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Regulation of *in vitro* gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers

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ABSTRACT

Starburst polyamidoamine (PAMAM) dendrimers are a new type of synthetic polymer characterized by a branched spherical shape and a high density surface charge. We have investigated the ability of these dendrimers to function as an effective delivery system for antisense oligonucleotides and 'antisense expression plasmids' for the targeted modulation of gene expression. Dendrimers bind to various forms of nucleic acids on the basis of electrostatic interactions, and the ability of DNA–dendrimer complexes to transfer oligonucleotides and plasmid DNA to mediate antisense inhibition was assessed in an *in vitro* cell culture system. Cell lines that permanently express luciferase gene were developed using dendrimer mediated transfection. Transfections of antisense oligonucleotides or antisense cDNA plasmids into these cell lines using dendrimers resulted in a specific and dose dependent inhibition of luciferase expression. This inhibition caused ~25–50% reduction of baseline luciferase activity. Binding of the phosphodiester oligonucleotides to dendrimers also extended their intracellular survival. While dendrimers were not cytotoxic at the concentrations effective for DNA transfer, some non-specific suppression of luciferase expression was observed. Our results indicate that Starburst dendrimers can be effective carriers for the introduction of regulatory nucleic acids and facilitate the suppression of the specific gene expression.

INTRODUCTION

For over 20 years, antisense inhibition of translation has been considered an effective technique for the modulation of gene expression (1). The general principle of antisense inhibition, whether employing synthetic oligonucleotides or antisense expression plasmid systems, relies on the specificity of Watson–Crick base pair formation (2,3). This enables complementary nucleic acid sequences to target and inhibit steps in the transcription and translation of specific genes (4). The ability of antisense techniques to down-regulate the expression of specific genes is well documented in both *in vitro* and *in vivo* systems (5,6). However, given the inefficiency of naked DNA entry into the cells, most of these studies have required micromolar concentrations of oligonucleotides with modified DNA structures (to increase the availability and the stability of the nucleic acid) in order to obtain a significant inhibitory effect (7). These requirements limit the utility and the potential therapeutic efficacy of this approach.

Several methods that enhance the transfer of DNA into eukaryotic cells offer the potential to facilitate antisense applications. Cationic lipid preparations have been demonstrated *in vitro* to improve the effectiveness of antisense oligonucleotides, presumably through increased transfer into cells (8,9). However, the currently available lipid preparations are not effective and have cytotoxic effects *in vivo* that often make it difficult to determine specific antisense effects (9). Other techniques for gene transfer rely on adenoviral and retroviral vectors (10,11) and may supply a means for transfer of antisense expression sequences incorporated into the viral genes. Unfortunately, these methods cannot be employed with synthetic oligonucleotides and pose problems with immunogenicity and cell targeting *in vivo*. Despite these difficulties, it seems likely that improving the efficiency of transfer of antisense oligonucleotides and targeting these agents to particular cells is crucial to the success of antisense technology.

PAMAM dendrimers are a new type of polymer with a molecular architecture characterized by regular, dendric branching with radial symmetry (12). This results in dendrimers having unusual physical and chemical properties. PAMAM dendrimers are currently the only class of dendritic macromolecules that are

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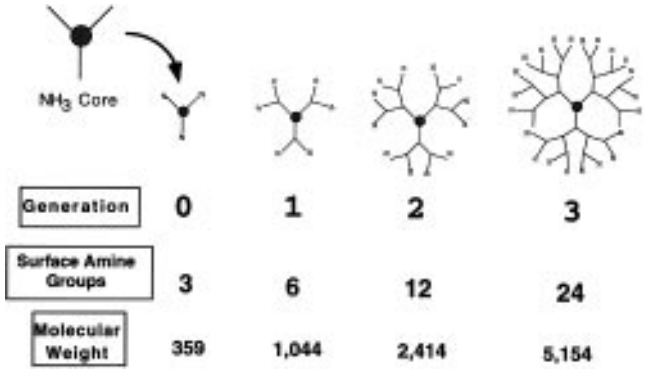

Figure 1. Schematic presentation of dendrimer structure and growth. Each generation, or layer of polymer, doubles the number of surface amine groups and approximately doubles the molecular weight. EDA core dendrimers have four starting arms instead of the three NH_3 core molecules. A generation 7 dendrimer EDA core dendrimer similar to what is employed in these studies has 512 surface amino groups and an approximate molecular weight of 90 000.

reliably produced in large quantities and that can be precisely synthesized over a broad range of molecular weights similar to that of proteins. These molecules range in size from 10 to 100 Å, with each generation, or layer, of the polymer adding ~10 Å to the diameter of the molecule (Fig. 1). The number of surface primary amino groups doubles with each generation, reaching 4096 for a tenth generation dendrimer (12,13). The defined structure of these molecules and their large number of surface amino groups has led to dendrimers being employed as a substrate for the attachment of antibodies, contrast agents and radionucleotides for applications in a number of different areas of biology and medicine (14–16). Studies using antibody/dendrimer conjugates *in vitro* and *in vivo* in experimental animals have documented these conjugates to be non-toxic and able to target biologic agents to specific cells (14,15).

