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ORIGINAL RESEARCH

Generation of stable cell line by using chitosan as gene delivery system

Emine Şalva · Suna Özbaş Turan · Ceyda Ekentok · Jülide Akbuğa

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Abstract Establishing stable cell lines are useful tools to study the function of various genes and silence or induce the expression of a gene of interest. Nonviral gene transfer is generally preferred to generate stable cell lines in the manufacturing of recombinant proteins. In this study, we aimed to establish stable recombinant HEK-293 cell lines by transfection of chitosan complexes preparing with pDNA which contain LacZ and GFP genes. Chitosan which is a cationic polymer was used as gene delivery system. Stable HEK-293 cell lines were established by transfection of cells with complexes which were prepared with chitosan and pVitro-2 plasmid vector that contains neomycin drug resistance gene, beta gal and GFP genes. The transfection efficiency was shown with GFP expression in the cells using fluorescence microscopy. Beta gal protein expression in stable cells was examined by beta-galactosidase assay as enzymatically and X-gal staining method as histochemically. Full complexation was shown in the above of 1/1 ratio in the chitosan/pDNA complexes. The highest beta-galactosidase activity was obtained with transfection of chitosan complexes. Beta gal gene expression was 15.17 ng/ml in the stable cells generated by chitosan complexes. In addition, intensive blue color was observed depending on beta gal protein expression in the stable cell line with X-gal staining. We established a stable HEK-293 cell line that can be used for recombinant protein production or gene expression studies by transfecting the gene of interest.

Keywords Chitosan · Beta gal · pDNA · Stable cell

E. Şalva (✉)
Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Inonu University, 44280 Malatya, Turkey
e-mail: emine_salva@yahoo.com; emine.salva@inonu.edu.tr

S. Ö. Turan · C. Ekentok · J. Akbuğa
Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

Introduction

Mammalian cells have been widely used to high-value recombinant protein manufacturing. Recombinant protein production in mammalian cells depends on endosomal uptake, cytoplasmic transport and integration of plasmid DNA into host cell genome (Wurm 2004). After transfection, eukaryotic cells uptake exogen DNA and a part of exogen DNA migrate and localize into nucleus. Gene expression can be transient or stable with transfection of different genes. The transport to nucleus and internalization of DNA are hindered with barriers such as size and charge of DNA, enzymatic activity inside or outside of the cell and cell membrane (Khalil et al. 2006). One of the most important criteria for successful generation of therapeutic proteins from recombinant cells is to obtain cell

lines that maintain stability of production (Saifudin et al. 2011).

The major challenge in usage of plasmid DNA for therapeutic approaches is to develop delivery systems which are biocompatible, and increase pharmacokinetic, biologic stability and cellular uptake of DNA. There are several studies to develop safe, efficient, biocompatible, easy to administrate and appropriate to therapeutic use gene delivery systems. By this time, efficient gene expression in different types of cells has succeeded with viral and non-viral systems (Elsabahy et al. 2011). Viral vectors are frequently used for DNA transfer but also suffer disadvantages, such as (1) retroviral vectors (RV) cannot infect non-dividing cells (2) adenoviral vectors (AV) induce inflammatory response and oncogenic effect (3) adeno-associated virus vectors (AAV) have limited capacity for transgene. In addition, toxicity, risk of recombination, high cost, difficulties in production and packaging are limiting the use of viral vectors while they have demonstrated a high transfection efficiency. Non-viral vectors are gaining popularity because of their lower toxicity and immunogenicity, safety, simpler production methods and no limitation in the size of the material to be transferred (Nayerossadat et al. 2012). Furthermore, non-viral gene transfer represents the preferred approach to generate stable cell lines (Wurm 2004).

