Enhanced Cellular Uptake of Oligonucleotides by EGF Receptor-Mediated Endocytosis in A549 Cells

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Purpose. The goal of this study was to investigate the feasibility of utilizing epidermal growth factor (EGF) receptor-mediated endocytosis to enhance cellular uptake and targeting of oligonucleotides in epithelial cancer cells. To overcome the problem of endosomal entrappment associated with receptor-mediated delivery, we also examined the effects of two fusogenic peptides, polymyxin B and influenza HA2 peptide, for their capability to promote cytoplasmic delivery of oligonucleotides.

Methods. A molecular conjugate consisting of EGF and poly-L-lysine (PL) was synthesized and complexed with 5′ fluorescently-labeled oligonucleotide. Cellular uptake of the complex in presence or absence of the fusogenic peptides was monitored fluorometrically. Microscopic studies were performed to visualize the intracellular distribution of the oligonucleotide.

Results. Cells treated with the complex exhibited intracellular fluorescence intensity significantly enhanced over free oligonucleotide-treated controls. The uptake of the complex was shown to occur via the EGF receptor-mediated pathway. Fluorescence microscopic studies revealed cellular internalization of the complex, however, the complex appeared to be accumulated in endocytic vesicles. Exposure of the cells to complex in presence of HA2 peptide and polymyxin B resulted in a more diffused intracellular fluorescence pattern and a corresponding increase in fluorescence intensity. These results are consistent with the known fusion and destabilizing activities of the peptides.

Conclusions. Since EGF receptors are overexpressed in many cancer cell types, the EGF-PL conjugate may potentially be used as an effective and selective delivery system to enhance uptake of oligonucleotides into cancer cells.

KEY WORDS: oligonucleotide; uptake; endocytosis; epidermal growth factor.

INTRODUCTION

Antisense oligonucleotides (ONs) offer considerable promise as sequence specific inhibitors of oncogene expression and have great therapeutic potential in cancer treatment. A major problem encountered with the therapeutic use of ONs deals with cellular uptake and targeting. ONs also show a tendency to remain trapped in endocytic vesicles and end up being degraded in lysosomes. In order to improve the uptake characteristics of ONs, numerous approaches have been investigated, including conjugation to polycations such as poly-L-lysine (1) and lipids such as cholesterol (2), and encapsulation in nanoparticles (3) and antibody-targeted liposomes (4). Most of these approaches, except the antibody-targeted liposomes, lack specificity for cell recognition.

Cell-surface receptors that undergo receptor-mediated endocytosis are good candidates to selectively target and enhance cellular uptake of ONs. Receptors for some vitamins and growth factors such as transferrin and folic acid are overly expressed in rapidly growing tumor cells and have been investigated for their potential to deliver ONs (5, 6). Many epithelial tumors and brain tumors of glial origin are known to overexpress the surface EGF receptor (7) while breast and ovary tumors overexpress the EGF-related receptor erbB2 (8).

We have described here a novel ON delivery system using the EGF receptor. Since many tumor cells derived from epithelial tumors overexpress the EGF receptor they can be preferentially targeted. Receptor-mediated ON delivery also offers the prospect of increased uptake efficiency. Since this system utilizes a naturally occurring cellular process, it is potentially non-toxic. Our delivery system comprises of a conjugate of EGF covalently linked to PL, which can then complex electrostatically with the polyanionic backbone of the ON. On recognition by the EGF receptor, the conjugate is internalized thus co-transporting the ON. Our results indicate that EGF receptor-mediated endocytosis significantly enhanced the uptake of ON in A-549 cells.

MATERIALS AND METHODS

Chemicals

EGF, bovine serum albumin, poly-L-lysine (MW ≈ 10,000), polymyxin B, and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were obtained from Sigma Chemical Company (St. Louis, Missouri). 5′-BODIPY FL labeled, 18-mer, phosphodiester ON (TGTAAAACGACGGCCAGT) was obtained from Molecular Probes Inc. (Eugene, Oregon). The labeled ON was purified by HPLC and was > 98% pure. The influenza fusogenic peptide (GLFEAIAGFIENGWEGMIDGGGYC) (9) was synthesized by Synpep Corp. (Dublin, California) using the Fmoc procedure. The peptide was purified by HPLC and characterized by amino acid analysis and mass spectrometry ($M+H^+$:2506).

