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Inhibition of Thymidylate Synthase Activity by Antisense Oligodeoxynucleotide and Possible Role in Thymineless Treatment

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ABSTRACT

Thymidylate synthase (TS) is an important target for chemotherapeutic treatment of cancer. However, efficacy of TS-targeted anticancer drugs is limited by the development of drug resistance as a result of TS gene amplification. In this work, a phosphorothioated antisense oligonucleotide (ODN), designated ATS-2, was used to suppress cellular synthesis of TS. ATS-2 at 0.2 μM concentration was mixed with lipofectin in a charge ratio of 1:1 and was used to treat the human embryonic kidney (HEK) cell line. A reduction of TS mRNA and protein was achieved. Furthermore, a dose-dependent reduction of cumulative viable cells of up to 98% was observed. Flow cytometer analysis of cell cycle progression indicates that ATS-2-treated cells were arrested and went into apoptosis at the S phase, possibly because of thymidine shortage, suggesting that ATS-2 is specifically effective for dividing cells. When used in combination with the anticancer drug FdUrd, ATS-2 exerted a additive inhibitory effect on cellular proliferation. To elucidate the possible role of cellular thymidine kinase (TdR kinase) in ATS-2 treatment, a second cell line, HeLa, was used. Both HEK and HeLa have similar rates of cell division and ODN uptake. In contrast to HEK, which was shown to have very low levels of TdR kinase activity in [^{3}H]thymidine incorporation experiments, [^{3}H]thymidine incorporation in HeLa was 15-fold greater than that of HEK. We found that HeLa cells were sensitive to FdUrd but were rather resistant to ATS-2. On the contrary, HEK cells were sensitive to ATS-2 but insensitive to FdUrd. Effects of ATS-2 and FdUrd are, therefore, complementary in thymineless treatment too.

Nucleotides participate not only in DNA and RNA synthesis but also in a number of metabolic pathways. However, the base thymine is used solely for DNA synthesis. Depletion of dTTP seldom causes cytotoxicity to nondividing cells because DNA replication has ceased in these cells. On the other hand, cells that are dividing actively, such as cancer cells, are usually sensitive to agents that inhibit the synthesis of thymidine or dTMP. Thymidylate synthase (TS) is an essential enzyme catalyzing the de novo biosynthesis of thymidylate (Danenberg, 1977). For this reason, TS is an important target for chemotherapeutic treatment for cancer patients (Hardy et al., 1987). For instance, antifolate drugs such as methotrexate and trimetrexate inhibit dihydrofolate reductase (Santi et al., 1974; Jackman et al., 1991) and in turn block TS activity. 5-Fluorodeoxyuridine (FdUrd), when converted to FdUMP by thymidine kinase (TdR kinase), is a suicidal inhibitor of TS (Tanaka et al., 1981; Heidelberger et al., 1983). In a thymineless state, FdUTP and dUTP, instead of dTTP, are incorporated into DNA (Ingraham et al., 1980; Mauro et al., 1993) leading to DNA strand breakages (Yin and Rustum, 1991) and replication errors (Sowers et al., 1988).

TS plays a significant role in the clinical development of FdUrd- and 5-FU-resistance in tumors. This suggestion arises from the observation that FdUrd- and 5-FU-resistance is associated with stable amplification of the TS gene (Johnston et al., 1994; Suzuki et al., 1994; Pestalozzi et al., 1997). In these instances, the synthesis rates of both the TS mRNA and protein are elevated. It is also known that TS protein negatively regulates its own translation by specifically binding with TS transcripts, thus blocking TS translation (Chu et al., 1993; Johnson, 1994). However, when FdTMP covalently interacts with TS protein, TS mRNA is

ABBREVIATIONS: TS, thymidylate synthase; TdR kinase, thymidine kinase; FdUrd, 5-fluorodeoxyuridine; 5-FU, 5-fluorouracil; FdUMP, 5-fluorodeoxy-UMP; ODN, oligodeoxynucleotide; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; RT, reverse transcription; kb, kilobase pair(s); ACP, acid phosphatase; PBS, phosphatase-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

released and TS translation is resumed (Keyomarsi et al., 1993). On the other hand, FdUrd-resistance may also be caused by changes in TdR kinase activity. Because TdR kinase plays a role in the salvage synthesis of dTMP from thymidine, abolition or reduction of TdR kinase activity leads to a blockage in the formation of the suicidal FdUMP (Rossana et al., 1982; Zhang et al., 1993).

Properly designed antisense ODNs are effective inhibitors of protein synthesis leading to lower levels of specific cellular proteins (Wagner, 1994; Lin et al., 2000; Simoes-Wust et al., 2000). In this approach, certain regions of a target mRNA are chosen to be annealing sites for antisense ODNs. Formation of RNA-DNA duplex leads to an abolition of protein synthesis by directly blocking translation or by mediating degradation of targeted mRNA through an RNase H action (Wagner, 1994; Kronenwett and Haas, 1998). To inhibit cancer cell proliferation and to enhance the efficacy of TS-targeted drugs, suppression of TS activity by antisense ODN has been attempted in cell-free systems (Schmitz et al., 1998). DeMoor et al. (1998) have further designed antisense ODNs targeted at the translation start and stop sites of the TS mRNA. However, the ODN used resulted only in an enhancement of TS gene transcription. More successful experiments have been achieved by Ferguson et al. (1999) using an antisense ODN complementary to a region in the 3′-untranslated region of the TS mRNA. TS mRNA levels were suppressed by 70% and cell proliferation was inhibited by up to 40%.

