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Exogenous Nurr1 gene expression in electrically-stimulated human MSCs and the induction of neurogenesis

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ABSTRACT

In this study, synergistic effects of electrical stimulation and exogenous Nurr1 gene expression were examined to induce the differentiation of human mesenchymal stem cells (hMSCs) into nerve cells in *in vitro* culture system. A two-step procedure was designed to evaluate the effects of electrical stimulus and exogenous gene delivery for inducing neurogenesis. First, an electrical stimulation device was designed using gold nanoparticles adsorbed to the surface of a cover glass. Gold nanoparticles, as an electrical conductor for stem cells, are well-defined particles adsorbed to a polyethyleneimine (PEI)-coated cover glass. The nanoparticle morphology was examined by scanning electron microscope (SEM). Second, a plasmid carrying Nurr1 cDNA was complexed with biodegradable poly-(DL)-lactic-co-glycolic acid (PLGA) nanoparticles to support neurogenesis. To evaluate the neuronal differentiation of stem cells mediated by the treatment with either electrical stimulation and exogenous Nurr1 gene delivery, or both, the expression of neuron-specific genes and proteins was examined by RT-PCR and Western blotting. Cells transfected with exogenous Nurr1 genes plus electrical stimulation (250 mV for 1000 s) showed the greatest level of neurite outgrowth with a mean neurite length of 150 μm. Neurite length in cells treated with only one stimulus was not significant, approximately 10–20 μm. These results indicate that electrical stimulation and exogenous Nurr1 gene expression together may be adequate to induce nerve regeneration using stem cells.

1. Introduction

Stem cells have been used in clinical applications by transplantation into damaged organs or tissues. However, stem cell-based tissue regeneration has many limitations, including undesired differentiation of the stem cells into heterogeneous cells. Several approaches have been used to differentiate stem cells into the desired type of cells. In particular, the use of biological stimuli, such as drugs and proteins, has been explored extensively [1–3].

In nerve axonal regeneration, electrical stimulation has played a pivotal role in neurite extension and the regeneration of transected nerve ends, and several types of materials for electrical stimulation have been effective in nerve regeneration [4–7]. Nanoparticle (NP)-modified surfaces have been used to construct homogeneous films for use as an electrical conductor [8]. To construct such devices, negatively-charged particles are complexed with positively-charged surfaces through layer-by-layer (LBL) assembly for the stimulation of cultured cells [9–11]. The construction of NP films by the LBL method has been successfully used to stimulate neurite outgrowth in nerve regeneration [12–15]. Surfaces homogeneously coated with NP-based materials have enabled the spatially controlled construction of conductor layers [16]. Furthermore, both organic and inorganic NPs coated on conventional surfaces have been used as electrical conductors. Electrical charges appear to focus on the stimulation of axonal regeneration; therefore, many electrical stimulating materials have been evaluated to determine if they can be used in the development of effective nerve regeneration. Although the exact mechanisms by which electrical stimulation enhances nerve regeneration are not well understood, it is well known that electrical stimulation enhances both neurite outgrowth *in vitro* and nerve regeneration *in vivo* [9,15].

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In this study, gold NPs coated onto a PEI-precoated cover glass were used to enhance neurite outgrowth and nerve cell differentiation by electrical stimulation. Nano-sized gold particles (20 nm) were used to characterize the potential capacity for stimulating neurite outgrowth *in vitro*. Although many researchers have focused on the nature and possible usage of electrical nano-sized apparatuses, attempts to maintain stable nano-structured surfaces for nerve regeneration have been little known [17–19].

Transfection with plasmid DNA (pDNA) has been also used to induce stem cell differentiation. As representative gene delivery vehicles, viral and non-viral vector systems have been commonly used due to their high transfection efficiency. As a delivery method, cationic polymers such as polyethyleneimine (PEI) and poly(L-lysine) (PLL) have been complexed with DNA and these complexes used for gene transfection both *in vitro* and *in vivo* [20–22].

