Folate-Mediated Targeting of Antisense Oligodeoxynucleotides to Ovarian Cancer Cells

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Purpose. Receptors for vitamin folic acid are frequently overexpressed on epithelial cancer cells, especially ovarian cancer cells. In this study, we examined whether this expression might be exploited to specifically deliver antisense oligodeoxynucleotides (ODN) to tumor cells.

Methods. A conjugate was prepared by directly coupling folic acid to the 3′ terminus of an anti-*c-fos* ODN and its cellular uptake and tumor inhibitory effect were evaluated using FD2008 cells that overexpress folate receptors.

Results. When a phosphorothioate (PS)/phosphodiester (PO) chimeric ODN was conjugated with folic acid, its uptake by FD2008 cells was increased by about 8-fold ($P < 0.01$). In contrast, conjugation of folate to the ODN did not increase its uptake by CHO cells that lack the expression of FBP ($P > 0.05$). Furthermore, the increase in the uptake of conjugated ODN by FD2008 cells could be blocked by adding an excess amount of folic acid. The PS/PO antisense ODN had some inhibitory effect on the growth of FD2008 cells. However, its activity was significantly increased following conjugation with folic acid ($P < 0.01$). ODN of scrambled sequences with and without conjugation with folic acid failed to inhibit the growth of FD2008 cells. Finally, the antisense effect of the conjugated ODN on FD2008 cells was inhibited by an excess amount of free folic acid, suggesting that the sequence-dependent effect of folate-antisense ODN conjugate was mediated by folate binding protein.

Conclusions. Direct derivatization of ODN with folate significantly improves their targeting efficiency to tumor cells *in vitro*. The folate-conjugated ODN, due to their small size and possibly efficient extravasation at tumor site, has the potential for treating solid tumors that overexpress folate receptors.

KEY WORDS: folate binding protein; antisense oligodeoxynucleotide; ovarian carcinoma; drug targeting.

INTRODUCTION

Antisense oligodeoxynucleotides (ODN) are potential therapeutics for the treatment of cancer, viral infections, and other pathological disorders (1–3). The specificity of ODN is due to a highly selective hybridization of these oligomers to their complementary target sequences on the mRNA, which causes inhibition of protein expression by at least two widely accepted mechanisms. The first is the degradation of RNA by RNase H, which selectively cleaves the RNA of the DNA-RNA heteroduplex (3–5). The second mechanism is the arrest of translation initiation caused by ODN hybridization to the 5′-untranslated region or the initiation site on the mRNA (3). In both mechanisms, protein expression is suppressed at the RNA level.

The mechanism by which antisense ODN enter cells is not fully understood. Some evidence suggests the existence of ODN receptors on the cell surface (6), and consequently, the uptake of ODN has been shown to depend on the time and concentration (7–8). However, cytoplasmic delivery of ODN without carriers is inefficient and many delivery vehicles have been developed to improve their intracellular delivery. These include phosphatidylcholine liposomes (9), cationic liposomes (10,11) and protein-polylysine conjugates (12,13). ODN can be entrapped inside liposomes (9). The liposomes can also be designed such that they are long circulating in the blood (14). However, the size of liposome required for achieving long circulation in the blood and efficient tumor localization is too small (~100 nm) for efficient entrapment of ODN. Cationic liposomes readily form complexes with ODN due to electrostatic interactions. Almost 100% of ODN can be recovered in complexed form. The resulting complexes usually contain a slight excess of positive charge, which allows efficient binding to the negatively charged cell membrane. Several liposomal formulations have been reported to enhance the intracellular delivery of ODN and change their intracellular distribution (10,11). ODN complexed with cationic liposomes exhibit a biological activity up to 1,000 fold higher than ODN alone (10). However, systemic administration of cationic liposome/ODN complexes remains problematic (15). No reports have been described so far of successful *in vivo* application of cationic liposome/ODN complexes. Protein-polylysine conjugates have been developed for the delivery of plasmid DNA and more recently for the delivery of ODN. *In vivo* injection of an asialorosomucoid protein-polylysine conjugate complexed with a phosphorothioate antisense ODN has been shown to inhibit the replication of hepatitis B virus in an animal model (16). However, delivery of ODN by protein-polylysine conjugates for the treatment of solid tumors might be hampered by its relatively large size. In addition, the efficiency could be further decreased following repeated injections due to the generation of anti-ligand antibody in the host. In an attempt to overcome these limitations, a different approach is investigated in this study by chemically conjugating an ODN with folic acid. The folate receptor-mediated cellular uptake of an anti-*c-fos* ODN is evaluated by using two cell lines with and without expression of folate binding protein as target and non-target cells, respectively. The activity of the ODN is also evaluated by cell growth inhibition.

