Quebec, Canada) and paclitaxel by Bristol-Myers Squibb (Canada) Inc. Dose-response curves were done to determine the appropriate dosages for each cell line. Cells (50,000) were seeded in six-well dishes and incubated overnight in regular medium. The appropriate drug was diluted in complete medium at various concentrations; the cells were overlaid with 2 mL of this mix and incubated overnight. Viable cells were counted the next day, using the Cell Counting Kit-8.

After the optimal drug concentrations were established, combined treatments with antisense oligodeoxynucleotide were done. After transfection for 5 hours with antisense oligodeoxynucleotide as described above, the solution was replaced with the drug of choice diluted in 2 mL of complete medium and cultured overnight. The same procedure was repeated the following day. Cell numbers were determined one day after the second round of transfection, using the Cell Counting Kit-8. Each experiment was done twice, in triplicate.

Western Blot Analysis for MTHFR and Poly(ADP-Ribose) Polymerase. To determine whether EX5 alters MTHFR expression, Western blotting was done to analyze the level of immunoreactive MTHFR protein after in vitro and in vivo treatment with antisense. Western blotting was also done on tumor tissue extracts to measure immunoreactive poly(ADP-ribose) polymerase (PARP); cleavage of PARP to an 85-kDa fragment by caspases is commonly used as an indicator of apoptosis.

Cells were harvested by centrifugation and washed twice in cold PBS. Cell pellets were resuspended in 0.1 mol/L KPO₄ (pH 6.3) with a protease inhibitor mixture tablet (Complete mini; Roche, Mannheim, Germany). Cell extracts were prepared by freeze thawing of cells (thrice) followed by centrifugation at 14,000 rpm for 20 minutes and collection of supernatant. Tumor extracts were prepared by homogenization (10 seconds, twice) in ice-cold 0.1 mol/L KPO₄ buffer (pH 6.3) with a protease inhibitor mixture tablet, followed by centrifugation at 14,000 rpm for 20 minutes and collection of supernatant. Protein concentration was assayed using the Bradford method according to the manufacturer’s instructions (BioRad, Mississauga, Ontario, Canada).

Western blotting for MTHFR and actin was done as previously described. Quantification of protein was determined by scanning the films with a flatbed scanner (Hewlett Packard Scan, Palo Alto, CA). The MTHFR and actin band areas were calculated; MTHFR protein is expressed as a ratio of MTHFR/actin. Western blotting for PARP using an anti-PARP antibody was done using the same procedures except that the anti-PARP antibody was diluted 1:100 and anti-mouse horseradish peroxidase–conjugated antibody (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) was used as a secondary antibody.

In vivo Experiments. Animal experimentation was approved by the Animal Care Committee of the Montreal Children’s Hospital. Female, athymic, 4- to 6-week-old nude mice (BALB/c and CD-1) were purchased from Charles River (Saint Constant, Quebec, Canada). All animal work was done under aseptic techniques. Solid tumors were first established in BALB/c nude mice. Approximately 3 × 10⁶ cells in PBS were injected s.c. into the mouse flank. Animals and tumor growth were monitored closely. After establishment of tumors, BALB/c nude mice were sacrificed, tumors dissected, and 3 × 3-mm pieces were transplanted s.c. unilaterally into the flank of CD-1 mice. Tumors were passaged in CD-1 mice thrice before use. Experiments were initiated when the tumor volume reached 100 mm³ (~7 days posttransplantation). Tumor volume was calculated using the formula (a × b²)/2, where a is the long diameter (mm) and b is the short diameter (mm; 16). Animals bearing human tumor xenografts were randomly divided into various treatment groups (six to eight mice per group); the mock-treatment group received PBS. The antisense oligodeoxynucleotides dissolved in PBS were administered i.p. at a dose of 3 mg/kg/d, one dose per day, for 14 days (experiments with antisense oligodeoxynucleotide alone) or 21 days (experiments with antisense oligodeoxynucleotide and drug). Cytotoxic drugs
were given i.p. as follows: cisplatin at a dose of 3 mg/kg, one dose per day, on days 1 to 3 or 5-FU at a dose of 20 mg/kg, one dose per day, on days 4 to 8 and 12 to 16.

Animals were inspected daily for possible treatment-related toxicity. After sacrifice, blood was collected for biochemical and hematologic analysis, and tumors were frozen.

**Statistical Analyses.** Mean and SEs are provided. Student’s *t* tests were used to measure statistical significance between two treatment groups for the *in vitro* studies. One-way ANOVA and *t* tests were done for analysis of the treatment groups for the *in vivo* studies. Data were considered significant for *P* values (<0.05).

