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Full Length Research Paper

Poly (amido amine) dendrimer silences the expression of epidermal growth factor receptor and p53 gene *in vitro*

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To understand more about the lower generations of poly(amido amine) dendrimer (PAMAM) as a non-viral vector for antisense (ANS) therapy, a 21-mer epidermal growth factor receptor (EGFR) ANS was delivered by generation five of PAMAM in T47D breast carcinoma cells in culture. The semi-quantitative polymerase chain reaction (PCR) and western blot analysis were used to quantify the expression of EGFR mRNA and protein, respectively. The results showed that PAMAM G5/ANS nanoparticles were able to decrease the level of EGFR mRNA more than 40% even at the lower dendrimer primary amine to the antisense phosphate groups (N/P) ratio of 0.5. But, only the data of western blot analysis at the higher N/P ratios of 10 and 20 showed a decrease of the protein expression level similar to the mRNA expression level. Moreover, PAMAM dendrimer had a positive effect on the EGFR ANS action to inhibit the EGFR mRNA and protein expression. Further studies revealed that PAMAM G5 dendrimer as such inhibits the expression of EGFR in a concentration-dependent manner. Since PAMAM as such was able to inhibit the mRNA expression of p53 gene, we speculated that the effect of PAMAM G5 on the EGFR is a kind of its non-selective effect on the transcription and/or translation machinery of the cell.

Key words: Poly(amido amine) dendrimer (PAMAM) dendrimer, epidermal growth factor receptor (EGFR) antisense, epidermal growth factor, RNAi, polyamidoamine dendrimer, toxicogenomics, gene delivery.

INTRODUCTION

Efficient treatment of cancer still remains a big challenge in science and medicine. Recent clinical trials for gene therapy of cancer have produced promising results which could lead to effective and acceptable treatment for many types of cancer (Guinn et al., 2008; Chawla et al., 2010).

One of these promising approaches is to utilize antisense (ANS) therapy to down regulate the expression of genes which are over expressed in cancer. There are already more than 20 ANS drugs in clinical trials worldwide. For example, Genasense®, which is a Bcl-2 antisense drug, is in phase III clinical trial (Juliano, 2006).

Epidermal growth factor (EGF) is a 170KD cell surface and integral protein receptor which is known to over express in many cancer types such as breast and lung cancer. Over expression of this receptor is related to the

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growth, division and invasiveness of many tumors (Arteaga, 2002). Down-regulation of the expression of this receptor by ANS is a promising approach to treat cancer (Hollins et al., 2004).

The administration of ANS as such is extremely challenging because ANS is a big hydrophilic and negatively charged molecule and is therefore eliminated rapidly after administration. Thus, it is necessary to design a proper vector for the delivery of ANS molecules (Tan et al., 2002; Cooper et al., 2005; Hartman et al., 2008). In general, delivery vectors are divided into two categories: viral and non-viral vectors. Viral vectors are efficient but have problems relating to their safety profile and large-scale production. Non-viral vectors are safer and easier to prepare but are inefficient *in vivo*. Among non-viral vectors, poly(amido amine) dendrimers have definite advantages because they are efficient and easy to prepare in large-scale (Haensler et al., 1993; Eichman et al., 2000; Esfand et al., 2001; Navarro et al., 2009; Cui et al., 2009). Earlier, we have shown (Nomani et al., 2010) that PAMAM G5 dendrimer is a safe and *in vitro* promising vector for the delivery of 21-mer antisense against epidermal growth factor receptor (EGFR ANS).

An ideal gene delivery vector should deliver its gene cargo to the target cells without affecting the normal functions of the cells. Earlier studies have clarified whether gene delivery vectors influence the expression profiles of the endogenous genes (Arteaga, 2002; Omidi et al., 2005a; Akhtar et al., 2007; Omidi et al., 2008; Gomase et al., 2008). Zhao et al. (2007) investigated the genomic changes after transducing two different mice strains, BALB/c and CD-1 (IRC), with lentiviruses, using DNA microarray and RT-PCR. They confirmed a significant up-regulation of 5 oncogenes in a CD-1 (IRC) model by RT-PCR analysis, whereas only moderate changes in the gene expression profile in BALB/c mice were detected after transduction. Omidi et al. (2003) studied the effect of cationic lipids, Lipofectin® and Oligofectamine®, on the gene expression profile of A431 human alveolar epithelial cell line by microarray technology. The results showed that cationic lipids as such and combined with DNA were able to change the mRNA level of different genes. The Oligofectamine® induced changes were more than Lipofectin® (eg. the dual specificity phosphatase-1, Cysteine dioxygenase type I, and similar to activator of s-phase kinase (h. sapiens) genes were shown to have more than 3-fold over expression changes after Oligofectamine® transfection). Therefore, it is important to understand how vectors, especially cationic carriers, influence the expression profile of the endogenous genes for instance, on the expression of the gene to be silenced when the silencing cargo is delivered.