Chitosan-based delivery systems are gaining importance because they are safer and more efficient by comparison with other non-viral vectors. Chitosan offers many advantages such as no-toxicity, low immunogenicity, high degree of biodegradability and biocompatibility. Moreover, because of positive charge, chitosan can easily form complexes with negatively charged nucleotides by electrostatic interactions (Lee et al. 2005; Mansouri et al. 2004; Şalva et al. 2011).

Production efficiency and stability of protein should be increased for therapeutic protein production in mammalian cells. For this reason, specific production and living cell biomass must be increased and protein production in culture must be stable for long period. With stable transfection, after gene of interest is introduced the cell, it integrates into the host cell genome. Thus, the cells produce recombinant proteins constantly while their cycle or division process. Establishing recombinant cell line processes based on isolation and development of transfected cell lines with a transgene. Stability of recombinant cell line is identified by observation of protein production and cell growth for several months (Wurm 2004; Reisinger et al. 2009).

In this study, we aimed to establish stable recombinant HEK-293 cell lines by transfection of chitosan complexes prepared with pDNA which contain LacZ and GFP genes. With this aim, recombinant protein and GFP expressions in stable cells were analyzed by different methods.

Materials and methods

Plasmids and chemicals

We used pVitro-2-neo-GFP/LacZ vector (Invitrogen, Carlsbad, CA, USA) which has an expression capability in eukaryotic cells. pVitro-2 is a 9838 base pairs length plasmid vector. The vector contains the *Escherichia coli* origin (ori), the 7SK and hCMV-HTLV mammalian promoters and the EM7 bacteria promoter, the neomycin (neo) resistance gene for selection and green fluorescent protein (GFP) reporter gene. When eukaryotic cells are transfected with this plasmid, the beta-galactosidase protein is expressed. In addition, by mean of the selection marker gene neo, the cells express LacZ gene stably. In addition, transfection control and stable cell selection is possible with GFP gene.

Plasmid DNA transformation and isolation

Escherichia coli DH5α strain (Invitrogen) was used to generate vectors with the LacZ gene coding the beta-galactosidase protein. Transformation was performed according to the manufacturer's instructions (Invivogen, USA). *Escherichia coli* DH5α competent cells were incubated on ice for 5 min. 1 ml chilled recostitutive solution was added in tubes and tubes were incubated on ice. Then cells were gently homogenized and incubated on ice for 25–30 min with purpose of complete rehydration. 1 µg supercoil pDNA was added to a chilled Eppendorf tube and tubes were placed in ice. The cells were homogenized and 100 µl of cell suspension was added to the tubes which contain pDNA. The tubes were shaken gently and incubated on ice for 30 min. Afterwards, the tubes were incubated in a water bath at 42 °C for 30 s and

placed on ice for 1–2 min. Then 900 µl LB medium (Sigma, St. Louis, MO, USA) was added to each tube and tubes were incubated at 37 °C for 1.5 h by gentle shaking. 100 µl LB medium was added to the dishes which were prepared with neomycin (Invitrogen) containing LB and agar. Thereafter, dishes were incubated overnight at 37 °C. Colonies in dishes were stored and by using these stocks pDNA was isolated and controlled.

Escherichia coli DH5α was tranformated with pVitro-2-LacZ plasmid, and was cultured in LB medium which contains 25 µg/ml of neomycin. These initial cultures were seeded in 1000 ml antibiotic containing LB medium and incubated at 37 °C for 18–24 h. Cultures were recovered by centrifugation (10,000 rpm, 10 min, +4 °C). Then cells were washed with TE buffer. Plasmid DNA isolation from stored *E. coli* strains was carried out using alkaline-lysis method according to the manufacturer's recommendations (Roche, Mannheim, Germany). Isolated pDNA was controlled and stored at −20 °C.

