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Photo-mediated internalization of nanocomplex for effective gene delivery to adipose tissue-derived stem cells

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ABSTRACT

To deliver efficiently osteogenic, chondrogenic or adipogenic induction genes, such as Runx2, SOX9 and C/EBP-α, to adipose tissue-derived stem cells (ADSCs), a photo-mediated nanocomplex internalization gene delivery system was designed using chlorin e6 as a photosensitizer (PS) and polyethyleneimine (PEI) as a gene delivery carrier. In this system, gene delivery efficacy was significantly increased in ADSCs by photo irradiation. The gene transfection efficiency of Runx2, SOX9 and C/EBP-α was increased by 8.6-, 6.7- and 9.3-fold, respectively, by applying 0.7 J/cm^2 of irradiation. Osteogenic, chondrogenic and adipogenic differentiation was confirmed by differentiation-related markers and histological analysis. ADSCs transfected with Runx2, SOX9 and C/EBP-α genes via photo irradiation indicated enhanced differentiation in comparison to the non-irradiated cells. These findings demonstrate that photo-mediated internalization is a promising system for efficient gene delivery and differentiation in ADSCs.

1. Introduction

In recent decades, photodynamic therapy (PDT) has been considered to be an attractive method for cancer treatment [1–3]. PDT is based on the photochemical reactions between photosensitizer (PS) and light irradiation. Upon appropriate red visible light irradiation ($\lambda \geq 600$ nm), the PS generate singlet oxygen molecules ($^1O_2$) by energy transfer from the ground singlet state to the triplet excited state [4,5]. The $^1O_2$ induces lipid peroxidation, which inactivates the cellular membrane, thereby leading to cell death [6–8]. Based on this PS property, few investigators have studied photo-mediated internalization techniques for the cytosolic delivery of chemotherapeutic drugs, proteins and genes trapped in the endo/lysosome [9–11]. Photo-mediated internalization is based on PS that localize in the endosomes, which induce endosomal membrane rupture such that the drugs are efficiently released into the cytosol following light irradiation. The Berg group has demonstrated the effectiveness of photo-mediated internalization through in vitro and in vivo models [12,13]. In addition, Høgset et al. developed photo-mediated internalization technology that improves gene delivery to colorectal carcinoma cells using an adenovirus [14].

In the meantime, cell therapy has been studied for treating degenerative diseases such as bone, cartilage, adipose tissue, nerve axon and cardiac diseases [15–17]. Among the various adult stem cell types, adipose tissue-derived stem cells (ADSCs) can differentiate into multiple lineages including osteoblasts, chondrocytes and adipocytes [18,19]. Unlike other adult stem cells such as bone marrow (BM) or cord blood-derived stem cells, ADSCs are abundant in adipose tissue, and they can be isolated by less invasive methods [20,21]. The lipo-aspiration of adipose tissue is used to routinely collect 400,000 ADSCs/mL with 90% cell viability. Additionally, ADSCs grow rapidly, and they can be cultured for longer than BM-derived stem cells (BMSCs) [22,23]. Non-viral gene delivery methods using cationic polymers have been approached to control ADSC differentiation [24,25]. However, non-viral systems have low efficiency in the ADSCs because of intracellular barriers, such as poor uptake and poor endosome escape by transfected genes [26]. In addition to non-viral gene delivery, mechanical or physical gene delivery methods have been developed such as electroporation, nucleofection, and ultrasound-mediated gene delivery [27,28]. Physical gene delivery methods enhance transfection efficiency in a wide range of cell types, and they induce the passage of exogenous genes through the cell membrane by forming micropores [27–30]. However, significant cytotoxicity is thought to be a limitation of physical gene delivery techniques [31]. Based on this situation, it is necessary to develop gene transfection materials for efficient and safe gene transfer to ADSCs.

In this paper, a photo-mediated gene complex internalization in which chlorin e6 (Ce6) was used as a PS was designed for enhanced gene delivery to ADSCs. Polyethyleneimine (PEI) and Ce6 were used together as a gene delivery vector, and PS delivered differentiated genes into ADSCs. Runt-related transcription factor 2 (Runx2), Sry-type HMG box 9 (SOX9) and CCAAT/enhancer binding

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protein-alpha (C/EBP-α) genes were delivered by photo-mediated internalization. These factors are important for osteogenic, chondrogenic and adipogenic differentiation [32–34], respectively. The efficacy of Runx2, SOX9 or C/EBP-α gene delivery was monitored by western blotting, RT-PCR, real-time PCR and confocal microscopy. Transfected ADSCs were cultured in 2D or pellet suspension for 4 weeks, and ADSC differentiation was later confirmed by RT-PCR, real-time PCR and histological analysis.

2. Experimental methods

2.1. Cell culture

Human ADSCs were obtained from Lonza (Lonza, Walkersville, MD) and cultured in DMEM/F12 (Hyclone, USA) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.2. Preparation of expression vectors

Plasmid DNA (pDNA) encoding enhanced green fluorescence protein (EGFP) was purchased from Clontech (pAcGFP-N1; Clontech, Palo Alto, CA, USA). pDNA encoding EGFP-Runx2, SOX9, dsRed1-SOX9, C/EBP-α and EGFP-C/EBP-α were kindly donated by Prof. Keun-Hong Park of CHA University, Republic of Korea.