The aim of the present study was to investigate whether G5 PAMAM dendrimer can deliver EGFR ANS to its target cells efficiently and whether the expression of EGFR is silenced both at mRNA and protein levels. Furthermore, it is clarified if PAMAM dendrimer as such has influences on the expression profiles of EGFR and p53 genes. This study shows for the first time that cationic PAMAM G5 dendrimer can non-selectively down-regulate the expression of the target gene (EGF) thus has synergistic effect on the therapeutic effect of its gene cargo.

MATERIALS AND METHODS

Cell culture

T47D, a human ductal breast epithelial tumor cell line, was obtained from the National Cell Bank of Iran, Pasteur Institute (Tehran, Iran). Cells were grown in RPMI 1640 (Gibco, UK) supplemented with 10% FBS (Gibco, UK), streptomycin (100 ng/ml), penicillin (100 units/ml) (Sigma-Aldrich Co., Germany) and incubated at 37°C, 5% humidified CO_2. Typically, the cells were subcultured 2 to 3 times per week.

Dendrimer synthesis

Poly(amido amine) dendrimer of generation five (PAMAM G5; Mw 28826Da) has ethylenediamine as initiator core and 128 primary amino groups at its surface. This dendrimer was synthesized via iterative reactions of Michael addition of methylacrylate to the ethylenediamine for half generations, followed by exhaustive amidation of half generations by high excess methanolic ethylenediamine at the next step for full generations, according to the previously reported method (Tomalia et al., 1986). The products at each step of the synthesis were purified by azeotropic distillation, overnight high vacuum and ultrafilteration with defined MWCO. The final product was freeze dried and characterized by ^{1}H-NMR and FT-IR. All solvents and reagents of either analytical or HPLC grade were purchased from Merck KGaA (Germany) and were used freshly.

Antisense against epidermal growth factor receptor (EGFR ANS)

The sequences of phosphorothioated EGFR ANS 5'-TTT CTT TTC CTC CAG AGC CCG-3' were chosen according to those reported by Petch et al (2003). All sequences were purchased from TIB MOLBIOL GmbH (Germany) or BIONEER (Korea).

Preparation of dendri- and polyplexes, and transfection

Dendriplexes composed of PAMAM G5 and ANS were prepared at different N/P charge ratios (0.5, 2.5, 5, 10 and 20), as described earlier (Nomani et al., 2010). Briefly, ANS stock solution (100 µM in DEPC-treated water) was diluted in 5% dextrose at 5 µM concentration. The dendrimer solution was also diluted from its stock solution in 5% dextrose at an appropriate concentration, and then an equal volume (100 µl) of dendrimer was added to the ANS solution. The concentration and volume of the dendrimer were calculated in such a way that the desired charge ratios at the fixed final ANS concentration of 500 or 1000 nM were present in the mixtures for incubation with the cells. After mixing and incubation for 10 to 15 min, 1 ml of serum-free RPMI 1640 was added to the dendriplexes.

Branched polyethyleneimine (PEI25kD) was purchased from

Sigma-aldrich Co. (Germany). Preparation of PEI/ANS polyplexes at N/P ratio of six was performed according to the method described by Boussif et al. (1995).

Two days before transfection, T47D cells were seeded onto 6-well plates (4×10^5 cells/well). On the transfection day, the medium was removed and the cells were once washed with PBS. The dendriplexes or PEI-polyplexes in serum-free medium were added to the cells and incubated for 4 h at 37°C. After incubation, the dendriplexes or PEI- polyplexes were removed, the cells were washed with PBS and fresh medium was added. After 48 h incubation, the expression of EGFR at mRNA and protein levels was evaluated using polymerase chain reaction (PCR) and western blot methods, respectively.

Effect of cationic PAMAM G5 on the endogenous expression of EGFR

PAMAM G5 dendrimer at the concentrations corresponding to the amount of dendrimer used in dendriplexes (0.17, 0.83, 1.66, 3.32 and 6.64 µM) was diluted in serum-free RPMI medium and incubated with T47D cells for 4 h. Two days (48 h) after removal of the dendrimers, the mRNA levels of EGFR and p53 genes were evaluated by PCR.