MATERIALS AND METHODS

Materials

^{125}I was purchased from DuPont-NEN (Boston, MA). [^{3}H]folic acid (25.2 Ci/mmol) was obtained from Moravek

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ABBREVIATIONS: ODN, oligodeoxynucleotides; PS, phosphorothioate; PO, phosphodiester; ATP, adenosine 5′-triphosphate; FBP, folate binding protein; HRP, horseradish peroxidase; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Sulfo-NHS, *N*-hydroxysulfosuccinimide; FD, folate deficient; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis.

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Biochemicals, Inc. (Brea, CA). Rabbit serum against FBP was generated by immunizing the rabbit with pure FBP (Sigma, St. Louis, MO). Goat anti-rabbit immunoglobulin G antibody labeled with horseradish peroxidase (HRP) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Monoclonal anti-*c-fos* antibody and goat anti-mouse immunoglobulin G antibody labeled with HRP were purchased from Oncogene Science (Cambridge, MA). ECL™ western blotting kit was obtained from Amersham Life Science (Buckinghamshire, UK). Acrylamide and *N'*, *N'*-methylene-bisacrylamide were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Urea and ammonium persulfate were obtained from Fisher Scientific (Fair Lawn, NJ). Folic acid, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDCI), *N*-hydroxysulfosuccinimide (Sulfo-NHS), deoxycholate, aprotinin, nonidet P-40 and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO).

Cells and Medium

Human ovarian cancer cell line 2008 was generously provided by Dr. Paul A. Andrews, Georgetown University, Washington, DC. A subline, FD2008, was established by culturing 2008 cells in folate-deficient RPMI 1640 (normal medium except without folic acid) supplemented with 10% fetal bovine serum and antibiotics (the final folate concentration in the serum-supplemented medium was approximately physiological). CHO cells were obtained from ATCC (Rockville, MD) and cultured in F12 medium supplemented with 10% (v/v) heat inactivated fetal calf serum.

Expression of FBP on FD2008 and CHO Cells (17)

2×10^4 FD2008 or CHO cells were plated in a 96 well tissue culture plate. After incubation at 37°C for 24 h, the cells were fixed with 0.25% glutaraldehyde in PBS at 37°C for 10 min. The cells were washed three times with PBS containing 0.2% Tween-20 and 0.1 ml of serially diluted rabbit anti-FBP serum was added. Following incubation at 37°C for 1 h, cells were washed three times with PBS containing 0.2% Tween-20. Then, 0.1 ml of goat anti-rabbit immunoglobulin G antibody labeled with HRP was added. The peroxidase reaction was initiated by the addition of *o*-phenylenediamine and the optical density at 490 nm of each well was recorded.

Synthesis of ODN

A 15-mer antisense ODN complementary to the *c-fos* gene and nucleotides 917–931 of the *c-fos* genome (18) was synthesized with a 3'-amino modifier CPG {[(1-dimethoxytrityl)-oxy]-3-[(fluorenylemethoxycarbonyl) amino]propan-2-succinoyl]-long chain alkyamino-CPG} (The Midland Certified Reagent Company, Midland, Texas). This yielded the following ODN: 5'-GAGGATGACGCCTCG-amino-3'. The size of this ODN was determined by the manufacturer by comparing migration distance with that of the parallel ODN standard using a 20% polyacrylamide/7M urea gel electrophoresis. Similarly, another ODN which was a scrambled version of the antisense ODN was synthesized. The sequence was: 5'-AAGGGTA-GCCCGTGC-amino-3'. Two PS/PO chimeric ODN with identical sequences to the two ODN above were also synthesized. The ODN were 5'-GsAsGsGATGACGCCsTsCsG-amino-3' and 5'-AsAsGsGGTAGCCCGsTsGsC-amino-3'.

