Antisense Targeting of Thymidylate Synthase (TS) mRNA Increases TS Gene Transcription and TS Protein: Effects on Human Tumor Cell Sensitivity to TS Enzyme-Inhibiting Drugs

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Thymidylate synthase (TS) catalyses the only de novo pathway to produce thymidylate for DNA replication and repair and is an important target for cancer chemotherapy. Preexisting or acquired drug resistance in tumor cells limits clinical efficacy of TS-targeting drugs. Cells selected for higher TS protein activity have decreased sensitivity to TS-targeting chemotherapeutic agents (5-FUdR and raltitrexed). New therapeutic strategies are required to overcome treatment resistance. Among these, upregulation of drug resistance mediators in normal, nontarget cells and/or antisense downregulation of those mediators (alone or in combination with protein-targeting drugs) are candidate strategies. We have targeted human TS mRNA with antisense oligodeoxynucleotides (AS ODNs), complementary to the translation start site (TSS), the coding region, and the 3' untranslated region. We report here that, in response to treatment with a novel TSS-targeting AS ODN 791, TS gene transcription in a human cervical carcinoma cell line (HeLa) was unexpectedly increased by 70%. Interestingly, the increased TS gene transcription and nuclear TS RNA did not elevate levels of total cellular TS mRNA, but did increase TS protein activity by 35% and TS protein level by 150%. Increased TS protein activity and level did not alter proliferation rate or sensitivity to TS-targeting drugs (5-FUdR or raltitrexed). To assess concentration-dependent effects of TS on sensitivity to TS-targeting drugs, incremental increases of TS protein levels were generated by transfection of a mammalian TS expression vector. Increases in TS protein of less than approximately 400% did not significantly affect sensitivity to TS-targeting drugs, while greater TS protein levels did. These data indicate that AS ODNs targeting TS mRNA can upregulate TS expression and activity in a manner dependent on the sequence being targeted, and that there exists a threshold increase (greater than approximately 400–700% in HeLa cells), required to initiate resistance to TS-targeting drugs.

Key words: Antisense oligodeoxynucleotides; Thymidylate synthase; Chemotherapy; Drug resistance; Run-on transcription; Threshold

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INTRODUCTION

Thymidylate synthase (TS) is an essential enzyme that produces thymidylate (dTMP) from deoxyuridine monophosphate (dUMP) by a reaction that is the sole intracellular pathway generating dTMP for DNA replication and repair (13). Because of the importance of dTMP in DNA precursor synthesis, TS is a target for many chemotherapy agents including 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), raltitrexed, pemetrexed, and others (10,13). 5-FU, since its development in the late 1950s, has been the standard treatment for colorectal cancer and is commonly used for treatment of head and neck, breast, and gastrointestinal cancers (25). TS is a negative prognostic marker for colorectal cancer and indicative of increased cellular proliferation, tumor invasiveness, and metastatic capacity (34). Despite the importance of TS as a molecular target for anticancer therapy, response rates to 5-FU remain low (in the 10–30% range) (38). Development of new and better agents to target TS or the dTMP pathway is required (21,38). A primary obstacle in the effectiveness of fluoropyrimidine chemotherapeutic agents is the development of resistance to this class of TS-targeting drugs in tumor cells, through a variety of mechanisms, including (and of primary importance) elevated TS protein levels in tumors (7,21). Acquired or intrinsic drug resistance reduces the clinical effectiveness of current anti-TS drugs (38), and tumor cell resistance to fluoropyrimidines (5-FU, 5-FUdR) and other antimetabolites (raltitrexed, pemetrexed) is thought to be mediated by TS (1,26). For example, TS gene amplification, increased TS protein stability, and translational derepression (increased TS mRNA translation after treatment with TS-targeting drugs due to drug-induced dissociation of TS from its own mRNA) all lead to increased TS protein levels and resistance to TS-targeting drugs (28,37). Variant TS proteins with lowered capacity to bind to TS-targeting drugs have been identified, and allow persistent TS activity and resistance to anti-TS drugs (6). Sensitivity of normal tissues to TS-targeting drugs is also considered to be a major impediment to TS-related therapy (33), and strategies to enhance TS expression in normal cells and/or selection of patients for TS-targeted treatment on the basis of polymorphisms in individuals with elevated TS in normal tissues that enhance therapeutic indices have been proposed (8).

