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Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol

(folate-binding protein/liposome-targeting)

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ABSTRACT **Antisense oligodeoxyribonucleotides targeted to the epidermal growth factor (EGF) receptor were encapsulated into liposomes linked to folate via a polyethylene glycol spacer (folate–PEG–liposomes) and efficiently delivered into cultured KB cells via folate receptor-mediated endocytosis. The oligonucleotides were a phosphodiester 15-mer antisense to the EGF receptor (EGFR) gene stop codon (AEGFR2), the same sequence with three phosphorothioate linkages at each terminus (AEGFR2S), a randomized 15-mer control of similar base composition to AEGFR2 (RC15), a 14-mer control derived from a symmetrized *Escherichia coli lac* operator (LACM), and the 5′-fluorescein-labeled homologs of several of the above. Cellular uptake of AEGFR2 encapsulated in folate–PEG–liposomes was nine times higher than AEGFR2 encapsulated in nontargeted liposomes and 16 times higher than unencapsulated AEGFR2. Treatment of KB cells with AEGFR2 in folate–PEG–liposomes resulted in growth inhibition and significant morphological changes. Curiously, AEGFR2 and AEGFR2S encapsulated in folate–PEG–liposomes exhibited virtually identical growth inhibitory effects, reducing KB cell proliferation by >90% 48 hr after the cells were treated for 4 hr with 3 μM oligonucleotide. Free AEGFR2 caused almost no growth inhibition, whereas free AEGFR2S was only one-fifth as potent as the folate–PEG–liposome-encapsulated oligonucleotide. Growth inhibition of the oligonucleotide-treated cells was probably due to reduced EGFR expression because indirect immunofluorescence staining of the cells with a monoclonal antibody against the EGFR showed an almost quantitative reduction of the EGFR in cells treated with folate–PEG–liposome-entrapped AEGFR2. These results suggest that antisense oligonucleotide encapsulation in folate–PEG–liposomes promise efficient and tumor-specific delivery and that phosphorothioate oligonucleotides appear to offer no major advantage over native phosphodiester DNA when delivered by this route.**

Antisense oligodeoxyribonucleotides have shown great efficacy in the selective inhibition of gene expression (1–4). However, the therapeutic applications of such antisense oligonucleotides are currently limited by their low physiological stability, slow cellular uptake, and lack of tissue specificity. The instability obstacles have been largely overcome by use of backbone-modified oligonucleotides that are more resistant to nucleases. Methylphosphonates, protein–nucleic acid conjugates, and phosphorothioates all appear to resist enzymatic digestion better than the corresponding natural oligonucleotides (5–8).

Problems with cellular uptake of antisense oligonucleotides have been more difficult to solve. Endogenous uptake pathways that rely on pinocytosis and related processes generally have insufficient capacity to deliver the quantities of antisense of oligonucleotides required to suppress gene expression (9). Hydrophobic modifications have also been undertaken to improve membrane permeability, but such derivatization strategies are most useful only for short oligonucleotides (9). Although complexes of antisense constructs with cationic liposomes (10, 11) and polylysine (12, 13) have significantly enhanced intracellular delivery, they have simultaneously introduced new disadvantages of their own. Thus, both methods exhibit some carrier cytotoxicity, and like other protocols, neither strategy allows for any tissue or cell targeting. In short, intracellular delivery and tissue specificity remain major obstacles to the implementation of antisense drugs in the treatment of human disorders.

We have observed that the vitamin folic acid can be used to deliver macromolecules into the cytoplasm of cells expressing a folate receptor. When folate is linked to a macromolecule via its γ-carboxylate moiety, the affinity of folate for its cell-surface receptor remains essentially unaltered ($K_d \approx 10^{-9}$ M) (14–19). Importantly, folate has already been exploited to nondestructively deliver antibodies, toxins, enzymes, small organic molecules, genes, and liposomes into receptor-bearing cells (14–19). We report here that folate–PEG–liposomes can deliver sufficient antisense against the growth factor receptor (EGFR) into KB cells to eliminate EGFR expression, to alter cell morphology (20), and to halt cell growth. Importantly, at similar concentrations, the free antisense oligonucleotides exhibit little effect.