Preparation of Folate-ODN Conjugate

For the conjugation with folate, 0.1 μmol of 3'-amino-ODN was mixed with 10 molar excess of folate and 15 molar excess each of sulfo-NHS and EDCI in 0.2 ml 0.02 M phosphate buffer pH 7.4. The reaction mixture was stirred at RT for 4 h. Following the reaction, the conjugate was separated from free folate by gel filtration on a Sephadex G-25 column (1.0 cm × 5.0 cm). Possible aggregates were then removed from the conjugate by gel purification. The concentration of folate was determined by measuring the absorbance at 363 nm, $\varepsilon = 13{,}600$. The concentration of ODN was then calculated by subtracting the contribution of folate at 260 nm from the A260 of the conjugate. Alternatively, [^{3}H]folic acid (2.52 mCi/mmol) was used to prepare the conjugate to follow the conjugation efficiency.

Preparation of ^{125}I-Labeled ODN

ODN, free or conjugated, were iodinated as described (19). Briefly, ^{125}I was added to ODN in 0.1 mM sodium acetate, pH 4.0 in a glass tube coated on the bottom with Iodogen reagent (Pierce, Rockford, IL). The mixture was incubated in a water bath at 50°C for 30 min. Iodinated ODN were then separated from free iodine by passing twice through a Bio-Gel P-4 spin column (Bio-Rad, Richmond, CA).

Uptake of ODN by FD2008 and CHO Cells

For uptake study, approximately 10^5 cpm of ^{125}I-labeled and 2μM of unlabeled ODN were added into each well. Following incubation at 37°C for 2 h, the culture medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4 containing 1% bovine serum albumin (BSA). After washing, the cells were resuspended with Versene (Gibco), washed again with PBS containing BSA and centrifuged at 1,000 rpm for 5 min. The cell pellet was suspended with a solution of 0.2 M acetic acid and 0.5 M sodium chloride, pH 2.5, at 4°C for 10 min to elute the surface-bound ODN and centrifuged at 1,000 rpm for 5 min. The cell-associated radioactivity resistant to the acid-salt treatment represented the intracellular ODN and was measured by using a gamma scintillation counter. The effect of 1 mM free unlabeled folate on the uptake of ODN, modified or unmodified, by the cells was also investigated.

In Vitro Cytotoxicity

The MTT tetrazolium colorimetric assay was employed to assess the inhibitory effect of ODN (20). FD2008 or CHO cells in 0.1 ml growth medium (3×10^3 cells/well) were plated in a sterile tissue culture plate with 96 flat-bottomed wells. ODN, modified or unmodified, were filtered through a 0.22 μm Millerpore filter, and various dilutions in 0.1 ml were added to the wells in triplicate. The cells were incubated for 96 h at 37°C in a 5% CO_2 atmosphere. After the incubation, an aliquot of 20 μl of MTT dye stock solution (5 mg/ml in PBS) was added into each well and the cells were incubated for another 4 h. The unreacted MTT and medium were removed by aspiration. To each well 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the MTT formazan and the absorbance at

565 nm was determined. The inhibitory effect of test agents was calculated according to the following formula:

$$\% \text{ growth inhibition} = \left(1 - \frac{\text{A565 test}}{\text{A565 control}}\right) \times 100\%$$

Western-Blot Analysis

FD2008 cells (5×10^4) were plated in a sterile tissue plate with 6 flat-bottomed wells and allowed to adhere for 12 hr. Folate-conjugated ODN (10 μM), antisense or scrambled, were applied in culture medium for 48 hr. The cells were then treated with lysis buffer (10 mM Tris chloride, 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 1% aprotinin, 0.25 mM phenylmethylsulfonyl fluoride [pH 7.4]) for 15 min at 4°C. Cell debris was removed by centrifugation at 4°C for 30 min at 37,000 g. Equal amounts of protein were subjected to 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Immunodetection of *c-fos* by anti-*c-fos* monoclonal antibody using ECL™ was performed according to a protocol described by the manufacturer. Subsequently, blots were densitometrically evaluated.