Dendrimers are able to interact on an electrostatic charge basis with biologically relevant polyanions, such as nucleic acids, because they have a surface covered with primary amino groups. We have demonstrated that Starburst dendrimers can be used *in vitro* to mediate the efficient transfer and expression of plasmid DNA in eukaryotic cells (17). The current studies document that dendrimers can deliver regulatory nucleic acids, both oligonucleotides and plasmids expressing antisense mRNA, and enhance their ability to modulate the expression of specific genes.

MATERIALS AND METHODS

Dendrimer synthesis

The synthesis of Starburst PAMAM dendrimers has been previously described in detail (13,17) and is presented schematically in Figure 1.

Plasmids

The luciferase reporter plasmid pCMVLuc was constructed using pcDNAI vector (Invitrogen) and *Hin*dIII–*Bam*HI fragment, containing the entire luciferase cDNA and SV40 polyadenylation signals of pGL-basic (Promega). Two types of antisense luciferase mRNA expressing plasmids were generated. The pCMVAsLuc, containing the ATG region was constructed by ligating the 0.66 kb *Hin*dIII–*Eco*RI fragment of luciferase gene (from pGL-basic) with *Bam*HI digested pcDNAI vector. The fragments were made compatible by filling in with Klenow polymerase (18). Transformants were screened using diagnostic digests with *Hin*dIII and *Nar*I to identify insert orientation, and sequenced to confirm insertion sites of the truncated luciferase gene. The pCMVAsLuc-∂ATG was generated by *Xba*I digest that deletes 48 nucleotides of the luciferase coding sequence of pCMVAsLuc. The pGEM-Luc (Promega) and pCMVβgal (19) (Clontech) were purchased from the suppliers.

Preparation of DNA–dendrimer complexes

Plasmid DNA was amplified in bacteria and then isolated by double cesium chloride gradient (18) to ensure the purity of the DNA preparation. Dendrimers were diluted to an appropriate concentration in buffer containing 20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol, all solutions stored at 4°C until required. DNA–dendrimer complexes were formed by incubating the two components together in 15–50 µl water for a minimum of 5 min at room temperature. Ratios of nucleic acid to dendrimer were based on the calculation of the electrostatic charge present on each component; the number of phosphate groups in the nucleic acid versus the number of terminal NH_2 groups on a dendrimer. For example, given that the number of bases in 1 µg DNA is 1.71×10^{15}, ~1.71×10^{15} negative charges are present per 1 µg DNA while a G7 (NH_3) dendrimer has ~2.65×10^{15} charges per microgram. Therefore, to obtain a 1:1 charge ratio, 1 µg DNA was mixed with 0.65 µg dendrimer.

Cells and media

A number of stable clones that demonstrated the long-term expression (4–6 months) of transfected luciferase cDNA were generated from D5 mouse melanoma and Rat2 embryonal fibroblast cell lines. Cells were transfected with G7 (NH_3) dendrimer and pCMVLuc plasmid DNA. Clones with integrated plasmid DNA were initially selected by incubation in neomycin (G418, Geneticin, Life Technologies) at 300 µg/ml for 3 weeks. The numbers of G418-resistant clones were determined by visually counting the colonies of cells after staining with either eosin or methylene blue. Neomycin resistant clones were assayed for luciferase expression by chemiluminescence as described below. Presence of integrated pCMVLuc DNA was confirmed by Southern hybridization of chromosomal DNA using a fragment of the luciferase gene as a probe.

Rat2 cells were maintained in D-MEM medium (Gibco-BRL) with 5% Nu-serum (Collaborative Biomedical Products), 1% penicillin–streptomycin and 2 mM L-glutamine. D5 cells were cultivated in CM medium RPMI 1640 with 10% fetal calf serum, 1% penicillin–streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 1 mM non-essential amino acids (Life Technologies). U937 human histiocytic lymphoma cells were maintained in RPMI 1640 with 5% fetal calf serum, 1% penicillin–streptomycin. All cells were incubated at 37°C in 5% CO_2.