Total RNA extraction and PCR analysis

Total RNA of the cells was extracted by using TRI Reagent® according to the manufacturer's instructions (Sigma-Aldrich Co., Germany). Briefly, after removal of the medium, the cells were lysed with TRI Reagent® (1 ml/per well). Then 200 µl of chloroform was added to each cell lysate, mixed for 5 min at RT and centrifuged at 12000 ×g at 4°C for 15 min. The aqueous upper phase was gently collected, mixed with 500 µl of isopropanol for 5 min at RT and centrifuged for 10 min at 12000 ×g at 4°C. The precipitated RNA was washed with 75% ethanol and centrifuged for 5 min at 7000 ×g at 4°C. Finally, the RNA was dissolved in 20 to 50 µl of DEPC-water, and its purity and concentration were measured by UV-spectroscopy.

In order to prepare cDNA, 2 µg of each mRNA sample was defolded by incubating for 5 min at 75°C, then mixed with the reverse transcription reagent solution containing 5.4 µl DEPC-treated water, 1.5 µl Oligoprimer (0.5 µg/µl), 5 µl reverse transcription buffer (5X), 1 µl dNTPs (10 mM), 0.5 µl RNase inhibitor (40 U/µl), and 0.1 µl M-MuLV (200 U/µl) (Fermentas, Hanover, MD, USA). The mixture was incubated first at 42°C for 60 min and then at 95°C for 10 min.

PCR was done by taking 1.5 µl of each cDNA sample, mixed with 18 µl of DEPC-treated water, 2.5 µl PCR buffer (10X), 1.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP (10 mM), 0.25 µl Taq DNA polymerase (5 U/µl) (Fermentas, Hanover, MD, USA), and 0.5 µl of proper dilution of EGFR forward primer 5'-CAA CAT CTC CGA AAG CCA-3' and 0.5 µl reverse primer 5'-CGG AAC TTT GGG CGA CTA T-3' or β-actin Forward 5'-GGC ATG GGT CAG AAG GAT T-3' and reverse 5'-GGG GTG TTG AAG GTC TCA AA-3' primer as housekeeping gene . The PCR program for EGFR and for β-actin is as follows: initial denaturing at 94°C for 5 min, then 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 45 s and finally, 8 min at 72°C for the final extension of the amplification. The p53 forward primer was 5'-CTG AGG TTG GCT CTG ACT GTA CCA CCA TCC-3' and the reverse primer was 5'-CTC ATT CAG CTC TCG GAA CAT CTC GAA GCG-3'. The PCR conditions for p53 gene were 35 cycles of 95°C for 1 min, 69°C for 1 min, 72°C for 80 s. Densitometry analysis was performed using ImageJ® software (Version 1.42, National Institute of Health, USA). The signals obtained for each sample were normalized to β-actin signal, and the mean ± SE of two independent experiments were plotted.

Western blot analysis

Two days (48 h) after transfection with dendriplexes, the T47D cells were lysed and the amount of total protein was measured by Bradford assay (Bradford, 1976). Equal amounts of each sample were loaded and separated on 8% resolving SDS-PAGE, and transferred by wet transfer device (BIO-RAD Co., USA) to the activated PVDF membrane. After overnight blocking with 1% BSA containing 0.1% Tween 20 in TBS buffer, the membrane was incubated separately with specific rabbit monoclonal antibody of EGFR (1:1000 diluted in 5% BSA+0.1% tween 20 in TBS) (Cell Signaling Technology, Inc., Danvers, MA , USA) and mouse monoclonal antibody of β-actin (1:2000 diluted in 1% BSA+ 0.1% tween 20 in TBS solution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), at 4°C for 10 h. After washing with TBS solution, the membrane was incubated with the HRP conjugated secondary antibody (1:10000 dilution in 1% BSA, Cell Signaling Technology Inc., Danvers, MA, USA) at RT for 30 min. After two 15 min washings with TBS buffer, the bands of EGFR and β-actin proteins were visualized using a chemiluminescence kit (Thermo Fisher Scientific Inc., USA) and Biomax film (Kodak Co., UK). Densitometry analysis was performed using ImageJ® software (Version 1.42, National Institute of Health, USA). The EGFR band intensity of each sample was normalized to its corresponding β-actin protein level on the radiographic film.

Statistical analysis

Student t-test was used for comparison of two means, and one-way ANOVA with Bonferroni post hoc for more than two means. P value < 0.05 was considered as significantly different.

