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ORIGINAL ARTICLE

Nonviral gene delivery to human ovarian cancer cells using arginine-grafted PAMAM dendrimer

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Abstract

Background: A specific and effective strategy is in demand to treat ovarian cancer successfully. Epidermal growth factor receptor (EGFR) is highly expressed in ovarian cancer, and thus EGFR antisense gene therapy can be a potential therapeutic strategy. *Method*: L-Arginine-grafted-polyamidoamine dendrimer (PAMAM-Arg) has been reported to be a novel nonviral gene delivery carrier. Therefore, the ability of PAMAM-Arg in transferring a luciferase gene to ovarian carcinoma SK-OV3 cells has been examined, and the cytotoxicity of the cationic polymer has been investigated. In addition, the suppression of cell proliferation has been evaluated by transferring an EGFR antisense gene to SK-OV3 cells using PAMAM-Arg. Polyethyleneimine (PEI) 25K was used as a positive control. *Results*: As a result, in vitro gene transfection efficiency of PAMAM-Arg was enhanced with increasing transfection time and N/P ratios. PAMAM-Arg transferred the luciferase gene into cells more efficiently than PEI. In addition, PAMAM-Arg was minimally toxic to the cells whereas PEI 25K was highly toxic. The polyplexes formed by the EGFR antisense gene and PAMAM-Arg significantly reduced thymidine incorporation into the cells suggesting the suppression of cancer cell proliferation. *Conclusion*: These results suggest that a PAMAM-Arg/EGFR antisense gene complex can be used as a safe and efficient therapeutic agent for cancer gene therapy.

Key words: Antisense gene, cancer gene therapy, epidermal growth factor receptor, l-arginine-grafted-polyamidoamine dendrimer, polyethyleneimine, thymidine incorporation

Introduction

Ovarian cancer is the fifth leading cause of cancer death in women because of the lack of specific symptoms and diagnostic technique[1]. Although the development of new chemotherapy regimens has been progressing continually, the efficacy of the available treatment has been limited by adverse reactions, tolerance, and multidrug resistance. Therefore, a specific and effective drug therapy strategy is required for a successful treatment of ovarian cancer[2].

Epidermal growth factor receptor (EGFR) is related to cell migration, proliferation, and differentiation. Increased EGFR signaling has been linked with tumor invasion, angiogenesis, and metastasis. EGFR is highly expressed in a variety of solid tumors, including ovarian cancer and breast cancer, and is often associated with poor prognosis and drug resistance in chemotherapy[3–6]. The downregulation of EGFR resulted in the reduction of cell proliferation, cell–cell attachment, and tumorigenicity[7]. Thus, EGFR antisense gene therapy can be a potential therapeutic strategy for the treatment of ovarian cancer.

Gene therapy is an attractive therapeutic approach for the treatment of hereditary or acquired diseases. However, the lack of safe and efficient gene delivery systems makes their clinical application difficult. During the last decades, viral vectors, such as adenoviruses and retroviruses, have been considered to be promising gene delivery carriers on account of their efficient gene delivery and expression. However, these vectors have been reported to have several problems, such as insertional mutagenesis, immunogenic and inflammatory

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responses, limited loading capacity, and difficulties in large-scale production[8]. Therefore, nonviral vectors, such as cationic lipids or cationic polymers, have recently received increasing attention as gene delivery carriers.

Cationic lipid–DNA complexes (lipoplexes) have relatively high transfection efficiency in vitro when locally delivered at low doses[9]. However, their in vivo transfection sites are limited to the lung and liver because of their large size, excessive positive charge, and considerable aggregation property[10,11]. Cationic polymers including poly-L-lysine, polyethyleneimine (PEI), and chitosan have several advantages, such as efficient condensation with DNA and simple formation of a relatively small and stable DNA–polymer complex at low cost[12,13]. However, their use as an in vivo gene delivery vector is limited because of some undesirable characteristics, such as inherent cytotoxicity and relatively low transfection efficiency in vivo as compared with viral vectors[14,15]. Nevertheless, cationic polyamidoamine (PAMAM) has been considered to be a promising gene delivery carrier with modification of the linear structure to dendrimers for enhancing transfection efficiency[16]. In addition, the introduction of arginine residues to the PAMAM dendritic surfaces significantly enhanced gene delivery potency compared with native PAMAM, resulting in high transfection efficiency with relatively low toxicity in primary cortical cultures[17,18].

