Effect of chitosan grafted polyethylenimine nanoparticles as a gene carrier on mesenchymal stem cells viability

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ABSTRACT

This study discusses the effect of complexes of chitosan grafted polyethylenimine(Ch-PEI) with plasmid DNA on viability of mesenchymal stem cells(MSCs) derived from human marrow. Ch-PEI/pDNA nanoparticles were synthesized through the complex coacervation method using pIRES plasmid containing Green Fluorescent Protein (GFP) gene. To confirm the complexation, samples were run through an agarose gel. Human bone marrow mesenchymal stem cells were studied for the cytotoxicity of the nanoparticles by MTT assay. MTT results indicated Ch-PEI does not have any significant cytotoxicity compared with PEI and Lipofectamine^{2000} leading to 40% cytotoxicity. According to the results it seems that grafting chitosan with PEI improves the MSCs viability.

Keywords: Chitosan; PEI; Cytotoxicity; Mesenchymal stem cell

INTRODUCTION

Mesenchymal stem cells are clonogenic and nonhematopoeitic cells in bone marrow with the ability of differentiating to different mesodermal cells such as osteoblast, chondrocyte and endothelial cells and even nonmesodermal cells such as neurons. They are the first stem cells used in clinical application because of their wide differentiating potential. Their low immunogenecity caused them to be used allogene [1]. Transfecting of some genes could lead to a better differentiation potential for example transfecting of TGF-β gene could lead to chondrogenic differentiation or hTERT gene could enhance the proliferation [2,3]. But the important obstacle is the selection of the best procedure for gene transfection. in a perfect gene delivery system ,the vector should be nontoxic and nonimmunogenic [4], should be small enough to enter nucleous [5].
Gene delivery vectors divide into two groups: Viral and nonviral but the simplest approach is using of naked DNA. Direct injection of naked DNA in some tissues such as muscle shows a high level of expression [6]. Although it causes gene expression but its expression level is so less than viral or liposomal vectors. Transfection efficiency will be higher using viral vectors. But also there are some defects that limited their clinical application.The first and most important problem is patient's immunity [7]. Viral vectors could only transfer small sizes of DNA. They are mutagen and oncogen [8,9].
Nonviral vectors could be administered frequently with minimum immune response. Targetability, stability during storage and ease of production are some of their advantages [10]. Cationic lipids and cationic polymers are the two main types of nonviral vectors.They both interact negativley charged DNA with electrostatic bonds through their positive charge and compose complexes. Cationic polymers condense DNA and inhibit their degradation by nucleases. The most impportant feature is their low toxicity[7] but in

contrast with viral vectors they yield lower gene expression [11]. chitosan and its derivatives are some of cationic polymers [12]. Chitosan is a linear aminopolysacharid of N-acetyl-D-glucose amine and D-glucose amine [13], biocompatible and biodegradable. Chitosan is nonimmunogen and low toxic and also can fully protect DNA against nucleases [14,15].

However, its application in biomedical experiments is restricted due to its low transfection efficiency, it is mainly resulted from its 1)low solubility at physiological pH,2) low endosomal release resulted from lack of buffering amines, and 3) very strong condensation of DNA resulting in inefficient unpackaging of transgene in the cytoplasm [16].

To eliminate these limitations Jiang et al [17] prepared Ch-PEI and observed a comparable transfection rate of Ch-PEI with that observed with Lipofectamine. Thus for a higher level of MSCs transfection we proposed introducing a cationic polymer(PEI) onto the chitosan. This grafting circumvents all the limitations (solubility, buffering and DNA binding properties) and also maintains the integrity of chitosan backbone.

As per the literature PEI is the most potent transfecting agent however, it has high toxicity [18]. It is necessary to use a nontoxic vector for transfecting target cells In a gene therapy process so since there is very rare experiments on gene delivery to MSCs by modified chitosan in the current experiment we studied the cytotoxicity of Ch-PEI nanoparticle as a gene carrier to MSCs.

