Evaluation of Generation 2 and 3 Poly(Propylenimine) Dendrimers for the Potential Cellular Delivery of Antisense Oligonucleotides Targeting the Epidermal Growth Factor Receptor

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Purpose. To evaluate low generation, G2 and G3, poly(propylenimine) dendrimers for the potential cellular delivery of antisense oligonucleotides (ODNs) targeting the epidermal growth factor receptor (EGFR) in A431 epidermoid carcinoma cells.

Methods. Cell cytotoxicity of the dendrimers was evaluated using trypan blue exclusion assays. Cellular uptake studies of fluorescently labeled ODNs were performed using fluorescence-activated cell sorting analysis. Intracellular fate of dendrimer-delivered ODNs was assessed in both fixed and live cells using fluorescent microscopy. Antisense ODN activity was assessed in terms of cancer cell growth, inhibition of target EGFR protein, and reduction in mRNA levels.

Results. G2 dendrimer (DAB-8) was less toxic than G3 (DAB-16) dendrimer in A431 cells, with IC_{50} of >175 and ≈30 μg/ml, respectively. Uptake of fluorescently labeled ODN:dendrimer complexes was increased by up to 100-fold compared to a marker of fluid-phase endocytosis and up to 9-fold over free ODN at the optimal dendrimer:ODN (w/w) ratio of 5:1. Uptake of dendrimer:ODN complexes was significantly reduced at 4°C ($p < 0.05$). Live cell fluorescent microscopy resulted in an intracellular distribution of dendrimer:ODN complexes that was suggestive of endocytic uptake; in contrast, cell fixation resulted in an artefactual nuclear localization. Treatment of A431 cells with anti-EGFR antisense ODN:dendrimer complexes inhibited cell growth, protein, and mRNA expression to levels comparable to Oligofectamine-mediated delivery.

Conclusions. G2 and G3 poly(propylenimine) dendrimers markedly improved the delivery and activity of ODNs and thus may represent general reagents for the delivery of ODNs to cells in culture.

KEY WORDS: A431 cells; antisense oligodeoxynucleotide; epidermal growth factor receptor; gene delivery; poly(propylenimine) dendrimer.

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INTRODUCTION

Delivery of gene and oligonucleotide (ODN)-based therapies by nonviral systems, though much studied, presents many challenges in matching the efficacy of natural viral systems (reviewed in Ref. 1). A relatively recent advance has been the use of cationic polymer complexes (polyplexes) that exhibit lower cytotoxicity than the widely used lipoplex delivery systems (reviewed in Ref. 2). Polyplexes are assembled through electrostatic interactions; for example, between cationic polymer and negatively charged nucleic acids (3). A number of cationic polymers, such as poly-L-lysine (4), poly(ethylenimine) (5), poly(amidoamine) dendrimer (6), and hyperbranched poly(amino ester) (7), can compact plasmid DNA into submicrometer-sized water-soluble particles (polyplexes) that are efficiently transferred into cells.

More recently, poly(propylenimine) dendrimers have been considered for macromolecular drug delivery. These offer the advantage of possessing 100% protonable nitrogens (8), which would suggest they are ideal for nucleic acid binding and potentially DNA transfection agents (Fig. 1). The use of dendrimers as gene delivery agents has largely focused on the high generation polyamidoamine (9–12), with little work being reported on the use of the lower generation poly(propylenimine) dendrimers for nucleic acid delivery. In recent years, high generation poly(propylenimine) dendrimers have been studied for their cytotoxicity (13), as pH-sensitive controlled release drug delivery systems (14), and as gene transfer agents (15). More recently, low generation poly(propylenimine) dendrimers have been studied as a delivery system for plasmid DNA (16). These authors (16) also compared poly(propylenimine) dendrimer delivery with the lipid, DOTAP and concluded that lower generation poly(propylenimine) dendrimers (specifically DAB 8) presented improved biocompatibility and *in vitro* transfection capability.

The purpose of this study was to investigate the efficacy of poly(propylenimine) dendrimers in the delivery of antisense oligonucleotides (ODNs) targeted to the epidermal growth factor receptor (EGFR); a receptor tyrosine kinase proto-oncogene that plays a central role in the initiation and development of several human malignancies, notably breast, brain, and lung tumors (17,18). We report that G2 and G3 poly(propylenimine) dendrimers were effective agents for the delivery of antisense ODNs resulting in a marked knockdown of EGFR expression in A431 cancer cells that was comparable to that achieved by Oligofectamine-mediated delivery—a commercially available cationic lipid-based transfection agent.

METHODS

Materials

A431 [a human epidermoid carcinoma cell line (passage 6)] was purchased from European Collection of Cell Cultures (ECACC; Salisbury, UK). Culture plastics were purchased from Corning-Costar (UK), and all tissue culture media and reagents were obtained from Life Technologies (UK). Oligofectamine, OptiMEM, MMLV reverse transcriptase, and dNTPs were purchased from Invitrogen (UK). Dendrimers (DAB 8 and DAB 16), Tri-reagent, and mouse anti-human β-actin monoclonal antibody were purchased from Sigma-Aldrich (Poole, UK). Rabbit anti-EGFR polyclonal antibody