Oligonucleotides

A 27 base single-stranded phosphodiester oligonucleotide 5′-GGC-GTCTTCCATTTTACCAACAGTACC-3′, complementary to the

ATG region of pCMV-Luc plasmid (antisense-ATG oligo) and its complement, identical to the coding strand of the ATG region of luciferase gene (sense-ATG oligo), were synthesized using 394DNA/RNA Synthetizer (Applied Biosystems) and purified by HPLC at the Biomedical Research Core Facility of the University of Michigan.

Transfection methods

Transfections with dendrimers were performed and analyzed using assays for luciferase activity reflecting the luciferase gene expression. DNA expression plasmids pCMV-Luc, or antisense expressors pCMVAsLuc and pCMVAsLucðATG as well as single-stranded oligonucleotides were complexed with dendrimers as described above. DNA (50–1000 ng DNA per well of transfected cells) was mixed with dendrimers at a variety of charge ratios, from 1:10 to 1:50 and were then allowed to complex for ≥5 min at room temperature. Twenty-four-well plates, seeded 24 h before the transfection with ~2×10^4 cells per well were washed once with serum-free media. Fifteen microliters of the solution containing 50–500 ng DNA–dendrimer complexes were added to 185 µl serum free-media in each well of cells and incubated for 3 h at 37°C. The serum-free medium containing the complexes was then washed out of the cells, and standard growth media was substituted. The cells remained in culture for 24–96 h before being harvested to analyze for expression of luciferase.

Analysis of the cellular retention and stability of DNA–dendrimer complexes

A single-stranded 27 base long oligonucleotide was radiolabelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. After separation from unincorporated label, ~5 ng radiolabelled DNA (2×10^6 c.p.m.) was mixed with 100 ng cold oligonucleotide and complexed with G5, G7 and G9 EDA core dendrimers in a 1:5, 1:10 and 1:100 DNA to dendrimer charge ratio. U937 cells were washed twice with serum-free media and plated at 2×10^5 cells/well in 24-well plates. Serum-free medium (0.2 ml) containing radioactive oligonucleotides or oligonucleotide–dendrimer complexes was added. The DNA was incubated with the cells for 4 h. The cells were washed with serum-free medium, 3 ml complete medium was added and cultures were incubated for 12, 24, 48 and 96 h. Total nucleic acids were isolated from cells with DNA-zol (MRC Inc., Cincinnati) with an additional phenol–chloroform extraction and precipitation with 5 vol EtOH in the presence of 0.3 M NaOAc. Precipitates were then washed twice with 80% EtOH at 4°C. All samples were resuspended in 20 µl buffer containing 0.5% SDS, 1 mM EDTA, 10 mM Tris–HCl pH 7.8 and radioactivity was quantified using a scintillation counter.

Expression assays

Luciferase expression was quantified in lysates of transfected cells at 24 h after transfection by measuring the light emission resulting from 10 µl cell lysate incubated with 2.35×10^{-2} µmol of luciferin substrate (Promega, Technical Bulletin No.101). Light intensity was measured in a chemiluminometer (LB96P; EG&G Berthold), and adjusted according to the total protein concentration of the sample. The total protein concentration in the cell lysate was measured in a standard protein assay (DC protein assay; BioRad, Richmond, CA). Expression of luciferase was also examined using a coupled transcription–translation system (TNT Coupled Reticulocyte Lysate System; Promega) containing 200 ng luciferase expression plasmid (either pCMV-Luc or pGEM-Luc). Luciferase activity was determined as previously described.

Statistical analysis

Statistical analysis was performed using Systat 5.2 software for Macintosh. Errors were calculated as standard deviations and differences between samples were analyzed by ANOVA.

RESULTS

Sequence-specific inhibition of luciferase expression by antisense oligonucleotides complexed with dendrimers in an *in vitro* transcription–translation system