We previously reported antisense oligodeoxynucleotides (AS ODNs) that target TS mRNA to decrease TS protein levels and overcome treatment resistance. Targeting the 3' untranslated region (3'-UTR) and the coding region of the TS mRNA with AS ODNs decreases TS mRNA levels, TS protein levels, and cel-

lular proliferation in human tumor cell lines grown in vitro and as xenografts in immunocompromised mice (5,17–19) (Jason et al., submitted). In addition to successful downregulation of TS mRNA and protein using AS, we have also reported that targeting the TS mRNA translation start site (TSS) with AS RNA vectors and AS ODNs unexpectedly increased TS gene transcription in HeLa cells (14). Increased TS protein levels have been observed in a human colon tumor cell line (HT-29) in response to an 18-mer antisense ODN targeting the AUG of the TSS (24), which, although gene transcription was not assessed in that study, is consistent with our observation of HeLa cell response to targeting the TSS with antisense reagents.

In the present study we show that treatment of HeLa cells with a novel TS AS ODN 791 (targeting the TSS) increased TS gene transcription (as measured by increased TS nuclear RNA). Increased TS gene transcription occurred without increasing steady-state levels of TS mRNA in total cellular RNA. Increased TS transcription was accompanied by increased TS protein and activity levels. The observed increase in TS protein following AS ODN 791 treatment did not mediate resistance to TS-targeting agents, nor did it alter proliferation rate, both of which have been reported to correlate with TS protein levels (1). On the other hand, transfection of a mammalian TS expression vector to increase TS protein to higher levels than those achieved after treatment with AS ODN 791 did enhance resistance to the TS-targeting agent, raltitrexed. These data suggest that resistance to TS-targeting drugs requires a threshold level of increased TS protein, and indicate that increases in TS protein levels (up to 700%) do not drive enhanced proliferation.

MATERIALS AND METHODS

Cell Culture

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were grown in Dulbecco's modified Eagle's medium. All media were supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Tissue culture reagents were from Invitrogen Canada (Burlington, ON, Canada).

Oligodeoxynucleotides and Transfection Methods

TS AS ODN 791 (5'-CGCGGTACGGACACCGGCCG-3') is complementary to bases 101 to 120 within the 5'-UTR and includes the translation start site of human TS mRNA. The scrambled control

ODN 25 (5'-CGGCACGCCCATAGGCGGCG-3') has the same base composition in a random order. The ODNs have phosphorothioated internucleotide linkages, and have a 2' ribose modification of methoxyethoxy (ODNs 791, 25; kindly provided by ISIS Pharmaceuticals, Carlsbad, CA) or 2'-*O*-methyl substitutions (ODN 791, 25 purchased from Eurogentec North America, San Diego, CA). Similar results were obtained in experiments using ODNs with either 2' ribose modification. Prior to ODN treatment, cells were plated in triplicate at a density of 1.5×10^5 cells/25-cm² flask. The following day, ODNs were mixed with 0.5 µg/ml Lipofectamine 2000 (Invitrogen) in serum-free medium for 20 min, after which FBS (0.1 volume) was added. The medium on the cells was then replaced with 2 ml of medium with ODN/lipid. Four hours later, a further 2 ml of growth medium was added, and the cells were incubated for up to 4 days. Due to some inconsistencies in nonspecific toxicity and antisense efficacy using Lipofectamine 2000, some of the results (shown in Fig. 4) were obtained using Oligofectamine (Invitrogen) as a transfection reagent. Cells were plated at densities of 1.5×10^5 cells/25-cm² flask. The following day, cells were transfected using Oligofectamine (according to the manufacturer's instructions).

Plasmid Preparation and Plasmid Transfection

TS-14 overexpressing plasmid was a generous gift from Dr. Maria Zajac-Kaye (Center for Cancer Research, National Cancer Institute, National Institute of Health, Bethesda, MD, USA). GFP and TIED and plasmids were obtained from Invitrogen and Berg et al. (4). HeLa cells were plated in triplicate at a density of 3×10^5 cells/25-cm² flask. The following day, plasmids were mixed with 2 µl/µg Lipofectamine 2000 (Invitrogen) in serum-free medium for 15 min and then 2 ml of medium with plasmid/lipid was added to each flask. Four hours later, a further 2 ml of growth medium was added, and cells were incubated for up to 4 days.