Cell Culture

The adenocarcinoma A-549 cells (ATCC# 185-CCL) were grown in F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 μg/ml streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Prior to use, the cells were washed and resuspended in HEPES buffer.

Synthesis of EGF-PL Conjugate

EGF and PL were conjugated through a reversible di-

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sulfide linkage using SPDP as a cross-linking agent, according to the modified method of Carlsson *et al.* (10). Briefly, EGF (10 μg) was dissolved in 0.5 ml phosphate-buffered saline (PBS), pH 7.4. An ethanol solution of SPDP (50 μl, 5 mM) was added to the stirred protein solution and the reaction allowed to proceed for 30 min. Excess reagent was removed by centrifugal filtration through the Durapore™ dialysis filter (CL5K, Millipore Corp.). The modified protein was then collected and reconstituted in PBS. The degree of substitution of pyridyl disulfide groups in the protein was determined, as previously described (10), and was found to be 0.8-1.0.

Modification of PL was similarly performed, except the protein was reconstituted in sodium acetate buffer, pH 4.5. The resulting PL-pyridyl derivative was then converted to a thiol derivative by treating with dithiothreitol (25 mM, final concentration) and reconstituted in PBS. The modified PL was then mixed with the EGF-pyridyl derivative and, after a 18 h incubation period at 4°C, the reaction mixture was filtered to remove unreacted proteins. The resulting EGF-PL conjugate was then collected, stored in HEPES buffer at 4°C, and used within 48 h. Analysis of the protein conjugate was performed using gel chromatography (Pharmacia Superose 12 column) with the aid of protein molecular weight markers. This study showed that the conjugate obtained was a monoconjugate.

Preparation of EGF-PL Oligomer Complex

The EGF-PL conjugate was complexed with the BODIPY-labeled ON in HEPES buffer for 30 min prior to use. In experiments designed to evaluate the effect of concentration of conjugate on ON uptake, various concentrations of conjugate (10-500 μg/ml) were used. In a separate study, the stability of the ON complex or ON alone in HEPES buffer containing 10^5/ml cells was also evaluated using gel electrophoresis. After a 3 h incubation period at 37°C, the samples were electrophoresed in 20% (w/v) polyacrylamide-7M urea sequencing gels and analyzed by fluorography. No detectable degradative products of ON were observed in this study.

Uptake Studies

The cells (10^5/ml) were plated and allowed to attach in 96-well plates. They were added with solution (100 μl) containing ON or ON:EGF-PL complex and incubated at 37°C for a specified period of time. After incubation, the cells were washed with HEPES buffer containing trypsin (0.25%) to remove non-internalized material. Subsequently, the cells were lysed with Triton X (0.1%, 100 μl) and measured for their fluorescence intensity using a microplate fluorometer (Cambridge Technology Inc., model 7630) at the excitation and emission wavelengths of 490 nm and 520 nm. To evaluate the structural requirement of EGF-PL conjugate on ON uptake, control studies using ON (10 μg/ml) in combination with PL (100 μg/ml) or EGF (100 μg/ml) were also conducted. For competition experiments to test for the specificity of the EGF-PL conjugate for the EGF receptor, cells were treated with the complex in the presence of excess free EGF (1 mg/ml) as a specific competitor or bovine serum albumin (1 mg/ml) as a non-specific competitor. In experiments employing fusogenic peptides, cells were treated with the complex in the presence of increasing amounts of peptides (10-100 μg/ml) and their fluorescence intensities were similarly determined. Differences between the test results were determined using analysis of variance at $p < 0.05$.