pDNA/chitosan complex preparation and control

Chitosan solution was prepared in 40 nM Tris–acetate solution (pH 5.0) at 0.25 % (w/v) final concentration. 1 mg/ml pDNA solution that was prepared in TE buffer (pH 8.0), was added to 2.5 mg/ml chitosan solution and vortexed. Complexes with different charge ratios (1/1–5/1; chitosan/pDNA; ±) were prepared by simple complexation method. Chitosan and pDNA solutions were vortexed and incubated at room temperature for 30 min for complex formation. Complexes were controlled by agarose gel electrophoresis method, agarose gel is used at a concentration of 0.7 % (w/v). After 30 min incubation, 10 µl of complexes were loaded into the gel. The gel was run at 80 V/40 mA for one hour, Then pDNA was dyed with ethidium bromide and pDNA was visualized by using UV light (Şalva et al. 2011).

In vitro transfection and generation of stable cell line

Human embryonic kidney cell line HEK-293 from American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in DMEM medium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10 % fetal bovine serum (Biological Industries) and antibiotics at 37 °C and 5 % CO_2. Before transfection, the cells were seeded into 6-well plates at a density of 1.2×10^6 cells per well and cultured overnight. The cells were transfected with chitosan–pDNA complexes (2/1) when grown to approximately 70 % confluence and the transfection reagent Lipofectamine (Invitrogen) used as a positive control. Transfection efficiency was determined by observation of GFP expression. After transfection, cultures were changed to complete medium containing G-418 (Invitrogen) as a selective reagent. Cell growth was followed for 2–3 days and medium containing selection drug was changed every week. After 2–4 weeks, isolated colonies began to appear. Cells that express beta-galactosidase were identified by β-Galactosidase activity and X-gal staining assays (see below).

Beta-galactosidase assay

At 48 h after transfection, cells were sampled and growth medium was removed. The cells were washed twice with PBS (pH 7.4) buffer. 200 µl Triton-X-100-Lysis buffer (1X) (Roche) was added and cells were incubated at room temperature for 30 min using an horizontal shaker. 25 µl cell lysate was transferred to a 96-well plate and 135 µl of a β-mercaptoethanol buffer solution (Sigma) was added. Cells were incubated at 37 °C for 15 min. Then, 50 µl ONPG solution was added and cells were incubated at 37 °C until a faint yellow color had developed. Color development continued for 19 h. After color development, the reaction was stopped by adding 90 µl 1 M Na_2CO_3 solution. The absorbance of the samples was read immediately at 420 nm (Özbas-Turan and Akbuğa 2011).

X-gal staining assay

Growth medium was removed from culture wells and cells were washed few times with buffered physiological saline. Then the cells were fixed for 10–20 min (2 % formaldehyde, 0, 2 % glutaraldehyde in PBS). After fixation, cells were washed with PBS and 5-bromo-4-cloro-3-indol-β-D-galactopyranoside staining solution (Roche) was added and incubated overnight. After staining, depending on gene expression blue

color had developed and cells were detected by light microscopy (Turan and Akbuğa 2011).

Results and discussion

Establishing recombinant cells that express a gene of interest stably is important for gene therapy studies. Plasmid vectors that can express a foreign gene in eukaryotic cells are designed in a manner that contain the gene of interest and an antibiotic gene as a selective marker (Akyüz et al. 2011). Many cationic polymers including poly-L-lysine (PLL), polyethylenimine (PEI), chitosan and their derivatives are widely investigated for stable and transient gene transfection studies. Nonviral gene transfer is very easy to perform, can accomodate large DNA vectors, is safe and does not depend on the cell cycle. Transfection with chitosan which is a non-toxic, biocompatible and biodegradable polymer, offers some advantages in comparision to transfection with commercial transfection reagents. In this study, the transfection potential of chitosan was investigated by using chitosan/pDNA complexes. Recombinant cell lines which express beta gal and GFP were generated by transfection of chitosan/pDNA complexes.