Run-on Transcription Measurement

HeLa cells were treated with the 100 nM ODN/liposome solution for 24 h. Nuclei were isolated and run-on transcription was carried out after addition of reaction buffer {dGTP, dATP, dUTP, [³²P]-dCTP (GE Healthcare, Baie d'Urfe, PQ, Canada), 1 mM DTT} for 30 min at 37°C. Radiolabeled RNA was isolated following TRIzol protocol (Invitrogen). Probes specific for TS, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 18S were immobilized on a Hybond-N membrane (GE Healthcare) and pre-hybridized for 1 h. Radiolabeled RNA was added to

the membrane, hybridized for 48 h, developed, and quantitated using Phosphorimager and Imagequant software.

mRNA Measurement

Cells were treated with ODNs as described above. After 4, 8, 16, or 24 h, total RNA was isolated using TRIzol or TRI Reagent (Sigma, Oakville, ON, Canada) and quantitated using a spectrophotometer. Reverse transcription of 1 µg of RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) was followed by polymerase chain reaction (PCR) using primers specific for GAPDH and TS as described previously (5). PCR products were visualized on 1% agarose gels stained with ethidium bromide. For real-time PCR amplification of TS, the PCR reaction mixture consisted of 600 nM human TS forward (5'-GGCCTCGGTGTGCCTTT-3') and human TS reverse (5'-GATGTGCGCAATCATGTACGT-3') primers, 200 nM human TS probe (5'-6FAM-AACATCGCCAGCTACGCCCTGC-MGBNFQ-3'), and TaqMan Universal PCR Master Mix (PE Applied Biosystems, Branchburg, NJ, USA). The level of 18S rRNA was measured as an internal standard, using Taqman Pre-Developed Assay Reagents (Applied Biosystems, Foster City, CA, USA). PCR reactions (50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min) were performed, and reporter fluorescent dye emission was monitored, using the ABI Prism 7700 System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). TS mRNA levels were normalized to 18S rRNA levels for each sample.

Protein Measurement

Cells were treated with ODNs as described above. After 24 h cells were washed twice with PBS, scraped in cold PBS with a rubber policeman, and centrifuged at $200 \times g$ for 10 min. Cell pellets were lysed in ice-cold lysis buffer; 20 mM Tris-HCl, pH 7.6, 0.1% SDS, 1% Triton X-100, 10 mM EDTA for 30 min at 4°C. Lysates were centrifuged at $10,000 \times g$ for 10 min and the supernatants collected. Protein concentrations were estimated using a BioRad protein assay kit (BioRad, Montreal, PQ). Proteins (40 µg per lane) were resolved on SDS-polyacrylamide (12%) gels and transferred to Hybond membranes (GE Healthcare). The membranes were blocked in 5% skim milk powder in TBS-Tween (1 h at room temperature), and incubated for 2 h with rabbit anti-human TS polyclonal antibody (the generous gift of Dr. Masakazu Fukushima, Taiho Pharmaceuticals, Tokushima Research Center, Hanno-City, Japan) followed by rabbit anti-actin antibody (Sigma) for 1 h.

Proteins were visualized using horseradish peroxidase-labeled anti-rabbit antibody and enhanced ECL-Plus (GE Healthcare). Intensity of bands was quantitated using AlphaEaseFC software. To quantitate TS protein activity, a [^3H]FdUMP binding assay was used, as described previously (17). Total protein (30 μg) was electrophoresed on a 12% polyacrylamide gel as described above. Gels were stained with Coomassie blue (2.5 g Coomassie brilliant blue, 45% methanol, 45% H_2O , 10% acetic acid) for 1 h with shaking at 25°C , washed twice in distilled water, and destained (10% acetic acid, 40% methanol) with shaking at 25°C . Densitometer scanning was performed to determine the total amount of blue staining in each lane (where staining indicated the amount of total protein). The relative amount of total protein in each lane was determined by dividing the densitometric volume of each lane by the cumulative densitometric volume of all compared lanes.

Growth and Drug Sensitivity Assay

Cells were treated with ODNs (50 nM) as described above. After 4 days cells were removed from the flasks by trypsin treatment, and counted in saline solution using an electronic particle counter (Beckman Coulter, Hialeah, FL). For drug sensitivity assays, cells were treated with ODN (50 nM) as above. After the initial 4-h ODN treatment, the appropriate concentration of drug was added. For plasmid treatment drug sensitivities, drug was added 24 h after transfection. Proliferation is expressed relative to treatment with control ODN 25 or ODN 791 in the absence of drug (Fig. 6) or plasmid in the absence of drug (Figs. 7B, C and 8A, B).

Flow Cytometry

HeLa cells were plated at 2.5×10^5 cells/75- cm^2 flask. The following day, cells were treated with ODNs (100 nM) as described above and incubated for 24 h. Analysis was carried out by the procedure supplied by Becton-Dickinson (BD-Canada, Oakville, ON, Canada). Flow cytometry was carried out on a Beckman Coulter Epics XL-MCL Flow Cytometer (Beckman Coulter) with at least 10,000 events collected.