Nurr1 is a transcription factor belonging to the orphan nuclear receptor superfamily and plays a crucial role in the development and maintenance of dopaminergic neurons, which have been implicated in the pathogenesis of Parkinson's disease [23–25]. Several studies have reported that Nurr1 overexpression can induce the differentiation of stem cells into dopaminergic neurons, suggesting the possibility that genetically-manipulated stem cells can be directed to the dopaminergic fate [26–28]. Recently, Nurr1 deficiency was shown to result in the rapid loss of dopamine, loss of neuron markers and neuron degeneration, indicating that Nurr1 is crucial for maintaining dopaminergic neurons [29].

Herein, PEI-complexed biodegradable PLGA nanoparticles and then mixed with pDNA were added into human mesenchymal stem cells (hMSCs) to induce neurogenesis. Furthermore, electrical stimulation was applied to Nurr1-transfected hMSCs adhered to gold NPs. These co-treatments, electrical stimulation and Nurr1 gene transfection, synergistically enhanced the neurogenesis of hMSCs. To verify hMSC neurogenesis by combined electrical stimulation and Nurr1 gene transfection, FACS, RT-PCR, Western blotting analysis and confocal laser microscopy were used. In addition, the characteristics of biodegradable PLGA nanoparticles were examined by scanning electron microscopy (SEM) and dynamic light scattering (DLS).

2. Materials and methods

2.1. Materials

The poly-(DL)-lactic-co-glycolic acid (PLGA) (Mw: 33,000) was purchased from Boehringer-Ingelheim (Petersburg, USA). Fibrinogen and aprotinin were purchased from the Mokam Research Center. Human MSCs (hMSCs) were purchased from Lonza Ltd. and then cultured (Lot numbers: 7F3674, 7F3675, and 7F3677). Polyvinylalcohol (PVA) (Mn: 1300–23,000), ascorbate, DMSO, Tween, ethidium bromide, HEPES/NaOH, 2.5% gluta-aldehyde, gold nanoparticles, sodium cacodylate, anti-flag and glutamine were purchased from Sigma (Minneapolis, MN). All other chemicals were of reagent grade, and were used as received. Alpha-minimum essential medium (α-MEM), Trypsin–EDTA, phosphate-buffered saline (PBS), fetal bovine serum (FBS), MTT (3-{4,5-dimethylthiazol-2yl}-2,5-diphenyl-2H-tetrazoliumbromide), and penstreptomycin were obtained from GIBCO BRL, Life Technologies (Grand Island, NY). The LIVE/DEAD® Cell Viability Assay Kit, and Pico Green® dsDNA Assay Kit (Molecular Probes-Invitrogen, Paisley, UK). The anti-Nurr1 were from Abcam (Abcam, Cambridge, MA). The polyethyleneimine (PEI) was purchase from Polyscience (Warrington, PA). All cell culture plastics were purchased from Nunc (Roskilde, Denmark).

2.2. Immobilization of gold nanoparticles onto glass

Cover glasses were coated with positively-charged PEI. The polymer was dissolved in distilled water at its native pH (7.4), with no additional salt. Under these conditions, the PEI (Mr: 1800 Da) side chain amines ($pK_a \approx 10$) would be extensively protonated. The cover glasses were soaked for 12 h in 0.1% PEI solution (1 mg/ml), rinsed 3 times in distilled water, and then submersed in gold nanoparticle solution (0.2 mg/ml) for 24 h with gentle stirring. The gold nanoparticle-coated substrate was then rinsed four times in distilled water. This protocol is summarized in Fig. 1.