RESULTS

The mRNA and protein levels of EGFR after silencing with dendriplexes

The expression of EGFR in T47D cells was silenced by utilizing dendriplexes at different N/P ratios (Figure 1a and b). β-actin could be considered as a housekeeping gene, since its mRNA expression level was affected by neither PAMAM, nor PAMAM/ANS dendriplexes (Figures 1 and 3). The mRNA expression of EGFR was normalized with mRNA expression of β-actin (Figure 1b). Figure 1a and b show that after transfection with dendriplexes, the mRNA expression of EGFR decreased by 45% already at N/P ratio of 0.5. Interestingly, increasing the N/P ratio did not significantly change the mRNA expression of EGFR, (one-way ANOVA, P value ≥ 0.05). PEI-polyplexes at a charge ratio of 6 had only moderate silencing effect (27%) on the mRNA expression of EGFR.

Figure 2a and b indicate that the level of EGFR protein expression was correlated with the level of mRNA expression after transfection by dendriplexes at N/P ratios of 5, 10 and 20. Normalization by their corresponding β-actin protein level for N/P ratios of 5, 10 and 20 showed a lower expression of EGFR protein than for the lower N/P ratios. The densitometery of N/P 0.5 and 2.5 showed different results for the western blot and

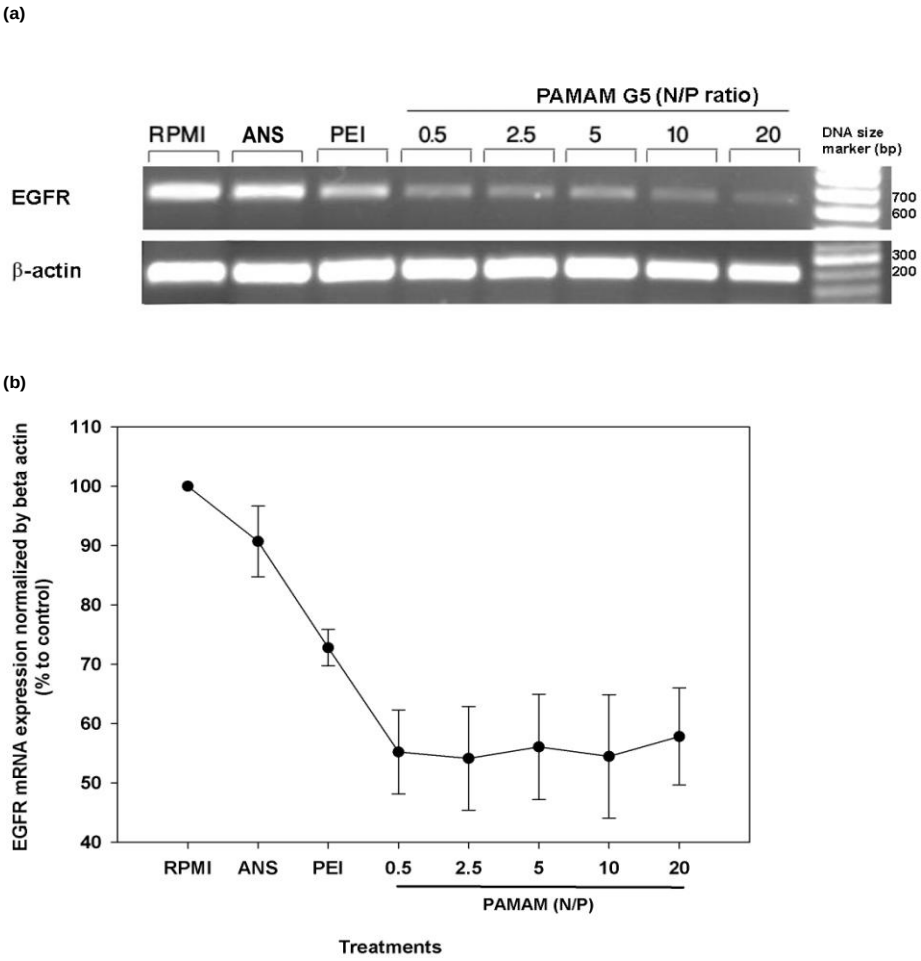


Figure 1. (a) Representative image of RT-PCR and agarose gel electrophoresis of mRNA expression of EGFR gene after transfecting the cells with PAMAM G5/EGFR dendriplexes at N/P ratios of 0.5, 2.5, 5, 10 and 20. Polyethylenimine/antisense (PEI) complexes at N/P ratio 6, antisense alone (ANS) 500 nM, were used as the control. RPMI symbol indicates the untreated cells. (b) Bar chart of the normalized EGFR mRNA expression after treating cells with the dendriplexes. The data points are the mean intensity of three independent measurements. The intensity of the EGFR band on the agarose gel after taking the picture was normalized by the intensity of the corresponding β-actin band. The data are shown as mean ± standard deviation.