MATERIALS AND METHODS

Materials. KB cells were from the Purdue Cancer Center. Other reagents and their suppliers were as follows: folic acid, mouse monoclonal antibody against the human EGFR, and fluorescein-labeled goat anti-mouse IgG antiserum (Sigma); phospholipids (Avanti Polar Lipids); the bicinchoninic acid (BCA) protein assay kit and long-chain alkylamine controlled-pore glass (Pierce); fluorescein phosphoramidite and 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) (Pharmacia); 5′-*O*-DMT-2′-deoxyribonucleoside cyanoethylphos-

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Abbreviations: AEGFR2, 15-mer antisense oligodeoxyribonucleotide against the stop codon in the human epidermal growth factor receptor gene; AEGFR2S, AEGFR2 bearing three phosphorothioate linkages at each terminus; DMT, dimethoxytrityl; EGF, epidermal growth factor; EGFR, EGF receptor; fluorescein–AEGFR2, AEGFR2 5′-labeled with fluorescein; fluorescein–AEGFR2S, AEGFR2S 5′-labeled with fluorescein; RC15, randomized control 15-mer of similar base composition to AEGFR2; LACM, mutant *Escherichia coli lac* operator 14-mer oligonucleotide.

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phoramidites, where DMT is dimethoxytrityl (Cruachem, Herndon, VA); and dimethylaminopyridine, acetic anhydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 5′-*O*-DMT-2′-deoxyribonucleoside-3′-*O*-succinates, and 1*H*-tetrazole (Aldrich). The tetrazole was recrystallized from acetonitrile before use. Acetonitrile and pyridine (Fisher Scientific), triethylamine (Baker), and 1,2-dichloroethane (Mallinckrodt) were distilled from CaH_2 before use.

Cell Culture. An attached monolayer of KB cells was continuously cultured at 37°C in a humidified atmosphere containing 5% CO_2 in folate-free minimum essential medium/10% heat-inactivated fetal calf serum, which provided a level of folate that is physiological for human cells. Before each experiment, cells were transferred to 35-mm culture dishes at 10^5 cells per dish and grown in culture medium for an additional 24 hr to ≈50% confluence unless stated otherwise.

Design of the Oligonucleotides. The AEGFR2 oligonucleotide sequence, 5′-CCGTGGTCATGCTCC-3′, is complementary to positions 3811–3825 of the EGFR cDNA (21), which contains the opal translation termination codon at residues 3817–3819. Incorporation of three phosphorothioate linkages at each terminus gave AEGFR2S (5′-$C_SC_SG_S$TGGTCATG$C_ST_SC_SC$-3′). The corresponding oligonucleotides bearing a 5′-fluorescein phosphoramidite gave fluorescein–AEGFR2 and fluorescein–AEGFR2S. Two control oligonucleotides were used, a phosphodiester 14-mer based on a symmetrized *E. coli lac* operator with the sequence 5′-TGTGTGCGCACACA-3′ (LACM), and a randomized phosphodiester 15-mer oligonucleotide with the sequence 5′-GCTGACGCACTGACT-3′ (RC15).

Synthesis of the Oligonucleotides. The 5′-*O*-DMT-2′-deoxyribonucleoside-3′-*O*-linked solid-phase supports were prepared from long-chain alkylamine controlled-pore glass using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and the appropriate 5′-*O*-DMT-2′-deoxyribonucleoside-3′-*O*-succinates (22) and packed into Pharmacia cassettes. All DNA sequences were synthesized by the solid-phase phosphoramidite method (23) on a Pharmacia GeneAssembler synthesizer, with the phosphodiester oligonucleotides synthesized according to the manufacturer's protocols. The phosphorothioate linkages in AEGFR2S and fluorescein-AEGFR2S were generated via sulfurization of the terminal P(III) to P(V) with the Beaucage reagent (24) in acetonitrile. The 5′-fluorescein-labeled sequences were synthesized by using the fluorescein phosphoramidite, yielding a 1:1 fluorescein/oligonucleotide stoichiometry. The finished sequences were cleaved from the support and deprotected with concentrated NH_4OH at 60°C for 14 hr. Each deprotection mixture was then chromatographed in reversed-phase mode on a 7 × 305-mm Hamilton PRP-1 column with a linear gradient of 30 mM (pH 7.2) triethylammonium acetate and acetonitrile. The pooled tritylated fractions were lyophilized, detritylated in 20% acetic acid at 55°C for 1 hr, relyophilized, and then rechromatographed on the same column. The pooled DNA fractions were lyophilized to dryness and then relyophilized from 2 ml of 10 mM NaP_i at pH 7.4. The samples were stored at −5°C until use. The fluorescein-labeled oligonucleotides were chromatographed once, lyophilized to dryness, and then relyophilized from 2 ml of 10 mM NaP_i at pH 7.4 and stored at −5°C until use.