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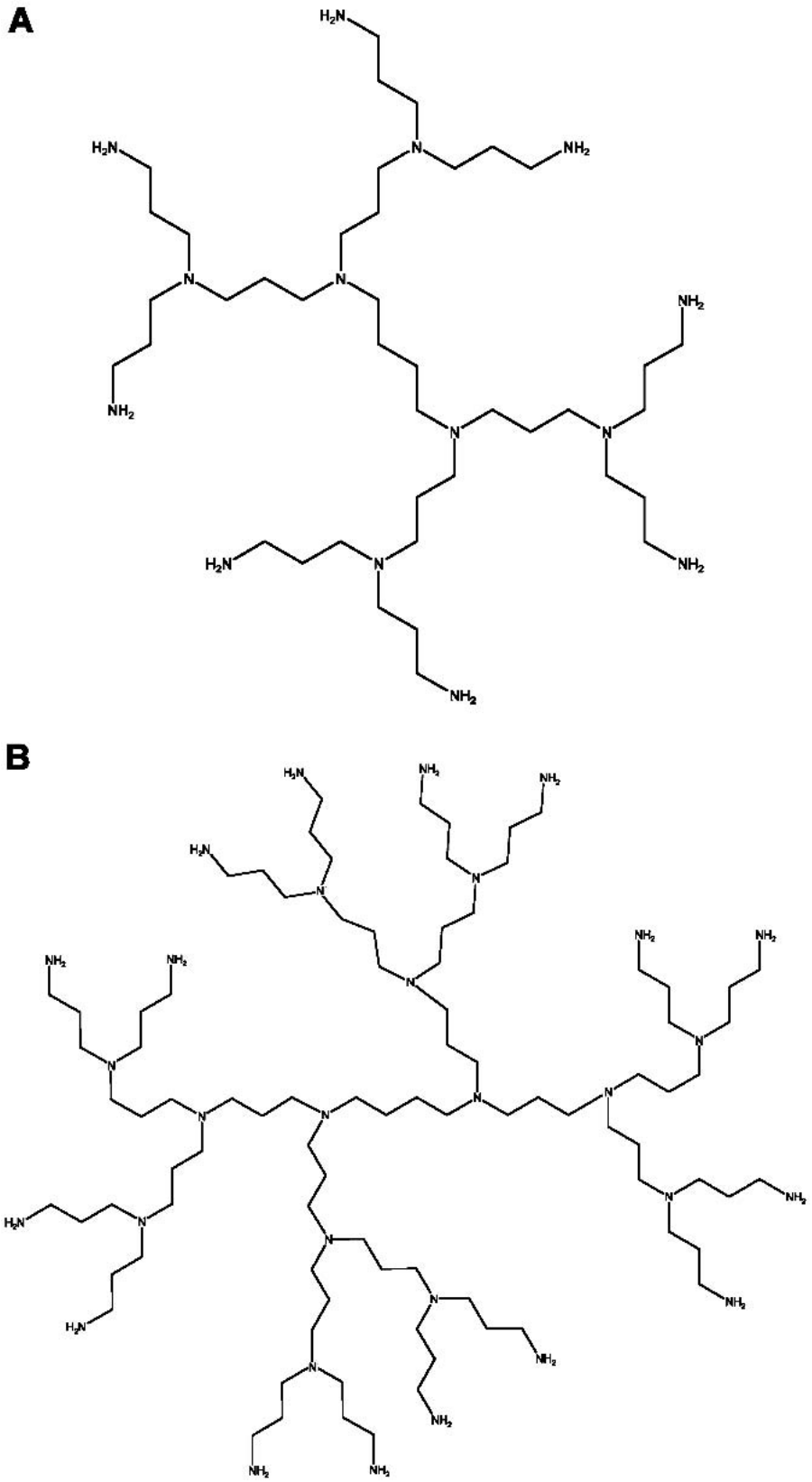

Fig. 1. Chemical structures of low generation polypropylenimine dendrimer. (A) DAB 8, generation 2 (molecular weight 773) with 8 surface amine groups. (B) DAB 16 (molecular weight 1684), a generation 3 possessing 16 surface amine groups.

was purchased from Upstate Cell Signaling (Milton Keynes, UK). Horse-radish peroxidase (HRP) conjugated donkey anti-rabbit and sheep anti-mouse antibodies, pdN6 random hexamers, and ethidium bromide were purchased from Amersham Biosciences (Chalfont St. Giles, UK). All flow cytometry materials were from Becton-Dickinson (Oxford, UK). All PCR primers were synthesized by MWG-Biotech (Milton Keynes, UK) (to a 0.2 nM scale and HPLC purity). RNasin was from Promega (Southampton, UK). Taq polymerase enzyme was from Qiagen (Crawley, UK). Unless otherwise stated, other reagents and chemicals used were purchased from Fisher Scientific (Loughborough, UK).

Cell Culture

A431 cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin/streptomycin. Cell were cultured in 75 cm^2 vented capped tissue culture flasks and placed in a 95% humidified incubator with an atmosphere of 5% CO_2 in air and at a temperature of 37°C. Confluent flasks were subcultured every 3 to 4 days.

Antisense

An anti-EGFR ODN 21mer d(TTT CTT TTC CTC CAG AGC CCG) and a scrambled 5′-CTG ATC CTG CTC TGA TCC TCT were designed using a scanning ODN array spanning the first 120 nt downstream of the AUG initiation codon of the EGFR mRNA [described in Petch *et al.* (19)]. ODNs were synthezised on an automated DNA/RNA syntheziser [Model 392, Applied Biosystems (ABI), UK] using standard cyanoethyl chemistry (reagents from Cruachem, UK). For fluorescent uptake studies, the anti-EGFR ODN was fluosecein labeled at the 5′-end as a terminal coupling step in the automated synthesis cycle by using FAM-amidite reagent according to the manufacturer's protocol [Applied Biosystems (ABI), UK]. The deprotected ODNs were then purified using NAP10 Sephadex 25 column (Pharmacia Biotech, UK), and the ODN eluted in water prior to drying under vacuum (DNA SpeedVac, Savant, UK) and stored at –20°C until use. ODN concentration was determined using an UltroSpec 3100pro UV/Vis Spectrophometer (Amersham Biosciences, Chalfont St. Giles, UK).

