Antisense inhibition of methylenetetrahydrofolate reductase reduces survival of methionine-dependent tumour lines

Folate derivatives participate in single-carbon transfers in several reactions, including the synthesis of nucleotides and methionine. Methionine is the precursor of S-adenosylmethionine which is utilised in numerous transmethylation reactions, including DNA methylation. Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate, the methyl donor in thymidine synthesis, into 5-methyltetrahydrofolate, the methyl donor utilised in methionine synthesis from homocysteine by vitamin B12-dependent methionine synthase. We hypothesised that methylenetetrahydrofolate reductase inhibition would affect cell viability through decreased methionine synthesis. Using medium lacking methionine, but containing homocysteine and vitamin B12, we found that nontransformed human fibroblasts could maintain growth. In contrast, four transformed cell lines (one colon carcinoma, two neuroblastoma and one breast carcinoma) increased proliferation only slightly in the M-H+ medium. To downregulate methylenetetrahydrofolate reductase expression, two phosphorothioate antisense oligonucleotides, EX5 and 677T, were used to target methylenetetrahydrofolate reductase in the colon carcinoma line SW620; 400 nm of each antisense oligonucleotide decreased cell survival by approximately 80% (P < 0.01) and 70% (P < 0.0001), respectively, compared to cell survival after the respective control mismatched oligonucleotide. Western blotting and enzyme assays confirmed that methylenetetrahydrofolate reductase expression was decreased. Two neuroblastoma and two breast carcinoma lines also demonstrated decreased survival following EX5 treatment whereas nontransformed human fibroblasts were not affected. This study suggests that methylenetetrahydrofolate reductase may be required for tumour cell survival and that methylenetetrahydrofolate reductase inhibition should be considered for anti-tumour therapy.

Keywords: methionine-dependence; cancer cells; methylenetetrahydrofolate reductase; antisense technology

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MATERIALS AND METHODS

Cell lines

Human fibroblasts MCH 51 and MCH 75 were obtained from the Repository for Mutant Human Cell Strains (Montreal Children’s Hospital, Montreal, Canada). Human colon carcinoma cell line SW620 and two neuroblastoma lines (BE(2)C and SKNF-1) were obtained from American Type Culture Collection (Rockville, MD, USA). The breast carcinoma cell lines, MCF7 and SKBr3, were a gift from Dr Morag Park (McGill University, Montreal, Canada). Genotyping for the MTHFR variant at bp 677 was performed by PCR amplification and HinfI digestion, as previously reported (Frost et al., 1995). The MCF7 cell line was grown in z-minimal essential medium (z-MEM) (Life Technologies, Rockville, MD, USA) and the SKBr3 cell line was maintained in D-MEM (Life Technologies). Media for both lines was supplemented with 10% foetal bovine serum (Intergen, Purchase, NY, USA). The other cell lines were grown in MEM (Life Technologies) supplemented with 5% foetal bovine serum (Intergen) and 5% iron enriched calf serum (Intergen). All media was also supplemented with 50 IU ml⁻¹ penicillin (Life Technologies), 50 μg ml⁻¹ streptomycin (Life Technologies), 0.5 μg ml⁻¹ fungizone reagent (Life Technologies). All cell lines were cultured in 75 cm² flasks in a humidified 37°C incubator in 5% CO₂.

Deficient culture media

MEM and MEM without folate and methionine supplemented with 100 mM sodium pyruvate (F-M⁻) were obtained from Life Technologies. For methionine-deficient (M⁻) media, 2.3 μM folate (Sigma-Aldrich, Oakville, ON, USA) was added to the F-M⁻ media. For all media, 5% foetal bovine serum (Intergen), 5% iron enriched calf serum (Intergen), 50 IU ml⁻¹ penicillin (Life Technologies), 50 μg ml⁻¹ streptomycin (Life Technologies), and 0.5 μg ml⁻¹ fungizone reagent (Life Technologies) were added. For methionine-deficient medium supplemented with homocysteine and vitamin B₁₂ (M⁻H⁺) media, 0.44 mm DL-homocysteine (Sigma-Aldrich) and 1.5 μM vitamin B₁₂ (Sigma-Aldrich) was added to the M⁻ media. Dialysed serum was used for all experiments with deficient media. These conditions were based on similar studies in fibroblasts and in transformed lines (Hoffman and Erbe, 1976; Rosenblatt and Erbe, 1977).