In this study, we investigated luciferase gene expression efficiency in human ovarian carcinoma SK-OV3 cells using a cationic arginine-grafted PAMAM dendrimer (PAMAM-Arg). The cytotoxicity of the cationic polymer was also tested in the cells. In addition, the suppression of cell proliferation was examined by transferring the EGFR antisense gene to SK-OV3 cells using PAMAM-Arg.

Materials and methods

Materials

A luciferase assay system was purchased from Promega Co. (Madison, WI, USA). A BCA™ protein assay kit was supplied by Pierce Co. (Rockford, IL, USA) and a QIAfilter plasmid maxi kit was obtained from Qiagen (Hilden, Germany). Ultima-gold scintillation cocktail and [methyl-^{3}H]-thymidine (81.7 Ci/mmol) were supplied by Perkin Elmer Life Science (Boston, MA, USA). The SK-OV3 human ovarian adenocarcinoma cell line was obtained from Korea Cell Bank (Seoul, Korea). PAMAM-Arg (Figure 1) was kindly gifted by Professor Jong Sang Park (Seoul National University, Seoul, Korea). Clone 790 and 882 plasmids were generously gifted by Dr. William M. Pardridge (University of California, Los Angeles, CA, USA). Clones 790 and 882 are a luciferase expression pCEP4 plasmid and an antisense hEGFR expression pCEP4 plasmid, respectively[19,20]. All other reagents were of analytical grade.

Plasmid preparation

Escherichia coli transformed with plasmid DNA was cultured in Luria–Bertani broth medium with 100 μg/mL ampicillin for 16–18 hours at 37°C. Plasmid DNA was purified by the QIAfilter plasmid maxi kit and dissolved in Tris–EDTA buffer. Plasmid DNA concentration was determined by the measurement of UV absorbance at 260 nm. The purity of plasmid DNA was evaluated by measuring the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}). The ratio of A_{260} to A_{280} was over 1.8.

In vitro transfection assay

SK-OV3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. The cells were seeded at a density of 1.0×10^5 cells per each well and grown in the medium for 24 hours at 37°C. The polyplexes were formed with plasmid DNA (clone 790) and PAMAM-Arg at various N/P ratios (the molar ratios of the amine groups of PAMAM-Arg to the phosphate groups of DNA: 0/1, 4/1, 6/1, and 8/1) for 30 minutes at room temperature. The amount of plasmid DNA used was fixed at 1 μg at each N/P ratio. Following incubation in serum-free medium for 4 and 8 hours, the cells were incubated in the medium containing 10% FBS for 48 hours. The cells were also treated with polyplexes at an N/P ratio of 6/1 for 1, 2, 4, 6, and 8 hours. PEI 25K was used as a positive control. Transfection efficiency was determined by the measurement of luciferase activity using a MicroLumat Plus Luminometer (Berthold Technologies, Bad Wildbach, Germany), which was normalized by the protein concentration.

In vitro cytotoxicity assay

SK-OV3 cells were grown at a density of 1.0×10^5 cells per well in the medium for 24 hours at 37°C. The cells were treated with the cationic polymer over a concentration range of 0.225–0.459 mg/mL in serum-free medium for 4 and 8 hours. Then, the cells were incubated in the medium containing 10% FBS for 48 hours. Moreover, the cells were treated with PEI 25K of a concentration range between 0.122 and 0.243 mg/mL for 4 and 8 hours. In vitro cytotoxicity was examined using a sulforhodamine B assay[21].

Thymidine incorporation in SK-OV3 cells

SK-OV3 cells were cultured at a density of 1.0×10^5 cells per well in the medium for 24 hours at 37°C. The cells were treated with polyplexes of clone 882 plasmid DNA and PAMAM-Arg formed at an N/P ratio of 8/1 for 8 hours at 37°C. The cells were then incubated in the medium containing 10% FBS for an additional 24 hours. A final concentration of 2 μCi/mL [^{3}H]-thymidine and 10 μM unlabeled thymidine was added to each well, followed by the incubation of the cells at 37°C for 24, 48, and 72 hours. The cells were fixed with 10% trichloroacetic