**RESULTS**

**Impact of Antisense and Chemotherapeutic Agents on Cell Survival In vitro.** The effects of MTHFR antisense on cell survival were examined in five human cancer cell lines: colon cancer (SW-620), lung cancer (A-549), prostate cancer (PC-3), neuroblastoma (SK-NFI), and breast cancer (MCF-7). We used an antisense oligodeoxynucleotide against a region in exon 5 of the MTHFR coding sequence (EX5) because we had previously showed the efficacy of this antisense oligodeoxynucleotide against SW-620, two breast tumor lines (MCF-7 and SKBR3), and two neuroblastoma lines [BE(2)C and SKNF-1].

To determine the efficacy of antisense therapy combined with classic cytotoxic treatment, we used 5-FU, cisplatin, or paclitaxel at a concentration that was determined from dose-response experiments to be intermediate with respect to cell toxicity. Previous experiments with antisense alone had shown dose dependence between 100 and 400 nmol/L. We elected to use an intermediate dose, 200 nmol/L, for antisense in the combination experiments.

As illustrated in Fig. 1, the number of SW-620 colon carcinoma cells after transfection with 200 nmol/L EX5 alone was ~40% of that seen with the CTS. After treatment with 5-FU at 10 μmol/L, cell numbers were ~65% of those with CTS. The combination of 5-FU with CTS was essentially similar to that seen with 5-FU alone. However, the combination of 5-FU and EX5 had the strongest inhibitory effect, with ~10% of cell numbers compared with CTS. Comparisons involving EX5 alone or EX5 with 5-FU versus all other treatment groups (and compared with each other) were statistically significant (*P* < 0.001, *t* test).

EX5 and cisplatin (10 μmol/L) were equally effective in reducing viability of A-549 lung carcinoma cells (Fig. 2), with 55% to 70% of cells remaining, compared with CTS. In contrast to other drugs and cell lines (SW-620, MCF-7, SK-NFI), we observed an interaction between cisplatin and the control antisense oligodeoxynucleotide in the A-549 cell line in reducing cell numbers. Nonetheless, the combination of cisplatin and EX5 showed the strongest inhibitory effect, with cell numbers reduced to <10% of those seen with CT6. Again, comparisons involving EX5 or EX5 with drug versus other treatment groups were statistically significant (*P* < 0.01).

Three other tumor lines were tested with a combination of EX5 and selected chemotherapeutic agents: breast cancer line MCF-7 with 100 μmol/L paclitaxel, prostate cancer line PC-3 with 100 μmol/L paclitaxel, and neuroblastoma line SK-NFI with 5 μmol/L cisplatin (data not shown). In all three cases, the inhibitory effect of EX5 alone was similar to that seen with the drug alone or with a combination of CTS and drug. The combination of EX5 and cytotoxic drug showed the strongest inhibitory effect. The percentage of viable cells after the combined treatment, compared with that with the control antisense oligodeoxynucleotide was 31%, 14%, and 29% in the breast cancer, prostate cancer, and neuroblastoma cells, respectively. As mentioned above, comparisons involving EX5 or EX5 with drug, versus other treatment groups, were statistically significant (*P* < 0.05) with the exception of EX5 versus cisplatin in the SK-NFI line, where *P* = 0.102.

The experiments discussed above for the five different tumor lines were all repeated in triplicate. Comparisons of EX5 or EX5 with drug were all statistically significant against other treatment groups and compared with each other (*P* < 0.05).

In earlier work with EX5 (12), we showed that the antisense decreased MTHFR immunoreactive protein, by Western analysis of SW-620 colon carcinoma cells. We did the same type of analysis in A-549 lung carcinoma cells and observed that treatment with EX5 at 200 nmol/L reduced MTHFR protein by ~60% compared with that seen in mock-transfected cells (data not shown).
Impact of Antisense on Tumor Growth In vivo. We did in vivo experiments in two human cancer xenograft models (SW-620 colon carcinoma and A-549 lung carcinoma). In the colon carcinoma xenograft model (Fig. 3), a single daily injection of EX5 (3 mg/kg) for 14 days was associated with decreased tumor volumes starting from ~day 7 until the final measurement at day 16. The difference in mean values among the treatment groups was statistically significant ($P = 0.005$, one-way ANOVA). Compared with the treatment with control antisense oligodeoxynucleotide, EX5 reduced tumor volume at day 16 by 60% ($P = 0.050$, $t$ test). Compared to the mock injections (PBS), EX5 reduced tumor volume at day 16 by 73% ($P = 0.001$, $t$ test).