Cell viability studies of cells in deficient media

Cell viability studies were performed in 6-well tissue culture plates starting with 30 000 – 50 000 cells per well and three replicates for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer. Cell survival in MEM was used as a control for each condition. The initial number of cells were estimated with a hemocytometer.
were visualised by enhanced chemiluminescence with an ECL kit (Amersham Pharmacia Biotech). Quantitation of protein was determined by scanning the films with a flat-bed scanner (Hewlett Packard Scan). MTHFR has been shown to have two isoforms (Froost et al, 1995). Both MTHFR bands and the actin band areas were calculated; MTHFR protein level is expressed as a ratio of MTHFR/actin.

Statistical analysis

One-way ANOVA was performed using SPSS software, Version 10.0 (SPSS Inc., Chicago, IL, USA), to analyse cell survival after treatment with EX5. Statistical analysis of cell survival data of SW620 cells following treatment with 677T was performed with the SAS-PC statistical software, Version 8.0 (SAS Institute, Cary, NC, USA). The mixed effects analysis of variance model was used. In this model two factors were considered: drug and concentration. The Student t-test was used to evaluate differences in MTHFR activity, and to analyse cell survival data of fibroblast cell lines treated with EX5, 677C and 677T antisense.

RESULTS

Growth studies in methionine-deficient media

Two fibroblast strains (Figure 1) and 4 transformed lines (Figure 2) were grown in MEM, MEM without methionine (M-), or MEM without methionine supplemented with homocysteine and vitamin B_{12} (M-H+). The latter medium served to examine de novo synthesis of methionine from homocysteine and 5-methyltetrahydrofolate, catalysed by vitamin B_{12}-dependent methionine synthase. 5-Methyltetrahydrofolate is the product of the MTHFR reaction. All six lines showed sensitivity to the M- medium; growth was significantly reduced in this medium compared to that in MEM. The fibroblasts (MCH 51, MCH 75) could maintain virtually normal growth in the M-H+ medium. However, the transformed lines (colon carcinoma SW620, breast carcinoma SKBr3 and neuroblastomas BE(2)C and SKNF-1) cultured in the M-H+ medium increased their proliferation only slightly through endogenous methionine synthesis (Figure 2). The cell numbers were just a small percentage (5–25%) of the values obtained in MEM. The SKBr3 line was also tested with a lower concentration of homocysteine in the M-H+ medium; the results were similar to those with the higher concentration (0.44 mM) (data not shown).

Treatment with the EX5 antisense

A BLAST search identified sequences in exon 5 of MTHFR that did not have any homology with other ESTs in the NCBI database. Figure 3a demonstrates a dose-dependent decrease in cell survival (P<0.01, one-way ANOVA) after treatment with SW620 carcinoma cells with EX5. At the maximal dose of 400 nM, cell survival decreased approximately 80% compared to that of cells treated with the scrambled control oligonucleotide.

To ensure that MTHFR expression was altered, Western blotting was used to analyse immunoreactive MTHFR protein, after three consecutive treatments with the EX5 ASO. In previous work, we showed that two MTHFR isoforms are present in human tissues (Froost et al, 1995); these isoforms differ only at the N-terminus of the protein encoded by the 5’ end of the mRNA. Consequently, the antisense should inhibit expression of both isoforms. Figure 3b demonstrates a significant decrease in MTHFR protein levels after EX5 treatment, compared to treatment with the scrambled control, CT5EX5, or compared to treatment with Lipofectin reagent only (mock transfection). After normalisation to actin, MTHFR protein levels following treatment with the control oligo were 94% of mock-treated cells, whereas treatment with 200 and 400 nM of EX5 oligo, MTHFR protein levels were 39% and 25%, respectively, of that in mock-treated cells (average of three Western blots).

After treatment with 400 nM of EX5, the two neuroblastoma cell lines (BE(2)C and SKNF-1) showed significant decreases in cell survival compared to control ASO treated cells; decreases of 80% (P<0.001) and 65% (P<0.01), respectively (data not shown). Similarly, the breast carcinoma cell lines SKBr3 showed a 80% (P<0.0001) decrease in cell survival and the MCF7 breast carcinoma line showed a 92% (P<0.0001) reduction in cell survival compared to control oligo treated cells (data not shown). Contrary to data obtained in transformed lines, two human fibroblast cell lines (MCH 75 and MCH 51) treated with 400 nM of EX5 did not exhibit significant differences in cell survival compared to control oligo treated cells (P>0.05; data not shown).