MATERIAL AND METHODS
Material
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (SIGMA), αMEM, FBS, Penicillin/Streptomycin (Pen/Strep), Trypsin/EDTA (Ethylene Diamine Tetraacetic Acid) (GIBCO), pIRES plasmid and Lipofectamine™ 2000(invitrogen)
Polyethylenimine and Low molecular weight chitosan (75-85% deacetylated)(aldrich), tris base, ethidium bromide, dimethyl sulfoxide (MERCK), Qiagene HiSpeed Plasmid Maxi Kit, Escherichia coli (DH5α), DNA ladder 1 kb (Fermentas)

Plasmid Purification
pIRES plasmid amplification was carried out in Escherichia coli DH5α and the purification process using Qiagene HiSpeed Plasmid Maxi Kit performed according to the manufacturer's instructions. In order to evaluate the concentration of purified plasmid DNA absorptiometry at 260nm was performed. Finally pIRES plasmid encoding GFP was alliqouted to the concentration of 0.2 µg/µl and stored at -20° c.

Synthesis of Ch-PEI
Synthesis was carried out with a process similar to Jiang et al [17]. Briefly potassium periodate (0.01M) and chitosan (0.1M) were dissolved in sodium acetate buffer (pH 4.5), degassed with N2 and adjusted to 4 ∘C. Reaction left for 48h and stopped by ethylene glycol (10% v/v). Then dialyzed against NaCl (0.2 M, pH 4.5) and deionized water (pH 4.5).

In the second step PEI (20mM) stirred for 2 days at 4 ∘C with the periodateoxidized

chitosan solution (10mM). Subsequently treated with sodium borohydride (2 g) and dialyzed against NaCl (0.2 M, pH 4.5) and deionized water at 4 ∘C. Finally the product was freeze dried.

Preparation of the solutions
1) Chitosan was dissolved in 1% acetic acid and was diluted to 1% (V/W) concentrations. PH of the solutions was adjusted to 5.5 -5.7.

2 and 3) PEI and Ch-PEI were dissolved in PBS to 1% concentration. Finally each solution was filtered through the 0.22µ filters.

Complex formation
Nanoparticles were prepared by using the complex coacervation process nearly according to what Chew and Gao et al. reported[19,20]. Equal volumes of each solution (Ch, Ch-PEI and PEI) and plasmid DNA (0.2 µg/µl concentration) warmed to 55°C and rapidly vortexed for 60s. Then complexes left for 30 min at room temperature to become stable.

Gel Retardation Assay
Naked DNA and different concentrations of Ch-PEI/pDNA nanoparticles were loaded onto a 0.8% agarose gel containing EtBr in Tris-borate EDTA buffer. The samples were

mixed with a loading buffer and run through the gel at 80V for 1hour. Finally the gel was stained with EtBr and photographed.

Isolation and culture

Human bone marrow obtained from healthy donors after informed consent and diluted with PBS and layered onto Ficoll. MSCs were isolated from bone marrow in a multi step process. So it centrifuged at 1500 rpm for 30 min at 4°C.

Then interphase mononuclear cells were collected and seeded into ratio of $10^6/CM^2$ in α-MEM medium supplemented with 15% fetal bovine serum, Pen/Strep(100U/ML) and 2mML-glutamine and incubated at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was exchanged 2 times a week and when cells reached more than 80% confluency, they were treated with 0.25% trypsin/ethylene diamine tetra acetic acid (EDTA) for 3 min and replaced for subculture. The cells were expanded for three passages.