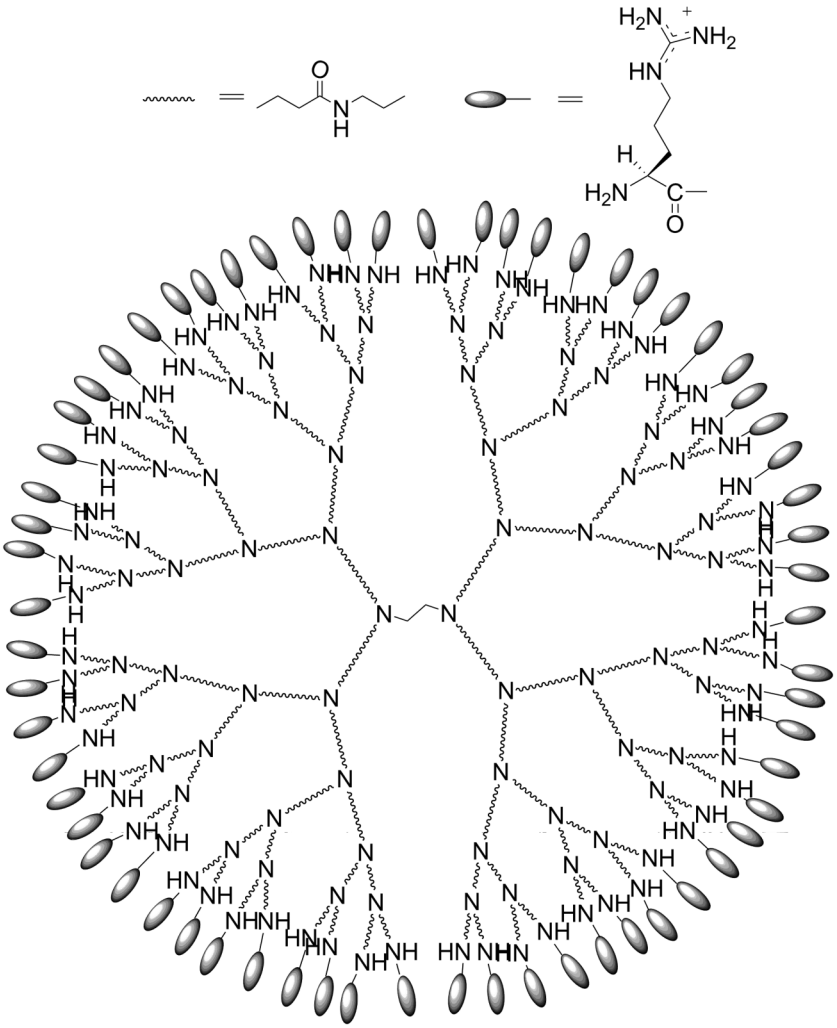

Figure 1. Chemical structure of L-arginine-grafted-polyamidoamine dendrimer (PAMAM-Arg).

acid (TCA) on ice for 10 minutes and centrifuged for 60 seconds. After the removal of the TCA supernatant, 0.5 mL of 1 N NaOH–0.25% SDS was put into the pellet and mixed thoroughly. Subsequently, the pellet was heated for 30 minutes at 60°C, and 350 µL of the solubilized cell solution was analyzed for ^{3}H-radioactivity using a liquid scintillation counter, LS2000 (Beckman Instrument Co., Fullerton, CA, USA)[20].

Statistical analysis

All the data were presented as mean ± SD, unless stated otherwise. The data were compared by one-way ANOVA followed by a Tukey's test among more than two groups and by unpaired Student's *t*-test between two groups. The differences were considered to be statistically significant when the *P* value was less than 0.05.

Results and discussion

Gene therapy with antisense technology has been considered to be an innovative and fundamental method to treat numerous cancers[22]. Because EGFR is highly expressed in ovarian cancer and often associated with poor prognosis[3–6], EGFR antisense gene therapy can be a novel therapeutic strategy for the treatment of ovarian cancer.

Among various cationic polymers, PEI has been reported as a potential nonviral vector for gene delivery. PEI has intrinsic endosome-buffering capacity, that is, this polymer can capture protons in acidic endosomal compartments, cause osmotic disruption of the endosome, and release DNA into cytosol[23,24]. PEI showed remarkable transfection properties in in vitro and in vivo studies suggesting a possibility of clinical application in human bladder cancer therapy[23,25]. Nevertheless, the toxicity of PEI such as inherent cytotoxicity, erythrocyte aggregation, and acute lung embolism has restricted its use as a gene delivery carrier for clinical therapy[24,26].