In this report, we show that an antisense ODN complementary to a sequence in the coding region of the TS gene suppressed TS protein level up to 90% and inhibited cell proliferation up to 98%. A possible reason for the hypersensitivity is discussed.

Materials and Methods

Cell Lines and Cell Culture. HEK (American Type Culture Collection; Manassas, VA) is a human embryonic kidney cell line transformed by adenovirus type 5 DNA. HeLa is a human cervical carcinoma cell line. Both cell lines were cultured in DMEM medium containing fetal calf serum (10%), Na-glutamate, Na-pyruvate, and nonessential amino acids at 37°C in 5% CO_2 atmosphere.

Antisense Oligodeoxynucleotide and Antisense Treatment. The anti-TS ODN designated ATS-2 was a phosphorothioated ODN with the sequence *d*-5′GGATCTGCCCCAGGTAC3′ which is complementary to nucleotide 201~217 in the coding region of the TS mRNA (Takeishi et al., 1985). This region was predicted to be an open region using the FOLDRNA program (GCG; Accelrys, Inc., Princeton, NJ). A BLASTN search of the GenBank database from National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD) indicates that the antisense sequence is not homologous with any other human cDNA sequences. The control ODN, *d*-5′ CATGGACCCCGTCTAGG 3′, carried the sequence in a polarity reversed to ATS-2. To prepare the ODNs for treatment of cells, 20 μl of Lipofectin (Invitrogen, Carlsbad, CA) was added to 0.38 ml of DMEM and the medium stood for 40 min. Then 18 μl of 50 μM ATS-2 (or control ODN) was added and mixed well. The charge ratio of ODN to lipofectin was 1:1. The mixture was stood for 10 min to allow complex formation. After that, 4.1 ml of DMEM was added. The final concentration of ODN was 0.2 μM. When lower concentrations of ODN were used, the amount of lipofectin was reduced proportionately. In the meantime, HEK (or HeLa) cells were cultured to a density of 20% confluence (2×10^5 cells/6-cm Petri dish). Old medium was aspirated and ODN/lipofectin-containing DMEM was added. After a 3.5-h incubation, 0.5 ml of dialyzed fetal calf serum (Invitrogen) and other supplements mentioned above were added. When 10-cm petri dishes were used, all materials were doubled. The cells were then cultured for 1 or 2 days.

Quantification of TS Protein by Western Blot Analysis. HEK cells were grown in 10-cm petri dishes to 20% confluence and were then treated with or without ATS-2 for 24 h. The cells were harvested and lysed in a lysis buffer as described previously (Chen et al., 1998). The protein content of the lysate was determined using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cell lysates containing 75 μg of protein were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and were then transferred to a nitrocellulose membrane. The membrane was then cut at the 40-kDa position. The lower and the upper portions of the membranes were immuno-blotted and reacted with monoclonal antibodies against TS (NeoMarkers, Union City, CA) or β-actin (Chemicon International Inc., Temecula, CA), at a 1000-fold dilution, followed by incubation with horseradish peroxidase secondary antibody at 1000-fold dilution. The immunoreactive bands were visualized by the enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and an exposure to X-ray film.

Quantification of TS mRNA by RT-PCR. mRNA was purified from treated HEK cells by the Poly(A)Pure kit (Ambion, Austin, TX) according to the supplier's manual. Then, 0.2 μg of the mRNA was reverse-transcribed using the SuperScript preamplification system (Invitrogen) with oligo(dT) as the primer. One fourth of the product was used as the template in 50-μl PCR reactions, which contained 0.2 mM dNTP, 2 units of *Taq* DNA polymerase, 0.1 μg of each primer and 1× reaction buffer. The 5′ and 3′ TS primers were *d*-5′GAGCCGCGTCCGCCGCAC3′ and *d*-5′CCGTGATGTGCGCAAT CAT3′, respectively. An amplicon of 0.65 kb was obtained. As an internal control, a 0.8-kb β-actin cDNA fragment was amplified using the primer pair, *d*-5′ATCTGGCACCACACCTTCTACAATGAG CTGCG3′ and *d*-5′CGTCATACTCCTGCTTGCTGATCCACATCTGC3′. The PCR program used was as follows: 95°C, 4 min; 55°C, 30 s; 72°C, 1 min; 94°C, 30 s for 25 cycles and 55°C, 1 min; 72°C, 2 min. After PCR, the PCR products were loaded onto a 1.5% agarose gel for electrophoresis followed by ethidium bromide staining.

Quantification of Viable Cells. Cells were grown in 6-cm petri dishes to 20% confluence and were treated with or without ODNs for 48 h. To quantify the number of viable cells, the medium was aspirated and the cells were washed with PBS and subjected to an acid phosphatase (ACP) assay, as described previously (Lin et al., 1997), except that 0.6 ml of the ACP reaction buffer was added. The data presented were the means derived from three independent experiments.

Flow Cytometry. HEK cells grown in 6-cm petri dishes were treated with ATS-2 for 24 or 30 h. The cells were harvested by trypsinization, washed with PBS, resuspended in 500 μl of PBS, and fixed by the addition of 500 μl of ice-cold absolute ethanol at −20°C. After incubating for 30 min, cell pellets were collected by centrifugation and were resuspended in 0.5 ml of PBS containing 100 μg/ml RNase for incubation at 37°C for 30 min. Then, 0.5 ml of propidium iodide solution (100 μg/ml in PBS) was added, and the mixture was allowed to stand on ice for 30 min. The cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Distribution of cell cycle phases was analyzed using the ModFit LT software.

