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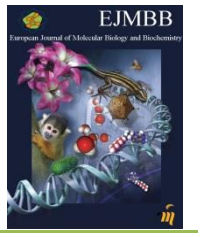

POLYETHYLENEIMINE-MEDIATED GENE DELIVERY ENHANCES TRANSFECTION EFFICIENCY IN NASAL INFERIOR TURBINATE-DERIVED STEM CELLS: IMPLICATIONS FOR REGENERATIVE MEDICINE

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ABSTRACT

New types of stem cells, derived from the tissues of the nasal inferior turbinates (hTMSCs), hold promise for regenerative medicine applications. These cells can be readily obtained from tissue donated during turbinectomy or conchotomy procedures. In this study, poly-ethyleneimine (PEI) was investigated as a gene carrier for hTMSCs using a non-viral delivery system. DNA-PEI nanoparticles (NPs) were generated via electrophoresis and characterized for particle size. The cytotoxicity and transfection efficiency of the DNA-PEI NPs were evaluated in hTMSCs through fluorescence imaging, flow cytometry, and MTT assays following a 4-hour treatment. While DNA-PEI NP treatment exhibited some cytotoxic effects, electrostatic interactions improved transfection efficiency. These findings demonstrate the potential of PEI as an efficient DNA carrier for hTMSCs, suggesting their suitability for gene therapy applications in regenerative medicine.

INTRODUCTION

The stem cells are classified as embryonic stem cells (ESCs), which are derived from blastocyst-derived inner cells [1], and adult stem cells (ASCs), which are obtained from adult tissues of mammals [2-3]. Cell harvesting of embryonic stem cells involves the destruction of embryos since they are pluripotent and have a unique ability to differentiate into various types of tissues including endoderm, mesoderm, and ectoderm [4, 5]. However, it is possible to isolate ASCs without any ethical issues from adult tissues and exhibit the same differentiation and self-renewal characteristics as other types of stem cells [6, 7]. ASCs were limited in what types of stem cells they can differentiate into. Transdifferentiation of stem cells in order to produce extraneous cells from the tissues of their origin has also overcome this issue [8–10].

Due to their easy isolation and rapid proliferation *in vitro*, mesenchymal stem cells (MSCs) are commonly used in regenerative medicine. Additionally, MSCs have been isolated from various adult tissues for tissue engineering and regenerative medicine, for example, fatty tissues, umbilical cord blood, and dental tissues, all of which are considered to be adult tissues. The inferior turbinate tissues in the nose are used to isolate human mesenchymal stromal cells from the turbinate tissues in the nose (hTMSCs). Bone marrow harvesting for hBM-SCs was associated with a high level of pain, so new approaches are needed. As a result of nasal inferior turbinate hypertrophy, it was possible to isolate hTMSCs from tissues that have been discarded after turbinectomy as the result of the hypertrophy. Using CD markers, previous studies have confirmed that hTMSCs have MSC-like characteristics [16]. Furthermore, hTMSCs multiply quickly in vitro, just like other types of stem cells, and they can differentiate into osteoblasts and chondrocytes in vitro and into bone-like tissues in vivo [19]. Considering these advantages, it can be said that hTMSCs are also useful in tissue engineering and regenerative medicine. It may cure cancer, chronic granulomatous disease, and immunodeficiency [20–22]. By attaching to DNA with a virus or non-viral system that attaches to DNA, foreign

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genes are introduced intra-cellularly. As DNA encodes information, mRNA converts it to mRNA, which then combines with tRNA to form the amino acid chain. In gene therapy, protein synthesis is controlled to create specific cells with a desired function. Additionally, this gene-based therapy can be used to treat specific diseases [23]. Gene transfer can be used to create desirable and tailorable stem cells, and gene delivery systems can be used to regenerate various tissues.

Non-viral gene delivery uses polymeric gene transfer carriers such as PEI [24, 25]. Molecular repeating units contain amine groups, which enable them to combine with DNA in order to form complexes when they are combined with DNA [26]. Endosomes contain protonated amino acids that promote ion inflow and membrane destruction as well. Using this method, DNA could be delivered into nuclei and PEI could then be eliminated [27]. A previous study compared linear PEI and branched PEI transfection efficiencies using the same concentration of PEI, and it was found that branched PEI performed better [28]. Based on these results, PEI's primary amine content influences gene transfection.