2.3. Preparation of PLGA nanospheres

PLGA nanospheres were prepared using a water-in-oil-in-water solvent evaporation technique. Briefly, 100 mg of PLGA was emulsified with 1 ml of methylene chloride by sonication for 30 s (sonifier: Bandelin electronic UW 70/HD 70, tip: MS 72/D, Berlin, Germany). After addition of 3 ml of a 7% (w/v) aqueous solution of PVA, the emulsion was sonicated again for 20 s. The resulting double emulsion was then poured into 50 ml of a 1% (w/v) aqueous PVA solution containing 2% isopropanol and then stirred for 1 h at 600 rpm. The residual methylene chloride was then evaporated under vacuum. Aliquots of the nanosphere suspension (1.8 ml) were washed twice with 20 mM HEPES/NaOH (pH 7.0) by centrifugation (7000 rpm, 10 min, 4 °C) and then resuspended. Also, fluorescein isothiocyanate (FITC) loaded PLGA nanospheres were prepared using a water-in-oil-in-water solvent evaporation technique. Briefly, 100 mg of PLGA and 1 mg of FITC were emulsified with 1 ml methylene chloride by sonication for 30 s (Bandelin electronic UW 70/HD 70; tip, MS 72/D, Berlin, Germany). After the addition of 3 ml of a 7% (w/v) aqueous solution of poly(vinylalcohol) (PVA), the emulsion was sonicated again for 20 s. The resulting double emulsion was then poured into 50 ml of a 1% (w/v) aqueous PVA solution containing 2% isopropanol and then maintained under mechanical stirring for 1 h at 600 rpm. The residual methylene chloride was then evaporated under vacuum. Next, aliquots of the nanosphere suspension (1.8 ml) were washed twice with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH (pH 7.0) by centrifugation (7000 rpm, 10 min, 4 °C) and then resuspended.

2.4. Nanoparticle characterization

Biodegradable PLGA nanoparticle size measurements were conducted using the Zetasizer Nano ZS apparatus (Malvern, Southborough, MA). In brief, the nanoparticles were suspended in deionized water at a concentration of 0.1 mg/ml. The mean hydrodynamic diameter was determined via cumulative analysis.

The ζ potential (surface charge) of the polymers and polyplexes was determined at 25 °C using a Zetasizer (Malvern, Worcestershire, UK). Samples were prepared in phosphate-buffered saline (PBS) and diluted 1:9 with deionized water to ensure that the measurements were performed under conditions of low ionic strength where the surface charge of the particles can be measured accurately. The final concentration of the polymer was 1 mg/mL. All data represent 15 measurements from one sample.

2.5. Scanning electron microscopic views of PLGA nanoparticles

Scanning electron microscopy (SEM, Philips 535M) was used to observe the size and morphology of PLGA nanoparticles. The morphology was observed following gold coating using a sputter-coater (HUMMER V, Technics, CA). The mean diameter and morphology of the PLGA nanoparticles, PEI-modified PLGA nanoparticles and PEI-modified PLGA nanoparticles/DNA complexes were characterized via SEM.

2.6. Preparation of PEI-modified PLGA nanoparticle/DNA complexes

The PEI-modified PLGA nanoparticle/DNA complex solutions was prepared as the following method. The particles were formulated using an N:P ratio of 10 (nitrogen (polymer):phosphate (DNA)). A total of 0.02 ml PLGA nanoparticle solution was mixed with 5 μg PEI in sterile HEPES-buffered saline, and the PEI-complexed nanoparticle solution was then added to a plasmid DNA solution (pDsRed-C1, Clontech) at different N:P ratios and vortexed gently. PEI-modified PLGA nanoparticle/DNA complexes at various N:P ratios were diluted into Opti-MEM and incubated for 20 min at room temperature.

2.7. Cell culture

Human MSCs (hMSCs) were cultured in a 75 cm^2 flask with minimum essential medium, α-modification (α-MEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, ascorbate, and glutamine at 37 °C in a humid atmosphere containing 5% CO_2. After 7 days, non-adherent cells were discarded and the adherent cells were cultured to confluence, with medium changes every 3 days.

2.8. Cell viability

The proliferation of cultured hMSCs was determined using the MTT (3-{4,5-dimethylthiazol-2yl}-2,5-diphenyl-2H-tetrazoliumbromide) assay. The medium was removed and 2 ml MTT solution was added to each well. Upon incubation at 37 °C for 4 h in a fully humidified atmosphere at 5% CO_2 in air, MTT was taken up by active cells and reduced in the mitochondria to insoluble purple formazan granules. Subsequently, the medium was discarded, the precipitated formazan was dissolved in DMSO (150 μl/well), and the optical density of the solution was evaluated using a microplate spectrophotometer at a wavelength of 570 nm. The analytical assays were performed every day, and at least 4 wells were randomly examined each time.

Cell viability was also determined following treatment with cytotoxic agents. The cells were washed prior to the assay to remove or dilute the serum esterase