TUNEL Assay. A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit from Boehringer Mannheim (Mannhein, Germany) was used to detect apoptotic cells according to the operation manual. In brief, HEK cells were treated with or without ATS-2 for 32 h. The cells were then trypsinized, fixed, and permeabilized, followed by terminal deoxynucleotidyl transferase reaction using fluorescein-dUTP as a substrate. Finally, the cells were analyzed by fluorescence microscopy.

Detection of Chromatin Condensation. HEK cells were treated with or without ATS-2 for 42 h. The cells were trypsinized and stained with Hoechst 33258 as described previously (Cynthia et al., 1998).

[^{3}H]Thymidine Incorporation. HEK or HeLa cells at 25% confluence (2.5×10^5 cells/6-cm Petri dish) were seeded and grown under normal culture conditions overnight. [^{3}H]Thymidine was

added to the medium (1 μCi/ml) and pulse-labeled for 4 h. Cells were washed twice with PBS and harvested by trypsinization with 1 ml of trypsin/EDTA buffer. Aliquots of 0.3 ml of the cell suspension were retained onto glass microfiber filter membranes by filtration (Whatman, Maidstone, England). The membranes were washed twice with PBS in the presence of 0.5% nonidet P-40, and twice with 75% ice-cold alcohol. The membranes were air-dried. The amounts of [^{3}H]thymidine were measured by β-counter with scintillation liquid.

Results

Uptake of ODN with Lipofectin as a Carrier. HEK cells are from a transformed human primary embryonic cell line with a high growth rate and are relatively insensitive to FdUrd; thus, HEK cells were chosen as a test for anti-TS experiments. To enhance the uptake of antisense ODN and