DNA Dendrimer Formulations

Following the method described by Zinselmeyer *et al.* (16), briefly, dendrimer:ODN formulations were made by mixing ODN and dendrimers in a 5% dextrose solution and allowing to stand for no longer than 5 min before use. The resulting colloidal dispersion was sized by photon correlation spectroscopy (Malvern Instruments, Malvern, UK).

Transfection

Dual transfection of A431 cells used cells seeded at 50,000 cells per well in 24-well plates (maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin). Cells were incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. The complexes and Oligofectamine (used at a determined optimal concentration of 1.5 μl per 24-well, prepared following manufacturers protocol) controls were prepared in OptiMEM. Cells were washed three times with 37°C sterile PBS. Complexes were added (200 ml per 24-well) and incubated with the cells for 4 h at 37°C in 5% CO_2. At the end of the incubation period, the incubation medium was replaced with serum containing DMEM culture media [containing 10% FBS, 2 mM L-glutamine, and both penicillin (100U/ml) and streptomycin (0.1 mg/ml)]. Cells were incubated at 37°C in 10% CO_2 for a further 20 h The cells were then transfected a second time (exactly as the first procedure). After the 20 h incubation of the second transfection, the cells were washed in PBS and analyzed with the various techniques.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting (FACS or flow cytometry) analysis of fluorescent anti-EGFR ODN (f-ODN) uptake by A431 cells in 24-well format was performed. After transfection, the cells were washed 3 times with ice-cold PBS before dissociation with Trypsin/EDTA at 4°C. Once cells were dissociated, ice-cold growth media was added, the cells were transferred to FACS tubes (Fahrenheit, Rotherham, UK), placed on ice, and analyzed immediately. Cell associated fluorescence distributions were obtained from 10,000 events per cell sample through a bandpass filter FL1 using a FACSCalibar flow cytometer (BD Biosciences, Oxford, UK). The fluorescence of gated cell populations was analyzed using validated analysis software, WinMDI. Statistical analysis was performed on the raw (median fluorescence intensity) data.

Fluorescent microscopy

Live Cell Microscopy

For live cell observations, cells were seeded into 30-mm dishes at 25,000 cell/cm^2 with coverslips set underneath a window in the dish. After transfection with appropriate DAB/ODN complexes, cells were washed 3 times with sterile phosphate buffered saline (PBS) and treated. Viewing was undertaken with the cells maintained in PBS.

Fixed Cell Microscopy

For studies involving fixation, cells were seeded onto 22-mm^2 coverslips at 25,000 cell/cm^2. Again, after transfection with appropriate DAB/ODN complexes, cells were washed 3 times with sterile PBS prior to fixation. Fixation involved washing the cells 3 times with PBS, followed by 10 min incubation with 2% formaldehyde in PBS at room temperature. Cells were then washed a further 3 times with PBS and mounted on slides using Vectashield Hardset mounting medium with or without DAPI nuclear stain (Vector Labs, Peterborough, UK).

All images were captured using a Leica DM IRB inverted epifluorescent microscope, fitted with a 12-bit cooled monochrome QImaging camera. Images were collected and handled using ImproVision, Openlab software (version 3.0.9)

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA extracted from treated A431 cells using Trireagent (following manufacturers instructions), the reverse transcription reaction comprised RNase/DNase free water, 8 μl 5× first strand buffer, 4 μl dNTPs (10 mM), 4 μl DTT (0.1 M), 4 μl pdN6 (100 ng), 1 μg of RNA, 1 μl RNasin (20 U), and MMLV-rt (200 U) in a total volume of 40 μl. Samples were heat denatured for 4 min before addition of reverse transcriptase, followed by further incubations of 10 min at 25°C, 50 min at 42°C, and a final termination step of 2 min at

99°C. Aliquots were subjected to PCR (primers were designed in house using the online design package, Primer3; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/), in a final volume of 25 μl using 8.5 μl RNase/DNase free ddH_2O, 2.5 μl Taq 10x buffer containing $MgCl_2$ (15 mM), 1 μl $MgCl_2$ (25 mM), 2 μl dNTPs (10 μM), 2 μl cDNA (100 ng/μl), 0.2–0.5 μl forward and reverse primers (10 pmol/μl), and 0.1 μl Taq polymerase (5 U/μl). The EGFR PCR program consisted of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C (30 cycles). The PCR products were electrophoresed through a 1.2% agarose gel in the presence of ethidium bromide and visualized under UV light. The housekeeping gene, β-actin, was amplified separately using the same reverse transcription products and thermal cycling parameters (appropriate negative control samples for all primers).