Fluorescence Microscopic Studies

Cells (10^5/ml) were plated on a glass cover slip. The solution (100 μl) containing ON (10 μg/ml) or ON:EGF-PL complex (10:100 μg/ml) in the presence or absence of fusogenic peptides (100 μg/ml) was added onto the cells and incubated for 1-3 h at 37°C. Subsequently, the cells were washed and immediately examined under the Nikon Diaphot® fluorescence microscope at the excitation and emission wavelengths of 490 nm and 520 nm. HEPES buffer was used to prevent shifts in extracellular pH and to maintain osmolarity which might change the sub-cellular distribution patterns.

RESULTS AND DISCUSSION

Uptake Efficiency of Free and Complexed Oligonucleotides

Cellular uptake of free ON and ON complexed with varying amounts of EGF-PL is shown in Fig. 1. The results indicated that despite their large molecular size the ON complexes were taken up by cells much more efficiently than free ON. The magnitude of enhancement was dependent on the concentration of the complexing agent. Increasing the concentration of EGF-PL resulted in a corresponding increase in cellular uptake of ON (upto 12-fold) as compared to free ON. The saturable nature of the uptake of ON:EGF-PL complex is indicative of receptor-mediated endocytosis.

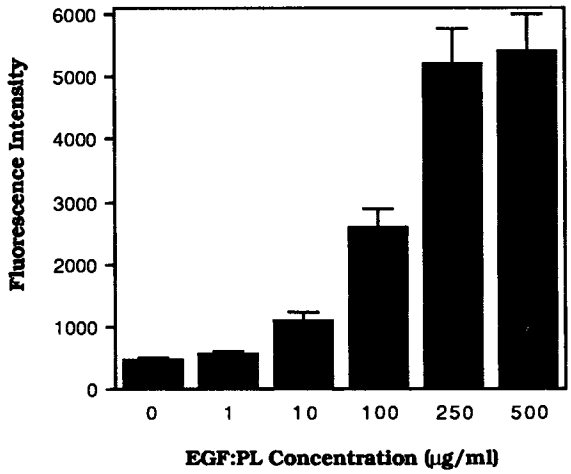

Fig. 1. Dose-dependent cellular uptake of oligonucleotide mediated by EGF-PL conjugate. A-549 cells were incubated with ON (10 μg/ml) in the presence of increasing doses of EGF-PL (0-500 μg/ml) in Hepes buffer for 3 h at 37°C. After incubation, the cells were washed and analyzed for intracellular fluorescence at the excitation and emission wavelengths of 490 nm and 520 nm. The data represent mean ± SE of four measurements after corrected for background autofluorescence.

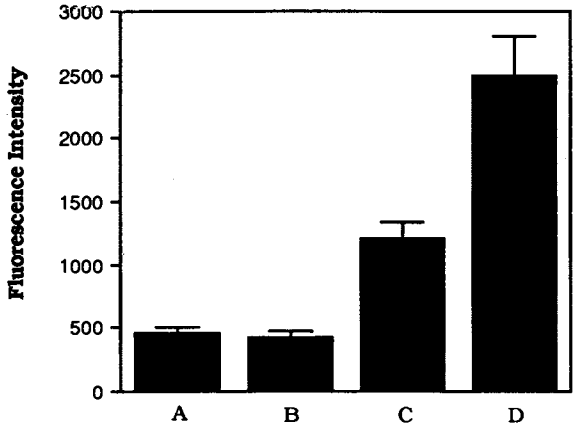

Fig. 2. Mechanism of oligonucleotide uptake mediated by EGF-PL conjugate. Cells were incubated with 10 μg/ml ON, alone (A) or in combination with 100 μg/ml of EGF (B), or PL (C), or EGF-PL conjugate (D) in HEPES buffer for 3 h at 37°C. The values represent mean ± SE, n = 4.