PEI-branched gene carriers were used in the transfection of genes encoding enhanced green fluorescence protein (EGFP) into human telomere stem cells (hTMSCs). This study addressed the following questions. (1) Can hTMSCs be used in gene therapy? (2) Could PEI be considered as a possible gene carrier for human induced pluripotent stem cells? Using this gene delivery system, can hTMSC protein expression be adjusted? Providing answers to these questions would demonstrate hTMSCs' potential for regenerative medicine as well as gene therapy.

METHODOLOGY

Purification and culture of hTMSCs: The inferior turbinate tissue was washed three to five times with gentamicin saline solution before removing it. There were then three more antibiotic-antimycotic washes and twice in phosphate-buffered saline (PBS). We incubated the tissue at 37°C in Dulbecco's Modified Eagle's Medium (Gibco) containing 10% fetal bovine serum after washing it. Every 2-3 days, fresh medium was changed. Tissue fragments were separated from floating cells on culture plates. Cultured HTMSCs were incubated with penicillin-streptomycin 1% and 10% FBS in minimal essential medium-alpha. CD34, CD90, and CD166 antibodies were used to confirm stemness in hTMSCs. Trypan blue staining was used to ensure viability of hTMSCs. hTMSCs were transfected at 70% confluence.

Using a nanodrop spectrophotometer, it was possible to measure DNA purity and concentration. In Tris-EDTA buffer, a dilution of 1 mg/mL of DNA was prepared. A charge ratio of 1, 2, 4, 8, 12, and 16 for the N/P charge was applied to 1 g of DNA dissolved in distilled water (DW). After vortexing DNA-PEI solution, 30 minutes of incubation formed NPs.

DNA-PEI NPs characterization: Gel electrophoresis of 1.2% agarose gels in Tris-acetate-EDTA buffer and imaging with ethidium bromide confirmed the formation of DNA-PEI NPs. Light scattering at room temperature was used to measure the size of the DNA-PEI nanoparticles and the surface zeta potential of the nanoparticles.

Test for cytotoxicity: A total of 48 human trophoblastic stem cells (hTMSCs) were seeded into 24-well plates with DNA-PEI NPs and then incubated for 24 hours before being treated with DNA-PEI NPs. In order to remove FBS, serum-free MEM was washed thoroughly and then replaced with fresh MEM in order to remove FBS. Before adding cell growth medium, DNA-PEI NPs were treated with hTMSCs for 4 h. A cytotoxicity test was performed at 24, 48, and 72 hours after the DNA-PEI NPs had been transfected with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric test that measures the activity of cellular enzymes that convert water-soluble MTT to insoluble purple formazan. Each well of hTMSCs was incubated at 37°C in 5% CO2 with the MTT solution (5 mg/mL in PBS). To dissolve the formazan crystals, the entire medium was removed, 500 l of dimethyl sulfoxide (DMSO) was added, and the plates were shaken at 100 rpm for 30 minutes. An enzyme-linked immunosorbent assay was used to measure optical density at 590 nm. Triplicates of all experiments were performed.

Efficiency of Transfection: hTMSCs were plated in 12-well plates (Nunc) at 104 cells per well before transfection. Each well was filled with two micrograms of DNA and DNA-PEI NPs with different N/P charge ratios. A different ratio of DNA-PEI nanoparticles was applied to hTMSCs for four hours. EGFP expression was monitored in treated hTMSCs after 24 h and 48 h using Axio Imager A1 and analyzed using AxioVision Rel. Transfection efficiency was assessed using the 4.8 software and trypsinization. hTMSCs were washed in PBS and then fixed in 4% paraformaldehyde for 24 hours following harvesting. In 100 liters of iced PBS, hTMSCs were resuspended. Using a flow cytometer equipped with a FACSCanto II, EGFP-transfected hTMSCs were detected. The data was collected from all samples with 10,000 events, and BD FACSDiva software was used to analyze the data.