Statistical Analysis

Statistical significance was determined using Student's *t*-test. All experiments were performed at least two times.

RESULTS

AS TS ODN 791 Induces TS Gene Transcription

We have previously reported that antisense targeting of TS mRNA sequences within and proximal to the TS mRNA TSS, but using antisense reagents (ODN 90 and pAS/TSS) complementary to sequences other than those specifically targeted by ODN 791 (Fig. 1), increased TS gene transcription (as reflected in increased nuclear TS RNA, including TS RNA with and without intron sequences) without concomitant increase in steady-state, total cellular, mature TS mRNA (14).

Treatment of HeLa cells with AS TS ODN 791 (100 nM), a 20-mer AS ODN complementary to the AUG translation start site (TSS) and sequences 5' and 3' to the TSS (Fig. 1), increased TS gene transcription (relative to GAPDH gene transcription) by $77 \pm 13.9\%$ compared to cells treated with scrambled control ODN 25, at 24 h posttransfection with antisense and control ODNs (Fig. 2). The increase in TS gene transcription relative to transcription from an alternative comparator (18S rRNA genes) was similar ($71 \pm 5.2\%$) and increased confidence in the validity of the observation. These data confirmed our earlier reported observation (14) as a basis for further investigation of the consequences of increased TS transcription for TS mRNA and protein levels, and effects on drug sensitivity.

It was not known whether total cellular mRNA levels were unaltered, as was the case when targeting with other antisense reagents that included sequences complementary and/or proximal to the TSS (14). To address this question, TS mRNA levels were assessed at multiple times after transfection with control ODN 25 or AS TS ODN 791. No changes in total cellular TS mRNA, relative to GAPDH mRNA or 18S rRNA, were induced by ODN 791 (100 nM) at 4, 8, 16, or 24 h posttransfection, when measured using semi-quantitative densitometric analysis of RT-PCR products separated by gel electrophoresis (Fig. 3A–C) or by quantitative real-time PCR (Fig. 3D).

AS TS ODN Increases TS Protein Without Altering Drug Sensitivity or Cell Cycle

Although AS TS ODN 791-induced changes in TS gene transcription did not affect steady-state TS mRNA levels, it was not known whether enhanced TS gene transcription resulted in increased TS protein levels or activity. To address this question, TS protein levels were measured 24 h after transfection with AS TS ODN 791. ODN 791 (100 nM) induced a $37 \pm 10\%$ increase in relative TS protein levels (i.e., relative to β -actin protein) (Fig. 4A) compared to cells

Figure 1. Portion of the TS mRNA targeted by antisense nucleic acids, ODN 791, ODN 90, pAS/TSS [from DeMoor *et al.* (14)] and AS-1 [from Ju *et al.* (24)].

treated with scrambled control ODN 25 (as measured by immunoblot). There were no significant differences in total protein levels among compared lanes (Fig. 5), confirming the observed increase in TS rela-

tive to β -actin. The increased TS level was accompanied by a $37 \pm 18\%$ increase in TS protein activity (as measured by a $[6\text{-}^3\text{H}]\text{FdUMP}$ in vitro TS binding assay to assess the capacity of TS protein to associate with radiolabeled FdUMP substrate) (Fig. 4B).

Increased TS protein and TS activity are associated with enhanced proliferation and resistance to TS-targeting drugs (1,29), and specific downregulation of TS leads to repressed proliferation and accumulation of cells at cell cycle checkpoint stages (5) and decreased resistance to TS-targeting drugs (17,18). To determine whether the increased level and activity of TS after treatment with AS TS ODN 791 was sufficient to alter HeLa cell sensitivity to chemotherapeutic drugs targeting TS (raltitrexed and 5-FUdR), and one that does not (cisplatin), the capacity of pretreatment of HeLa cells with control ODN or AS TS ODN 791 to alter tumor cell growth was assessed. AS TS ODN 791 treatment, at concentrations that significantly increased TS (100 nM), had no effect on the capacity of HeLa cells to increase in number over a 4-day growth period in culture (Fig. 6A) nor did it alter the number of cells in G₁, S, or G₂/M phases of cell cycle (Fig. 6B). Furthermore, AS TS ODN 791 treatment (sufficient to induce TS protein level and activity) did not affect the sensitivity of HeLa cells to raltitrexed (Fig. 7Aii) or at concentrations higher than those that increased TS protein and TS gene transcription (150 and 200 nM) (Fig. 7Aiii, Aiv). There was no change in the sensitivity of HeLa cells to 5-FUdR, raltitrexed, or cisplatin at concentrations lower (50 nM) than those that resulted in increased TS (Fig. 7Ai, Bi, Bii). Thus, increased TS resulting from treatment with antisense ODNs targeting the TS mRNA TSS did not affect cell cycle distribution and proliferation or sensitivity to either TS-targeting or non-TS-targeting cytotoxic drugs.