Mechanism of Oligonucleotide Uptake Mediated by EGF-PL Conjugate

To determine the mechanism of ON uptake mediated by the EGF-PL conjugate, the contribution of each moiety of the conjugate in promoting ON uptake was first evaluated. The ON in the presence of EGF, PL, or EGF-PL conjugate was evaluated for their ability to promote cellular uptake of ON (Fig.2). The complex consisting of the ON and EGF-PL conjugate was taken up more efficiently by cells than did the complexes consisting of ON in combination with PL or EGF. The ON:PL complex mediated ON uptake significantly greater than did the ON alone or in combination with EGF. These results demonstrated that effective ON uptake mediated by the conjugate requires functional domains capable of both ON binding and cell surface recognition. The observation of the partial enhancing effect of PL on ON uptake may be attributed to non-specific adsorptive endocytosis of the polycationic PL:ON complex. In this regard, PL has been shown to promote cellular uptake and biological activity of antisense ONs (1).

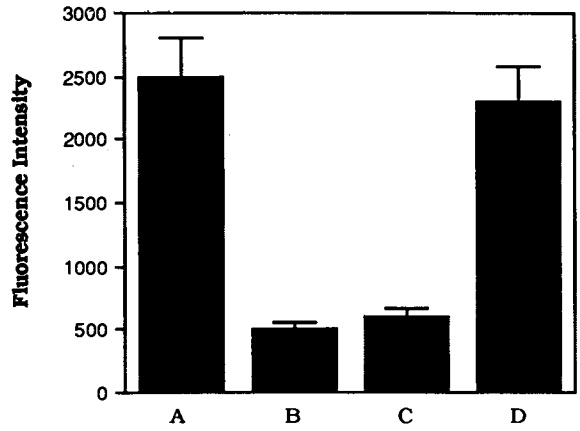

Fig. 3. Effects of temperature and inhibition for EGF receptor on oligonucleotide uptake mediated by EGF-PL conjugate. Cells were incubated with ON:EGF-PL complex (10:100 μg/ml) at 37°C (A) or 4°C (B). Experiments at 37°C were also repeated but in the presence of free EGF (1 mg/ml) (C) or bovine serum albumin (1 mg/ml) (D). The values represent mean ± SE, n = 4.

To test whether the cellular internalization of the ON:EGF-PL complex was mediated through the receptor endocytosis pathway, uptake studies were carried out at 4°C. Under this condition, uptake was seen to be inhibited (Fig.3). In addition, to further confirm that the uptake of the complex was indeed mediated through the EGF receptor, experiments employing the complex in the presence of competition for the EGF receptor were carried out. In these experiments, the specific competitor EGF was found to inhibit cellular uptake of the complex whereas the non-specific competitor albumin had no effect (Fig.3). Thus, our results indicated that ON uptake mediated by the EGF-PL conjugate occurred via the EGF receptor-mediated endocytosis pathway.

Microscopic Studies of Oligonucleotide Uptake

To provide morphologic evidence of ON uptake, cells were incubated with free ON or ON:EGF-PL complex at 37°C for 1-3 h and examined for their intracellular ON distribution using fluorescence microscopy (Fig.4). As can be