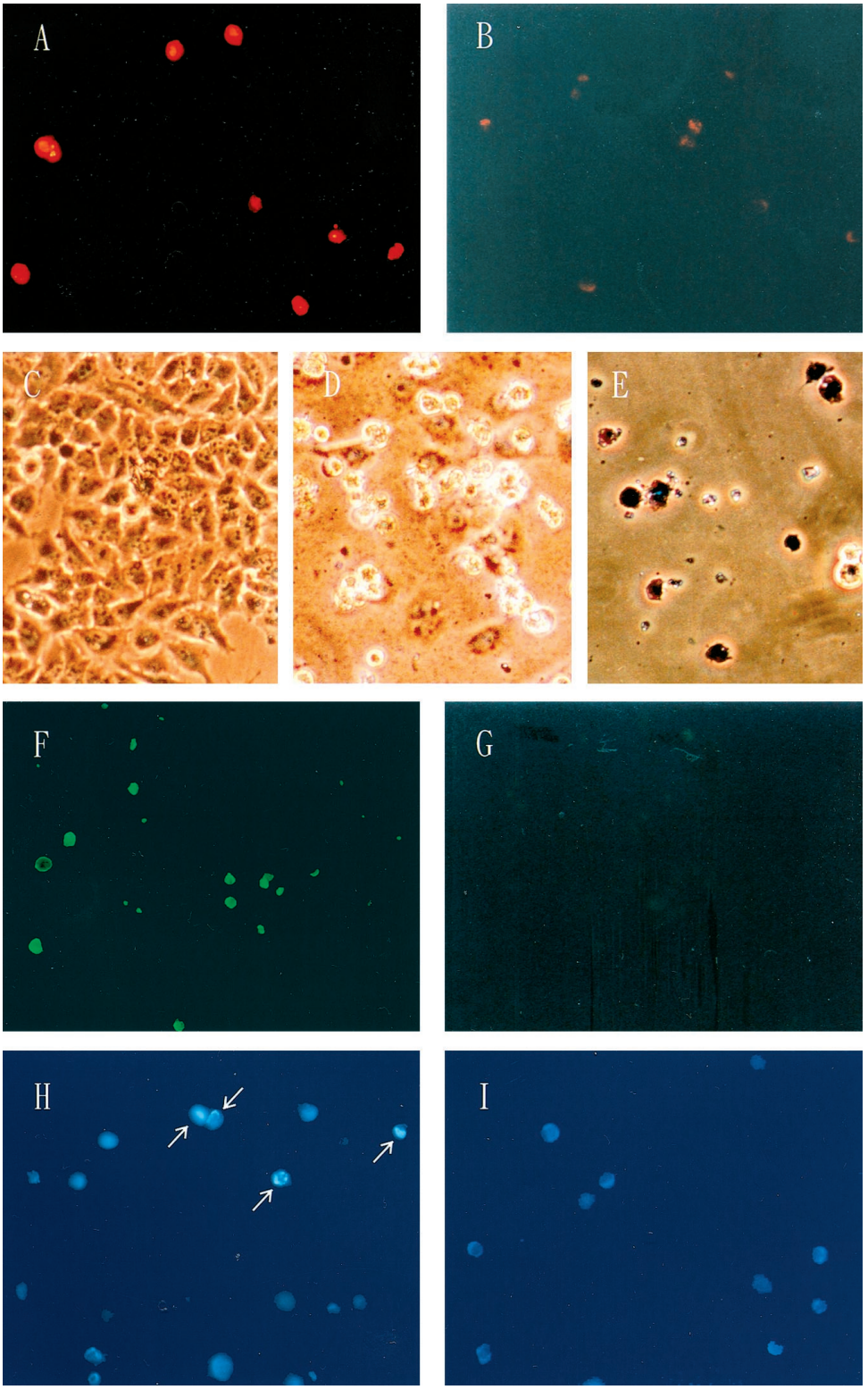

Fig. 1. Images of HEK cells under various conditions. ATS-2 ODN was labeled with rhodamine at the 3′ end. The ODN in complex with lipofectin (A) or ODN alone (B) at 0.2 μM concentration was incubated with HEK cells for 3.5 h for uptake experiments. HEK cells at 20% confluence were treated with 0.2 μM control ODN (C) or ATS-2 treated cells (D) with lipofectin for 48 h. The detached cells collected from the ATS-2 treated cells (D) were stained with trypan blue (E). Also shown are images of HEK cells when treated with 0.2 μM ATS-2 with lipofectin (F) or lipofectin only (G) for 32 h. These cells were then trypsinized and subjected to TUNEL assay. The nuclei of apoptotic cells are green. HEK cells were also treated with 0.2 μM ATS-2 with lipofectin (H) or lipofectin only (I) for 42 h followed by trypsinization and Hoechst staining. Arrows indicate cells with condensed chromatin. All pictures were taken at 100× magnification. The image was then processed by Fuji Bio-imaging Analyzer System (BSA-1000; Fuji, Tokyo, Japan).

proportionally reduce the amount of ODN needed, lipofectin was used as a carrier. ATS-2 was labeled with rhodamine at the 3′ end as a marker for uptake. The transfection efficiencies with or without lipofectin can be shown from the images of the exposed cells (Fig. 1, A and B). The images indicate that lipofectin enhanced the uptake of ODN by HEK cells substantially. These images (Fig. 1A) also show that the extent of uptake of the fluorescence-labeled ATS-2 is similar in all the cells. Thus, all subsequent antisense experiments were carried out in the presence of lipofectin unless stated otherwise.

Suppression of TS mRNA and Protein Synthesis by ATS-2. To elucidate the effects of ATS-2, HEK cells were grown in 10-cm Petri dishes to 20% confluence and were then treated with or without ATS-2 for 24 h. The cellular levels of TS protein and TS mRNA were determined. As shown by Western-blot assays (Fig. 2A), ATS-2 antisense ODN caused a 90% reduction in TS protein level in a 24-h treatment. In contrast, no suppression was found in cells treated with control ODN and with lipofectin only. Furthermore, there was an apparent reduction of TS mRNA level on ATS-2 treatment as revealed by RT-PCR assays, using β-actin mRNA as an internal control (Fig. 2B).

Inhibition of Cell Proliferation. After confirmation of specific effects of the ATS-2 on the TS enzyme and mRNA level, the effects on cell growth were studied. HEK cells at 20% confluence were cultured in medium with or without ODNs for 48 h. The total number of viable cells were then measured by an ACP assay. A dose-dependent suppressive effect of ATS-2 on cell proliferation was observed (Fig. 3 columns 3–5). At a dosage of 0.2 μM, the increase of the total number of viable cells was reduced as much as 98% compared with the number in control culture, in which the number of cells was increased by 6-fold during incubation (column 1). Lipofectin elicited no inhibition (column 2). Control ODN at 0.2 μM elicited a low level (35%) of inhibition (Fig. 3, column 6). This inhibition was attributed to the complex of ODN/lipofectin, because other ODN sequences in complex with lipofectin also showed inhibition to some extent (data not shown). ATS-2 alone without lipofectin carrier at a concentration of 10 μM, which is 50-fold higher than that used for the culture shown in column 5, resulted in 60% inhibition (column 7), but no inhibition was found for the control ODN alone at the same concentration (column 8). Upon microscope examination, the ATS-2-treated cells began to detach after 40 h of treatment. Figure 1 D shows the detachment of HEK cells treated with 0.2 μM ATS-2 for 48 h. On the contrary, cells of the control group looked healthy (Fig. 1C). The detached cells were nonviable and fragmented as revealed by trypan blue staining (Fig. 1E).

S-Phase Arrest and Apoptosis. Some intracellular changes seem to have taken place before detachment. After a 24-h treatment and flow cytometry analysis, we observed that 70% of the cells had DNA content higher than 2N but lower than 4N; the cell population was in an apparent S-phase but did not pass on to G_2/M phase (Fig. 4C). This cell population distribution pattern from culture treated with ATS-2 differs from the pattern obtained from the control culture (Fig. 4A) and from culture treated with control ODN (Fig. 4B). Increase in the treatment time from 24 to 30 h caused more cells to be arrested in the apparent S-phase with concomitant reduction in cells in the G_1-phase population (Fig. 4D). The onset of apoptosis for the ATS-2 treated cells was identified: (1) by TUNEL assay (at 32 h) (Fig. 1F), which indicated breakage of the chromosomal DNA, and (2) by Hoechst staining (at 42 h), which showed chromatin condensation (Fig. 1H). These phenomena were not found in cells in the control groups (Fig. 1, G and I).