Western Blotting for Total EGFR

Cells were seeded into 24-well plates as for growth assays and were lysed by the addition of lysis buffer [50 mM Tris-base, 5 mM ethylene glycol-*bis*(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 150 mM NaCl, 1% Triton 100, 2 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 20 mM phenylarsine, 10 mM sodium molybdate, 10 mg/ml leupeptin, and 8 mg/ml aprotinin; pH 7.6 (all purchased from Sigma-Aldrich)]. Cell debris removed by centrifugation at 13,000 rpm for 15 min at 4°C. Supernatants were analyzed for protein content using the standard BCA assay (Bio-Rad, Hercules, CA, USA). After mixing with sample loading buffer [60 mM Tris, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol, and 250 mM DTT], samples were boiled for 10 min at 100°C and equal amounts of protein (50 mg/lane) were loaded onto an SDS-PAGE (5% stacking gel and an 8% resolving gel) and run at 100 V until the dye reached the bottom of the gel. Protein patterns were transferred onto Protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and the free sites were blocked with 5% BM chemiluminescence blotting buffer POD (Roche, Diagnostics, Indianapolis, IN, USA). To detect total EGFR, nitrocellulose membrane was probed with rabbit polyclonal anti-EGFR antibody and followed by donkey anti-rabbit IgG-HRP-conjugated secondary antibody. Immunreactive bands were detected with SuperSignal chemiluminescent substrate (Perbio Science, Tattenhall, UK) using Kodak autoradiography film (G.R.I., Rayne, UK). To ensure equal loading of proteins, β-actin levels were detected using primary mouse anti-human β-actin monoclonal antibody, followed by the sheep anti-mouse IgG-HRP-conjugated secondary antibody. Image acquisition and band quantification were undertaken on GS-700 densitometer with Molecular Analyst software (Bio-Rad, UK).

RESULTS

In our previous studies on cationic lipid-mediated delivery of antisense ODNs targeting the EGFR, we have demonstrated that a double transfection protocol was necessary to obtain a sustained knockdown of the relatively long-lived EGFR protein in A431 cancer cells (19). We showed that two 4 h incubations of Lipofectin:ODN complexes administered at 24 h intervals over a 48 h treatment period resulted in 60–80% inhibition of EGFR mRNA and protein (19). Here, we used a similar protocol in our evaluation of DAB dendrimers as an effective delivery system for anti-EGFR antisense ODNs.

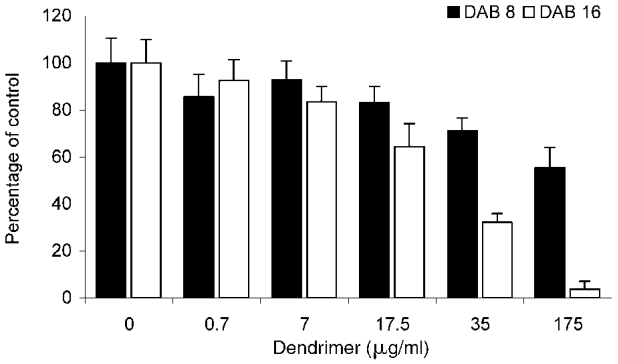

Fig. 2. Toxicity of both of the DAB 8 (solid bars) and DAB 16 (open bars) dendrimers, viable cell counts of A431 treated with six dendrimer concentrations. DAB 8 $IC_{50} \approx 175$ μg/ml, DAB 16 $IC_{50} \approx 20$ μg/ml, therefore DAB 16 is approximately 9-fold more toxic than DAB 8. Cells seeded at 50,000 cells/well, grown for 24 h prior to treatment of 4 h with AS-1 complexes (serum free), returned to full media for 20 h before carrying out the treatment and culture cycle once more (dual transfected). Cells were counted at the completion of the second cycle (n = 4); cells were also assessed for viability using Trypan blue dye exclusion (with all treatments cells were >97% viable).

DAB Toxicity

In order to assess the cellular toxicity of the DAB dendrimer delivery systems, we incubated increasing concentration of DAB 8 (generation 2) or DAB 16 (generation 3) dendrimers with A431 cells for 48 h using the double transfection protocol described by us previously for lipid transfection (19) and determined the number of viable cells remaining after the treatment period. Figure 2 shows that DAB 8 did not markedly reduce A431 cell viability up to a dose of 17.5 μg/ml whereas DAB 16 did not affect cell viability up to a concentration of approximately 10 μg/ml. The approximate IC_{50} for DAB 16 and DAB 8 was estimated as 30 μg/ml and >175 μg/ml, respectively, implying greater toxicity of the generation 3 dendrimer in A431 cells. A similar trend was observed by Zinselmeyer *et al.* (16), who reported slightly higher IC_{50} values for the DABs when only a single 4 h exposure of the DAB delivery systems was used for gene delivery to cells in culture. Based on the cell viability data presented here, we subsequently used subtoxic concentrations of DABs for ODN delivery; for example, 8.75 μg/ml was used for both dendrimers at a 5:1 DAB:ODN ratio and 17.5 μg/ml at the 10:1 ratio.

AS-1 Cellular Uptake

Fluorescence-activated cell sorting was used to assess the cellular uptake of ODNs in the presence of DABs and to determine the optimal dendrimer:ODN ratio for efficacy studies. Using 500 nM FITC-labeled ODN (f-ODN), uptake was determined over a range of DAB:ODN ratios, 2:1, 5:1, and 10:1 (DAB:ODN w/w) at 37°C for both DAB 8 and DAB 16. There was an increase in cell-associated fluorescence with increasing ratio of dendrimer in the complexes for DAB 16. The median fluorescence intensity for the respective treatments showed an approximate 9-fold increase in ODN uptake with dendrimer delivery compared to f-ODN alone (solid bars, Fig. 3). This represented an increase of approximately 100-fold greater uptake of ODN as compared to that of the