for the PCR experiments (Figures 1b and 2b): for the western blot, there was lower silencing of EGFR protein after dendriplex transfection. For example at N/P ratio of 0.5, the mRNA expression was silenced to about 45% but the protein expression silencing was negligible (about 8%). On contrast, at higher N/P ratios (N/P ratio higher than 5), the protein levels of EGFR are declined by more than 45% which correlates with mRNA expression.

Effect of PAMAM G5 and PEI carriers on the mRNA expression of EGFR and p53

Subsequently, this research studied whether dendrimer or PEI have influence on the endogenous expression of EGFR. Dendrimer had a concentration-dependent effect on the expression of EGFR at mRNA level (Figure 3a and b). At lower dendrimer concentrations (0.17 and 0.83 µM) corresponding to the lower N/P ratios (N/P of 0.5 and 2.5) of dendriplexes, the influence on mRNA expression of EGFR was relatively weak (20 to 37% decrease in the mRNA expression). However, at higher dendrimer concentrations (3.32 and 6.64 µM), the silencing effect on the mRNA expression of EGFR was almost at the same level with the silencing effect of dendriplexes (maximum 46% EGFR mRNA expression decrease at 6.64 µM concentration). On the contrary, free PEI only affected up to 20% of the mRNA levels of EGFR.

In addition, this paper studied whether free dendrimer had silencing effect on the expression of p53 gene, which