Osteogenic, Adipogenic, and Chondrogenic Differentiation

For Osteogenic and Adipogenic induction cells of third passage were seeded at a density of 3000 cells/cm^2 and were cultured in αMEM medium with 10% FBS (FBS, Gibco /BRL), Pen/Strep and osteogenic supplements(10^{-8}M dexamethasone, 10mM betaglycerophosphate (Sigma-Aldrich) and 50 μg/ml ascorbic acid-2-phosphate (Wako Chemicals) or adipogenic supplements (1 mM dexamethasone and 60 mM indomethacin; Sigma-Aldrich). Cells were incubated in 37°C and 5% CO_2. Culture medium was changed every 3 days for a period of 3 weeks.

Osteocytes were fixed with methanol at 5 min, and treated with Alizarin Red for 2 min and washed with H_2O. Adipocytes were washed with DPBS and fixed in 10% formalin solution and finally differentiation was confirmed by oil red staining.

Chondrogenic induction: A micro mass culture system was used [21,22]; 2×10^5 of third passage cells were pelleted under 300g for five minutes in a 15-ml polypropylene tube. The pellet was cultured at 37°C with 5% CO_2 in 500 μl of chondrogenic medium containing 10 ng/ml transforming growth factor-β$_3$ (TGF-β$_3$, Sigma, Germany) for 3 weeks. The pellets were embedded in paraffin and cut into 5-μm sections and finally stained with toluidine blue.

Cell viability assay

In order to evaluate the effect of ch-PEI/pDNA nanoparticles on MSCs viability, 24h prior to treatment cells were seeded at 10^4 cell/well in a 96 well plate and feeded with 100 μl αMEM supplemented with 15% FBS and 0.1% Pen/Strep. The next day medium was replaced by fresh medium. Subsequently cells received chitosan/DNA nanoparticles containing 1μg DNA and incubated in 37°C and 5%CO_2 for 12hours. Untreated cells used as positive control. Lipofectamine also used for comparison of viability ratio. At the end of incubation time medium was removed and MTT solution(5mg/ml)/medium to the ratio of 1/5 added to the culture and incubated for 2h in 37°C and 5%CO_2. Then medium was replaced with 100 μl DMSO. Viability was assesed by absorbance measurment at 570nm with microplate reader.

RESULTS

Complex formation

Composition of nanoparticles through complex coacervation process was confirmed by retardation of the complexes in agarose gel electrophoresis(Fig.1). Naked DNA indicated no retardation. Ch ,Ch-PEI and PEI complexes were completely retarded in agarose gel indicating the neutralization of plasmid DNA negative charge.

Mesenchymal Stem Cell Isolation and differentiation

In primary cultures, some cells tended to be adhered. These cells of elongated morphology proliferated to form colony which then grew larger and became confluent(Fig.2)

The osteogenic culture undergoes mineralization following osteogenic differentiation and confirmed by alizarin red staining.

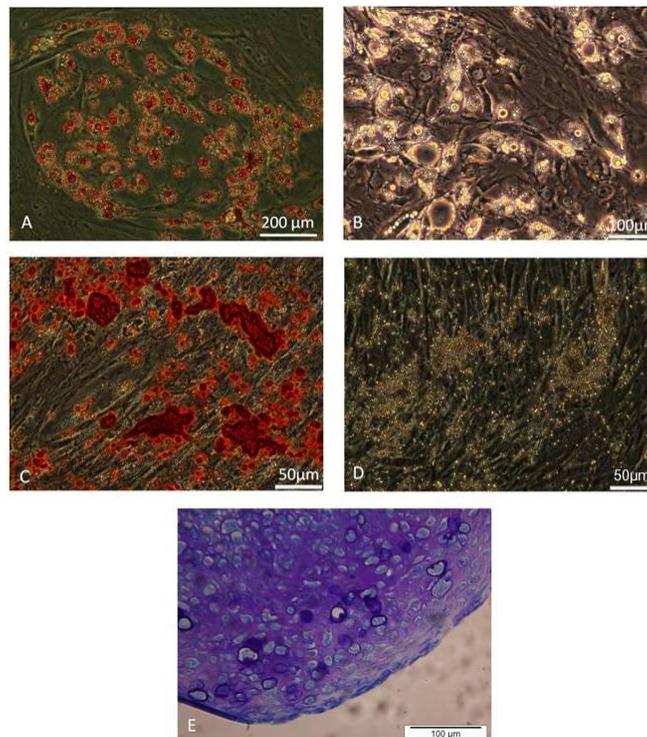By oil red staining of adipogenic cultures, lipid droplets were observed in differentiating cells.