The development of a system to evaluate the efficiency of the transfer of antisense regulatory DNA using Starburst PAMAM dendrimers began with an analysis of the specific effect of antisense oligonucleotides on *in vitro* transcription–translation of luciferase in a cell-free expression system. Increasing amounts (50 ng–1.0 µg) of the antisense-ATG oligonucleotide were added to the coupled transcription–translation system containing 200 ng of either pCMV-Luc (Fig. 2A) or pGEM-Luc (Fig. 2B) as templates and suppressed luciferase expression in dose-dependent fashion to 1.5% of baseline control level (98.5% inhibition, Fig. 2A). In contrast, identical concentrations of sense oligonucleotides had no effect on the luciferase expression. High concentrations of dendrimers (130 µg/ml) caused minimal non-specific suppression of luciferase synthesis in the coupled transcription–translation reaction, but did not interfere with the specific inhibitory effect of the antisense oligonucleotide. The expression of a control gene (β-galactosidase from pCMVβgal plasmid) was not altered by the presence of either the sense or antisense oligonucleotide (data not shown). The sequence specificity of inhibition was additionally confirmed by the expression of luciferase from a pGEM-Luc plasmid. This plasmid differs from pCMV-Luc by 6 base pairs in the plasmid 5′ of the initiating ATG of the luciferase cDNA, creating a six base mismatch with the antisense oligonucleotide designed for pCMV-Luc. Expression from pGEM-Luc was inhibited by the pCMV-Luc antisense oligonucleotide to a lesser degree than expression from pCMV-Luc (Fig. 2B).

Sequence-specific inhibition of luciferase transcription by antisense oligonucleotides complexed with dendrimers *in vitro* in cultured cells

A series of stable cell lines constitutively expressing luciferase from integrated pCMV-Luc DNA were generated in Rat2 and D5 cell lines (unpublished results). Changes in the expression of luciferase in response to oligonucleotides delivered into these cells were analyzed. The effect of increasing amounts (50, 250, 500 and 1000 ng) of antisense oligonucleotides transfected to D5-CMV-Luc#30 cells with or without G7 EDA-core dendrimers was compared (Fig. 3). Luciferase expression in cells transfected with antisense-ATG oligo complexed with G7 EDA was ~30% lower compared to cells cultured with antisense oligonucleotide alone, where no significant inhibition on luciferase expression was observed. Similar results were obtained in Rat2 derived, stable cell lines (data not shown).

To evaluate which generations of dendrimers were optimal carriers for the *in vivo* delivery of the antisense oligonucleotides,

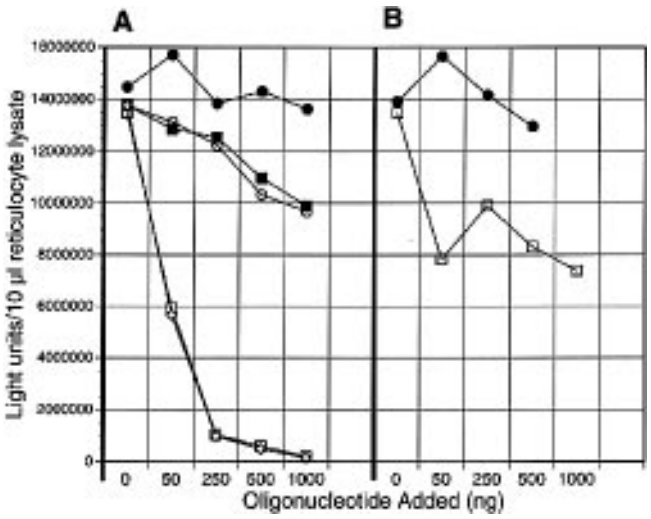

Figure 2. Inhibition of luciferase synthesis with antisense-ATG oligonucleotide in a cell-free, coupled transcription–translation system. (**A**) The synthesis of luciferase from a pCMV-Luc as a template is completely inhibited by either the antisense oligonucleotide (open squares) or the antisense oligonucleotide complexed with dendrimer (open diamonds). Sense oligonucleotide (solid circles) did not alter luciferase production. Sense oligonucleotide complexed with dendrimer (open circles) minimally suppressed luciferase production in a manner identical to dendrimer alone (solid squares). (**B**) Synthesis of luciferase from a pGEM-Luc template, which has a six base mismatch with the antisense oligonucleotide sequence, is incompletely inhibited by the antisense oligonucleotide (open squares) compared to the sense oligonucleotide (solid circles) demonstrating the sequence specificity of inhibition.