Treatment with the 677T antisense

A second oligonucleotide was chosen to target a different region of the mRNA – exon 4, which is the location of the common variant of MTHFR at bp 677. The SW620 line carries the valine allele with a T at bp 677. The 677T antisense should target the valine allele in SW620 whereas the 677C antisense, with one mismatch, should be less effective. The control oligonucleotide, CT677, has a 6 bp mismatch compared to the experimental oligonucleotide.

The cell survival of SW620 colon carcinoma cells treated with 677T differs significantly from cells treated with the mismatched control, CT677, at all four tested concentrations between 100 and 400 nM (P<0.02 for 100 nM, P<0.0001 for 200–400 nM) (Figure 4a). Cell survival decreased in a dose-dependent manner. At the highest concentration, 400 nM, cell survival was approximately 70% less than that of cells treated with CT677.

Cell survival after transfection with the 677C oligonucleotide, which differs from 677T by a single mismatch, was significantly higher at concentrations of 200–400 nM (P<0.01) than that seen with the 677T ASO (Figure 4a). At a concentration of 100 nM, cell survival does not differ significantly between 677C and 677T treated cells (P>0.05). Cell survival after treatment with 677C is not significantly different from cells treated with the control CT677 at concentrations of 100 and 200 nM (P>0.05), and shows only small but borderline significant differences at 300 and 400 nM (P=0.05 and P=0.04, respectively). At the maximal dose of 400 nM, cell survival after 677T transfection is decreased by 70% compared to cells treated with the control ASO, whereas survival after 677C transfection was decreased by only 30%.

Western blotting (Figure 4b) demonstrated that MTHFR protein levels were reduced following a 24-h transfection with the 677T oligonucleotide. MTHFR protein levels following transfection of the 677C or 677T were 81% and 17%, respectively, of the values obtained in cells treated with the control CT677 oligo. In addition, we measured enzyme activity after 24 h of treatment with Lipofectin only (mock treatment), cells treated with 400 nM CT677 (control), and cells treated with 400 nM of 677C or 677T (data not shown). Treatment with 400 nM of 677C reduced MTHFR enzymatic activity significantly (P<0.01) compared to mock treatment, to values that were approximately 20% (data not shown). Treatment with 400 nM of the CT677 or 400 nM of the 677C ASO, with the 1 bp mismatch to 677T, shows a borderline significant difference in enzymatic activity compared to mock treated cells (P=0.02), but the decrease was minimal (86% of mock activity).

One fibroblast cell line (MCH 75) was used to test the effect of treatment with either 677C or 677T on normal methionine-independent cells. This line is heterozygous for the polymorphism at bp 677. Treatment of MCH 75 cells with either 677C or 677T does not significantly affect cell survival compared to control ASO treated cells (P>0.05, data not shown).
This study demonstrates, in vitro, the potential of using antisense technology directed against MTHFR to decrease the cell viability of methionine-dependent transformed cells. The analysis of cell survival of nontransformed fibroblasts and of several transformed lines cultured in methionine-deficient medium, with and without homocysteine/vitamin B12 supplementation, supports earlier reports of the high methionine requirement of transformed lines (Hoffman, 1984); in that review, 23 human lines and 19 animal cell lines were reported to be methionine-dependent. Although normal fibroblast lines showed the ability to restore their growth by restoring endogenous methionine synthesis, the transformed cell lines only exhibited a slight increase in survival under the same conditions. The amount of recovery may in fact be a reflection of the degree of transformation of the colon carcinoma lines (Breillout et al, 1990).