Encapsulation of Antisense Oligonucleotides in Liposomes. Liposomes encapsulating the antisense oligonucleotides were prepared by the extrusion method (25). Briefly, 50 mg of egg phosphatidylcholine and 16.5 mg of cholesterol (giving a molar ratio of 3:2) were dissolved in 3 ml of chloroform. For folate-targeted liposomes, 5 mg (0.5 mol%) of folate–PEG–distearoylphosphatidylethanolamine, synthesized as described (19), was also included in the lipid mixture. This construct provides a ≈250-Å PEG spacer between the folate and the liposome surface, allowing formation of what we term folate–PEG–liposomes. The lipids were dried under reduced pressure to a thin film and then rehydrated overnight in 0.5 ml of oligonucleotide at 2 mg/ml dissolved in phosphate-buffered saline (PBS, 136.9 mM NaCl/2.68 mM KCl/8.1 mM Na_2HPO_4/1.47 mM KH_2PO_4, pH 7.4). After 10 cycles of freezing and thawing, the liposome suspension was extruded 10 times through a 100-nm polycarbonate membrane (Nucleopore, Pleasanton, CA). The liposome-encapsulated oligonucleotides were then separated from free oligonucleotides on a 1.5 cm × 20 cm Sepharose CL-4B column (Pharmacia) preequilibrated with PBS. The liposome fractions eluted in the void volume and were stored at 4°C until use (within 2 weeks). Encapsulation efficiencies of 30–40% were routinely achieved.

Uptake of Liposome-Encapsulated Antisense Oligonucleotides by Cultured KB Cells. KB cells grown to 2.5 × 10^6 cells per dish in 35-mm culture dishes (≈80% confluent) were incubated with 1 ml of either free or liposome-encapsulated 5-fluorescein-labeled oligonucleotide (2 μM final concentration) for 4 hr at 37°C. The cells were washed four times with 2 ml of PBS and dissolved in 2 ml of 1% Triton X-100. The amount of cell-associated fluorescence was then measured on a Perkin–Elmer MPF-44A fluorescence spectrophotometer. These data were then used to calculate the number of antisense molecules by using a standard plot of fluorescence versus fluorescein–AEGFR2 concentration. Competition between the folate-derivatized liposomes and free folate for cell-surface receptors was examined by including 1 mM folic acid in the incubation mixture.

Intracellular Distribution of Folate–PEG–Liposome-Encapsulated Oligonucleotides. To determine the intracellular distribution of cell-associated oligonucleotides, KB cells were incubated for 4 hr at 37°C with 1 ml of 2 μM folate–PEG–liposome-encapsulated fluorescein–AEGFR2. At this point, the cells were examined either immediately or after 20-hr further incubation using a MRC-600 confocal imaging system (Bio-Rad) attached to a Nikon Optiphot epifluorescence microscope. Images of the cells were collected under the photon-counting mode with a ×60 objective using the 488-nm line of a krypton/argon mixed gas laser as the excitation source and were processed on a Macintosh IIsi computer using the public domain National Institutes of Health image program (written by Wayne Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868) and printed on a Codonics NP-1600 photographic network sublimation printer.

Effect of Liposome-Encapsulated Antisense Oligonucleotides on KB Cell Proliferation. To determine the effect of anti-EGFR oligonucleotides on cell growth, KB cells in 35-mm culture dishes were treated at 37°C for 4 hr with 1 ml of either free or folate–PEG–liposome-encapsulated oligonucleotide at 3 μM final concentration in culture medium/10% heat-inactivated fetal calf serum. The cells were then washed once with 1 ml of PBS to remove unbound liposomes and incubated in fresh medium for a further 48 hr. At this point, the cells were washed again with PBS, solubilized in 1% Triton X-100, and quantified with the BCA protein assay. The dependence of growth inhibition on antisense concentration was determined by using the same protocol, except that oligonucleotides concentrations were varied. To determine the long-term effects of antisense oligonucleotide treatment on cell growth, the cells were treated at a confluency of 30% and then transferred to fresh medium for up to 10 days while the cellular protein content was monitored with the BCA protein assay.