Toluidin blue staining of the sections prepared from chondrogenic pellets demonstrated the presence of a metachromatic matrix (Fig.3)
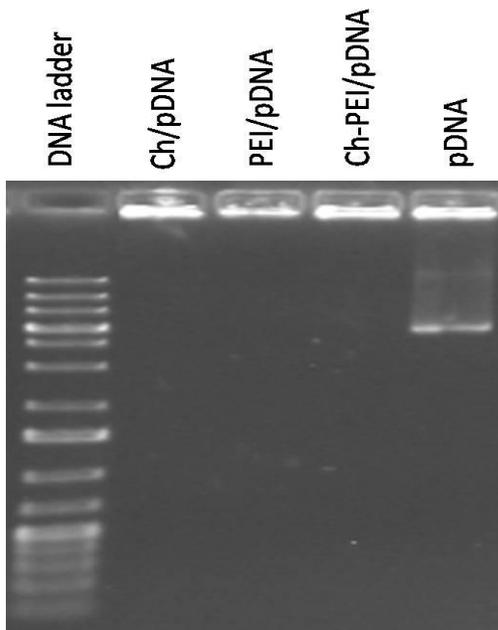
Figure1. Electrophoretic analysis of chitosan, PEI, Ch-PEI complexes and free plasmid
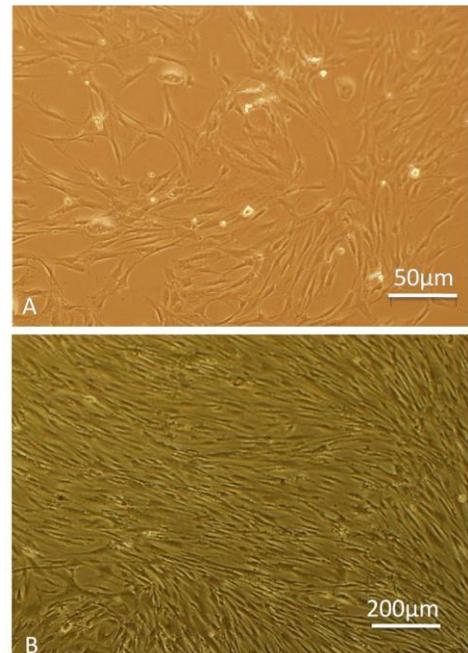
Figure2. Human bone marrow derived mesenchymal stem cell culture. A) Bone marrow cell at primary culture. B) confluent culture of passage 3 cells

Figure3. Human bone marrow derived mesenchymal stem cell differentiation. A) Oil red staining of the adipogenic culture. B) The same culture before staining. C) Alizarin red staining of the osteogenic culture. D) The same culture before staining. E)Toluidin blue staining of chondrogenic culture
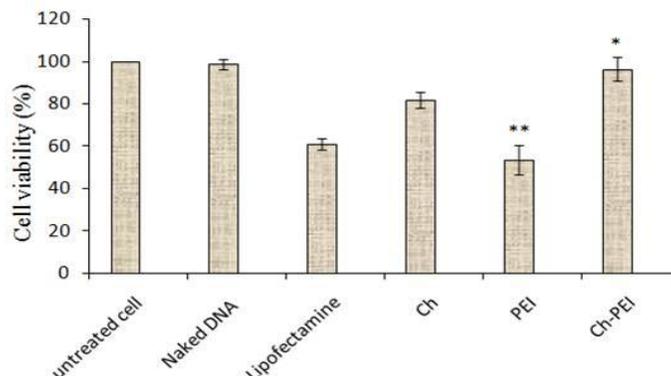
Figure4. (Mean Viability of hMSCs under treatment with naked DNA, lipofectamine and 3 different polyplexes. Untreated cells used as control. *indicates significant difference, p value<0.01, **indicates significant difference, p value<0.01)

Cell viability

The effect of ch-PEI/pDNA complexes on MSCs viability was evaluated using MTT assay. Cells were treated with nanoparticles under experimental condition employed in this paper. As shown in Figure 4.