AS TS Expression Vector Increases TS Expression and Sensitivity to a TS-Targeted Drug

While increased TS expression resulting from antisense targeting of the TS mRNA TSS did not alter proliferation or drug sensitivity, the association of TS with proliferation and resistance to TS-targeting drugs suggests that enhanced TS levels achieved by

Figure 2. Effects of ODN treatment on gene transcription in HeLa cells. HeLa cells were treated with 100 nM ODN 25 (white columns) or 100 nM AS TS ODN 791 (black columns) for 24 h. Cell nuclei were isolated and transcription of genes for TS, GAPDH, and 18S rRNA measured by run-on transcription as described in Materials and Methods. (A) Dot blots of probes for TS and GAPDH mRNA, and 18S rRNA, hybridized to radiolabeled nuclear RNA isolated from cells transfected with AS TS ODN 791 or scrambled control ODN 25. Dot blot data were quantified to determine TS gene transcription relative to 18S rRNA gene transcription (B) or GAPDH gene transcription (C) in cells transfected with ODN 791 (black columns) or control ODN 25 (white columns). Data are means $\pm$ SD of values from a representative experiment. *Different from cells treated with ODN 25 ($p < 0.05$, Student's *t*-test).

Figure 3. Effects of ODN treatment on TS mRNA levels in HeLa cells. (A) HeLa cells were treated with 100 nM ODN 791 (black columns) or control ODN 25 (white columns) for the indicated times. TS mRNA and GAPDH mRNA were measured in total cellular RNA by RT-PCR or real-time PCR at 4, 8, and 16 h post-transfection, and TS mRNA (relative to GAPDH mRNA) values are shown. (B) RT-PCR products of TS mRNA and GAPDH mRNA 24 h after transfection with control ODN 25 (lanes 1, 3, 5) or ODN 791 (lanes 2, 4, 6) visualized by gel electrophoresis and ethidium bromide staining. Reduction in TS mRNA by an antisense TS ODN previously shown to reduce TS (ODN 83, lane 8) is shown as a positive control. Its scrambled control ODN 32 (lane 7) had no effect on TS mRNA. (C) TS mRNA levels (relative to GAPDH mRNA) in cells transfected with ODN 791 (black column) or control ODN 25 (white column, normalized to 1.0). Data were assessed by quantification of RT-PCR products separated by gel electrophoresis. Means $\pm$ SD ($n = 3$) are shown. (D) TS mRNA levels (relative to 18S rRNA) measured by real-time PCR. Means $\pm$ SD are shown ($n = 3$).

other means might modulate these events and, consequently, provide a system to compare with antisense-mediated TS upregulation. We therefore transiently transfected HeLa cells with TS-14 (a plasmid directing expression of TS) to generate cells with TS protein levels enhanced between fourfold (mediated by 1.5 μ g plasmid transfected into 3×10^5 cells) and sixfold (mediated by 6 μ g plasmid transfected into 3×10^5 cells), compared to cells transfected with TIED [a plasmid directing expression of TIED, the expression of which is known to be irrelevant to sensitivity to cytotoxic drugs and proliferation (4)]. The trans-

Figure 4. Effects of ODN 791 treatment on TS protein levels in HeLa cells. HeLa cells were treated with 100 nM ODN 25 (white columns), 100 nM 2'-O-methoxyethoxy-AS TS ODN 791 (black columns), or 100 nM 2'-O-methyl-AS TS ODN 791 (gray column) for 24 h. (A) Immunoblot of TS and β -actin from a representative experiment. TS protein levels relative to β -actin protein levels, after treatment with methoxyethoxy-substituted ODN 791 or control ODN 25 (mean $\pm$ SD, $n = 3$). (B) TS protein activity (percent of activity in cells identically transfected with control ODN 25) measured by [3 H]FdUMP binding activity assay as described in Materials and Methods. Data are shown as means $\pm$ SD ($n = 3$), from a representative experiment. *Different from cells treated with ODN 25 ($p < 0.05$, Student's t -test).