Evaluation of EGFR Expression by Indirect Immunofluorescence. The effect of EGFR antisense oligonucleotides on EGFR expression was examined by indirect immunofluorescence with mouse monoclonal antibody against the human EGFR (26). KB cells were treated for 4 hr with 1 ml of 2 μM

free or folate–PEG–liposome-encapsulated oligonucleotides and then incubated for a further 24 hr in fresh culture medium. The cells were chilled to 4°C for 30 min, fixed in 3% *p*-formaldehyde, and permeabilized with 1 ml of 0.1% Triton X-100 for 5 min. After incubation with 1 ml of anti-EGFR at 20 μg/ml in blocking buffer (0.2% bovine serum albumin/PBS, pH 7.4) at 4°C for 1 hr, the cells were washed once with PBS and then similarly incubated with 1 ml of fluorescein-labeled goat anti-mouse IgG at 10 μg/ml. The cells were washed five times with 1 ml of PBS and then examined under an Olympus BH-2 fluorescence microscope and photographed under both the fluorescence and phase-contrast modes.

RESULTS

Uptake of Free and Encapsulated Oligonucleotides by Cultured KB Cells. Cellular uptake of the various oligonucleotide formulations is illustrated in Fig. 1. After incubation at 37°C for 4 hr, uptake of the free 5′-labeled phosphodiester species, fluorescein–AEGFR2, was barely detectable (bar a), whereas 1.3×10^6 molecules of the free exonuclease-resistant analog (fluorescein–AEGFR2S, bearing the 5′ and 3′ phosphorothioate linkages) were internalized by the KB cells under similar conditions (bar b). The uptake of oligonucleotides was greatly enhanced by liposome encapsulation and folate targeting. Approximately 2.1×10^7 molecules of folate–PEG–liposome-encapsulated fluorescein–AEGFR2 entered each cell, an amount exceeding uptake of the free fluorescein–AEGFR2S by ≈16-fold (bar d). The cellular uptake of fluorescein–AEGFR2 was also enhanced by encapsulation in nontargeted liposomes (bar c), presumably due to the protective effect of the liposomal membrane against nuclease digestion. Still, the level of uptake was only one-ninth of that for the folate-targeted oligonucleotides. The uptake of folate–PEG–liposome-encapsulated oligonucleotides could be competitively inhibited by 1 mM free folic acid, indicating the cellular delivery is mediated by folate receptors (bar e).

Intracellular Distribution of Folate–PEG–Liposome-Encapsulated Oligonucleotides. The intracellular distribution of fluorescein-labeled folate–PEG–liposome-encapsulated antisense oligonucleotides was examined by confocal fluorescence microscopy. After 4-hr incubation at 37°C, oligonucleotides were distributed primarily on the cell surface and within endocytic vesicles (data not shown). However, by 24-hr incubation at 37°C, the fluorescein-labeled oligonucleotides were observed throughout the cytosol, with some enrichment in the nucleus. The kinetics of these uptake events corresponds closely to that seen for other liposome-encapsulated material (18).

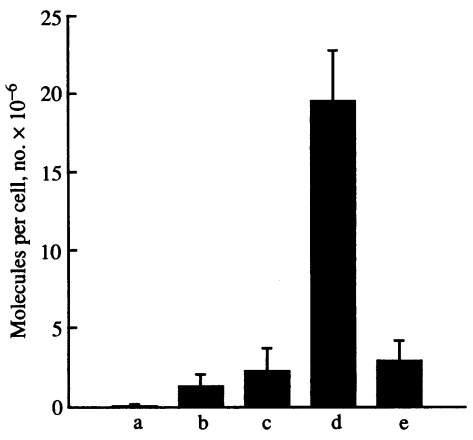

FIG. 1. Uptake of free and liposome-encapsulated EGFR antisense oligonucleotides by KB cells. KB cells were treated with free fluorescein–AEGFR2 (bar a), free fluorescein–AEGFR2S (bar b), fluorescein–AEGFR2 encapsulated in nontargeted liposomes (bar c), fluorescein–AEGFR2 encapsulated in folate–PEG–liposomes (bar d), or fluorescein–AEGFR2 encapsulated in folate–PEG–liposomes in the presence of 1 mM free folate (bar e). The number of cell-associated antisense molecules was determined by fluorimetry as described. Error bars indicates 1 SD ($n = 2$).