By utilising antisense phosphorothioate deoxyoligonucleotides, in vitro, to specifically suppress the expression of MTHFR, the cell viability of a methionine-dependent colon carcinoma cell line was substantially decreased. 677T was able to decrease cell survival by 70% at the maximal dose of 400 nM, while EX5 decreased cell survival by 80% under similar conditions. The 677T ASO appears to exhibit a high specificity for the valine form of MTHFR present in SW620 cells; the 677C ASO, with only a single mismatch (at the polymorphic nucleotide), did not have as great an effect on cell survival. The differences in cell survival between the 677T and 677C oligos were significantly different for concentrations of 200, 300 and 400 nM. These results are consistent with previous reports demonstrating the specificity for one form of a target gene using phosphorothioate oligonucleotides with single base mismatches as controls (Duroux et al, 1995; Bennet et al, 1996; Basilion et al, 1999). Our observations also suggest that allele-specific targeting may be possible for MTHFR. This approach has been proposed as a method for targeting tumour tissues, which often undergo loss of heterozygosity (LOH), without significantly compromising the gene and its product in non-tumour cells (Basilion et al, 1999). For genes that have a common variant and are essential for cell
survival, the single remaining allele could be targeted in vivo in tumour cells that have undergone LOH, whereas the non-tumour tissue, with two alleles, should still retain the activity of the non-targeted allele and, consequently, remain viable. We have recently demonstrated that MTHFR undergoes 15–20% LOH in colorectal tumours (Pereira et al., 1999); LOH for MTHFR may be as high as 45–50% in ovarian tumours (Viel et al., 1997).

Treatment of two neuroblastoma cell lines and two breast carcinoma cell lines showed significant decreases in cell survival after treatment with 400 nM of EX5 compared to control oligo treated cells. These results point to the applicability of downregulating MTHFR expression in various types of tumour lines in order to decrease cell survival. To test the effects of targeting MTHFR in nontransformed cells, two human fibroblast cell lines (MCH 75 and MCH 51) were treated. Both cell lines did not show significant decreases in cell survival. To test the effects of targeting MTHFR in nontransformed cells, two human fibroblast cell lines (MCH 75 and MCH 51) were treated. Both cell lines did not show significant decreases in cell survival. To test the effects of targeting MTHFR in nontransformed cells, two human fibroblast cell lines (MCH 75 and MCH 51) were treated. Both cell lines did not show significant decreases in cell survival.

The downregulation of MTHFR in methionine-dependent tumour cells may cause decreases in methionine levels to an extent that significantly affects cell survival. The same decreases in methionine production in methionine-independent lines may not substantially affect cell survival since these cell lines are not reliant on such high levels of methionine to sustain cell growth.

ASOs are designed to affect the information transfer from gene to protein, by altering the metabolism of RNA (Crooke, 1999). The end result is expected to be a decrease in levels of the gene product. We therefore examined MTHFR protein levels after antisense treatment. For the EX5 experiments, we performed Western blotting under the same conditions that were used for analysis of cell numbers i.e. following three rounds of transfection. For the 677T experiments, we found that the nonspecific toxicity of the phosphorothioate oligonucleotides was quite high; the toxicity of these oligos has been well documented (Stein and Cheng, 1993; Stein, 1999). Due to the greater toxicity of the 677T oligos (control and experimental ASOs), cell survival was considerably reduced after three rounds of transfection, even though the specific oligo was always associated with lower cell numbers than the control oligo. Consequently, we used one 24-h transfection, rather than three consecutive transfections, to generate enough cells for Western blotting. The conclusions from both sets of experiments (EX5 and 677T oligos) were similar – MTHFR protein was significantly reduced compared to that of the control oligonucleotides at the studied concentration (400 nM). MTHFR
enzyme activity was also measured following the 677T transfection; this experiment demonstrated that the 677T oligo had a direct effect on MTHFR activity.

Though reducing MTHFR expression to decrease cancer cell survival is a novel hypothesis, other groups have successfully used different means to decrease methionine availability for transformed cells. Guo et al. (1993a) deprived Yoshida sarcoma-bearing nude mice of dietary methionine resulting in tumour regression and extended survival of the mice. Other investigators have used the enzyme methioninase in vivo (Tan et al., 1996a); this enzyme from Pseudomonas putida catalyses the conversion of methionine to methanethiol (Weimer et al., 1999). It has been shown to deplete circulating methionine levels in mice and in humans (Lishko et al., 1993; Tan et al., 1996b). In vivo studies injecting purified methioninase into nude mice bearing either rodent or human tumours have successfully arrested the growth of these methionine-dependent tumours with no apparent toxic side effects (Tan et al., 1996a).

This study has shown that a reduction in MTHFR levels reduces cell viability in vitro. To evaluate the validity of this approach for chemotherapy, these techniques need to be transferred to an in vivo model. Inhibition of MTHFR through antisense technology or through other means, such as pharmaceutical agents, should be considered alone or in conjunction with other antifolate compounds, such as methotrexate or 5-fluorouracil, to increase our arsenal of chemotherapeutic reagents.

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