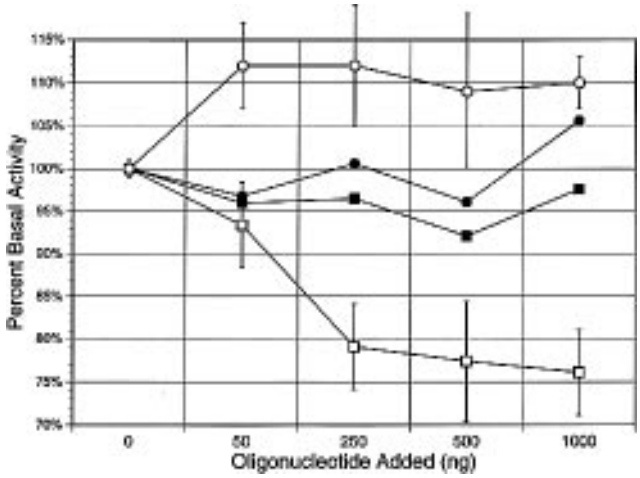

Figure 3. Inhibition of constitutive luciferase expression in D5Luc#30 cells. Progressive inhibition is noted when cells were transfected with increasing amounts of antisense-ATG oligonucleotides complexed to G7 EDA dendrimers (open square). No inhibition is observed with either sense-ATG oligonucleotides conjugated to dendrimer (open circle), or free antisense-ATG (solid square) and sense-ATG (solid circle) oligonucleotides.

D5 cells were co-transfected with 200 ng (0.031 pmol) pCMV-Luc and 50 ng (5.7 pmol) or 500 ng (57.0 pmol) of sense- or antisense-ATG oligonucleotides, with a molar excess of oligonucleotide DNA to plasmid DNA ~184- and 1840-fold respectively. The mixed DNA was complexed with G6EDA, G7EDA or G10EDA in 1:10 (Fig. 4A) and 1:4 (Fig. 4B) charge

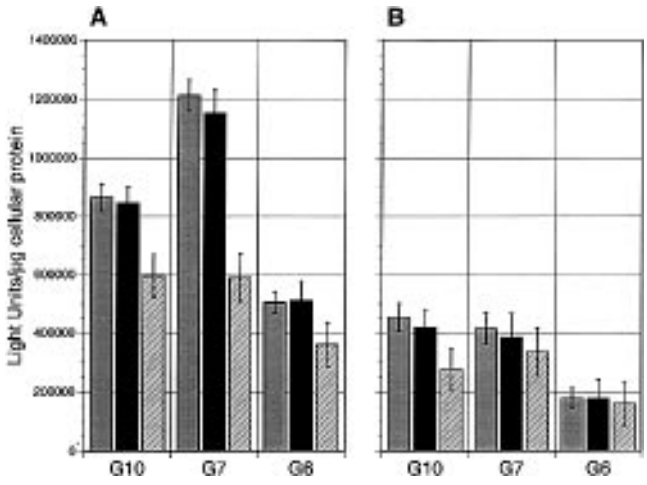

Figure 4. Inhibition of transient expression of luciferase from an expression plasmid (pCMV-Luc) cotransfected with antisense-ATG oligonucleotides. Both types of DNA were complexed with various generations of EDA core dendrimers. (**A**) Transfections performed using complexes formed at a 1:10 DNA–dendrimer charge ratio. (**B**) Transfections performed using complexes formed at a 1:4 DNA–dendrimer charge ratios. As compared to plasmid alone transfected without oligonucleotides (grey bars) or plasmid transfected with sense-ATG oligonucleotides (black bars), plasmid co-transfected with antisense-ATG oligonucleotides (striped bars) had significantly decreased luciferase expression that was greatest with G7 dendrimer at a 1:10 charge ratio.

ratios. All antisense-ATG–dendrimer complexes were effective in inhibiting luciferase expression however the greatest specific inhibition, ~50%, was achieved when 50 ng of anti-ATG oligo was complexed with G7 EDA dendrimer at a 1:10 charge ratio. This result correlates well with the transfection efficiency determined for D5 cells using pCMV-Luc alone and G10, G7 and G6 EDA core dendrimers (17). Prolonging the time of DNA complexation with dendrimers from 5 min up to 5 h did not improve inhibition efficiency (data not shown). Inhibition by antisense oligonucleotides was more dependent on the overall efficiency of transfection than the molar excess of oligonucleotides (Fig. 4), except when transfection was carried out in media containing >20% fetal calf serum (rather than serum free media). In this situation, efficiency decreased ~50% in the presence of serum, but improved when DNA–dendrimers complexes were formed at charge ratios >1:50 (data not shown).