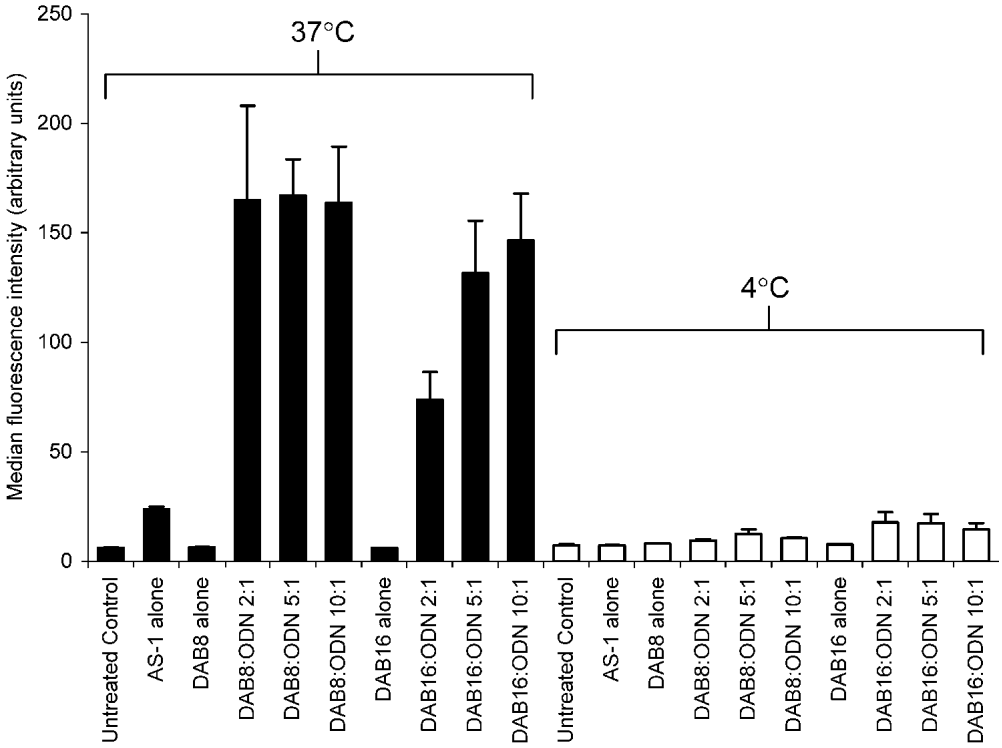

Fig. 3. Uptake assessed by flow cytometric analysis of fluorescently labeled AS-1 (f-ODN), comparing 37°C vs. 4°C controls. f-ODN was used in all treatments at 500 nM. The fluorescence of gated cell populations was analyzed using validated analysis software, WinMDI. Statistical analysis performed on the raw (median fluorescence intensity) data (n = 4).

fluid phase marker, FITC-dextran (4.4 kDa). A similar overall increase in ODN uptake was observed with DAB 8 at the three different ratios studied. ODN uptake was significantly reduced at 4°C for both DABs compared to that observed at 37°C (open bars, Fig. 3), implying that the uptake of the DAB:ODN complexes involved an active or energy-dependent mechanism.

Subcellular Distribution

Fluorescently labeled ODN-DAB complexes were incubated with A431 cells in serum-free medium, and the cell associated fluorescence and subcellular distribution was assessed by fluorescent microscopy in both fixed and nonfixed (live) cells. These studies confirmed that DAB 8 and DAB 16 improved the cellular association of f-ODN in A431 cells (Fig. 4, panels C, E, and F, compared to panel B). Cellular uptake of the naked f-ODN appeared modest with a punctate subcellular distribution characteristic of endocytic uptake that was similar to that described by us previously (20–22). In fixed cells, DAB-mediated delivery of f-ODN significantly increased the uptake of f-ODN. Subcellular distribution of f-ODN appeared to be localized in both the cytosol and the nucleus whereas Oligofectamine-mediated delivery of f-ODN resulted in a predominantly cytosolic localization. However, further examination of the subcellular distribution of ODN in nonfixed (live) cells showed the opposite effect. In the case of live cells, DAB-mediated delivery of f-ODN resulted in enhanced cytosolic uptake of ODN similar to that observed by FACS but with only a little fluorescence appearing in the nucleus, suggesting that the improved ODN uptake observed was probably due to a more efficient endocytic mechanism of entry than that used by the naked f-ODN (Fig. 4, panels E and F). These data highlight the artefactual results that may arise following fixation of cells prior to imaging and which have also recently been observed with ODN-peptide conjugates (23–26). Furthermore, live cell imaging of Oligofectamine delivered f-ODN showed that nonfixed cells retained the same cytosolic distribution observed in fixed cells, further suggesting that the artefactual cytosolic to nuclear change in distribution during fixation is not universal but is dependent on the delivery system or specific conditions used.