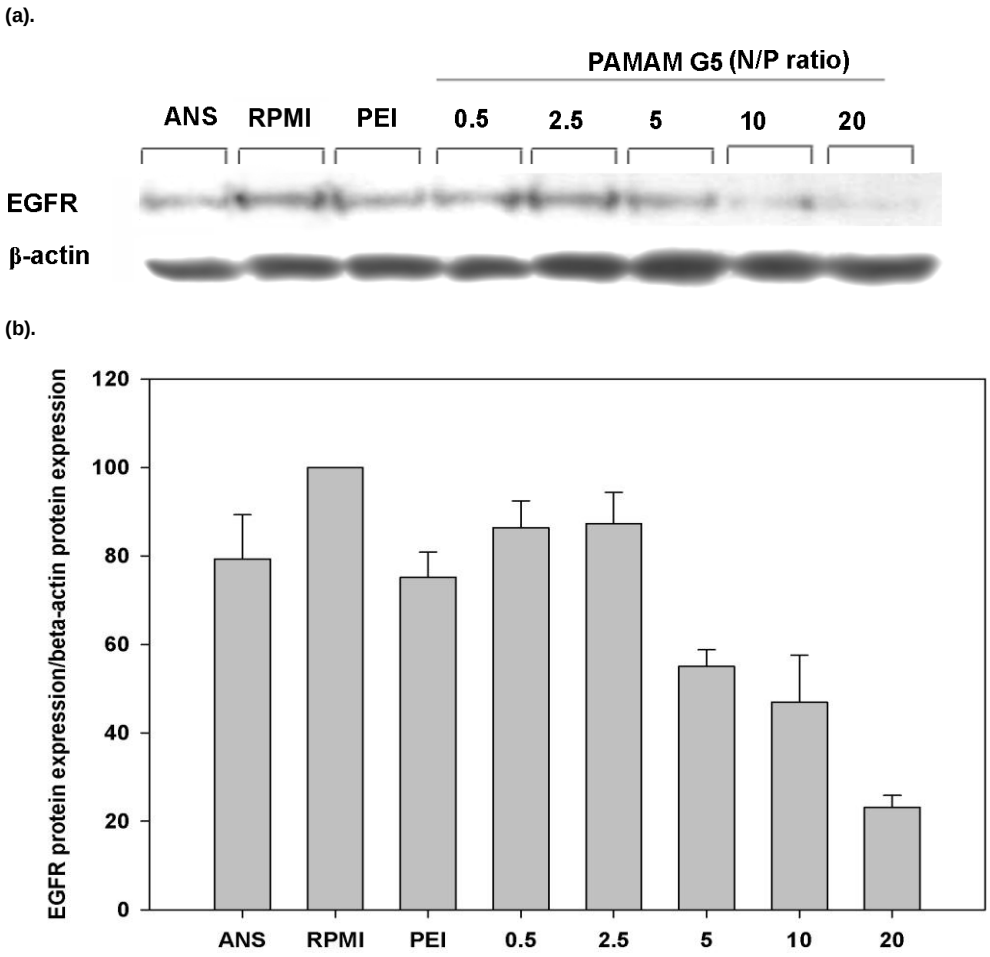


Figure 2. Evaluation of EGFR protein expression in T47D cells by western blot analysis after treatment with PAMAM/EGFR ANS dendriplexes. In Figure 2a, lane ANS is antisense alone at 500 nM concentration, lane RPMI indicates the untreated cells and lane PEI is polyethylenimine formulation at N/P ratio of 6. Other lanes are PAMAM/ANS dendriplexes at N/P ratios of 0.5, 2.5, 5, 10 and 20. Figure 2b shows the normalized intensities of EGFR to the β-actin bands of two independent experiments and the error bars are the maximum and minimum band intensities.

has a different role than EGF in the progression of cancer. Interestingly, p53 mRNA expression was also decreased by dendrimer especially at higher concentrations (3.32 and 6.64 µM) (Figure 4a and b).

DISCUSSION

PAMAM dendrimers have been utilized as gene delivery vectors since 1993, when Szoka's group (Haensler et al., 1993) tested different generations of intact PAMAM dendrimers for *in vitro* gene delivery. Later, the transfection efficiency of PAMAM dendrimers was improved when fractured PAMAM G6 and G8 were introduced (Tang et al., 1996). Bielinska et al. (1996) reported for the first time the use of different generations of PAMAM dendrimers for antisense delivery. Earlier studies have shown that in general the transfection efficiency correlates with the generation of PAMAM dendrimer, that is, the higher the generation, the better the *in vitro* transfection efficiency. Unfortunately, the higher generations of dendrimers are more toxic than lower generations; therefore, lower generations of dendrimers are more desirable vectors for gene delivery. Hollins et al. (2004) reported that the lower generations of poly(propyleneimine) dendrimers (G2 and G3) can markedly improve the transfection of EGFR antisense in cell cultures while having lower cytotoxicity in comparison with Oligofectamine® as cationic lipid vector. In our previous work (Nomani et al., 2010), we showed that different lower generations of PAMAM dendrimers form stable nanoparticles with 21-mer antisense against EGFR. At lower N/P ratios, the formed nanoparticles were rather big and heterodisperse, but they were still taken up by the cells rather efficiently. In this work, the PAMAM G5/EGFR antisense nanoparticles silenced