FdUrd and ATS-2 Treatment. The effects of addition of FdUrd or thymidine on cells treated with ATS-2 treatment were studied (Fig. 5). An additive effect was found when HEK cells were treated concurrently with ATS-2 and FdUrd. Cellular inhibition by ATS-2 0.1 μM plus FdUrd 3 μM (Fig. 5, column 5, more than 100% inhibition) was much higher than that found using FdUrd alone at 3 μM (Fig. 5, column 2) and ATS-2 singly at 0.1 μM (Fig. 5, column 4). Unexpectedly, addition of thymidine (3 μM) did not relieve the inhibition on proliferation caused by ATS-2 (column 6). We then compared the responses of the HEK cells with the responses of HeLa, a cervical cancer cell line, to treatments by ATS-2, FdUrd, and ATS-2 plus thymidine. HeLa cells were chosen for the comparative studies because HeLa cells have similar cell division rate and ODN uptake rates as HEK. The results of inhibition of proliferation are shown in Fig. 6. HeLa was more sensitive to FdUrd compared with HEK (Fig. 6, columns 1 and 2). On the contrary, HeLa was relatively insensitive to ATS-2 (Fig. 6, columns 3 and 4). Addition of thymidine (100 μM) partially relieved the inhibition of proliferation caused by ATS-2 in HeLa cells but not in HEK cells (Fig. 6, column 5). This observation could be interpreted by the mechanism that conversion of thymidine to dTMP (in relieving the inhibition caused by ATS-2) and conversion of FdUrd to FdUMP (in activating the inhibition caused by FdUrd) via TdR kinase activity is relatively strong in HeLa and relatively weak in HEK cells. This interpretation is further collaborated by the observation that HeLa cells incorporated more [^{3}H]thymidine (15-fold) from the culture than HEK cells (Fig. 6, column 6). This interpretation is predicated on the assumption that the internal pool sizes of thymidine of these two cell lines are similar in this culturing situation.

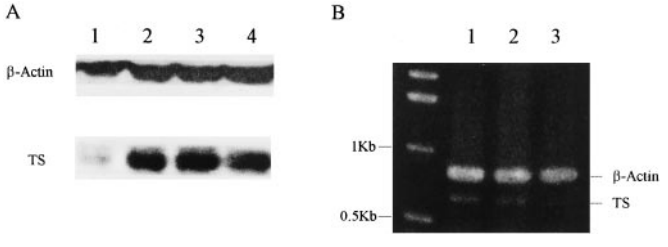

Fig. 2. Effects of ATS-2 on the cellular levels of TS protein and TS mRNA. A, HEK cells in 20% confluence were treated with 0.2 μM ATS-2 (lane 1) or control ODN (lane 2) with lipofectin, lipofectin only (lane 3), or no treatment (lane 4) for 24 h. Cell lysates containing 75 μg of protein were electrophoresed into a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was cut at the 40-kDa position. The lower and upper portions of the membranes were reacted with monocloned antibodies to TS and to β-actin, respectively, followed by reaction with a horseradish peroxidase secondary antibody. The immunoreactive bands were revealed by enhanced chemiluminescence reagent and exposure to X-ray films. B, HEK cells that were not treated (lane 1) or treated with 0.2 μM control ODN (lane 2) or ATS-2 (lane 3) with lipofectin for 24 h. mRNAs of these cells were purified by poly(A)pure kit and was reverse-transcribed using oligo(dT) primer. The products were subjected to PCR to amplify a 0.65-kb TS and a 0.8-kb β-actin cDNA fragment using their respective primers. The RT-PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining, and monitoring on a UV table.

Discussion

The aim of this study was to develop an effective anti-TS ODN to be used as an alternative drug for thymidineless treatment. We demonstrated that ATS-2 was such a candidate. Reasons for a higher inhibitory efficiency of ATS-2 sequence are as follows. ATS-2 is complementary to nucleotides 201 to 207 in the coding region of the TS mRNA (Takeishi et al., 1985) that was predicted not to reside in the stem of the hairpin structure of the TS mRNA. The sequence is not located in two mRNA regions (nucleotides 1–188 and 434–634) interacting with the TS protein (Chu et al., 1993). Antisense ODN sequence per se, a higher G+C content (65%) of ATS-2 could have resulted in a higher binding affinity with the TS mRNA. The ATS-2 sequence does not itself form dimer or hairpin structure as predicted by free energy (ΔG) calculations. Moreover, ATS-2 does not contain runs of more than three consecutive Gs and other sequence motifs unfavorable to antisense ODNs as demonstrated statistically by Matveeva et al. (2000).

The antisense ODN ATS-2 delivered by lipofectin resulted in an effective suppression of the TS level in 24 h in HEK

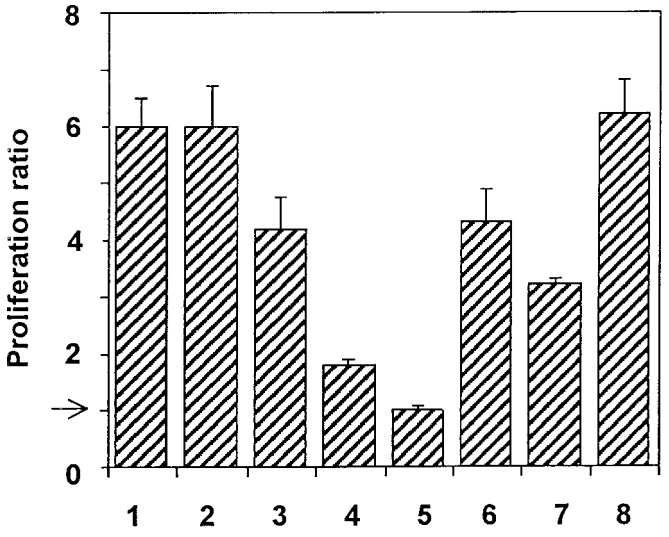

Fig. 3. Inhibition of cellular proliferation by ATS-2. HEK cells at 20% confluence were treated as follows for 48 h and subjected to ACP assays for quantitation of the viable cells. The ACP value obtained at 0 h is adjusted to be one (as indicated by an arrow). Column 1, blank; column 2, lipofectin (20 μl) only; columns 3 to 5, ATS-2 at 0.05, 0.1, and 0.2 μM with lipofectin, respectively; column 6, control ODN 0.2 μM with lipofectin; column 7, ATS-2 10 μM without lipofectin; column 8, control ODN 10 μM without lipofectin; error bars indicate S.D.