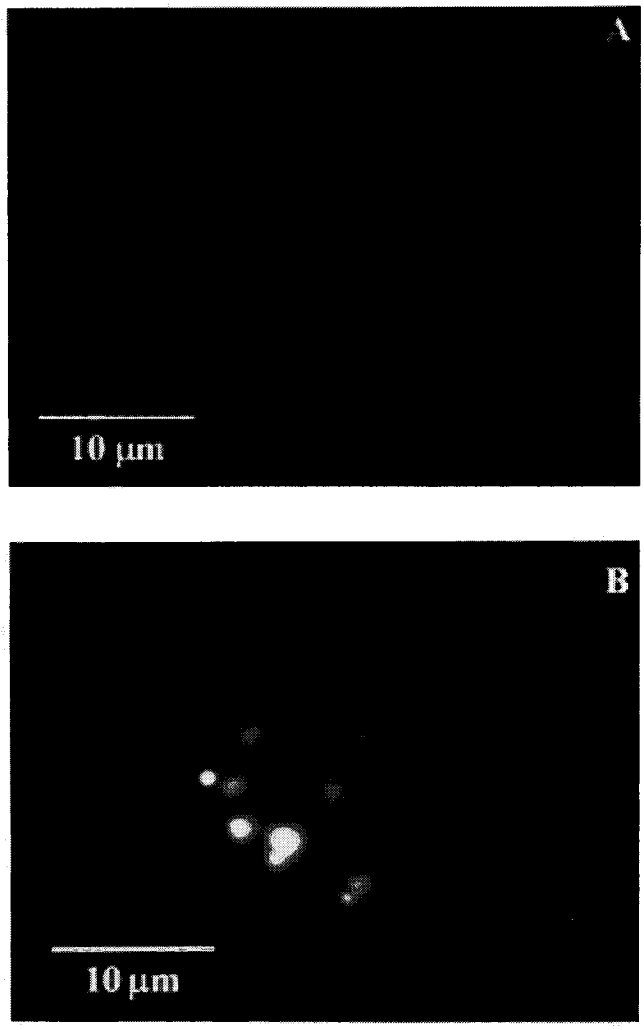

Fig. 4. Fluorescence micrograph of a single cell showing uptake of free ON (A) or ON:EGF-PL complex (B). Cells were incubated with ON (10 μg/ml) or ON:EGF-PL complex (10:100 μg/ml) at 37°C for 1 h.

seen in Fig.4A, cells treated with free ON show a weak fluorescence intensity with patchy pattern. Cells treated with ON:EGF-PL complex exhibit a similar fluorescence distribution but with a much more pronounced intensity (Fig.4B). In both cases, increasing the incubation time from 1 to 3 h did not significantly alter the overall fluorescence pattern. In the presence of excess free EGF, cells treated with the ON:EGF-PL complex showed very little intracellular fluorescence intensity (result not shown).

Enhanced Cytoplasmic Delivery of Oligonucleotide

As demonstrated above, ON internalization via the EGF receptor pathway resulted in the distribution of ON largely confined in endocytic vesicles. However, to be effective as an antisense agent the ON must be able to exit the endosomes to reach its target site in the cytosol. As an effort to enhance endosomal exit of the ON, we investigated the effect of two fusogenic peptides, the influenza hemagglutinin HA2 peptide and polymyxin B, on cellular uptake and distribution of ON in A-549 cells. The HA2 peptide has been shown to mediate fusion of viral and endosomal membranes (11). This peptide changes conformation at acidic pH and destabilizes the endosomal membranes, allowing leakage of viral contents into the cytoplasm of the host cells (12). Polymyxin B is an amphipathic peptide which has been shown to induce fusion of acidic liposomes (13). Its activity to promote cellular delivery of ONs, however, has not been demonstrated.

When the cells were incubated with the ON:EGF-PL complex in the presence of either of these peptides, a significant dose-dependent increase in intracellular fluorescence was observed (Fig.5). At the concentration of 100 μg/ml, the HA2 peptide and polymyxin B induced ≈ 4- and 6-fold increase in fluorescence intensity. To test whether these effect was due to increased cytoplasmic entry of ON, fluorescence microscopic studies were also conducted. As can be seen in Fig.6, both peptides induced a more diffused fluorescence pattern as compared to the non-treated control (Fig.4). When the experiments were repeated at 4°C, very little intracellular fluorescence was observed (result not shown), suggesting that the promoting effect of the two peptides was dependent on endocytic uptake of the ON complex.

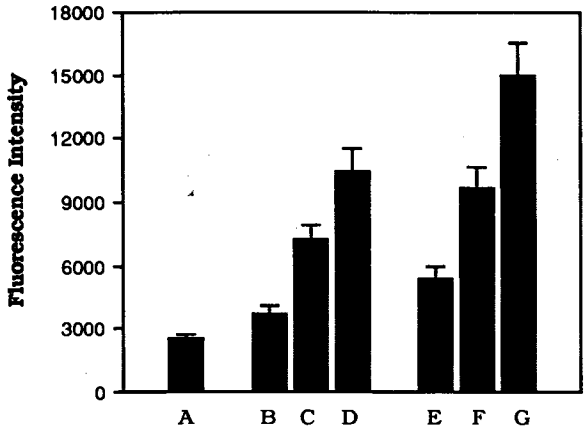

Fig. 5. Dose effects of HA2 peptide and polymyxin B on intracellular uptake of oligonucleotide complex. Cells were incubated with ON:EGF-PL complex (10:100 μg/ml) in the absence (A) or presence of varying concentrations of HA2 peptide (B-D) or polymyxin B (E-G) at 37°C for 3 h. (B-D) and (E-G) indicate HA2 peptide and polymyxin concentrations of 10, 50, and 100 μg/ml respectively. The values represent mean ± SE, n = 4.