Anti-EGFR ODN Activity in Cancer Cells

The antisense efficacy of dendrimer-delivered ODN was determined in A431 cells by a combination of cell growth and protein and mRNA expression assays similar to those described by us previously (19,27). Using a 500 nM concentration of ODN that had previously been determined to be optimal for down-regulating EGFR with cationic-lipid-ODN complexes (19), we evaluated the activity of DAB:ODN complexes at three different ratios. Although all three ratios gave a marked reduction in cell number for DAB 8-mediated ODN delivery, the 5:1 ratio was found to be optimal for both DAB 8 and DAB 16, (Fig. 5) and was therefore subsequently used in the studies assessing EGFR protein and mRNA expression. In cell growth assays, both the DAB 8 and DAB 16 dendrimers showed comparable efficacy resulting in up to 60% inhibition of cell growth for the DAB 8-mediated delivery of anti-EGFR ODN. The scrambled ODN:DAB showed little or no effect on cell growth, suggesting this was a sequence-specific effect of the anti-EGFR ODN as demonstrated by us previously using cationic lipid (Lipofectin) delivery (19). For comparison purposes, in this study we also examined the antisense activity of anti-EGFR ODN delivered

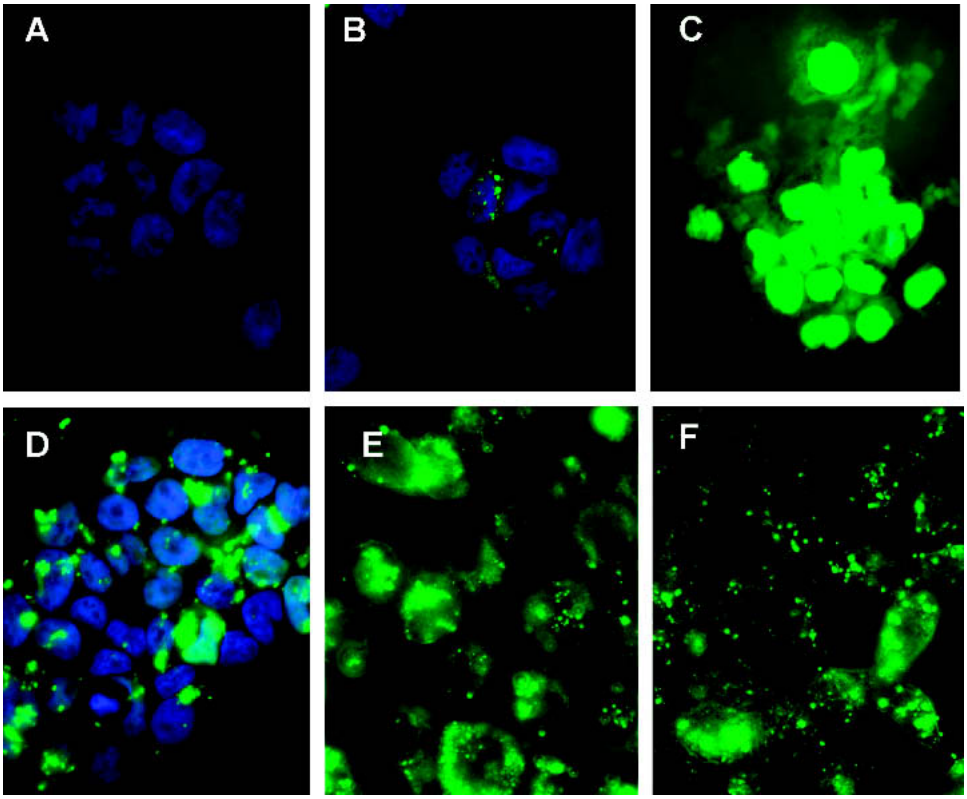

Fig. 4. Fluorescent microscopy showing distribution of f-ODN in A431 cells (f-ODN concentration 500 nM) before either fixation and mounting in Vectashield containing DAPI nuclear counterstain (A–D) or washed and cells viewed live (E, F). (A) untreated cells; (B) f-ODN alone; (C) DAB 16 f-ODN; (D) Oligofectamine (OF) and f-ODN; (E) DAB 8 with f-ODN (5:1) live cell; (F) DAB 16 with f-ODN (5:1) live cell.

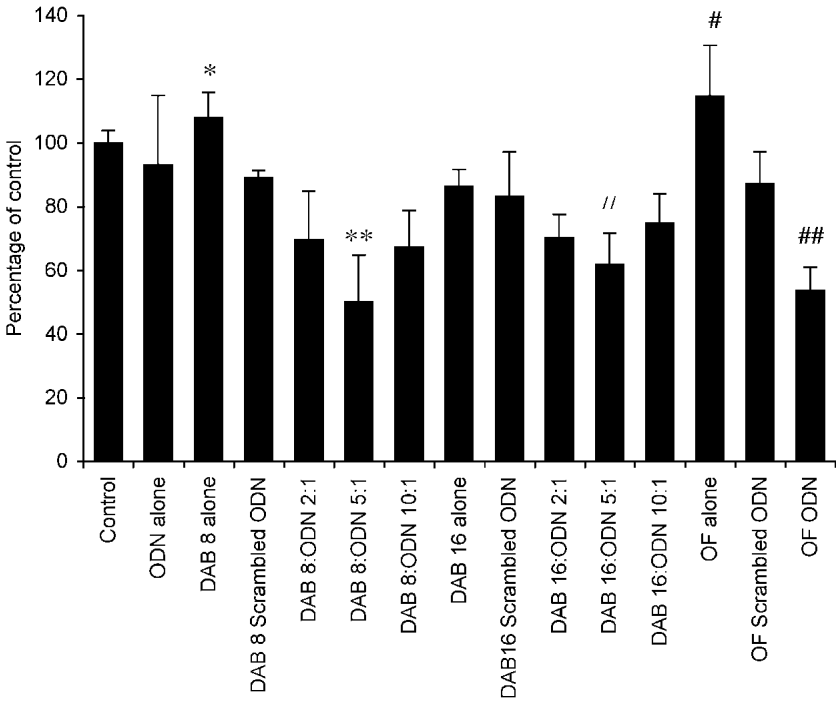

Fig. 5. Effect on cell proliferation of ODN at three DAB:ODN ratios, comparing to Oligofectamine (OF); also included was a scrambled ODN control. The three ratios were 2:1, 5:1, and 10:1 dendrimer:ODN for each dendrimer with the ODN concentration fixed at a previously determined optimal (500 nM) ($n = 4$). Statistical differences are marked using single and double symbols to show the respective differences ($p < 0.05$).

