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Characterization of glycol chitosan grafted with low molecular weight polyethylenimine as a gene carrier for human adipose-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) have a great capacity for self-renewal while still maintaining their multipotency, and can differentiate into a variety of cell types. The delivery of genes to a site of injury is a current and interesting field of gene therapy. In the present study, we describe a nonviral gene delivery carrier, glycol chitosan-methyl acrylate-polyethylenimine (GMP) polymer targeted towards human adipose-derived mesenchymal stem cells (AD-MSCs). Transfection efficiency, using luciferase (Luc) and a pDNA encoding enhanced green fluorescent protein (EGFP), along with cytotoxicity assays, were performed in human AD-MSCs. The results show that the transfection efficiency of the GMP polymer was similar to that of PEI25kD, and the cytotoxicity was lower. Moreover, human AD-MSCs were treated with the GMP polymer/pDNA polyplex and its cellular uptake and distribution were analyzed by flow cytometry and confocal microscopy. Furthermore, we performed endosomal escape analysis using LysoTracker Red, and found that the conjugated GMP polymer could escape from the endosome to the cytosol. Human AD-MSCs treated with the GMP polymer maintained their potential for osteogenic differentiation and phenotypic expression of human AD-MSCs based on flow cytometry analysis. The present study demonstrates that the GMP polymer can be used as a potential targeted–delivery carrier for effective gene delivery.

1. Introduction

Gene therapy is the treatment of a disease *via* the efficient delivery of genetic material to target cells or organs, with low cytotoxicity. Over the past few decades, many studies have focused on the development of efficient and safe gene delivery systems (Luo & Saltzman, 2000; Vijayanathan, Thomas, & Thomas, 2002), which can be divided into viral and nonviral. Viral vectors for gene delivery exhibit high transfection efficiency, however, they have many drawbacks such as toxicity, high immunogenicity, and tumorigenicity. With a view to overcoming these problems, there has been increased interest in nonviral vector systems (Engelhard, 2000; Pathak, Patnaik, & Gupta, 2009). The most commonly used nonviral vectors can be classified into cationic lipids and cationic polymers, which have several advantages over viral vectors such as low immunogenicity, increased biocompatibility, and reduced cytotoxicity. Consequently, the current development of high transfection efficiency and low toxicity vectors of this type has high potential for success in the field of nonviral gene delivery (Al-Dosari & Gao, 2009; Jones, Chen, Ravikrishnan, Rane, & Pfeifer, 2013).

Chitosan is a linear cationic polysaccharide composed of *N*-acetyl glucosamine and glucosamine linked by β-1,4-glycosidic bonds, which is a non-toxic, biodegradable, and biocompatible material used as a nonviral gene delivery system (Gao et al., 2010; Sato, Ishii, & Okahata, 2001). Chitosan forms complexes with DNA through ionic interactions between the negatively charged phosphate backbone of DNA and its primary amine groups (Mansouri et al., 2004; Toh, Chen, Lo, Huang, & Wang, 2011). The polymer effectively interacts with DNA and protects the complexed DNA against nuclease degradation (Guang Liu & De Yao, 2002; Jiang et al., 2007). Glycol chitosan (glycol chitin, deacetylated) is a polysaccharide chitosan derivative with enhanced hydrophilicity, acquired by modification of the chain (Yamada & Imoto, 1981). Glycol chitosan

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is known for its biocompatibility and is used in biomedical applications such as drug delivery, carriage of si-RNA, and diagnostic and therapeutic imaging (Mansur & Mansur, 2015; Sun et al., 2014).

Polyethylenimine (PEI) is one of the most studied cationic polymers used as a nonviral vector due to its higher gene expression compared with other polymers. Since its positively charged primary amine groups can interact with negatively charged DNA and promote cellular uptake, the tertiary amine groups of the polymer act as a proton sponge. However, the transfection efficiency and toxicity of PEI/DNA complexes are associated with molecular weight, with the high molecular weight PEI having high transfection efficiency but also high cytotoxicity *in vitro* and *in vivo* (Forrest, Koerber, & Pack, 2003; Hu, Peng, Chen, Zhong, & Zhuo, 2010).

Very recently, we demonstrated the low cytotoxicity and enhanced transfection efficiency of the glycol chitosan grafted with low molecular weight polyethylenimine (GMP polymer) in cancer cells (Lee, Park, & Choi, 2016), confirming its potential use in cancer therapy. Therefore, we hypothesize that the GMP polymer could efficiently encapsulate plasmid DNA and transfer it into human adipose-derived mesenchymal stem cells (AD-MSCs). Here, we show that the effectiveness of a gene carrier for human AD-MSCs requires efficient intracellular uptake and transfection, reduced cell toxicity, and the maintenance of the multi-lineage potential of MSCs (Pandita et al., 2011; Santos, Oramas, Pego, Granja, & Tomas, 2009). Adult bone marrow derived-MSCs have self-renewal capabilities and the potential to differentiate into various lineages including fat, bone, cartilage, and neural cells. Moreover, MSCs are of interest in the field of gene therapy and regenerative medicine, possessing properties that can be exploited to deliver therapeutic genes to sites of injury or diseased tissue (Pittenger et al., 1999; Tanabe, 2014). Furthermore, MSCs have tissue repair and migratory properties, homing to areas of injury, making them efficient vehicles for targeted delivery systems (Hwang, Varghese, & Elisseeff, 2008; Sudo et al., 2007).