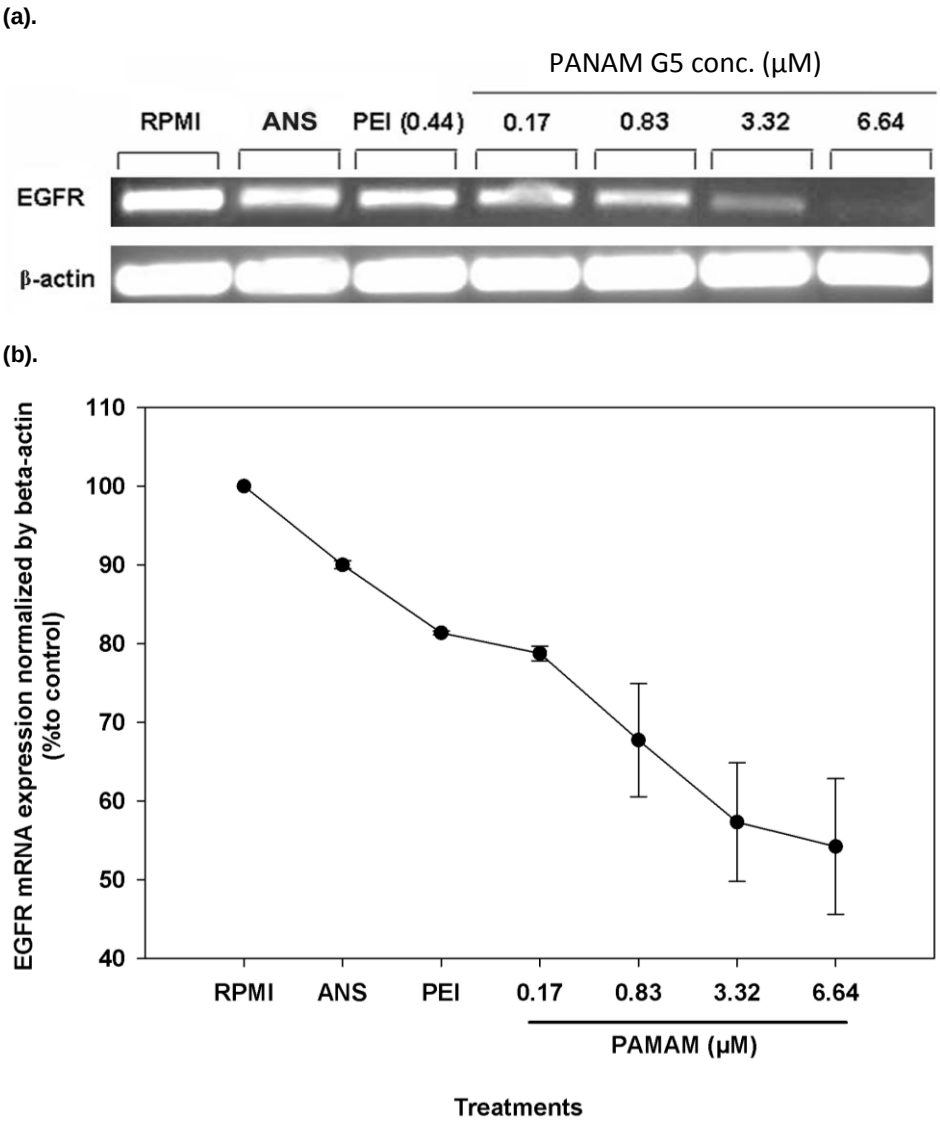
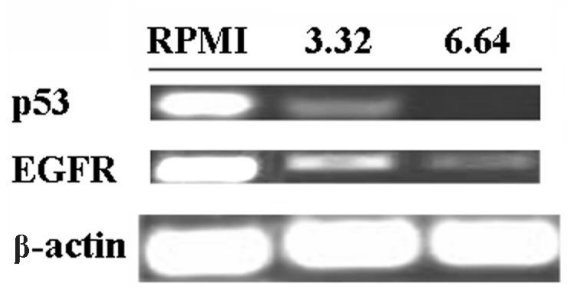


Figure 3. (a) Representative image of the effect of different PAMAM concentrations on EGFR mRNA expression. Lane RPMI is only the medium, lane ANS is antisense alone at 500 nM concentration and lane PEI is a concentration of 0.44 µM (equal to a concentration of PEI when it was used at N/P ratio of 6). Other lanes are concentrations of 0.17, 0.83, 1.66, 3.32 and 6.64 µM of PAMAM dissolved in serum-free RPMI, which are at the same concentrations of PAMAM used to prepare dendriplexes at N/P ratios of 0.5, 2.5, 5, 10 and 20, respectively. (b) The normalized EGFR mRNA expression after treating cells with the free dendrimer at different concentrations. The data points are the mean intensity of three independent measurements ± standard deviations. The intensity of the EGFR band on the agarose gel after taking the picture was normalized by the intensity of the corresponding β-actin band.

significantly the expression of EGFR both at mRNA and protein levels which was comparable with branched-PEI transfection.

One interesting finding in this study was that at low N/P ratios, the silencing effect of dendriplexes could be detected only at mRNA level; whereas, at higher N/P ratios the silencing effect could be seen both at mRNA and protein levels. This indicates that mRNA expression would not be completely sufficient for concluding the silencing effect of RNAi therapeutics and it seems that evaluation of protein expression is a more reliable method, although it is more tedious than mRNA investigation. However, in our experiment, more investigation is necessary for the better understanding of the relationship between PCR and western blot results specially at N/P ratios of 0.5 and 2.5.

(a).



(b).

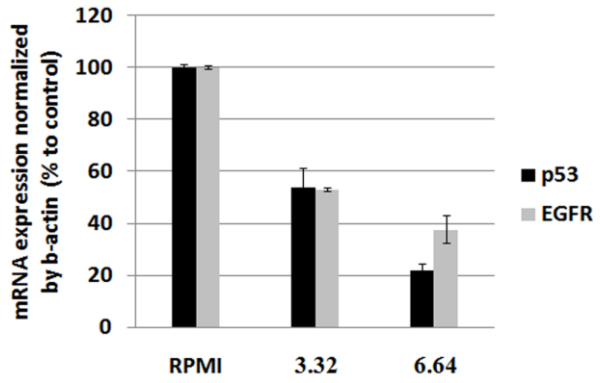


Figure 4. (a) The effect of PAMAM G5 dendrimer on the mRNA expression of p53, EGFR and β-actin genes at concentrations of 3.32 and 6.64 µM of free PAMAM G5. Lane RPMI shows the untreated cells. (b) The graph of normalized (to the β-actin) mean intensities of three independent measurements ± standard deviations.