In a second experiment with the colon tumor model, the combination of antisense and 5-FU was examined, as well as each agent alone, with appropriate controls. In the combination treatments, the antisense was injected once per day at 3 mg/kg for 21 days and 5-FU was injected once per day at 20 mg/kg for 10 days (days 4-8 and days 12-16; Fig. 4). The same conditions were used for each agent alone. Highest rates of tumor growth were seen with the mock treatment and with the CTS. The difference in mean values among the treatment groups was statistically significant ($P = 0.012$, one-way ANOVA). Additional analyses showed statistically significant differences between inhibitions of tumor growth by CTS or by EX5 at day 21 (40%; $P = 0.017$, $t$ test). The impact of EX5 alone, 5-FU alone and CTS + 5-FU were very similar, with reductions in tumor growth of ~40% compared with CTS at day 21. Similar to the in vitro experiments, the combination of EX5 and 5-FU showed the greatest degree of growth inhibition, with an additional reduction of ~30% compared with EX5 or other single agent alone.

In lung carcinoma, with daily injections of EX5 alone (3 mg/kg) for 14 days (Fig. 5), inhibition of tumor growth was first observed at day 7. One-way ANOVA of mean values on the final day of measurements (day 18) showed differences that had borderline significance ($P = 0.087$). Tumor volume was reduced by 45% with EX5 compared with the control antisense oligodeoxynucleotide at day 18 ($P = 0.068$; $t$ test). The reduction with EX5 compared with mock injections at day 18 (46%) was statistically significant ($P < 0.05$).

The combination of EX5 with cisplatin in the lung carcinoma model (data not shown) also displayed some inhibitory trends. Whereas cisplatin alone did not affect tumor growth, presumably because of the low doses, the combination of cisplatin + EX5 reduced tumor growth by 40% compared with cisplatin alone on day 22 and by 30% compared with cisplatin + CTS. However, none of these differences were statistically significant.

To assess potential toxicity of the administered agents, weight loss and comfort status were monitored regularly. Hematologic and biochemical parameters were evaluated in each host at the end of the experiment. Mice treated with cytotoxic agents alone or with a combination of drugs and antisense displayed the expected mild changes in hematologic parameters. Otherwise, we did not observe any significant thrombocytopenia, anemia, or neutropenia. Liver and renal function measurements were within the reference ranges. No treatment-related death, weight loss, or signs of treatment-associated discomfort (gastrointestinal toxicity, local reaction, or pain) after treatment with antisense alone or in combination experiments were observed. On autopsy, we observed occasional splenomegaly and reactive lymphadenopathy.
Lung carcinoma tumors were examined for MTHFR expression by Western blot analysis (Fig. 6, top). The MTHFR bands were quantified relative to the intensity of the internal control (actin; Fig. 6, middle). In the tumors treated with EX5, the MTHFR band was reduced by 60% compared with the samples treated with CTS, and by 85% compared with the mock-treated tumors.

To determine whether apoptosis was involved in tumor cell death, we also measured PARP protein by Western blotting (Fig. 6, bottom). PARP protein (116 kDa) is cleaved by caspases, which are activated in apoptotic cells. The digested PARP band at 85 kDa was clearly observed in the tumors that had been treated with EX5, with virtually no digested PARP product in the other lanes. The amount of undigested PARP protein was decreased by 60% in the EX5-treated tumor compared with the CTS-treated tumor.

**DISCUSSION**

Malignant tumors are known for various metabolic differences compared with normal cells. Sensitivity to a limited supply of methionine is one of the dissimilarities that have been confirmed *in vitro* and *in vivo* in many types of cancers (colon, liver, lung, brain, kidney, and melanoma; 8, 9). Molecular mechanisms underlying methionine dependence of cancer cells have not been fully clarified. However, methionine is necessary to maintain nucleotide pools and synthesize polyamines. It is also the immediate precursor of S-adenosylmethionine, the major methyl donor for methylation of DNA, RNA, and other molecules (9). The analysis of cell cycle kinetics in methionine-dependent tumor cell lines has revealed that methionine depletion causes an arrest of the cell cycle at S and G2-M phase and that this blockage is reversible (3–6). After methionine replenishment, cells enter the cell cycle phase as a relatively homogenous cohort. The synchronizing effect of methionine depletion sensitizes the cancer cells to the cell cycle-specific chemotherapeutic agents that are subsequently administered; methionine restriction with standard cytotoxic chemotherapy can therefore show synergistic effects (9, 17).