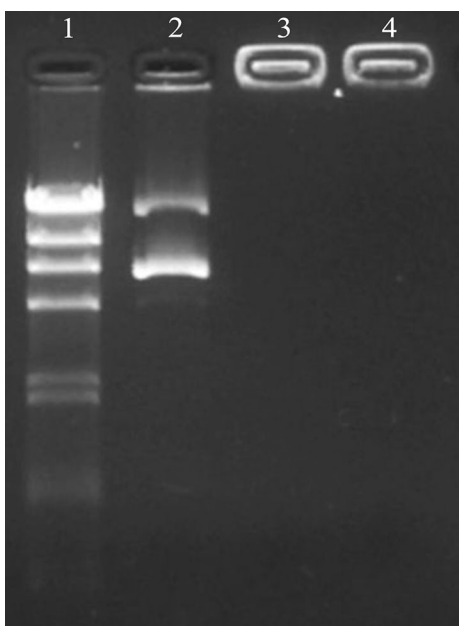

Fig. 1 Gel retardation assay of chitosan/pDNA complexes. *Lane 1* λDNA/HindIII marker, *lane 2* Free pDNA, *lane 3*, *4*. 0, 5/1 an 1/1 (±) complexes

To determine complex formation, chitosan/pDNA complexes were prepared in the 0,5/1 and 1/1 N/P ratios and loaded onto gel. While preparing complexes, pDNA ratio was stable, chitosan ratio was changed. Full complexation was obtained with both N/P ratios (Fig. 1). High positive charge density of chitosan helps to form stable complexes with anionic pDNA by ion–ion interactions (Şalva et al. 2011; Köping-Höggard et al. 2001).

The choice of the gene delivery method is an important factor that affects total transgene expression, target gene expression level and cell viability. These factors are critical in sensitive cells, protein production and the generation of stable cell lines (Dong et al. 2006). HEK-293 cells were transfected with chitosan/pDNA complexes at 1/1 ratio. This nonviral transfer method is safe and it is easy to transfected cells with large DNA vectors. Özbas-Turan and Akbuğa (2011) suggested that chitosan is an efficient delivery system to transfer pDNA. By using this method, HEK-293 cells can be transfected independently from cell cycle. Brunner et al. (2000) indicate that mitotic activity enhances transfection not only by polyplexes but also by lipoplexes.

The HEK-293 cell line used for in vitro transfection studies has been extensively used as an expression tool for recombinant proteins. HEK-293 cells provide a robust and reliable platform to express proteins with high fidelity. This cell line is able to perform most of the post-translational modifications and folding required for the functionality of the recombinant proteins. HEK cell is amenable to rapid amplification of protein product, it too can be used to generate tens of milligrams of protein in weeks. These features of

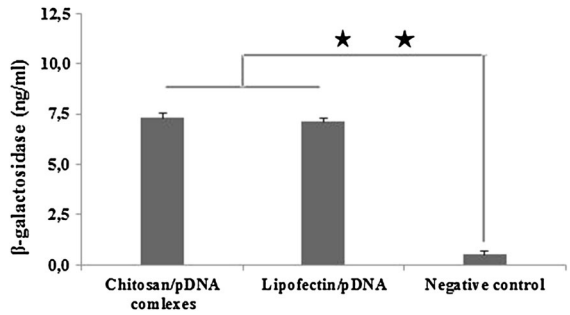

Fig. 2 Beta-galactosidase expression level 48 h after transfection of HEK-293 cells with chitosan/pDNA (1/1) complexes and Lipofectin/pDNA complexes. Negative control is non-transfected HEK cells