Analysis of the retention and stability of oligonucleotides complexed to dendrimers

U937 cells were incubated with 5′ radiolabelled single-stranded antisense ATG oligonucleotides, either alone or complexed with either G5, G7 or G9 EDA core dendrimers in varying charge ratios (1:5, 1:10 and 1:100). The cells were collected after 4 h of transfection (Time 0) and after incubation in culture for 12, 24, 48 and 96 h. Total DNA was extracted and radioactivity analyzed using a scintillation counter. At all charge ratios tested the presence of dendrimer of any generation facilitated the uptake of oligonucleotide 4–5-fold over that of oligonucleotides alone (Fig. 5). The retention of the radiolabelled oligonucleotide also increased after complexing with dendrimers. Radioactivity in DNA isolated from cells transfected with DNA–dendrimer complexes was detected up to 4 days after administration, although at lower levels than at Time 0 (Fig. 5). In contrast,

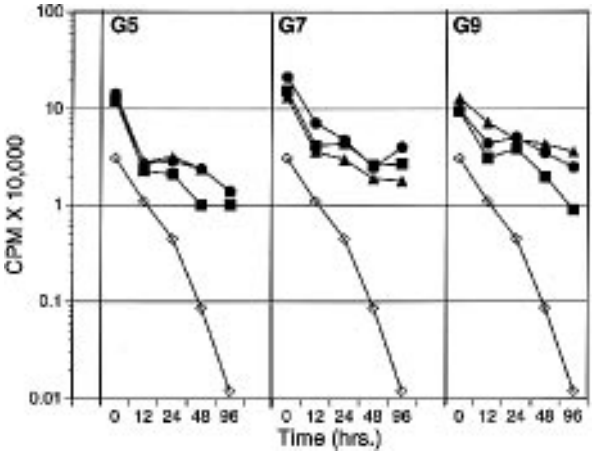

Figure 5. Retention of radiolabelled DNA in U937 cells after oligonucleotide transfection. Cells were transfected with a radiolabelled 27 base oligonucleotide complexed with either G5, G7 or G9 generation EDA core dendrimers. As compared to cells transfected with free oligonucleotide (open diamonds), radioactivity was retained in cellular DNA for a longer period of time when the cells were transfected with oligonucleotides complexed with all three dendrimers at charge ratios 1:5 (solid squares), 1:10 (solid circles) or 1:100 (solid triangles).

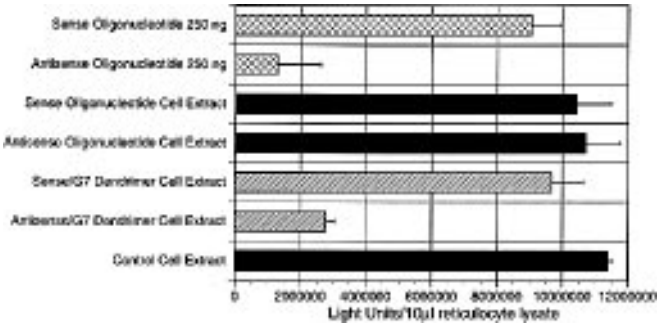

Figure 6. Persistence of intracellular antisense activity 24 h after transfection with oligonucleotides. Extracts obtained 24 h after transfection from cells transfected with 1.0 µg antisense-ATG oligonucleotides complexed to G7 EDA dendrimers (striped bars) retained the ability to inhibit luciferase expression in an *in vitro* transcription–translation assay. In contrast, extracts from cells transfected with free oligonucleotide (grey bars) had no activity. The degree of inhibition was approximately equal to that observed with the addition of 250 ng of added antisense oligonucleotide (hatched bar).

radioactivity recovered at Time 0 in DNA from cells cultured with free oligonucleotides was 300 times lower than in DNA from cells transfected with complexes and was essentially undetectable after only 24 h (Fig. 5). Similar results were observed in D5, Rat2 and Jurkat cells (data not shown).

To determine if the radioactivity in the DNA isolated from cells transfected with oligonucleotide–dendrimer complexes was the result of retained intact oligonucleotide, Rat2 cells were incubated with 1.0 µg oligonucleotide, either as free oligonucleotide or complexed at a 1:10 ratio with G7 EDA dendrimer, for 4 h and then washed and placed in culture for 24 h. The cells were then lysed and the lysate added to the coupled transcription–translation system containing 200 ng pCMV-Luc. Lysate from cells treated with antisense-ATG oligonucleotide complexed with dendrimer inhibited luciferase expression as compared to control lysate (Fig. 6). This inhibition was equivalent to that achieved with 100–250 ng antisense-ATG oligonucleotide added to the lysate. Lysate from cells incubated with either sense-ATG oligonucleotide complexed with dendrimer or free antisense-ATG oligonucleotide did not inhibit luciferase synthesis (Fig. 6).