Figure 5. Total protein in HeLa cell lysates assessed for changes in TS protein. HeLa cells were treated with control 100 nM ODN 25 (white column) or 100 nM ODN 791 for 24 h. Cells were lysed and total protein analyzed as described in Materials and Methods. (A) Coomassie stained polyacrylamide gel from a representative experiment; 20 μ g total protein per lane. (B) Quantification of Coomassie gel. Data are presented as mean $\pm$ SD ($n = 9$).

fection efficiency of the different doses of plasmid was similar, as verified by cotransfection of equal amounts of a β -galactosidase expression vector followed by measurement of β -gal levels (data not shown).

HeLa cells transfected with TS-14 had significantly increased TS (compared to control cells transfected with TIED) when the dose of plasmid was 0.5 μ g or greater: 0.25 μ g of plasmid had no effect on TS levels (Fig. 8A). HeLa cell resistance to raltitrexed was unchanged in cells transfected with 0.25 μ g of TS-14 (which did not increase TS protein levels). Cells transfected with 0.5 μ g of TS-14 plasmid had significantly increased TS protein levels (Fig. 8A), but the increase was insufficient to increase HeLa cell resistance to raltitrexed (Fig. 8B). Higher levels of TS protein in response to 1.5 or 2.0 μ g TS-14 plasmid (Fig. 8A) significantly decreased HeLa cell sensitivity to a range of raltitrexed concentrations (Figs. 8B, 9A). Increased TS mediated by transfection of 0.5 or 1.5 μ g TS-14 did not affect HeLa cell

sensitivity to the non-TS-targeting drug cisplatin (Figs. 8C, 9B).

DISCUSSION

Thymidylate synthase is well recognized as an appropriate target for cancer treatment (20) and current TS-targeted treatments have positive effects on human tumors and cancer survival. However, there is need for improvement. To increase anti-TS effectiveness, new TS-targeting agents, including raltitrexed, pemetrexed, and Thymitaq, have been developed. Changes in dose and timing of administration have been explored and implemented (35,38). Several physiological events, including resistance of tumor cells and sensitivity of normal cells to therapy, continue to be barriers to effectiveness of TS-targeting drugs, and improved strategies to overcome drug re-

Figure 6. Proliferation and cell cycle analysis of HeLa cells treated with ODN 791. (A) HeLa cell numbers were measured before (day 0, gray column) and 4 days after treatment with ODN 791 (black column) or control ODN 25 (white column) (mean $\pm$ SD, $n = 3$). (B) HeLa cells were treated with 100 nM ODN 791 (black columns) or 100 nM control ODN 25 (white columns) and harvested after 24 h. Cell cycle analysis was carried out as described in Materials and Methods. The fraction of cells in designated cell cycle phases (percent of total) is shown (mean $\pm$ SD, $n = 3$).

Figure 7. Effect of AS TS ODN 791 on HeLa cell sensitivity to raltitrexed, 5-FUdR, and cisplatin. Cells were transfected with different amounts of control ODN 25 (white squares) or ODN 791 (black squares) and proliferation in the presence of cytotoxic drug measured as described in Materials and Methods. (A) Proliferation of cells transfected with (i) 50 nM, (ii) 100 nM, (iii) 150 nM, (iv) 200 nM control ODN 25 or ODN 791 followed by exposure to various concentrations of raltitrexed. (B) Proliferation of cells transfected with 50 nM control ODN 25 or ODN 791 followed by exposure to various concentrations of raltitrexed. (C) Proliferation of cells transfected with 100 nM ODN 791 or control ODN 25 followed by exposure to various concentrations of cisplatin. All data points show proliferation relative to that observed in cells treated with ODN 25 in the absence of drug (mean $\pm$ SD, $n = 3$).

sistance and circumvent toxicity to normal cells are needed (9).

We have reported that, although AS ODNs targeting some TS mRNA regions reduce TS mRNA and protein and enhance sensitivity to TS-targeting drugs (2,3,5,17–19), targeting TS mRNA at or near the translation start site increases TS gene transcription (Fig. 1) (14). To explore unexpected antisense-mediated enhancement of TS gene activity we treated HeLa cells with AS TS ODN 791 (targeting the TSS) (Fig. 2). This increased TS gene transcription in iso-

lated HeLa cell nuclei, relative to either GAPDH or 18S rRNA gene transcription, compared to cells treated with a scrambled control AS ODN 25 (Fig. 2). Interestingly, the increased nuclear TS RNA induced by ODN 791 did not lead to an increase in total cellular TS mRNA (Fig. 3). The mechanism underlying the disparity is not known, but several possibilities may be relevant. First, the hybridization method used to detect nuclear TS mRNA in the present study does not discriminate between unprocessed, immature TS RNA and mature TS mRNA, while the