As shown in Figure 2, PAMAM-Arg showed a several-fold increase in reporter gene transfection efficiency as compared with PEI, a positive control, except for the N/P ratio of 4/1 after 8 hours incubation. Luciferase activity

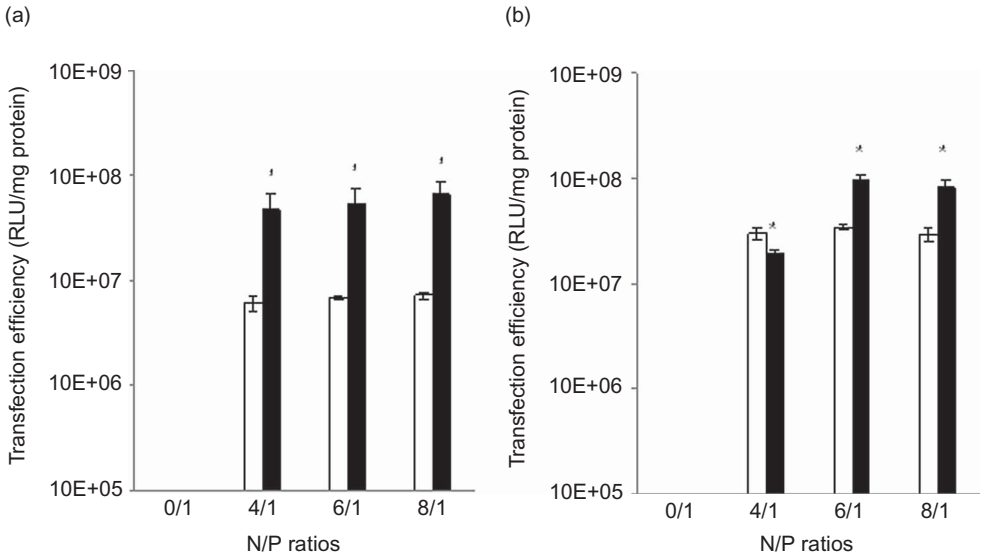

Figure 2. Dose-dependent transfection efficiency of PAMAM-Arg (black bar) and PEI 25K (white bar) in SK-OV3 cells after (a) 4- and (b) 8-hour incubation. The cells were transfected with polyplexes at various N/P ratios. Each bar represents the mean ± SD ($n = 3$). *$P < 0.05$ compared with PEI 25K.

was increased with increasing N/P ratio up to 6/1 and reached a plateau. In addition, in vitro gene transfection efficiency was augmented with increasing incubation time and reached a plateau after 6 hours of incubation (Figure 3). PAMAM dendrimers, a new class of highly branched spherical polymers, have been reported to have high aqueous solubility, transfection efficiency, safety, and stability of complexes with DNA[27]. In particular, PAMAM-Arg used in this investigation is a cationic arginine-grafted PAMAM dendrimer in which arginine residues were coupled to the surfaces of PAMAM dendrimers[17]. PAMAM-Arg showed significantly greater transfection efficiencies in primary cortical cultures in the presence or absence of serum, as compared with PEI, lipofectamine, and native PAMAM[18], which was consistent with our findings.

It has been reported that the toxicity of cationic polymers can be produced by the interaction between the polymers and cell membranes and is related to the efficiency of cellular uptake[28,29]. The cytotoxicity of gene delivery carriers is one of the major obstacles for its application. As presented in Figure 4, PAMAM-Arg showed almost no in vitro cytotoxicity against the SK-OV3 cells up to the concentration of 0.459 mg/mL (N/P ratio of 8/1) for 4- and 8-hour incubation periods. In the

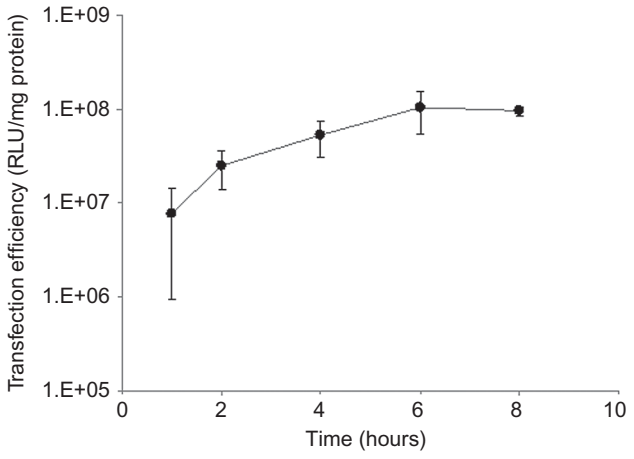

Figure 3. Time-dependent transfection efficiency of PAMAM-Arg in SK-OV3 cells. The cells were transfected with polyplexes at an N/P ratio of 6/1. Each data point represents the mean ± SD ($n = 3$).