Effect of Liposome-Encapsulated Oligonucleotides on KB Cell Proliferation. The growth and morphology of KB cells treated with various oligonucleotide formulations were examined under the phase-contrast microscope. Fig. 2 *Lower* displays KB cells that were grown for 48 hr in fresh medium after 4-hr exposure to antisense oligonucleotides. KB cells treated with folate–PEG–liposome-encapsulated AEGFR2 (data not shown) or AEGFR2S (*Lower Left*) developed a fibroblast-like morphology with a much lower cell density than observed for the untreated controls. However, cells treated with free AEGFR2 or AEGFR2S (data not shown) remained similar in appearance to the untreated cells (*Lower Right*). As shown in Fig. 3, treatment of KB cells with folate–PEG–liposome-encapsulated AEGFR2 resulted in an ≈90% reduction in proliferation compared with untreated cells, whereas free AEGFR2 had almost no effect on growth inhibition. Folate–PEG–liposome-encapsulated AEGFR2S, the more nuclease-resistant oligonucleotide, showed roughly the same efficacy (92% inhibition) as the folate–PEG–liposome-entrapped unmodified oligonucleotide, whereas free AEGFR2S caused a 15% growth inhibition. AEGFR2 encapsulated in nontargeted liposomes (lacking folate) also inhibited growth but was only ≈20% as effective. Oligonucleotide-free folate–PEG–liposomes had virtually no growth inhibitory effect. Finally, to demonstrate the sequence specificity of these effects, two control folate–PEG–liposome-encapsulated oligonucleotides (RC15 and LACM) were also delivered into KB cells, but both oligonucleotides showed very little growth inhibitory potency.

To evaluate the long-term effect of antisense delivery on KB cell proliferation, KB cells were treated for 4 hr with antisense oligonucleotides and then transferred to fresh medium for up to 10 days. Fig. 4 shows that maximal growth inhibition by folate–PEG–liposome-encapsulated AEGFR2 or AEGFR2S occurred during the first 4 days after treatment, and then the cellular growth rate returned to normal. The duration of growth inhibition by encapsulated AEGFR2S was slightly greater than AEGFR2, but otherwise no difference was seen.

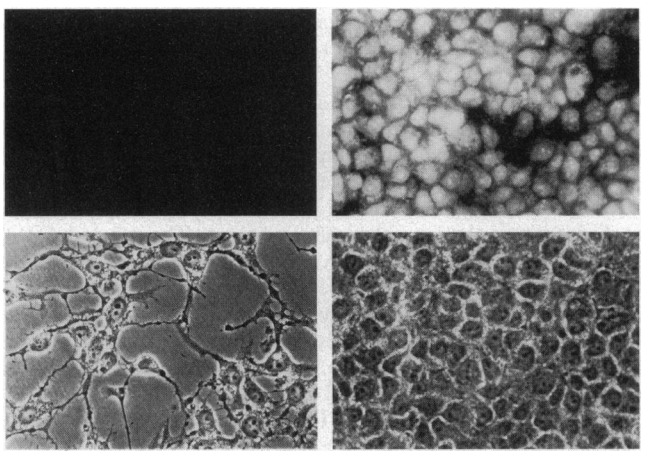

FIG. 2. Effect of free and folate–PEG–liposomal antisense to the EGFR on receptor expression and cell morphology. (*Upper*) Indirect immunofluorescence staining of KB cells with a monoclonal IgG to the EGFR. (*Lower*) Phase-contrast images of same cells. (*Left*) KB cells treated with AEGFR2S encapsulated in folate–PEG–liposomes as described. (*Right*) Untreated KB cells. Cells treated with either free antisense constructs or RC15 and LACM encapsulated in folate–PEG–liposomes gave qualitatively similar results to the untreated cells (data not shown).