RT-PCR methods used to measure total cellular RNA detect only mature TS mRNA. Thus, ODN 791-mediated increased TS gene transcription might enhance the level of nuclear TS RNA products without increasing mature TS mRNA. Alternatively, mRNA translation has been functionally linked to mRNA

Figure 8. Effect of transfection of TS-14 on TS protein levels and drug sensitivity in HeLa cells. Cells were transfected with the amounts of TIED plasmid or TS-14 plasmid indicated and TS protein and cell proliferation assessed as described in Materials and Methods. (A) TS protein relative to β -actin protein after transfection with control TIED plasmid (white columns) or TS-14 plasmid (black columns). Results from a representative experiment are shown. (B) Cell proliferation in the presence of raltitrexed after transfection with TIED (white columns) or TS-14 (black columns). Proliferation was normalized to 1.0 in cells transfected with TIED, and proliferation of cells transfected with TS-14 are relative to that normalized value (mean $\pm$ SD, $n = 3$). *Different from cells treated with TIED ($p < 0.05$, Student's t -test).

Figure 9. Effect of a TS expression vector on HeLa cell sensitivity to raltitrexed and cisplatin. Cells were transfected with control GFP plasmid (2.0 μ g, open symbols) or TS-14 plasmid (2.0 μ g, solid symbols) and proliferation assessed in the presence of a range of concentrations of raltitrexed (A) or cisplatin (B) as described in Materials and Methods. Proliferation of cells transfected with TS-14 is shown relative to proliferation of cells transfected with GFP. Data are presented as mean $\pm$ SD ($n = 3$). *Different from cells treated with GFP ($p < 0.05$, Student's t -test).

degradation in yeast (31) and in HeLa cells (12). If increased TS gene transcription is accompanied by increased TS mRNA that undergoes concurrent translation and degradation, then increased TS protein and TS gene transcription against a background of apparently unchanged overall TS mRNA would be expected. In fact, this is the pattern we observed: increased TS gene transcription following AS TS ODN 791 transfection was accompanied by increased TS protein levels (Fig. 4A) and in vitro TS protein activity (Fig. 4B).

A further formal possibility is that AS TS ODN 791 increased TS protein levels by enhancing TS protein stability. However, pulse-chase experiments to assess that possibility revealed no significant or reproducible changes in TS protein half-life (data not shown). Thus, ODN 791-induced enhancement of TS protein stability is unlikely to explain changes in TS protein levels and activity.

A stem-loop structure thought to be a binding site for TS protein that encompasses the TSS of TS mRNA has been proposed, based on recognition that complementary sequences flanking the TSS are expected to hybridize to generate a loop containing the TSS (28). TS protein/TS mRNA association at the TSS has been confirmed, and binding of TS protein to its substrates or TS-targeting drugs leads to dissociation of TS protein from mRNA and increased translation (11,28). AS TS ODN 791 is complementary to sequences within the stem and, if bound to those sequences, could interfere with stem-loop formation. If so (and if AS TS ODN 791 binding did not appreciably reduce functional association of the translation machinery with TS mRNA), the result could be reduced TS protein binding, enhanced translation, and increased TS protein levels. The possibility of antisense-mediated enhancement of translation is in agreement with our observation of increased TS protein levels in cells in the absence of increased TS mRNA, and is being actively investigated in our laboratory.

Overall, and regardless of mechanism, increased TS gene transcription resulted in increased TS protein and TS protein activity. The question of whether the increased levels affected physiological events known to be associated with increased TS function remained. Increases in TS protein levels in tumor cell lines and in normal rat tissues have been reported to accompany increased proliferation rate and accumulation of cells in G₁/S of the cell cycle (15,22,30,32,34). Furthermore, an approximately 200% increase in TS protein activity in a human colon tumor cell line (HT29) treated with antisense ODN targeting the TSS has been reported to enhance resistance to 5-FUdR (24).

We assessed the capacity of AS TS ODN 791 treatment to alter HeLa cell proliferation and/or accumulation of cells in different cell cycle compartments. Although AS TS ODN 791 increased TS protein activity by approximately 35% (Fig. 4B) and TS protein level by approximately 37% (Fig. 4A), there was no effect on proliferation (Fig. 6A) or distribution into cell cycle compartments (Fig. 6B). In addition, AS TS ODN 791 treatments that elicited the same increase in TS protein level and activity had no effect on sensitivity of HeLa cells to a range of concentrations of the TS-targeting drug raltitrexed (Fig. 7B) and non-TS-targeting cisplatin (Fig. 7C). The lack of capacity of increased TS to enhance resistance to TS-targeting drugs is qualitatively at odds with the observation of increased resistance to 5-FUdR after antisense induction of increased TS activity (24), but suggests that a threshold TS activity is necessary to mediate resistance.