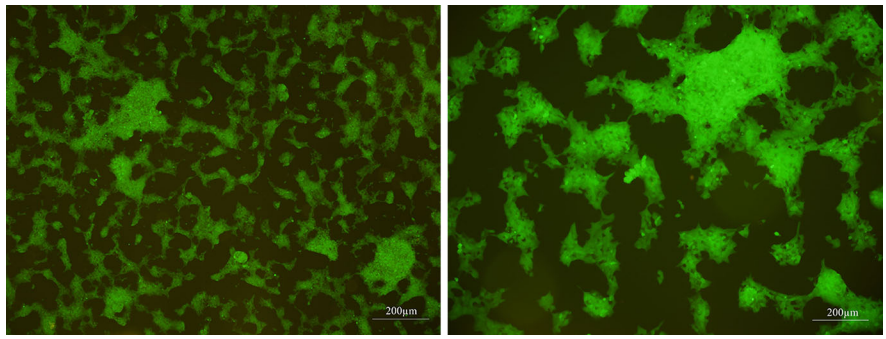

Fig. 3 Fluorescence microscopy images of GFP expression in stable HEK-293 cell line (original magnification, *left* picture ×200, *right* picture ×400)

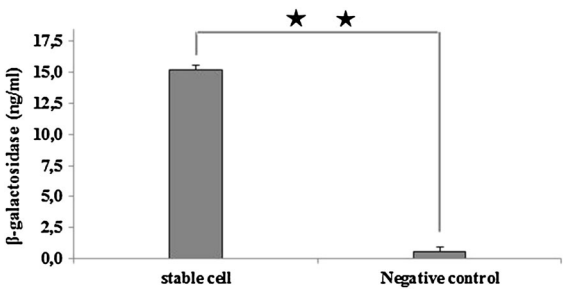

Fig. 4 Beta-galactosidase expression levels in stable HEK-293 cells and endogenous beta-galactosidase expression in non-transfected HEK-293 cells (Negative control)

HEK-293 cell allowed the use of this cell line as a suitable vehicle for the expression of the gene of interest (Thomas and Smart 2005).

To determine the transfection efficiency, GFP which is encoded by plasmid, expression was observed by fluorescence microscopy. At 48 h after transfection, GFP expression was increased and transfection efficiency was detected by counting GFP-positive cells and total cells under the microscope. We achieved a transfection efficiency of approximately 70 %.

In vitro transfection efficiency of chitosan complexes and beta gal protein expression were determined by using an enzymatic method. In addition, lipofectin which is a commercial transfection reagent, was used to compare transfection efficiency of chitosan complexes. Results of in vitro transfection studies with chitosan complexes show that adequate in vitro transfection efficiency was obtained with chitosan. Comparison of gene expression and in vitro transfection efficiency of chitosan and lipofectin complexes are shown in Fig. 2. Chitosan complexes resulted in levels of transgene expression approaching those of the positive controls. Chitosan complexes were almost equivalent to our positive control, Lipofectamine and achieved high levels of transgene expression. Surendra et al. (2010) suggested development of chitosan/pDNA complexes for use in therapeutic gene transfer applications.

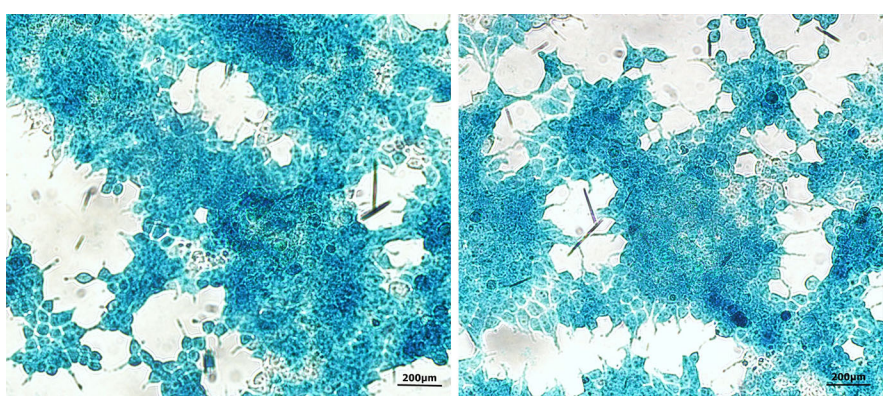

Fig. 5 X-gal staining of beta gal expression in stable HEK-293 cell line. (original magnification, *left* picture ×400, *right* picture ×200)