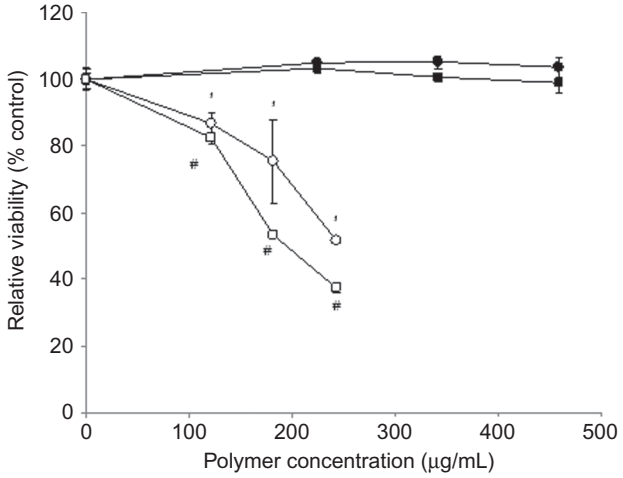

Figure 4. Cytotoxic effect of PAMAM-Arg (●, 4-hour incubation; ■, 8-hour incubation) and PEI 25K (o, 4-hour incubation; □, 8-hour incubation) in SK-OV3 cells. Cell viability was determined by a sulforhodamine B staining assay. Each data point represents the mean ± SD ($n = 3$). *$P < 0.05$ compared with PAMAM-Arg for 4-hour incubation, #$P < 0.05$ compared with PAMAM-Arg for 8-hour incubation.

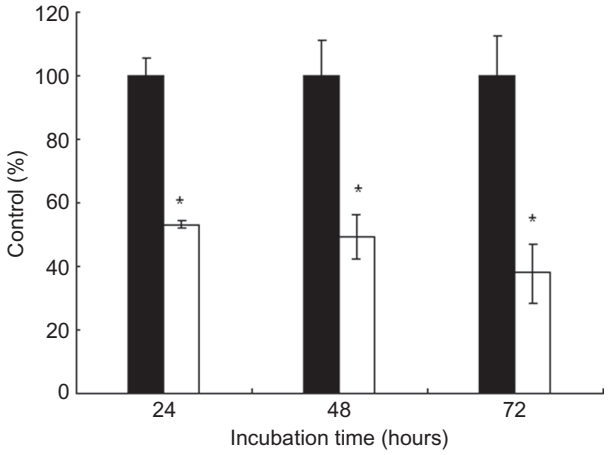

Figure 5. Inhibition of thymidine incorporation by polyplexes at an N/P ratio of 8/1 (white bar) in SK-OV3 cells. Each bar represents the mean ± SD (n = 3). *P < 0.05 compared with control (plasmid DNA only; black bar).

case of PEI 25K, approximately 48.4% and 62.6% reductions in cell viability were observed at 0.243 mg/mL (N/P ratio of 8/1) after 4 and 8 hours of incubations, respectively. Therefore, PAMAM-Arg may possess advantages over PEI because of higher or comparable gene transfection efficiency and much lower toxicity.

To examine the potential of PAMAM-Arg-mediated antisense gene delivery to ovarian cancer, thymidine incorporation assay was performed using a clone 882 plasmid. Clone 882 is a plasmid encoding for hEGFR antisense mRNA[19]. When SK-OV3 cells were transfected with clone 882 plasmid/PAMAM-Arg complexes at an N/P ratio of 8/1 for 8 hours, there was more than a 60% reduction in the thymidine incorporation into the cells after 72-hour incubation (P < 0.05), suggesting the suppression of cancer cell proliferation (Figure 5). Our results suggest that a PAMAM-Arg–EGFR antisense gene complex may be used as a potential therapeutic agent for the treatment of ovarian cancer with high efficacy and safety. In addition, these in vitro results are required to be confirmed in vivo in xenograft animal models in the future study.

Conclusions

PAMAM-Arg transferred the luciferase gene into ovarian cancer cells efficiently without cytotoxicity. The polyplexes formed by the EGFR antisense gene and PAMAM-Arg suppressed cancer cell proliferation successfully. These observations suggest that a PAMAM-Arg–EGFR antisense gene complex can be used as a safe and efficient therapeutic agent for cancer gene therapy.

Declaration of interest

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