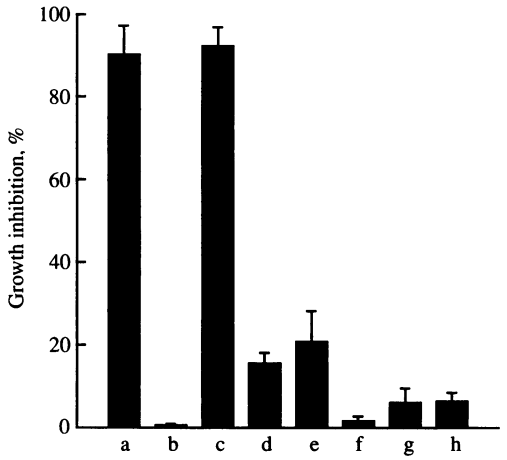

FIG. 3. Growth inhibition by EGFR antisense oligonucleotides. KB cells were treated with folate–PEG–liposome-encapsulated AEGFR2 (bar a), free AEGFR2 (bar b), folate–PEG–liposome-encapsulated AEGFR2S (bar c), free AEGFR2S (bar d), nontargeted liposome-encapsulated AEGFR2 (bar e), empty folate–PEG–liposome (bar f), folate–PEG–liposome-encapsulated RC15 (bar g), or folate–PEG–liposome-encapsulated LACM (bar h), as described. Growth inhibition was calculated from the equation: % inhibition = $[(P_{final}° - P_{final})/P_{final}° - P_0)] \times 100$, where $P_{final}°$ is the protein content of untreated cells after 48-hr incubation, P_{final} is the same value for antisense-treated cells, and P_0 is the protein content of the cells directly before antisense treatment—i.e., at time 0. The error bars indicate 1 SD ($n = 2$).

Free AEGFR2S was almost ineffective, and treatment with free AEGFR2 exerted no effect at all (data not shown).

Fig. 5 shows that folate–PEG–liposome-encapsulated AEGFR2S caused a concentration-dependent decrease in KB cell growth, whereas free AEGFR2S and the folate–PEG–liposome-encapsulated control oligonucleotides (RC15 and LACM) again showed lower efficacies.

Effect of Folate–PEG–Liposome-AEGFR2 on EGFR Expression. The expression of EGFR by KB cells in the presence or absence of folate–PEG–liposome-encapsulated antisense oligonucleotides was analyzed by indirect immunofluorescence 24 hr after incubation with the encapsulated antisense oligonucleotides. As shown in Fig. 2 *Upper*, cells treated with the folate–PEG–liposome-encapsulated antisense oligonucleotides displayed virtually no EGFR expression; in contrast,

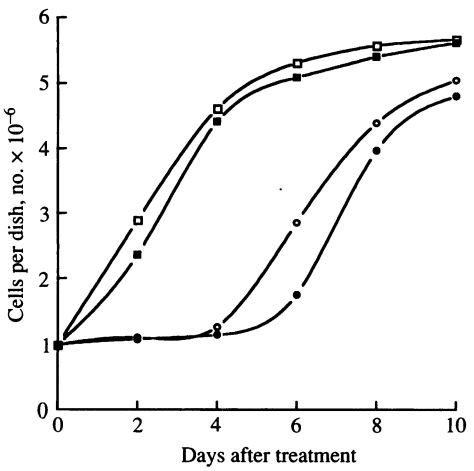

FIG. 4. Long-term growth inhibitory effect of EGFR antisense oligonucleotides. KB cells were either treated for 4 hr with 3 μM of AEGFR2 encapsulated in folate–PEG–liposomes (○), AEGFR2S encapsulated in folate–PEG–liposomes (●), free AEGFR2S (■), or remained untreated (□), as described. Each data point represents the average of two experiments.

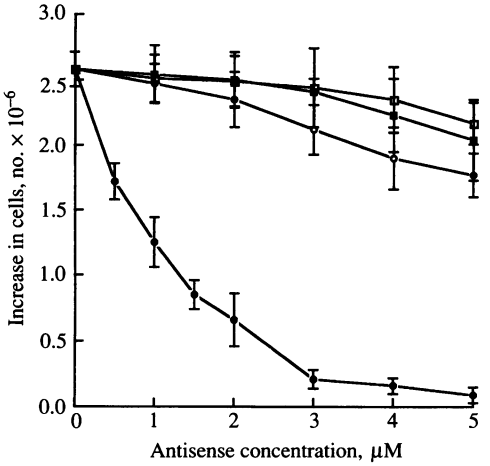

FIG. 5. Effect of oligonucleotide concentration on KB cell growth. Cells were plated in 35-mm dishes, grown to ≈1.0 × 10^6 cells per dish, and then treated for 4 hr with various concentrations of AEGFR2S encapsulated in folate–PEG–liposomes (●), free AEGFR2S (○), RC15 encapsulated in folate–PEG–liposomes (■), or LACM encapsulated in folate–PEG–liposomes (□), and assayed for protein content 48 hr later. Error bars indicate 1 SD ($n = 2$).