Beta gal expression in all stable cells was observed by using different methods. At 2–4 weeks after transfection, neomycin-resistant clones were picked, expanded and screened for GFP expression by fluorescence microscopy. GFP expression was observed in all cells (Fig. 3). In vitro transfection efficiency of chitosan complexes and beta-galactosidase protein expression in stable cell lines were determined by enzymatic method. Results showed that efficient gene expression (15.17 ng/ml) in stable HEK-293 cell was obtained with chitosan complexes (Fig. 4). In addition, with X-gal staining after fixation of stable cell line, blue color was observed depending on beta gal protein expression (Fig. 5). Our results suggest that chitosan is an effective and safe vector for delivery of plasmid DNA and can be used to generate stable cell lines producing recombinant protein.

Conclusion

We established a stable HEK-293 cell line by transfection with chitosan complexes. This method can be used to obtain stable cell lines for production of recombinant proteins. This study showed that chitosan complexes can be suggested as gene delivery system because of high transfection efficiency and low-toxicity properties in the generating stable cell lines.

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References

Akyüz MD, Hayta BB, Dinçer PR (2011) An efficient method for stable transfection of mouse myogenic C2C12 cell line using a nonviral transfection approach. Turk J Med Sci 41:821–825

Brunner S, Sauer T, Carotta S, Cotten M, Saltik M, Wagner E (2000) Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther 7:401–407

Dong W, Jin GH, Li SF, Sun QM, Ma DY, Hua ZC (2006) Cross-linked polyethylenimine as potential DNA vector for gene delivery with high efficiency and low cytotoxicity. Acta Biochim Biophys Sin 38:780–787

Elsabahy M, Nazarali A, Foldvari M (2011) Non-viral nucleic acid delivery: key challenges and future directions. Curr Drug Deliv 8:235–244

Khalil IA, Kogure K, Akita H, Harashima H (2006) Uptake pathways and subsequent intracellular trafficking in non-viral gene delivery. Pharmacol Rev 58:32–44

Köping-Höggard M, Tubulekas I, Guan H, Edwards K, Nilsson M (2001) Chitosan as a non viral gene delivery system: structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. Gene Ther 8:1108–1121

Lee KY, Kwon IC, Jo WH, Jeong SY (2005) Complex formation between plasmid DNA and self-aggregates of deoxycholic acid-modified chitosan. Polymer 46:8107–8112

Mansouri S, Lavigne P, Corsi K, Benderdour M, Beaumont E, Fernandes JC (2004) Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. Eur J Pharm Biopharm 57:1–8

Nayerossadat N, Maedeh T, Ali PA (2012) Viral and nonviral delivery systems for gene delivery. Adv Biomed Res 1:1–11

Reisinger H, Steinfellner W, Katinger H, Kunert R (2009) Serum-free transfection of CHO cells with chemically defined transfection systems and investigation of their potential for transient and stable transfection. Cytotechnology 60:115–123

Saifudin N, Ibrahim N, Anuar N (2011) Optimization in transfection and stable production of β-galactosidase in Chinese hamster ovary cells. Biotechnology 10:86–93

Şalva E, Turan SO, Akbuğa J (2011) The increased of in vitro cell proliferation by using chitosan/pGM-CSF complexes. Indian J Pharm Sci 73:131–138

Surendra N, Thibault MM, Lavertu M, Buschmann MD (2010) Enhanced gene delivery mediated by low molecular weight chitosan/DNA complexes: effect of pH and serum. Mol Biotechnol 46:182–196

Thomas P, Smart TG (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharmacol Toxicol Methods 51:187–200

Özbas-Turan S, Akbuğa J (2011) Plasmid DNA-loaded chitosan/TPP nanoparticles for topical gene delivery. Drug Deliv 18:215–222

Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22:1393–1398