Transfection with antisense cDNA plasmids complexed with dendrimers specifically inhibits luciferase expression

Various generations of dendrimers were tested to determine whether these polymers can be utilized for transient transfection and expression of antisense genes. Expression plasmids containing either a antisense sequence to luciferase mRNA (pCMVAs-Luc) or a truncated antisense sequence to luciferase mRNA with a deletion in the ATG region (pCMVAsLucðATG) were complexed with G7EDA dendrimer in a 1:20 charge ratio and transfected into D5 Luc#47-D1 cells. Transfection of the AsLuc plasmids resulted in the dose-dependent inhibition of the luciferase expression (Fig. 7A). In contrast, the reduced activity associated with AsLucðATG suggests that inhibition of luciferase

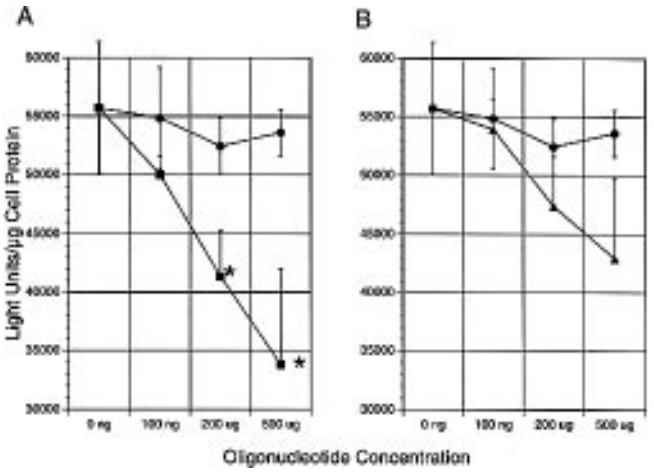

Figure 7. Transfection of luciferase antisense expression plasmid specifically inhibits constitutive luciferase expression in D5Luc#47A-D1 cells. (**A**) pCMV-AsLuc plasmid transfected with G7 EDA dendrimer (solid squares) inhibits luciferase production in a dose-dependent manner as compared to transfection with a control pCMVβgal plasmid (solid circles). (**B**) Transfection of a plasmid with a truncated luciferase antisense sequence (pCMV-AsLucðATG, solid triangles) demonstrates diminished ability to inhibit luciferase expression ($P \leq 0.05$, ANOVA).

expression involves a sequence specific RNA–RNA interaction (Fig. 7B).

Transient transfection of Rat2Luc#4A7-4 cells with pCMV-AsLuc plasmid and control pCMVβgal complexed in 1:10 charge ratio was performed with two generations of dendrimers (G5 and G7 EDA). G7 EDA transfection of the antisense expression plasmid resulted in ~40% inhibition of constitutive luciferase expression (Fig. 8) while transfection with G5 EDA dendrimer, previously shown to be not effective in transfecting Rat2 cells (17), did not alter luciferase expression.

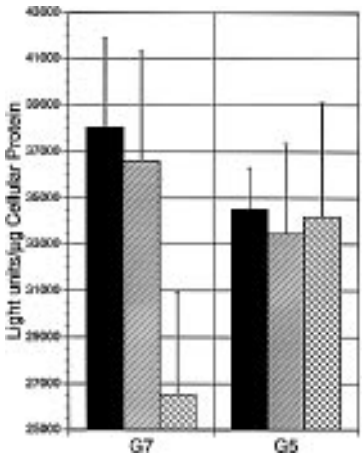

Figure 8. Inhibition of constitutive luciferase expression in Rat2Luc#4A-7D cells with an antisense expression plasmid. In comparison to cells administer only dendrimer (black bars) or dendrimer and control plasmid (pCMVβgal, striped bars) the antisense expression plasmid (pCMV AsLuc, hatched bars) was able to inhibit constitutive luciferase expression when transfected with G7 generation dendrimers that previously have been shown to efficiently transfect Rat2 cells. Transfection of the antisense oligonucleotide with G5 generation dendrimers, that are less efficient in transfecting Rat2 cells, was not able to inhibit luciferase expression.

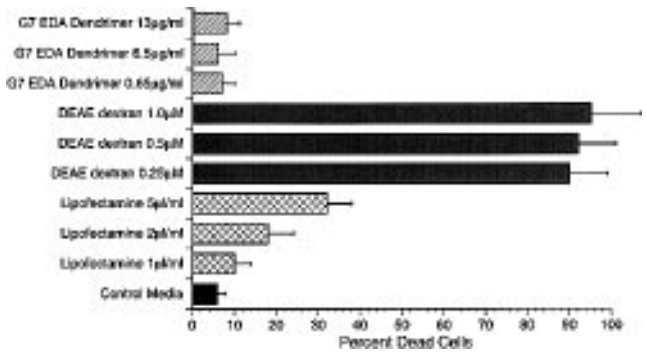

Figure 9. Cytotoxicity of various transfection agents on Rat2 cells. Dendrimers in all concentrations used for transfection (striped bars) demonstrated little cytotoxicity over media control (black bars). Lipofectamine (hatched bars) and DEAE dextran (grey bars) showed higher levels of cytotoxicity. Similar results were observed in the other cell lines employed in these studies.