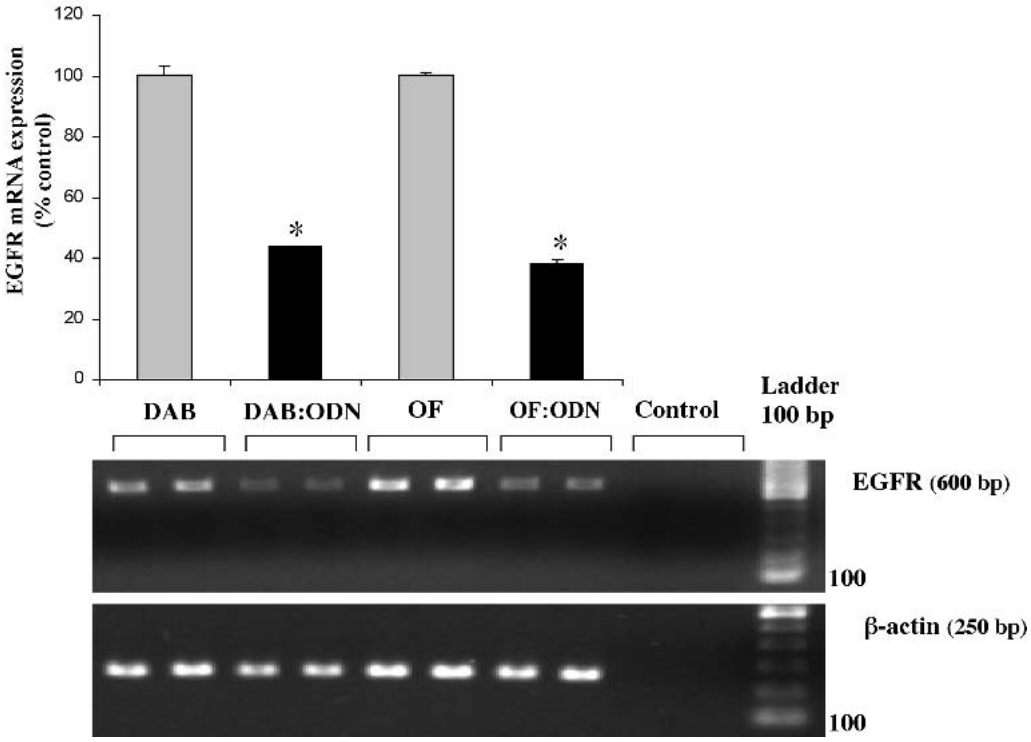

Fig. 6. RT-PCR for EGFR (product size 600 bp; primers: fwd 5′CAA CAT CTC CGA AAG CCA; rev 5′CGG AAC TTT GGG CGA CTA T). Housekeeping gene β-actin (product 254 bp; primers: fwd 5′GGC ATG GGT CAG AAG GAT T; rev 5′GGG GTG TTG AAG GTC TCA AA) was probed for using the same conditions and cycle program as the EGFR RT-PCR.

by another cationic lipid, Oligofectamine. The inhibition of cell growth obtained with DAB 8 and 16 mediated delivery of anti-EGFR ODN was comparable to that observed with Oligofectamine (Fig. 5).

Inhibition of EGFR mRNA levels (Fig. 6) and inhibition of protein expression by Western blotting (Fig. 7) with up to 60% inhibition observed in EGFR mRNA levels with DAB 16. In both cases, AS1-mediated reduction in mRNA and protein expression was comparable with DAB 16 delivery to that obtained with Oligofectamine delivery (Figs. 6 and 7).

DISCUSSION

Antisense ODNs are poorly transported into cells by an endocytotic mechanism that is dependent on a number of factors including oligonucleotide chemistry and charge, cell type, stage of cell cycle, pH of media, and the nature of any delivery system used (21,28,29). Several different delivery systems have now been explored for improving ODN delivery including the use of cationic lipids (lipoplexes), cationic polymers (polyplexes), and dendrimers (for reviews, see Refs. 28–30). Of these, the cationic lipids are still the most widely used despite their known toxicity to cells. As an alternative delivery system for antisense ODNs, in this study we have evaluated the use of low generation poly(propylenimine) dendrimers, DAB 8 and 16, as these have been demonstrated to have lower toxicity than a proprietary cationic lipid while exhibiting similar gene transfection capability (16).

Here we report that G2 and G3 poly(propylenimine) dendrimers can effectively deliver ODNs to cells in culture and inhibit target gene expression at levels comparable to those observed with Oligofectamine—a cationic lipid delivery system. Antisense ODN uptake was markedly enhanced (approximately 10-fold) when delivered as either DAB 8 or DAB 16 by a mechanism that appears to be energy dependent and results in cytosolic localization of the ODN as determined by a combination of FACS and fluorescent microscopy studies. Although it is not clear from this study whether the ODN is free or still present as a complex with the poly(propylenimine) dendrimers, our observations are consistent with an endocytic uptake mechanism that ultimately results in significant amounts of free or bioavailable antisense ODN to exert a marked (up to 60%) knockdown in EGFR mRNA and protein as determined by RT-PCR and Western blotting, respectively. Also, the degree of antisense inhibition appeared comparable to that obtained with Oligofectamine delivery (Figs. 6 and 7 of the current study) or, as shown in a parallel study, comparable to a novel, nontoxic, pentaerythritol-based dendrimer (31).