PEI causes a great cytotoxicity comparable with lipofectamine on mesenchymal stem cells. But also Ch-PEI at 1% concentration resultet in 96.35% viability,shows no significant difference with Naked DNA. On the other hand results show that grafting of PEI to chitosan reduces the cytotoxicity of chitosan for 14.5%.

DISCUSSION

For the treatment of orthopedic disorders such as osteoporosis, arthritis, or bone tumors one of the best approaches is to deliver the intact gene to the targeted area [23,24]. Here, the combination of nanoparticles with an "osteoprotective" gene is an obvious strategy. For example, Fernandes et al used folate-chitosan nanoparticles in combination with an interleukin-1 receptor antagonist (IL-1Ra) gene to decrease inflammation and reverse alterations in bone turnover in an arthritic rat model. But the most important problem of gene therapy is to find a biocompatible, non immunogenic and efficient gene delivery system to protect gene and safely deliver it to the target cell. In this regard many nanoparticles used to deliver gene to MSCs [25,26]. Nanoparticle-based gene therapy offers great opportunities for fine

modulation and treatment of bone diseases of different origin.

Nanosized complexes are formed upon self-assembly of negatively charged DNA with positive charge of polymer through electrostatic interaction, and excessive amine groups contribute to the surface positive charge. The cationic characteristic of polymer is necessary for complex formation and also for the complexes to bind to anionic cell surfaces to facilitate cellular uptake [27].

HMSCs were successfully isolated and cultured to form a fibroblast –like morphology and differentiation to three mesenchymal lineages (adipocytic,osteocytic and chondrocytic). They confirmed the mesenchymal stem cells characteristics. It was mentioned in our previous report that these cells are mononuclear nonphagocytic cells with fibroblast-like phenotype and colongenic potential capable of adhering to the culture surface in a monolayer culture [28].

One of the most important factors in selection of polymeric gene carrier is its Cytotoxic effect on the target cells. In this study a combination of two different polymeric vectors(chitosan and polyethyleneimine) was studied considering their cytotoxicity. The cytotoxicity of cationic polymers is probably caused by polymer aggregation on cell membrane and interactions with components [29,30]. Also these polymers may interfere with intracellular processes of cells

for example primary amine was reported to disrupt protein kinase C function through disturbance of protein kinase activity [31].

Chitosan is known as biocompatible polymer with a low toxic effect. It is an ideal carrier but it's most important deficiency is the poor transfection.The biophysical characteristics of chitosan/pDNA nanoparticles such as size, zeta potential and N/P ratio are the inevitable factors for a favorable cellular uptake [32,33].

On the other hand, polyethyleneimine could help chitosan for a favorable cellular uptake but also it has has severe toxicity due to a high density of primary amine groups [34] Therefore befor administration this derivative of chitosan and PEI should be carefully checked for any cytotoxiccity.

In this experiment Ch-PEI nanoparticles showed much higher viability of MSCs than PEI or Ch separately. Its low cytotoxicity may be caused by the increase in the charge density due to increase in the number of primary amine groups [35]. In addition, Ch-PEI may degrade into nontoxic PEI and chitosan units in cells resulting in lower cytotoxicity [36].

CONCLUSION

Mesenchymal stem cells viability in treatment with Ch-PEI nanoparticles shows that these nanoparticles could be a promising gene carrier gene therapy of bone and cartilage disorders.

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