2.3. Preparation and characterization of PEI/pDNA complexes

PEI/pDNA complexes were prepared by mixing PEI and pDNA in serum-free DMEM/F12 medium with an N/P ratio of 5, which indicates the proportion of nitrogen in PEI to the phosphate in pDNA. The mixture was incubated at room temperature for 15 min. The particle size and surface charge were determined by dynamic light scattering (DLS; Zetasizer Nano ZS; Malvern Instruments Ltd., UK). For the gel retardation assay, the PEI/pDNA complex was analyzed on a 1% agarose gel containing 0.5 μg/mL ethidium bromide.

2.4. Photo-mediated transfection in ADSCs

ADSCs were seeded in a 35-mm dish at a density of 1×10^5/well and incubated for 24 h. The culture media was replaced with fresh serum-free media containing 0.5 μg/mL Ce6 (Frontier Scientific, Logan, UT) and PEI/pDNA complex. After 4 h, the cells were irradiated with a He–Ne laser (633 nm), and FBS was added. After 24 h, the cells were harvested to confirm the transfection efficiency.

2.5. Transfection efficiency determination

Western blot, RT-PCR, real-time PCR and confocal microscopy were used to confirm the transfection efficiency. For the western blot, the transfected cells were lysed in RIPA buffer (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, Germany). The proteins were separated on an SDS polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked in 5% skim milk and incubated with anti-EGFP (Clontech, USA) or anti-β-actin (Santa Cruz Biotech, Santa Cruz, CA) primary antibody. The membrane was then incubated with an HRP-conjugated secondary antibody and developed by chemiluminescence (Ab Frontier, Seoul, Korea).

RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) for RT-PCR and real-time PCR analysis. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA and random hexamer primers. The subsequent RT-PCR was performed with cDNA, 2× PCR Master mix solution (Intron Biotechnology, Seoul, Korea) and gene-specific primers (Table S1). The real-time PCR was performed with cDNA, SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and gene-specific primers in a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). The primers used in this study are shown in Table S2.

To visualize the transfected cells, all cells were fixed with 4% paraformaldehyde, counter-stained with DAPI, and mounted on glass slides. Cells were observed with a confocal laser-scanning microscope (LSM 510 Meta; Zeiss, Germany).

2.6. Cell cytotoxicity

To confirm cell cytotoxicity, Live/Dead, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and CCK-8 assays were performed. For the Live/Dead assay, 2 μM calcein AM and 4 μM EthD-1 were added to the cells. The live cells were stained fluorescent green and the dead cells were red. For the TUNEL, cells were fixed and treated with 0.1% Triton X-100. Cells were incubated with 50 μL of TUNEL mixture for 1 h in 5% CO_2 at 37 °C. Cells were counter-stained with DAPI, mounted, and observed by fluorescence microscopy.

For the CCK-8 assay, cells were treated with 100 μL of CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD), and the optical density of the solution was measured using a microplate reader at 450 nm.

2.7. Histological analysis

To induce osteogenesis, chondrogenesis or adipogenesis, the genes Runx2, SOX9 or CEBP/α were transfected into ADSCs. These cells were incubated for 4 weeks in osteogenic medium (DMEM/F12 supplemented with 10% FBS, 100 nM dexamethasone, 50 mM ascorbic acid, and 10 mM β-glycerol phosphate), chondrogenic medium (DMEM/F12 supplemented with 100 nM dexamethasone, 170 mM ascorbic acid, 0.35 mM L-proline, 1 mM sodium pyruvate, ITS and 10 ng/mL recombinant human transforming growth factor-b1) or adipogenic medium (DMEM/F12 supplemented with 10% FBS, 100 nM dexamethasone, 0.2 mM indomethacin, 0.01 mg/mL insulin and 0.5 mM 3-isobutyl-1-methylxanthine) for induction. The differentiated cells were stained with von Kossa, Alizarin Red S, Alcian Blue, Safranin O, Sudan Black B, and Oil Red O.

For the pellet cultures, the transfected ADSCs were trypsinized, collected in 15 mL conical tubes and centrifuged at 500 × *g* for 5 min. The treated cells were then incubated for 4 weeks in osteogenic, chondrogenic or adipogenic induction media. The pellet-cultured cells were embedded in O.C.T. compound and frozen. The specimens were sliced to 6 μm thick on a cryostat.

3. Results

3.1. EGFP transfection efficiency by photo-mediated internalization

To determine the optimal transfection conditions for photo-mediated gene complex internalization, ADSCs were transfected with a plasmid encoding reporter gene EGFP with various irradiation powers, and EGFP expression was subsequently detected via western blot, RT-PCR, real-time PCR and confocal microscopy. Western blot analysis showed that there were high levels of EGFP protein in irradiated ADSCs (Fig. 1a and b). EGFP expression was increased with irradiation up to 0.7 J/cm^2. However, when the light exceeded 0.7 J/cm^2, there was a decrease in the EGFP expression. To measure EGFP expression at the mRNA level, RT-PCR and real-time PCR were performed. As shown in Fig. 1c and d, ADSCs irradiated with 0.7 and 1.0 J/cm^2 of photo power had increased EGFP mRNA expression in comparison to the non-irradiated cells (maximum