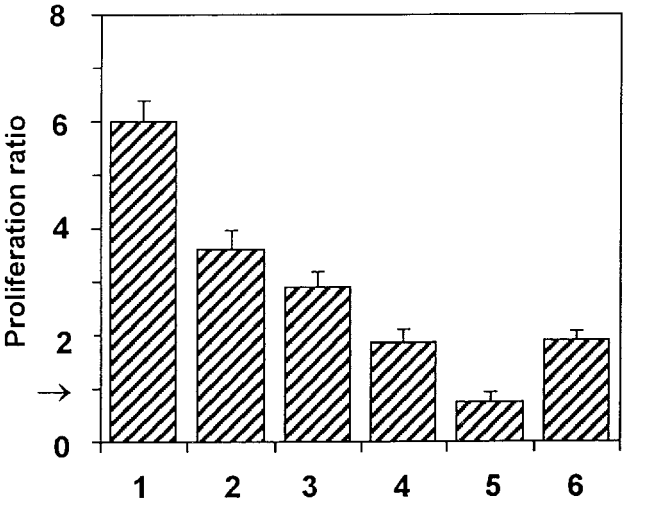

Fig. 5. Comparison of the effects of FdUrd and ATS-2 on cell proliferation. HEK cells at 20% confluence were treated as follows for 48 h and subjected to ACP assays. The value of ACP obtained at 0 h is adjusted to be one (as indicated by an arrow). ATS-2 was always added with lipofectin. Column 1, blank; column 2, 3 μM FdUrd; column 3, 9 μM FdUrd; column 4, 0.1 μM ATS-2; column 5, 3 μM FdUrd + 0.1 μM ATS-2; column 6, 3 μM thymidine + 0.1 μM ATS-2. Error bars indicate S.D.

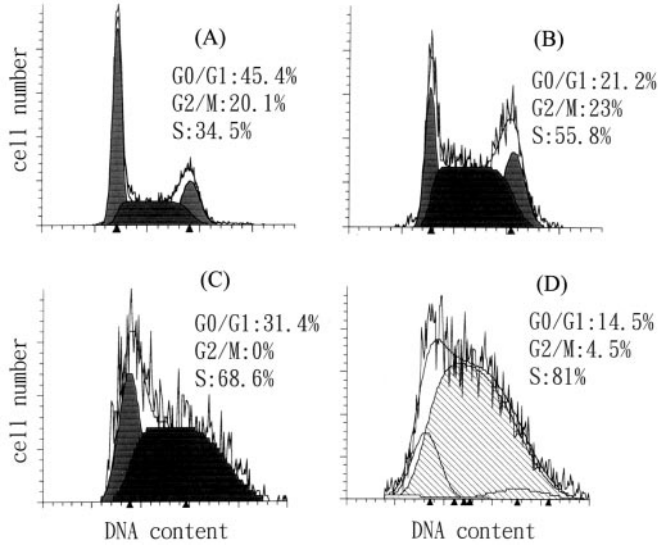

Fig. 4. Effects of ATS-2 on cell cycle. HEK cells at 20% confluence were treated with lipofectin alone (A), 0.2 μM control ODN (B), or ATS-2 (C) with lipofectin for 24 h, or 0.2 μM ATS-2 with lipofectin for 30 h (D). The cells were then trypsinized and washed with PBS. After fixation with alcohol, the cells were treated with RNase followed by staining with propidium iodide. The cells were analyzed with a FACScan flow cytometer. Cell cycle phase distribution was analyzed using the ModFit LT software.

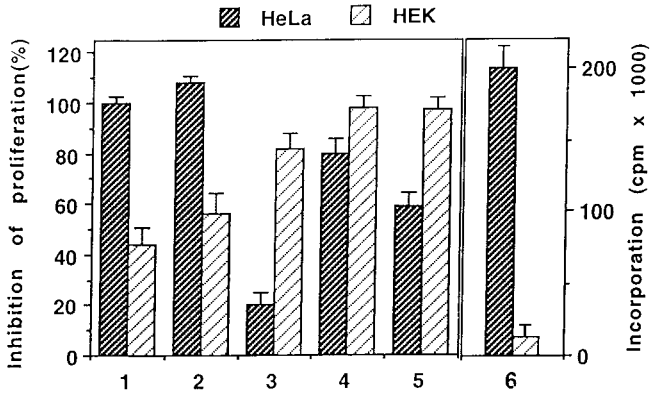

Fig. 6. Effects on proliferation of HEK and HeLa cells. HEK and HeLa cells both in 20% confluence were treated as follows for 48 h and subjected to ACP assays. ATS-2 was always added with lipofectin. Column 1, 3 μM FdUrd; column 2, 9 μM FdUrd; column 3, 0.1 μM ATS-2; column 4, 0.2 μM ATS-2; column 5, 0.2 μM ATS-2 + 100 μM thymidine. Inhibition (%) = $[(A_{blank} - A_{treatment}) / (A_{blank} - A_{0h})] \times 100$. For [^{3}H]thymidine incorporation experiments (column 6), 2.5×10^5 HEK or HeLa cells were seeded and grown in 6-cm Petri dishes under normal culture conditions overnight. The cells were pulse-labeled with [^{3}H]thymidine (1 μCi/ml) for 4 h. The cells were then washed with PBS and trypsinized. The cells were retained on glass microfiber filters by filtration. The filters were washed with PBS in the presence of 0.5% Nonidet P-40, followed by 75% ice-cold alcohol. The amounts of [^{3}H]thymidine were measured by β-counter with scintillation liquid.