Rhod-PEI Synthesis: In order to determine when DNA-PEI NPs had been inserted into hTMSCs, Rhod B ITC was used to label the 5% primary amine groups of PEI. The PEI was dissolved in 0.1 M sodium carbonate at a concentration of 2 mg/mL. At 4°C, 50 microliters of Rhod B ITC solution in dry DMSO (1 mg/mL) were slowly added to PEI solution. To quench the reaction, ammonium chloride solution in DW was added at a final concentration of 50 mM after 24 h. The dialyzed Rhod-PEI was then diluted to the same concentration as normal PEI in DW. DNA nanoparticles containing Rhod-PEI were inserted into transfected hTMSCs in real-time and assessed for intracellular expression of pEGFP and DNA-Rhod-PEI NP

insertion time. The human TMSCs were seeded in 3× 104 wells on a 2-chamber slide and incubated for 24 hours. After staining the nuclei of hTM- SCs with 33342 in culture medium, we washed the cells in serum-free MEM. After 4 hours, DNA-Rhod-PEI NPs were removed and hTMSCs were treated with culture medium. The Rhod-PEI, pEGFP, and Hoechst 33342 expressions in human stromal cells were monitored with a Motic Images Advanced inverted fluorescence microscope.

STATISTICS

Using SPSS 12.0 software, a one-way ANOVA was conducted with Bonferroni being used as the post hoc test for all statistical analyses.

RESULTS

They are very easy to isolate from donated tissue after they have been turbinectomy or conchotomized. In passage 5, CD34 was found to be negative, while CD90 and CD166 were found to be positive. Only 0.4% of hTMSCs were CD34-positive, which indicates that they are not blood-derived. Cells were almost all CD90- and CD166-positive, suggesting MSC-like characteristics. From passages one to five, hTMSCs proliferated rapidly, according to previous research. hTMSCs can be used in gene therapy and other fields that require MSCs due to their properties. One of the most powerful mechanisms for non-viral gene transfer is the endocytosis mechanism. DNA-PEI NPs were charged 1-16 to assess complex formation and transfection efficiency. An agarose gel retardation assay was used to evaluate DNA complexation with PEI. Electrophoresis showed that complexation with PEI at all N/P ratios completely delayed DNA migration. DNA-PEI nanoparticles were characterized by their size and zeta potential in order to determine their properties. PEI concentration-dependently decreased DNA-PEI NP particle size Detection of DNA-PEI NP cytotoxicity in hTMSCs: A 590 nm MTT assay was used to measure DNA-PEI NP cytotoxicity in hTMSCs. After treatment with DNA-PEI NPs, hTMSCs were found to be viable 24 and 48 hours after treatment. The N/P ratio increased the cytotoxicity of DNA-PEI NP. The hTMSCs showed significant toxicity from N/P 1 to 8, but they continued to proliferate for 48 h after DNA-PEI NP treatment. In contrast, hTMSC viability at 24 h and 48 h was less than 50%; at a charge ratio of 16, the optical density of the hTMSCs at 48 h was similar to that at 24 h. It was the DNA-PEI NPs having a charge ratio of 16 that were the most toxic to hTMSCs. Efficacy of transfection into hTMSCs: The membrane of cells synthesized green fluorescence protein in response to EGFP transfection, and this allowed for the measurement of the green fluorescence expression level to be used to assess the efficiency of gene transfection. Fluorescence microscopy and flow cytometry were used to detect green fluorescence after hTMSCs were treated with DNA-PEI NPs at N/P charge ratios of 1-16 before and after their treatment with DNA-PEI NPs. In comparison to nontreated hTMSCs, hTMSCs treated with DNA-PEI showed enhanced green fluorescence as N/P ratio increased. In spite of this, the efficiency of transfection was the same whether the charge ratio was 16 N/P or 12 N/P. DNA-PEI nanoparticles with N/P charge ratio 16 are toxic to hTMSCs, reducing transfection efficiency. These results are accurate based on cell viability analysis. Rhodamine B ITC was used to label PEI, and DNA was treated with Rhod-PEI NPs. On the images, we can observe a blue fluorescence (nuclei) and phase fluorescence (phase) immediately after the treatment. Two hours after adding DNA-Rhod-PEI NPs, a red fluorescence appeared near the nuclei (Rhod-PEI). DNA-Rhod-PEI nanoparticles were found attached to the membrane of the hTMSCs after 2 hours and close to the nucleic acids at 5 h. Rhod-PEI red fluorescence was weaker at 7 h. Plasmid DNA was detected after 11 hours on the membrane of the hTMSCs. In less than 24 hours, the red fluorescence had left the nucleus and had disappeared. DNA-PEI was inserted two to seven hours after DNA-Rhod-PEI NP treatment, and green fluorescence protein was formed nine hours later. 24 h later, Rhod-PEI leaked from the cells and remained in the cytoplasm. After addition of PEI, DNA-PEI NPs have a higher zeta potential when PEI is added because of the phosphate groups in DNA. DNA-PEI NPs were condensed into positively charged particles by PEI. By endocytosis and attraction, nanosized and positively charged particles could insert DNA into hTMSCs.