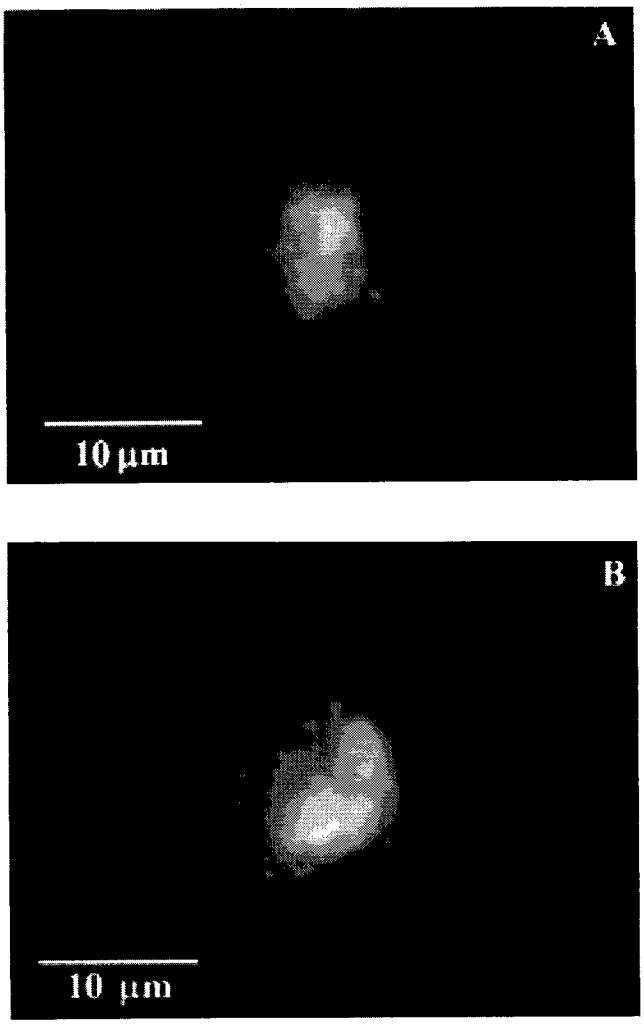

Fig. 6. Cellular distribution of oligonucleotide complex in the presence of fusogenic peptides. Cells were incubated with ON:EGF-PL complex (10:100 μg/ml) in the presence of HA2 peptide (100 μg/ml) (A) or polymyxin B (100 μg/ml) (B) at 37°C for 1 h.

Cytotoxicity Studies

We also evaluated the potential toxicity of the system using the trypan blue viability assay. Cells were incubated with experimental amounts of ON (10 μg/ml), ON:EGF-PL complex (10:100 μg/ml), HA2 peptide (100 μg/ml), or polymyxin B (100 μg/ml) at 37°C for 3 h. Our results indicated that, except for polymyxin B, none of these test agents caused apparent toxic effects to the cells, i.e., $<3\pm1\%$ *vs* $2\pm1\%$ for the untreated controls. At the high concentration of 100 μg/ml, polymyxin B caused a slight but significant damage to the cells ($5\pm1\%$). At lower concentrations (10 and 50 μg/ml), however, polymyxin B had no significant effect on the cells. The potential utilization of the delivery agents and

their toxicity *in vivo* remain to be established. Although our *in vitro* studies indicated the relative safety of the system in A-549 cells, other cell types may be more sensitive to toxicity caused by the system, especially when polymyxin B is to be used.

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