Cytotoxicity of dendrimers

Cytotoxicity of various transfection agents was assessed by trypan blue exclusion test 24 h after transfection of U937 cells. Dendrimers in concentrations used for transfection demonstrated little cytotoxicity over media control (Fig. 9). Lipofectamine and DEAE dextran showed higher levels of cytotoxicity (Fig. 9). Similar results were observed for the other cell lines employed in these studies (data not shown).

DISCUSSION

Antisense technology has been proposed as one means for modulating the expression of specific genes (20,21). The mechanism for this is thought to be the formation of hetero-duplexes with the mRNA for a specific protein that either prevent the translation of the message or accelerate the degradation of the mRNA (22). One of the major problems with the use of antisense regulatory nucleic acids is the difficulty in achieving functional concentrations of oligonucleotides in cells and the rapid degradation of these molecules by cellular nucleases. The low efficiency of oligonucleotide cellular uptake is usually compensated through the use of very high concentrations of oligonucleotides, while chemical modifications to the phosphodiester bond improve stability of the genetic material (23,24). The efficiency of antisense nucleic acids can be greatly enhanced when delivered into cells using transfecting agents. Cationic liposome-mediated transfection of 100 nM phosphorothioate antisense ICAM-I oligonucleotide resulted in 90% reduction of protein synthesis in endothelial cells (25), and 50–80% reductions of human protocollagenase mRNA expression in fibroblasts by 200 nM phosphorothioate specific oligonucleotides (26). However these transfer techniques have proved problematical in delivering oligonucleotides *in vivo*. As a result, the practical application of antisense technology awaits more efficient delivery systems.

Our studies indicate that Starburst dendrimers can be used as a transfection reagent for the delivery of antisense oligonucleotides and plasmid expression vectors coding antisense mRNA. Inhibition of the expression of the reporter luciferase gene by antisense oligonucleotides as well as antisense mRNA ranged from 30 to 60% of the control depending on the DNA concentration, type of dendrimer used and charge ratios of DNA–dendrimer complexes. In contrast to the nanomolar or micromolar concentrations of oligonucleotides needed for inhibition reported by others (25–27), we have achieved specific inhibition of targeted gene expression with picomolar concentrations of specific oligonucleotides when delivered by dendrimers. Sequence specificity of inhibition was documented by using sense control oligonucleotides and a derivative of the antisense luciferase expression plasmid that lacked the ATG region of luciferase gene. The efficiency of transfer was aided by the finding that dendrimers were not toxic to cells in the concentrations required for gene transfer. We have failed to obtain consistent effects on luciferase expression in Jurkat, Rat2 and D5 cells when antisense oligonucleotides were transfected with liposomes, predominantly due to cytotoxicity. This suggests that complexing DNA with dendrimers results in highly efficient oligonucleotide delivery and avoids sequence-independent, carrier-mediated alterations on cellular function (28–31), and can potentially lead to *in vivo* applications of regulatory nucleic acids, that are not possible with the currently-available delivery techniques (32,33).

Stability of oligonucleotides is a necessary requirement for the application of antisense technology to inhibit gene expression *in vivo*. This often precludes the use of 'naked' phosphodiester oligos because of either degradation in serum or intracellular destruction by endosomes or nucleases (8,9). Significant efforts have been made toward the development of nuclease-resistant oligonucleotides; in particular phosphorothioates and methylphosphonates (34,35). From our studies, phosphodiester oligonucleotides appear to have the increased stability when complexed to dendrimers. This may allow the use of non-modified oligonucleotides in place of costly and sometimes toxic modified phosphoderivatives (36). In addition, it is of interest that oligonucleotides bound to dendrimer appear to function as specifically and effectual as free oligonucleotides. It is possible

that the binding of the oligonucleotide phosphate backbone to the dendrimer surface does not interfere with the ability of the bases to form hydrogen bonds with the complementary sequences.

Antisense nucleic acids (oligonucleotides and plasmids coding antisense mRNA) show great promise for the specific inhibition of gene expression. Our studies indicate that the complexing of oligonucleotides with dendrimers increases cellular uptake and prolongs the activity of regulatory sequences while not altering the functional capability of those molecules. Application of dendrimers as a delivery system for nucleic acid molecules may lead to improvements in this technology and may facilitate the development of therapeutic antisense techniques.

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