RESULTS

Expression of FBP on FD2008 and CHO Cells

Initially, 2008 cells grew very slowly in a medium with approximately physiological concentration of folate. Some of the cells looked like megaloblastic cells indicating a folate deficiency in those cells. About 2 months later, 2008 cells adapted to the folate deficient condition suggesting an upregulation of FBP expression in the cells. No obvious difference was found between these 2008 cells and the parent 2008 cells in either morphology or doubling time (about 30 h). This subline was designated FD2008 cells. The amount of FBP expressed on the cells was measured by ELISA using rabbit antiserum against purified FBP. As shown in Fig. 1, a high level of FBP expression was found on FD2008 cells. In contrast, FBP was almost non-detectable on CHO cells. Consequently, FD2008 and CHO cells were used as target and non-target cells respectively in all subsequent studies.

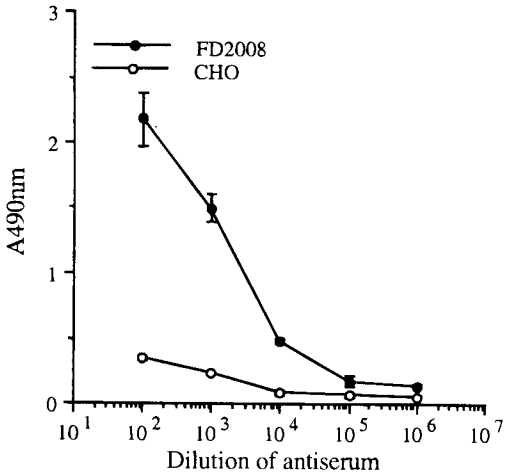

Fig. 1. Enzyme linked immunosorbent assay of the FBP expression on FD2008 and CHO cells. Cells were incubated with various dilutions of rabbit antiserum against FBP for 1 h followed by the addition of goat antirabbit immunoglobulin G antibody labeled with HRP. The HRP reaction was initiated by the addition of *o*-phenylenediamine.

Conjugation of Folate with 3′-Amino-ODN

ODN obtained from the manufacturer were repurified from PAGE gel before conjugation. They manifested as a single peak on HPLC and also showed as a single band on PAGE. Following reaction with folic acid in the presence of carbodiimide, the folate-ODN conjugates were removed from free folate by Sephadex G-25 and then further separated from possible impurities by PAGE. A conjugation efficiency of 85–90% was routinely obtained with approximately a 60% yield.

Uptake of Folate-ODN Conjugates by Cells

An initial study was performed to compare the difference in the uptake of 4 different, unmodified ODN by FD2008 cells, i.e. antisense and scrambled ODN in both PO and PS/PO form. No obvious difference was noticed among the 4 ODN (data not shown). Therefore, further uptake studies were focused on the PS/PO antisense ODN. Figure 2 shows the uptake by FD2008 and CHO cells of the ODN with and without conjugation with folate. The uptake of conjugated ODN by FD2008 cells was about 8 fold higher than that of unmodified ODN ($P < 0.01$). The improvement in the uptake was blocked by an excess amount of free folate. When non-target cells (CHO) were used, only a slight increase was observed for conjugated ODN ($P > 0.05$). No difference was found if 1 mM of free folate was added. Similar results were obtained when ^{32}P-labeled ODN was used to follow the cellular uptake (data not shown).