Methionine depletion has been attempted by dietary methionine deprivation (18, 19) and by the use of methioninase to degrade methionine and deplete circulating methionine levels (8, 20). Inhibition of an enzyme critical for methionine production, such as MTHFR, is another means of limiting methionine supply and affecting proliferative potential. Previous studies in our laboratory with MTHFR antisense resulted in significant reductions of MTHFR protein that affected survival of methionine-dependent cancer cell lines, whereas non-transformed cells (human diploid fibroblasts) were not affected. EX5 of the human MTHFR mRNA was determined to be quite effective and examined in this study, although an antisense against exon 4 also had growth inhibitory properties (12).

Drugs that inhibit folate metabolism or transport are some of the pillars of anticancer treatment due to the role of folate in nucleotide synthesis. Methotrexate and 5-fluorouracil are the more classic agents that disrupt folate pools through inhibition of dihydrofolate reductase or thymidylate synthase. More recent anti–folates include other dihydrofolate reductase inhibitors such as edatrexate or other thymidylate synthase inhibitors such as raltitrexed, or inhibitors of the glycaminide ribonucleotide formyltransferase, such as LY309887 (21). Some agents such as pemetrexed (ALIMTA) are multitarget antifolates that affect thymidylate synthase, dihydrofolate reductase, and glycaminide ribonucleotide formyltransferase (22).

In the present study, we tested EX5 on several cell lines *in vitro* and on two tumors *in vivo*, alone or in combination with cytotoxic drugs. The overall rationale behind the use of combination therapy is that agents with different mechanisms of action should minimize toxicity and drug resistance. Furthermore, additive or synergistic effects may be achieved. Our *in vitro* data showed additive effects between EX5 and 5-FU, cisplatin, or paclitaxel, suggesting that combination therapy with a variety of cytotoxic agents may be useful in combination with MTHFR inhibition. 5-FU is of particular interest with respect to MTHFR because it inhibits thymidylate synthase. The substrate of MTHFR, methylenetetrahydrofolate, is also the substrate for thymidylate synthase. If MTHFR is inhibited, the increased availability of thymidylate synthase substrate should enhance 5-FU efficacy because this pyrimidine analogue forms a ternary complex with thymidylate synthase and its substrate. This hypothesis was substantiated in our recent study (2), in which we examined 5-FU response in patients with colorectal cancer that have a common mutation in MTHFR, an alanine to valine substitution at bp 677. In that study, we showed a significant increase in response to 5-FU in patients that carried the mutation, as compared with those without the mutation. A decrease in MTHFR activity, through naturally occurring mutations or through antisense or other types of small molecule inhibitors, should be particularly beneficial in cancers that respond to 5-FU.

*In vivo*, we evaluated the activity of EX5 in colon and lung tumors. This treatment was effective alone and in combination with cytotoxic drugs. We used oligomers that did not contain nonmethylated CpG motifs, which are potential immune stimulators. We can therefore exclude the possibility that the antitumor efficacy of EX5 was due to immunologic effects.

Western blotting analysis showed a significant decrease in MTHFR protein *in vitro* and *in vivo* after treatment with antisense. Thus, there is a correlation between down-regulation of MTHFR expression and inhibition of tumor growth. We also
observed a correlation between the level of digested PARP, an indicator of apoptosis, and growth inhibition. This finding suggests that MTHFR inhibition may be associated with increased apoptosis. The mechanism for this phenomenon is not clear, although MTHFR inhibition could result in elevated homocysteine levels or decreased methylation potential because MTHFR generates the 5-methyltetrahydrofolate derivative that provides the methyl group for homocysteine remethylation to methionine. Homocysteine has been shown to increase apoptosis in several cultured cell systems such as cortical neurons, lymphocytes, myocardial cells, vascular endothelial cells, and others, possibly through an increase in oxidative stress (23, 24). The well-characterized decrease in DNA methylation in individuals with deficient activity of MTHFR (25) suggests that antisense-mediated inhibition of MTHFR should also affect DNA methylation. The changes in DNA methylation could contribute to gene expression changes that might affect cell survival.

Our experiments in vivo were done with relatively low doses of EX5 (at 3 or 5 mg/kg) compared with studies with other antisense. For example, antisense targeting the mouse double minute-2 oncogene was injected at 25 mg/kg/d for 25 days (26). The cell survival gene clusterin was targeted with oligonucleotides at a dose of 12.5 mg/kg/d for 28 days (16) and oligonucleotides targeting thymidylate synthase were injected at 11.3 mg/kg/d for 14 days (27). In our study, the 21-day treatments with antisense injected at 3 mg/kg/d did not result in any obvious side effects or overt toxicity at the relatively low doses that were administered.

In conclusion, our results suggest that the MTHFR gene is a useful target for anticancer treatment. Our earlier work reported on any obvious side effects or overt toxicity at the relatively low

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