DISCUSSION

Human inferior turbinate stromal cells were isolated and characterized as MSCs by this study. TMSCs can be differentiated into osteoblasts, chondrocytes, or adipocyte depending on the purpose of differentiation is possible [16–19, 29]. As a result of their characteristics, hTMSCs are considered crucial for tissue engineering. In order to evaluate hTMSCs as potential gene therapy sources, we used PEI as a non-viral gene carrier in order to evaluate their potential efficacy. Since PEI contains a large number of positively charged amine groups in its main chain, it has been used as a gene carrier. This allows the transfer of genes into cells and protection of DNA through the proton sponge effect [27]. Following the addition of PEI solution to DNA, DNA-PEI NPs were evaluated by agarose gel electrophoresis and zeta potential measurements. As PEI concentration increased, DNA-PEI NPs decreased from 1145 nm to 140 nm, and zeta potential increased from 20 mV to 30 mV. There was evidence that DNA-PEI NPs are formed as a result of electrostatic interactions [30, 31]. hTMSCs were treated with DNA-PEI NPs for 4 hours and their transfection efficiency was assessed. As compared to low charge ratio DNA-PEI NPs, high charge ratio DNA-PEI NPs showed greater cytotoxicity; however, DNA enhanced green fluorescence in hTMSCs. Due to disruption of pH regulation and depolarization of cell membranes, PEI may exhibit cytotoxicity in gene delivery [32–35]. As NPs are taken up

by adhesion, they may interact strongly with membranes when they are taken up [36]. Previously, DNA-PEI NPs were observed to be more cytotoxic to hTMSCs than other MSCs. According to studies conducted under similar conditions, hTMSC transfection efficiency was about twice that of other MSCs [25, 37]. Based on these results, hTMSCs are an excellent cell source for gene transfection. To effectively transfect hTMSCs, PEI, a polymeric gene carrier, needs to be resolved because of its cytotoxicity. The hTMSCs were treated with DNA-Rhod-PEI nanoparticles in an 8:1 charge ratio to measure uptake and green fluorescence expression intracellularly. hTMSCs began to fluoresce red after a few hours after Rhod-PEI was added. In colocalization with Hoechst stain, hTMSCs expressed green fluorescent protein 9 h after being treated with DNA-Rhod-PEI nanoparticles for 8 hours. HTMSCs expressed EGFP in their cytoplasm and nuclei as incubation time increased, and Rhod-PEI dissociated from their nuclei. DNA-carrier complexes dissociated after being absorbed into nucleic acids by DNA polymerase [38]. In hTMSCs, DNA-Rhod-PEI NPs were dissociated and Rhod-PEI fluoresced in the cytoplasm of the cells as a result. Lentiviral vector transfection and stem cell differentiation have been few [39]. As far as we are aware, DNA-PEI NPs can transfect hTMSCs for the first time. However, further studies are currently being conducted in order to examine osteogenic differentiation of hTMSCs transfected with BMP-2 using nonviral gene carriers.

CONCLUSION

DNA was introduced via PEI, one of the most widely used gene carriers, into hTMSCs in order to verify their suitability for gene therapy. As a result, DNA-PEI nanoparticles were formed by interacting both the DNA molecule and PEI molecule with different ratios of charge N/P between the two molecules. hTMSCs transfected with DNA-PEI nanoparticles showed a higher N/P charge ratio when compared to those transfected with NPs that were not DNA-PEI. About 30% of human TMSCs were transfected with PEI at N/P 12. This study found that PEI can be used to transfect hTMSCs without viruses, and hTMSCs are useful for gene therapy since they are highly transfected. hTMSCs may be useful in vitro as a method of gene transfection, and this system may be used to control protein expression, according to the findings of this study. DNA and PEI formulations must be optimized to be able to transfect cells efficiently and with the least amount of toxicity.

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