Growth Inhibitory Effects of Conjugated ODN

A MTT colorimetric assay was used to evaluate the biological activity of ODN with and without conjugation with folate. Figure 3 shows the result of PS/PO ODN. Antisense ODN alone had some inhibitory effect on the growth of FD2008 cells, which was dose-dependent. However, after conjugation with folate, the activity was significantly increased ($P < 0.01$). The activity was also sequence-dependent since ODN of scrambled sequence had little effect on the growth of FD2008 cells. Conjugation with folate hardly increased their inhibitory effect ($P > 0.05$). The specific inhibition of FD2008 cells by conjugated antisense ODN could be blocked by adding an excess amount of free folate (Fig. 4). Addition of 1 mM free folate into the folate-ODN conjugates decreased their activity to the level of free ODN. Compared with PS/PO ODN, PO ODN had significantly less activity in inhibiting the growth of target cells (Fig. 5). Attachment of a folate molecule to those ODN resulted in only a slight increase in their inhibitory activity. ODN, conjugated or unconjugated, had no effect on the growth of CHO cells (data not shown).

Down-Regulation of *c-fos* by Folate-ODN Conjugate

To see whether inhibition of FD2008 cells by ODN was target sequence-specific, the effect of folate targeted ODN on the expression of *c-fos* was evaluated by immunoblotting. The

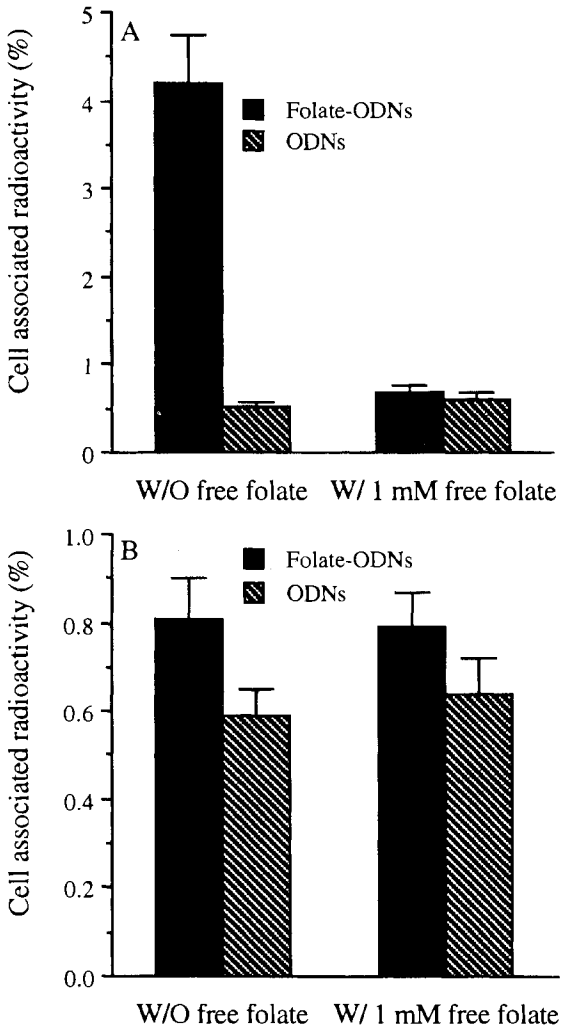

Fig. 2. The uptake of folate-ODN (PS/PO) conjugates by FD2008 (A) and CHO (B) cells. ODN, conjugated or unconjugated, were incubated with the cells for 2 h as described in Materials and Methods and the cell associated radioactivity was expressed as the percentage of the total counts added to the well. Data are shown as mean and SD (n = 3).

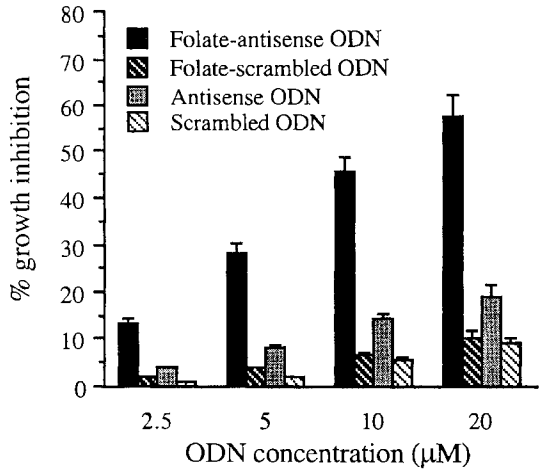

Fig. 3. Inhibitory effect of folate-ODN (PS/PO) conjugates on the growth of FD2008 cells. Various concentrations of ODN, conjugated or unconjugated, were incubated with FD2008 cells for 96 h and the cytotoxicity was evaluated by a MTT colorimetric assay. Data are shown as mean and SD (n = 3).

result was shown in Fig. 6. Densitometric analysis showed that 53.4% of *c-fos* reduction was achieved in the cells treated with antisense ODN. In contrast, only 6.7% of *c-fos* reduction was found in the cells treated with scrambled ODN.