cells. TS depletion in turn led to a shortage in thymidylates, and consequently resulted in an S-phase arrest. On the other hand, ATS-2 seemed not to hamper the treated cells crossing G_2/M to G_1 and G_1 to S phase (see Fig. 4). The results imply that ATS-2 is specifically effective for dividing cells. The S phase-arrested cells, as documented by many authors (Chen et al., 1997; Cynthia et al., 1998; Zhang et al., 2000), then entered into apoptosis as verified by DNA breakage and chromatin condensation (see Fig. 1, F and H).

However, in our attempts to detect apoptotic DNA laddering, electrophoretic smears rather than the anticipated ladders were found (data not shown). One possible explanation is that, because dTTP was depleted as a consequence of the action of the anti-TS ODN, dUTP accumulated and was incorporated into the replicating DNA instead (Ingraham et al., 1980; Mauro et al., 1993). This in turn had triggered the DNA repairing system involving uracil-DNA glycosylase, which removes the uracil bases in double-stranded DNA. Subsequently, the action of apurinic/apyrimidinic endonuclease had then brought about massive but random strand breakages (Ingraham et al., 1980; Yin and Rustum, 1991; Mauro et al., 1993). Such mechanism would be the one that confers major cytotoxic effect observed in ATS-2-treated cells.

Because FdUMP inactivates existing TS while the anti-TS ODNs suppress the synthesis of new TS, a combined treatment at certain concentration produces an additive effect as anticipated. However, in a comparative study between HEK cells and HeLa cells on their relative responses in cellular proliferation inhibition to the impacts of FdUrd and anti-TS ODN, HEK cells were shown to be more sensitive to anti-TS ODN than HeLa cells, whereas HeLa cells are more sensitive to FdUrd than HEK cells. We propose the key factor in the difference between these two cell lines is the relative activities of the TdR kinase system. The relative strength of the TdR kinase activity can be shown by the effect of added thymidine on the relief of the inhibitory effects of the anti-TS ODN on these two cell lines and by their relative rates of [^{3}H]thymidine incorporation into DNA in the culture.

Thus, HEK cell proliferation is sensitive to anti-TS ODN but insensitive to FdUrd inhibition, whereas HeLa cell proliferation is sensitive to FdUR but insensitive to anti-TS ODN. Cancer cells resistant to FdUrd (or possibly 5-FU) can be good candidates for treatment with anti-TS ODNs, and the combination of both treatments could be very effective over a broad spectrum of cancer cells.

Acknowledgments

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References

Chen CC, Wang JK, Chen WC and Lin SB (1998) Protein kinase C-η mediates lipopolysaccharide-induced nitric oxide synthase expression in primary astrocytes. *J Biol Chem* **273:**19424–19430.

Chen G, Shu J and Stacey DW (1997) Oncogenic transformation potentiates apoptosis, S-phase arrest and stress-kinase activation by etoposide. *Oncogene* **15:**1643–1651.

Chu E, Voeller D, Koeller DM, Drake JC, Takimoto CH, Maley GF, Maley F and Allegra CJ (1993) Identification of an RNA binding site for human thymidylate synthase. *Proc Natl Acad Sci USA* **90:**517–521.

Cynthia M, Dean SR, Sudha I, A Hamid Boulares and Mark ES (1998) Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. *J Biol Chem* **273:**13703–13712.

Danenberg PV (1977) Thymidylate synthase: A target enzyme in cancer chemotherapy. *Biochim Biophys Acta* **473:**73–92.

DeMoor JM, Vincent MD, Collins OM and Koropatnick J (1998) Antisense nucleic acids targeted to the thymidylate synthase (TS) mRNA translation start site stimulate TS gene transcription. *Exp Cell Res* **243:**11–21.

Ferguson PJ, Collins O, Dean NM, DeMoor JM, Li CS, Vincent MD and Koropatnick J (1999) Antisense down-regulation of thymidylate synthase to suppress growth and enhance cytotoxicity of 5-FUdR, 5-FU and Tomudex in HeLa cells. *Br J Pharmacol* **127:**1777–86

Hardy LW, Finer-Moore JS, Montfort WR, Jones MO, Santi DV and Stroud RM (1987) Atomic structure of thymidylate synthase: target for rational drug design. *Science (Wash DC)* **235:**448–455.

Heidelberger C, Danenberg PV and Moran RG (1983) Fluorinated pyrimidines and their nucleosides. *Adv Enzymol* **54:**58–119.

Ingraham HA, Tseng BY and Goulian M (1980) Mechanism for exclusion of 5-fluorouracil from DNA. *Cancer Res* **40:**998–1001.