In the present study, we compared the GMP polymer with the control vector, PEI25kD, since it has the most effective gene expression due to entry into cellular membranes following a proton buffering capacity (Sun, Xiao, Cheng, Zhang, & Zhuo, 2008). The synthesis of the GMP polymer was analyzed by ^{1}H and ^{13}C NMR. In addition, we performed polyplex formation, cytotoxicity, and transfection efficiency analysis of the GMP polymer. The cellular uptake and intracellular distribution of the GMP polymer were analyzed by flow cytometry and confocal microscopy. Furthermore, we examined the osteogenic and adipogenic differentiation of human AD-MSCs following the use of the GMP polymer as a gene delivery carrier.

We think that this report is in line with some recent reports of chitosan-based biopolymers for various biological studies of MSCs (Cheung et al., 2014; Dumont et al., 2016). And, as a first study, we demonstrate that the glycol chitosan-based GMP polymer showed much reduced toxicity with robust transfection efficiency in human AD-MSCs and the polymer-treated human AD-MSCs maintained the phenotypic characterization and induced osteogenic differentiation. Since MSCs are multipotent non-hematopoietic cells capable of self-renewal and are able to differentiate into multiple lineages such as osteogenic, adipogenic, and chondrogenic lineages, we believe that this paper shows in depth studies of bioanalysis for the relevant drug delivery and tissue engineering studies for human AD-MSCs.

2. Materials and methods

2.1. Materials

Glycol chitosan (GC, purity ≥60%, degree of deacetylation = 91.6%,) (Lavertu et al., 2003), methyl acrylate, and PEI 800 Da (water free, low molecular weight polyethylenimine) were purchased from Sigma-Aldrich (Seoul, South Korea). Adipose-derived human mesenchymal stem cell growth medium, osteogenic differentiation medium, adiopogenic differentiation medium, and penicillin/streptomycin were purchased from Cell Engineering for Origin (Seoul, South Korea). The luciferase reporter plasmid DNA was prepared as reported previously (Choi et al., 2004). The luciferase assay kit and 5 x Reporter Lysis Buffer, and Cell-Titer glow luminescent assay kit were purchased from Promega (Madison, WI, USA). The Micro Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). EZ-Cytox reagent was purchased from Daeil Lab Service (Seoul, South Korea). PicoGreen reagent, LysoTracker, and Alexa fluor 488 5-SDP ester were purchased from Invitrogen (Seoul, South Korea). CD31 was purchased from BD Pharmingen and CD73 and CD105 were obtained from Millipore (Seoul, South Korea). Oil Red O and Alizarin Red were purchased from Sigma-Aldrich (Seoul, South Korea).

2.2. Synthesis of the GMP polymer

The molecular weight of glycol chitosan (M_n = 198 kDa, M_w = 490 kDa, polydispersity index = 2.47) was determined by Waters GPC (Gel permeation chromatography) system using TSKgel G5000PWxl-CP and TSKgel G3000PWxl-CP columns (Korea Polymer Testing and Research Institute). The GMP polymer was synthesized as described in our recent study (Lee et al., 2016). Briefly, glycol chitosan was reacted with excess methyl acrylate for 4 days at 37 °C with shaking at 180 rpm to prepare glycol chitosan-methyl acrylate (GC-MA), which was followed by dialysis against distilled water using a dialysis membrane with a molecular weight cut-off (MWCO) of 10 kDa and freeze drying. Then, the dried GC-MA was dissolved in water to be conjugated with excess PEI 800 Da by amidation for 5 days at 37 °C in methanol/water with shaking at 180 rpm. The reaction solution was dialyzed against water using a dialysis tubing (MWCO 10 kDa) and the GMP polymer was obtained after freeze-drying the aqueous solution under vacuum overnight. The conjugation yield defined as the ratio of PEI-conjugated unit of glycol chitosan polymer was determined by ^{1}H NMR spectroscopy (>95%), and ^{13}C NMR data of the polymers were obtained (Korea Basic Science Institute).

2.3. PicoGreen assay

Polyplex formation was performed using HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.0) and the PicoGreen reagent, diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The polyplexes were prepared by mixing 1 μg pDNA with GC, PEI 800 Da, and GMP polymer. The PicoGreen reagent was added to the polyplex sample and further incubated for 2 min. Fluorescence (ex 480 nm, em 520 nm) was measured with a filter fluorometer (QUANTEC, Thermo Scientific).

2.4. Cytotoxicity assay

Cytotoxicity of GC, PEI 800 Da, and GMP polymer was measured using EZ-Cytox Reagent (Daeil Lab Service, Seoul, South Korea) based on the WST assay. Human AD-MSCs were seeded in 96 well plates at a density of 1.3×10^4 cells/well and incubated in 100 μL of stem cell growth medium, 100 u/mL penicillin, and streptomycin under 37 °C and 5% CO_2 for 12 h. The cells were treated with various concentrations of GC, PEI 800 Da, and GMP polymer, and then the cells were incubated for 24 h. PEI25KD was used a control group. Thereafter, 10 μL EZ-Cytox Reagent was added to each well and the cells were incubated for an additional 2 h. The absorbance was