DISCUSSION

Despite of the general opinion that cellular uptake of ODN without carriers was inefficient *in vitro*, several studies showed that free ODN were indeed effective *in vivo* (21). One recent study by Nesterova and Chochung demonstrated that a single injection of antisense ODN against protein kinase A could inhibit the tumor growth *in vivo* (21). The efficient tumor inhibition with minimal toxicity of free ODN might be due to, among other factors, the small size of ODN and the efficient extravasation in the tumor tissue. It is difficult to predict whether the existing delivery vehicles (anionic liposomes, cationic liposomes and protein-polylysine conjugates) could further increase the *in vivo* activity of the ODN since the distribution of the complexed ODN may be significantly different from that of free ODN in the tumor bearing host. A feasible approach to improve the already effective ODN is to modify the ODN such that the modified ODN remain in the free form but possess tumor targeting properties. Direct derivatization of free ODN with folic acid might meet this requirement. Folate binding protein (or folate receptor), a glycosylphosphatidylinositol anchored cell membrane protein, is vastly overexpressed in a wide variety of human tumors, especially ovarian carcinomas (22–24). Folate binds with its receptor with high affinity (Kd about 1 nM) and high specificity as folate receptor is not found in most normal tissues. Folate-conjugated macromolecules and liposomes have been shown to be specifically taken up by cultured receptor-bearing tumor cells as well as implanted xenografts *in vivo* (9,25,26). Therefore, it might be possible to render ODN tumor-specific while not changing its physicochemical property by direct conjugation with folate, a low molecular weight moiety.

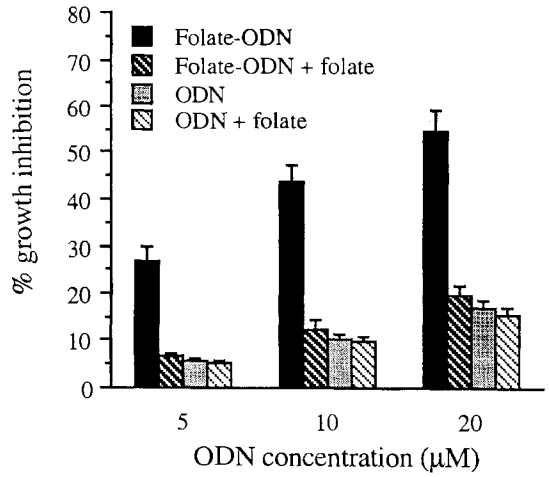

Fig. 4. The effect of free folate on the inhibitory activity of folate-ODN (PS/PO) conjugates. Folate was added into each well to a final concentration of 1 mM, the rest was the same as described in Fig. 3.

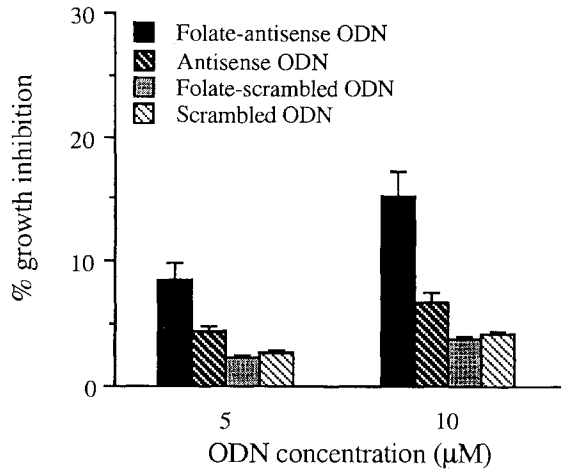

Fig. 5. Inhibitory effect of folate-ODN (PO) conjugates on the growth of FD2008 cells. The conditions in this experiment were the same as that in Fig. 3.