Jackman AL, Taylor GA, Gibson W, Kimbell R, Brown M, Calvert AH, Judson IR and Hughes LR (1991) ICID1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumour cell growth in vitro and in vivo: a new agent for clinical study. *Cancer Res* **51:**5579–5586.

Johnson LF (1994) Posttranscriptional regulation of thymidylate synthase gene expression. *J Cell Biochem* **54:**387–392.

Johnston PG, Fisher ER, Rockette HE, Fisher B, Wolmark N, Drake JC, Chabner BA and Allegra CJ (1994) The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* **12:**2640–2647.

Keyomarsi K, Samet J, Molnar G and Pardee AB (1993) The thymidylate synthase inhibitor, ICI D1694, overcomes translational detainment of the enzyme. *J Biol Chem* **268:**15142–15149.

Kronenwett R and Haas R (1998) Antisense strategies for the treatment of hematological malignancies and solid tumors. *Ann Hematol* **77:**1–12.

Lin SB, Hsieh SH, Hsu HL, Lai MY, Kan LS and Au LC (1997) Antisense oligodeoxynucleotides of IGF-II selectively inhibit growth of human hepatoma cells overproducing IGF-II. *J Biochem* **122:**712–722.

Lin SB, Wu LC, Huang SL, Hsu HL, Hsieh SH, Chi CW and Au LC (2000) In vitro and in vivo suppression of growth of rat liver epithelial tumor cells by antisense oligodeoxynucleotide against protein kinase C-α. *J Hepatol* **33:**601–608.

Matveeva OV, Tsodikov AD, Giddings M, Freier SM, Wyatt JR, Spiridonov AN, Shabalina SA, Gesteland RF and Atkins JF (2000) Identification of sequence motifs in oligonucleotides whose presence is correlated with antisense activity. *Nucleic Acids Res* **28:**2862–2865.

Mauro DJ, De Riel JK, Tallarida RJ and Sirover MA (1993) Mechanisms of excision of 5-fluorouracil by uracil DNA glycosylase in normal human cells. *Mol Pharmacol* **43:**854–857.

Murakami Y, Kazuno H, Emura T, Tsujimoto H, Suzuki N and Fukushima M (2000) Different mechanisms of acquired resistance to fluorinated pyrimidines in human colorectal cancer cells. *Int J Oncol* **17:**277–283.

Pestalozzi BC, Peterson HF, Gelber RD, Goldhirsch A, Gusterson BA, Trihia H, Lindtner J, Cortes-Funes H, Simmoncini E, Byrne MJ, et al. (1997) Prognostic importance of thymidylate synthase expression in early breast cancer. *J Clin Oncol* **15:**1923–1931.

Rossana C, Gollakota Rao L and Johnson LF (1982) Thymidylate synthetase overproduction in 5-fluorodeoxyuridine-resistant mouse fibroblasts. *Mol Cell Biol* **2:**1118–1125.

Santi DV, McHenry CS and Sommer H (1974) Mechanisms of interactions of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* **13:**471–481.

Schmitz JC, Agrawal S and Chu E (1998) Repression of human thymidylate synthase mRNA translation by antisense 2′-O-methyl oligoribonucleotides. *Antisense Nucleic Acid Drug Dev* **8:**371–378.

Simoes-Wust AP, Olie RA, Gautschi O, Leech SH, Haner R, Hall J, Fabbro D, Stahel RA and Zangemeister-Wittke U (2000) Bcl-xl antisense treatment induces apoptosis in breast carcinoma cells. *Int J Cancer* **87:**582–590.

Sowers LC, Eritja R, Kaplan B, Goodman MF and Fazakerly GV (1988) Equilibrium between a wobble and ionized base pair formed between fluorouracil and guanine in DNA as studied by proton and fluorine NMR. *J Biol Chem* **263:**14794–14801.

Suzuki M, Ohwada M, Tamada T and Tsuru S (1994) Thymidylate synthase as a prognostic factor in ovarian cancer. *Oncology* **51:**334–338.

Takeishi K. Kaneda S. Ayusawa D. Shimizu K. Gotoh O, Seno T (1985) Nucleotide sequence of a functional cDNA for human thymidylate synthase. *Nucleic Acids Res* **13:**2035–43.

Tanaka M, Yoshida S, Saneyoshi M and Yamaguchi T (1981) Utilization of 5-fluoro-2′-deoxyuridine triphosphate and 5-fluoro-2′- deoxycytidine triphosphate in DNA synthesis by DNA polymerases alpha and beta from calf thymus. *Cancer Res* **41:**4132–4135.

Wagner RW (1994) Gene inhibition using antisense oligodeoxynucleotides. *Nature (Lond.)* **372:**333–335.

Yin MB and Rustum YM (1991) Comparative DNA strand breakage induced by FUra and FdUrd in human ileocecal adenocarcinoma (HCT-8) cells: relevance to cell growth inhibition. *Cancer Commun* **3:**45–51.

Zhang Y, Rishi AK, Dawson MI, Tschang R, Farhana L, Boyanapalli M, Reichert U, Shroot B, Van Buren EC and Fontana JA (2000) S-phase arrest and apoptosis induced in normal mammary epithelial cells by a novel retinoid. *Cancer Res* **60:**2025–2032.

Zhang ZG, Malmberg M, Yin MB, Slocum HK and Rustum YM (1993) Isolation and characterization of a human ileocecal carcinoma cell line (HCT-8) subclone resistant to fluorodeoxyuridine. *Biochem Pharmacol* **45:**1157–1164.

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