Studies with other dendrimers have also shown similarly improved cellular uptake of ODNs *in vitro* and *in vivo* (5,32,33), though it is difficult to make direct comparisons of studies using delivery systems in different cell types. Bielinska *et al.* (9), working with polyamidoamine dendrimers, reported a 98% inhibition of luciferase production in a cell-free system with antisense ODN, as well as up to 50% inhibition in transfected Rat2 embryonal fibroblast cells. Work with low generation polyamidoamine starburst dendrimers by Delong *et al.* (33) in cultured U251 astrocytes presented a 50-fold increase in uptake, while also reporting some nuclear, with mainly cytoplasmic, cellular distribution in live cells, noting that the relative distribution between the two locations varied

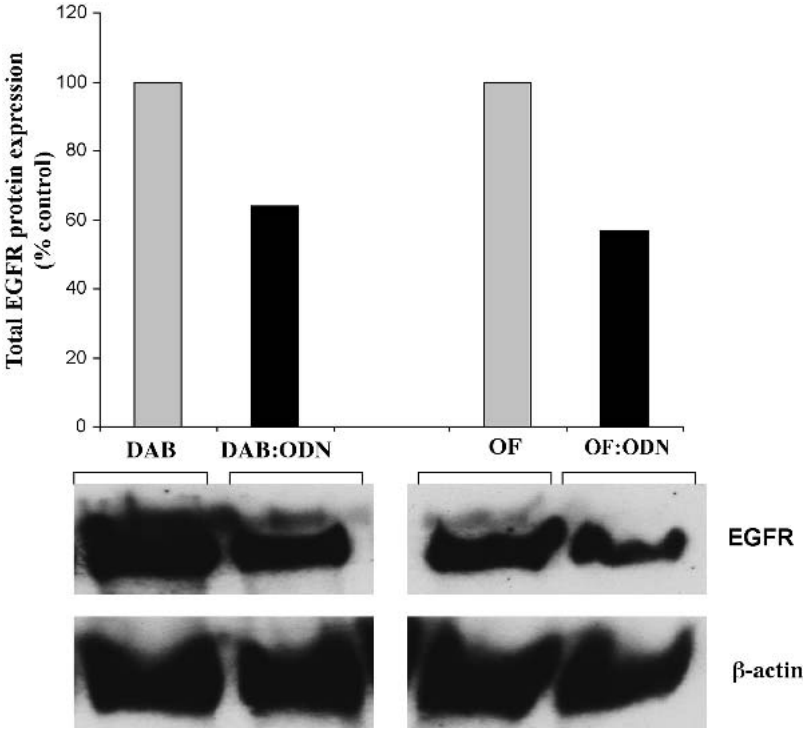

Fig. 7. Panel of Western blots showing ODN activity against total EGFR protein expression, compared to the housekeeping protein β-actin. Use of optimal ratio of DAB 16 to ODN as well as DAB 16 alone and Oligofectamine (OF) as a comparison.

markedly through the cell population studied. Sato *et al*. (34) have shown in a mouse model that generation 4 polyamidoamine dendrimers could also effectively deliver ODNs to tumors *in vivo* following i.p. administration.

Poly(ethylenimine) has also been examined for ODN and gene delivery (32,35). Boussif *et al.* (5) compared poly(ethylenimine)-mediated delivery of an 18-mer ODN with Transfectam (a cationic lipid delivery system) for a range of human and avian cells. These authors reported that poly(ethylenimine) was comparable to or better than the lipid in all but one cell line in terms of transfection efficiency (5). Poly(ethylenimine)-mediated delivery is thought to deliver ODNs and genes into the nucleus when examined in both fixed (5) and live cells (35). However, the exact mechanism and kinetics of uptake of free and complexed ODN *in vitro* and *in vivo* are unknown and is an area of ongoing study (29,36,37; reviewed in Ref. 38). The exact intracellular fate may be dependent on cell type (39) and/or use of fixation techniques.

Here we report that intracellular fate of ODNs complexed with poly(propylenimine) dendrimers was dependent on whether cells were fixed or not prior to micropscopic examination. Fixation of A431 cells with 2% formaladehyde suggested that DAB:f-ODN complexes localized generally within the cells in the cytoplasm and nucleus. However, live cell studies showed mostly cytosolic localization with little nuclear localization of the DAB:f-ODN in A431 cells. A similar artefactual nuclear localization with fixation was recently reported for cell-penetrating peptides (23–26). Furthermore, we have found that the artefactual nuclear localization observed with fixation is not a universal phenomenon with all ODN delivery systems. Indeed, in contrast to DAB-delivered f-ODN, the intracellular fate of f-ODN delivered by Oligofectamine remained cystolic irrespective of fixation, further suggesting that the artefactual cytosolic to nuclear change in distribution is dependent on the delivery system and/or specific conditions used. Thus, this study supports avoiding the use of fixed cells wherever possible for intracellular trafficking studies with drug delivery systems.

In conclusion, the data presented herein show poly(propylenimine) (DAB) dendrimers represent a delivery system capable of delivering antisense oligonucleotide, producing a comparable gene expression knockdown as a proprietary lipid-based system, with the notable advantage of reduced toxicity. Thus, PPI dendrimers are worthy of further examination as potential delivery systems for antisense ODNs *in vitro* and vivo.

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