Results from the uptake study clearly demonstrated an improvement in the uptake of ODN by FD2008 cells following conjugation with folate. This is highly cell type-specific as increase in the uptake of folate-ODN conjugates was not found in CHO cells that lack the expression of FBP. Furthermore, 1 mM free folate blocked the uptake of folate-ODN conjugates by FD2008 cells, suggesting that intracellular delivery of folate-ODN conjugates was mediated by FBP. Unmodified ODN, either in PO or PS/PO form, showed no difference in their cellular uptake (data not shown). However, a slight increase in the uptake of folate-ODN conjugates was consistently found for CHO cells, which could not be blocked by excess amount of free folate. This is probably due to the increase in the hydrophobicity of ODN after conjugation with folate.

C-fos was chosen in this study as a model to evaluate the biological activity of folate conjugated ODN because it is overexpressed in 2008 cells. *C-fos* protooncogene is involved in cell growth and signal transduction pathways. It is one of the earliest genes activated in response to mitogenic stimuli (27). *C-fos* also plays a role in drug resistance and in mediating DNA synthesis and repair processes by modulating expression of genes such as dTMP synthase, DNA polymerase β, and topoisomerase I (18). Down-regulation of *c-fos* protein by ribozyme-mediated cleavage of *c-fos* mRNA resulted in the reverse of drug resistance in A2780DDP cells (18). Recently, down-regulation of *c-fos* by ribozyme has also been shown in this laboratory to be associated with growth inhibition of 2008 cells (Hofland and Huang, unpublished data). Similar results were found in this study with antisense ODN. As shown in Fig. 3,

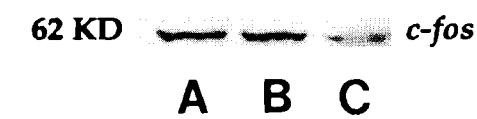

Fig. 6. Suppression of *c-fos* by folate targeted antisense ODN in FD2008 cells. After incubation for 48 hr with ODN (10 μM), cells were solubilized. Equal amounts of protein (40 μg/well) were subjected to 10% PAGE followed by immunoblotting using monoclonal antibody against *c-fos*. A: control cells; B: cells treated with folate-scrambled ODN; C: cells treated with folate-antisense ODN.

anti-*c-fos* ODN alone had some inhibitory effect on FD2008 cells. However, their activity was significantly increased following conjugation with folate. The improvement in their biological activity could be blocked by adding excess amount of free folic acid (Fig. 4), suggesting that the inhibitory effect of folate conjugated ODN was mediated by FBP. ODN of scrambled sequence was not effective in inhibiting the cell growth, indicating that the biological effect of ODN was sequence-specific. This was confirmed by immunoblotting. Folate targeted antisense ODN drastically inhibited the expression of *c-fos*. Scrambled ODN, ineffective in growth inhibition, also failed to down-regulate the expression of *c-fos* in FD2008 cells. Anti-*c-fos* ODN did not have much effect on the growth of CHO cells (data not shown), uptake of ODN might be too low in these receptor negative cells to affect their growth. In addition, *c*-fos might not be highly important in governing the growth of CHO cells.

It should be noted that PO ODN were not effective in inhibiting the growth of FD2008 cells. No benefits were noticed after conjugation with folate. This indicates that folate plays a more important role in improving the intracellular delivery of ODN than protecting the ODN from degradation by 3′-exonuclease. It is also possible that mechanism other than 3′-exonuclease are important in inactivating the PO ODN. Co-delivery of PO ODN with a lytic peptide might facilitate their release from lysosomes to cytoplasm and improve their tumor inhibitory effect.

In summary, we have developed a targeting strategy for free ODN which is potentially applicable in treating human tumors that overexpress folate receptor. Compared with other delivery systems, this approach possesses several advantages such as lack of toxicity to the host and low antigenicity. Also, since the overall size of the conjugated ODN is small, extravasation of the conjugate in the tumor tissue would be less problematic. These advantages make the folate-ODN conjugate a targeting agent worthy of further exploration.

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