untreated cultures revealed a high distribution of EGFR throughout the cell. The free antisense constructs and two control oligonucleotides entrapped in folate–PEG–liposomes gave qualitatively similar results to the untreated cells (data not shown).

DISCUSSION

We have compared the cellular uptake of free antisense oligonucleotides with the same antisense constructs encapsulated in nontargeted and folate receptor-targeted liposomes. The latter liposome formulation was found to promote internalization of nine times more oligonucleotide than its nontargeted counterpart and 16 times more than the free oligonucleotide. Because the functional assay—i.e., inhibition of cell growth, suppression of EGFR expression, and induction of fibroblast morphology (20)—also reflected the same relative potencies, we conclude that the folate–PEG–liposome antisense is delivered intracellularly in functionally active form. Importantly, this conclusion agrees with previous data (14, 19), showing that folate endocytosis does not culminate in lysosome deposition but rather leads to nondestructive release of much of the internalized material into the cytosol.

Deprivation of growth stimuli for several days can often induce a cell to undergo apoptosis. Unfortunately, our antisense-mediated suppression of EGFR expression inhibited cell proliferation for only 4 days. Although the reason for this transient effect was not investigated, several possible explanations are worth considering. (*i*) Other growth stimuli may have maintained the KB cells in a quiescent state until the antisense oligonucleotides could be degraded. (*ii*) The KB cells used in our studies may have been incapable of apoptosis. (*iii*) The level of EGFR suppression achievable with our antisense construct may have been inadequate to induce the suicide response.

Because of the sensitivity of native phosphodiester DNA to nucleases, significant effort has been devoted to developing more stable oligonucleotide backbone chemistries (5–8). In our study, however, folate–PEG–liposome-encapsulated phosphodiester and phosphorothioate oligonucleotides showed essentially identical potencies up to 4 days after treatment (Figs. 3 and 4). These similar inhibitory potencies may have arisen because release or escape of the antisense oligonucleotide from the internalized liposomes may be rate-limiting in oligo-

nucleotide degradation. Although discovery of methylphosphonates, phosphorothioates, and protein–nucleic acid constructs have greatly improved antisense stability, problems with cytotoxicity, enantiomeric purity, and cost have prompted researchers to continue their search for the ideal backbone chemistry. Obviously, a single study should not be extrapolated to apply to all antisense research, but if differences between targeted liposomal formulations of chemically different oligonucleotides continue to prove negligible, then the emphasis in antisense research may more appropriately be focused on delivery instead of stability.

Overexpression of the EGFR has been observed in a variety of human cancers and may be related to cell transformation (20, 27). Recent studies also show that many cancers overexpress a high-affinity receptor for folic acid on their cell surfaces (28–33). Although the conjugation of folic acid to an antisense oligonucleotide should permit its cell-specific targeting, we feel the folate–PEG–liposome formulation offers several advantages not shared by the isolated oligonucleotide. (*i*) As noted above, the protection provided by the liposomal membrane may obviate the need for unnatural backbone chemistries. (*ii*) Because of its carrying capacity, the targeted liposome can deliver multiple antisense constructs at each receptor or receptor cluster, thereby enhancing the quantity of antisense delivered. (*iii*) The liposome should retard loss of the antisense through the kidneys and avoid rapid degradation by extracellular nucleases. (*iv*) Because of its potential for multiple attachments to adjacent receptors, folate–PEG–liposomes can avoid significant competition from endogenous folates (18, 19). Although other means of liposome targeting are also possible, folate represents an attractive candidate because the ligand is inexpensive, compatible with either organic or aqueous solvents, easy to conjugate to PEG and other liposome tethers, and relatively specific for many human tumors.

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