It is typical in the gene delivery literature that only the silencing effect of the antisense/carrier nanoparticles are discussed, but the influence of the carrier on the expression of the gene to be silenced is not commonly studied (Petch et al., 2003; York et al., 2009). Hollins et al. (2007) reported the effect of two commercially available transfection kits of PAMAM dendrimer (Polyfect® and Superfect®) on the silencing of EGFR by a designed siRNA against that. The cellular uptake induced by both transfection kits was similar but the final effect of anti-EGFR siRNA/carrier nanoparticles was different. They were explained this observed difference by the effect of carrier structure where, Superfect® which had a more flexible structure, lower density of surface positive charges per molecule thus, had a lower gene expression change effect on the target cells, had better apparent siRNA potency.

In this study, PAMAM G5 dendrimer showed concentration-dependent silencing effect on the expression of EGFR at mRNA level (Figure 3a and b).

This silencing effect was not only limited to EGFR but the mRNA expression of p53 gene was also dramatically reduced at the selected PAMAM concentrations (Figure 4). This suggests that positively charged dendrimer molecules can interact with negatively charged cellular molecules and thus disturb the normal functions of the cells. The intrinsic effects of the gene delivery vectors used on the cellular function and genomics as well as their efficacy are important and should carefully be considered. These effects can be divided into positive and negative effects. Dufes et al. (2005) reported the intrinsic antitumor activity of PAMAM dendrimer, polypropyleneimine dendrimer and PEI. These polycations were interestingly effective in inhibiting the growth of A431 epidermoid carcinoma murine xenografts model in a plasmid-independent manner when they were given by a single tail-vein injection. This can be considered as a positive effect for treating cancer. On the other hand, the vectors could have undesired effects on their targets. For example, toxicogenomic evaluations show that the acute impact of vector on cell genome content is a serious problem that can finally lead to cellular dysfunction and morphological changes, which have been reported in the literature for cationic dendrimers, PEI and cationic lipids (Tan et al., 2002; Omidi et al., 2003; Omidi et al., 2005b; Akhtar et al., 2007; Hartman et al., 2008; Omidi et al., 2008).

The mechanism of genotoxicities of cationic polymers such as PAMAM and PEI is not fully understood, but the role of surface primary amines and cationic charges in some experiments have been proposed to be the main reason (Hunter, 2006). Since the interaction of these surface amines with complement systems (e.g. C3) have been clearly demonstrated, the probability of interaction between these highly active groups and the other activators, nucleic acid structures (DNA and RNA) and other molecules inside the cells, should be noted as well. Moreover, most of the macromolecules present in the cell structure are negatively charged and therefore the interaction between them and cationic carriers is inevitable.

The use of an excess of non-viral vector in efficient transfection is necessary (Boeckle et al., 2004) but this study again emphasizes that care should be taken to choose an appropriate ratio for a minimum amount of undesired effects with better transfection efficiency.

Conclusion

This study shows that PAMAM G5 dendrimers are promising vehicles for the delivery of EGFR ANS molecules. It seems that silencing the EGFR gene is achieved already at the lower N/P ratios, whereas the higher N/P ratios are required to achieve silencing at protein level. While the lower N/P ratios were able to silence mRNA, it seems that a higher N/P (eg. N/P of 5) is necessary to decrease the protein level efficiently. We show that PAMAM G5 dendrimers have a synergistic effect on the silencing capabilities of EGFR ANS.

Moreover, this study reveals that PAMAM as a cationic polymer suffers from the same non-selective genotoxicity problems as the other cationic polymers which are commonly used as gene delivery vectors. Therefore, careful monitoring of the intrinsic impact of this vector on the other genomic contents is necessary to select an optimized excess charge for efficient gene delivery and to avoid more toxicity on the cells. For this monitoring, high-throughput screening technology (Anderson et al., 2005) beside the routine methods of specific evaluation of the target genes and proteins such as PCR and western blot can complement each other to achieve a better understanding of the gene delivery problems and to solve them.

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