A necessary minimum increase in TS enzyme ac-

tivity to mediate enhanced TS-targeting drug resistance has not been reported, but evidence of a requirement for a threshold level of other endogenous proteins to mediate a detectable increase in drug resistance does exist. Aldehyde dehydrogenases (specifically, ALDH1A1 and ALDH3A1) mediate sensitivity to cyclophosphamide and other oxazaphosphorines used to treat breast cancer (36). High ALDH1A1 and ALDH3A1 levels have been reported to predict resistance to cyclophosphamide, but the correlation is not apparent at lower ALDH levels in human breast tumors: an observation that has been suggested as evidence that the lower levels are below a threshold level necessary to mediate cyclophosphamide resistance (36).

To assess whether lower levels of TS fell below a threshold necessary to mediate resistance to TS-targeting drugs, HeLa cells transfected with increasing amounts of TS-14 expression vector were assessed for increased TS protein levels and enhanced sensitivity to raltitrexed. Transfection of 0.25 μ g TS-14 into HeLa cells neither increased TS protein levels nor enhanced resistance to raltitrexed. However, transfection of 1.5 or 2.0 μ g TS-14 both increased TS protein levels and enhanced raltitrexed resistance. Interestingly, transfection with intermediate amounts of TS-14 (0.5 and 1.0 μ g) significantly increased TS protein but had no effect of sensitivity to raltitrexed (Fig. 8). The enhanced resistance was specific to the TS-targeting raltitrexed, with no change in resistance to cisplatin: an observation consistent with our reports (17,18) of antisense-mediated downregulation of TS increases sensitivity to TS-targeting but not TS non-targeting drugs.

An inducible TS expression system has been reported to increase TS protein levels in a human breast tumor cell line (MDA-435) by approximately sixfold, and to concomitantly increase resistance to 5-FU and raltitrexed (29). In that study, increased TS had no effect on proliferation rate or cell cycle, consistent with the lack of effect on cell cycle and proliferation in response to TS-14-mediated increased TS in HeLa cells (Fig. 6).

A threshold level of TS mRNA in colorectal tumors in patients $[(\text{TS mRNA})/(\beta\text{-actin mRNA}) > 4.1 \times 10^3]$ has been recognized as a predictor of lack of response to treatment with 5-FU (16,26,27). However, the heavy reliance of TS regulation on posttranscriptional events reduces the strength of the correlation between TS mRNA and protein, and the reliability of TS mRNA measurement as a surrogate for TS protein and TS activity measurement. To our knowledge, our observation of a threshold level of TS protein required to mediate resistance to TS-targeting drugs is the first such report. We believe that this

Figure 10. Comparison of TS protein in cells transfected with a TS expression vector or AS TS ODN 791. TS protein levels in cells transfected with TS-14 (white columns) or ODN 791 (black columns) are shown, relative to TS in cells transfected with their respective control plasmid TIED plasmid or ODN 25 (gray columns) (mean $\pm$ SD, $n = 3$). By definition, TS levels in cells transfected with TIED or ODN 25 are normalized to 1.0. *Different from cells treated with TIED or ODN 25 ($p < 0.05$, Student's t -test).

may be the basis of future measurement of TS protein in human tumors as a tool to more accurately predict sensitivity to drugs, and development of this concept is being actively pursued in our laboratory.

In summary, our study extends the observation that antisense TS ODNs targeted to the TSS of TS mRNA can induce TS gene transcription, and reveals that TS protein levels are also increased by TSS targeting. Although the AS TS ODN-induced increase was statistically significant, it was not sufficient to enhance

resistance to TS-targeting drugs: an observation that we have further extended to reveal that a threshold level of TS protein appears to be necessary to initiate resistance to TS-targeting drugs (Fig. 10). Furthermore, and in spite of the association of increased cellular TS levels with proliferation and the G₁/S compartment of cell cycle, increased TS protein levels in response to transfection of AS TS ODN 791 or the TS expression vector TS-14 had no effect on proliferation or the fraction of cells in different cell cycle compartments, even when those levels were sufficient to mediate resistance to TS-targeting drugs. Overall, the phenomenon of antisense-mediated upregulation of TS expression should be taken into consideration when designing antisense strategies to target TS, and consideration of threshold TS protein levels may be of future value in selecting therapeutic regimens for treatment of human tumors.

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