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Extremely low frequency electromagnetic fields enhance neuronal differentiation of human mesenchymal stem cells on graphene-based substrates

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ABSTRACT

Graphene is a non-cytotoxic and biocompatible material which serves as a powerful platform for cell growth, differentiation, and fate conversion. The unique features of this nanomaterial are exploited in regenerative medicine and tissue engineering as a scaffold for biological tissues. Graphene-based substrates support neuronal differentiation of stem cells and hence can potentially be applied in nerve regeneration. Since the exact differentiation mechanism mediated by graphene substrate is not fully understood, the efficiency of the process remains to be improved. Herein, the extremely low frequency electromagnetic fields (ELF-EMF; 50 Hz, 1 mT) exposure synergistically increased biological efficacy of neuronal differentiation in bone marrow-derived human mesenchymal stem cells (hMSCs) grown on graphene-coated substrate. We show that such enhancement in neurogenesis is achieved by altering global gene expression profile thereby up-regulating cell adhesion through intracellular calcium influx and activated focal adhesion kinase signaling pathway, which is stimulated by extracellular matrix production. Our findings may provide the framework for a useful therapeutic strategy in regenerative medicine.

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1. Introduction

Graphene, a single-atom-thick sheet of carbon atoms arranged in a hexagonal lattice, is widely used in various fields including energy technology, biosensors, and photothermal anticancer therapy. The high biocompatibility and stability of graphene attracted attention as a potential scaffold material in tissue engineering [1–4]. In addition, graphene-based materials are non-cytotoxic, and can mediate proliferation, and differentiation, and an alteration of cell fate [5–8]. For example, graphene-based substrates promoted differentiation and reprogramming of cells, such as adult stem cells differentiating into osteoblast, cardiac and kidney cells, and reprogramming of fibroblasts into induced pluripotent stem cells [6,7,9,10]. Furthermore, the neurogenic capacity of stem cells on graphene substrate has attracted attention [11–13].

Bone marrow-derived human mesenchymal stem cells (hMSCs) are capable of self-renewal and are multipotent cells that can differentiate into various lineages [14,15]. MSCs are naturally induced to differentiate into mesodermal cells such as chondroblasts, adipocytes and osteoblasts *in vivo*, but can also transdifferentiate into nerve cells by treating various compounds [16,17]. The neurogenic capacity of MSCs is being applied in stem cell therapies on spinal cord injuries and neurodegenerative disorders such as stroke [15,18]. Recent studies demonstrated that electrophysiological stimulations can also be applied to treat neurodegenerative disorders, along with conventional treatments with numerous factors including growth factor, cytokine and neurotrophins [19–21]. Among different electrophysical stimulations, extremely low-frequency electromagnetic fields (ELF-EMF) affect plethora of cellular functions, and this unique property drew attention in non-invasive therapies [22–27]. Indeed, ELF-EMF stimulated neuronal differentiation of stem cells through the up-regulation of calcium signaling and phosphorylation of cAMP response element binding (CREB) pathway [28]. Calcium signaling triggered by ELF-EMF is highly specific and effective for regulation of neuronal differentiation and survival [23,28,29]. Interestingly, the conductivity generated by ELF-EMF at a level which would not affect most cell types can still alter the neuronal membrane potential leading to the activation of intracellular signaling to neurogenesis [30].

Cell-substratum adhesion regulates cell differentiation,

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proliferation and survival, which is increased in graphene-coated surfaces [7,9,31,32]. In neuronal cells, a high level of cell-substratum adhesion leads to up-regulation of cell adhesion molecules, thereby enhancing axonal alignment and neurite outgrowth [33–35]. Cells secrete extracelluar matrix (ECM) to allow cells to adhere to various substrate, and the secretion and deposition patterns of ECM are affected by the substrate, which in turn alter cellular behavior on the substrate [7,36–38]. Cell attachment to the ECM stimulates integrin-mediated intracellular signal pathway which leads to phosphorylation of focal adhesion kinase (FAK) [33]. FAK-mediated signaling pathway is involved in cell adhesion, invasion, and migration that have crucial roles in neurogenesis [34,39,40]. Along with the activation of FAK via cell-substratum adhesion, neural cell adhesion molecule (NCAM), a cell–cell adhesion molecule, is also known to activate FAK [39–41]. In this study, we examined the combinatorial effects of ELF-EMF and graphene-coated substrate on differentiation of an adult stem cell line (hMSCs) into neuronal cells with emphases on cell-substratum adhesion, calcium influx, intracellular signaling pathway, and the global gene expression profile.

2. Materials and methods

2.1. Preparation of graphene substrate for cell culture

The preparation of graphene was performed on a copper foil film by using a typical chemical vapor deposition (CVD) technique. The exposed copper foil was dissolved in 0.5 M $FeCl_3$ solution for several hours. After the copper foil was completely removed, the graphene sample was picked up by a target substrate. The graphene on glass was rinsed in iso-propyl alcohol and D.I. water for 20 min in order to remove residual Fe^{3+} ions.

2.2. Cell culture and neuronal induction

hMSCs were purchased from Lonza (USA) and cultured on glass or graphene substrate in non-hematopoietic (NH) stem cell medium (Miltenyi Biotech, Germany) in standard culture conditions. Cells were detached by using accutase (Millipore, USA) and cells at passage 5 were used throughout the experiment. At ~80% confluency, differentiation was induced by incubating cells in neuronal differentiation medium (NM) composed of Dulbecco's modified Eagle medium (DMEM)/F12 with 5 mM KCl (Sigma, USA), 1 μM hydrocortisone (Sigma), 10 μM forskolin (Sigma), 2 μM valproic acid (Sigma), and 5 μg/ml insulin (Welgene, Korea) for 14 days.

2.3. ELF-EMF exposure

We employed a previously described ELF-EMF exposure system and cells were uniformly exposed to the field [40,41]. Non-exposed control cells were grown in a different CO_2 incubator under the same conditions without exposure to ELF-EMF. Cells were exposed to continuous sinusoidal ELF-EMF ($B_m = 1$ mT; $F = 50$ Hz sinusoidal) during entire period of differentiation in NM medium or 30 min in specified media.

2.4. Cell adhesion assay

Cells were seeded on glass or graphene substrate at a density of 7×10^5 cells/cm^2. To measure the relative adhesion ratio of cells on the two substrates at 24 h after seeding, the cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) for 1 min. The images of cells on the two substrates were captured by a confocal microscope (Nikon, Japan) and the number of cells stained with DAPI was scored. The relative adhesion ratio was calculated by normalizing the number of DAPI-stained cells on the glass or graphene substrate exposed to ELF-EMF and non-exposed control cells, respectively.

2.5. Calcium influx assay

To measure calcium influx level, cells were seeded and cultured on the two substrates as described in adhesion assay. Cells were maintained in NM or DMEM/F12 medium in the presence or absence of ELF-EMF exposure for 30 min, respectively. The cells were then washed twice with Dulbecco's phosphate buffered saline (DPBS) (Welgene), and loaded with 5 μM Fluo-3/AM (Invitrogen, USA) for 30 min at 37 °C in the dark. Cells were rinsed twice with DPBS to remove the extracellular Fluo-3/AM and observed by a confocal microscope.

2.6. Immunocytochemistry analysis

Cells cultured on the two substrates were fixed with 4% formaldehyde (Sigma), permeabilized with 0.25% Triton X-100 (Sigma) and treated for blocking with 1% bovine serum albumin (Welgene). Cells were then incubated with primary antibodies against different neuronal markers including nestin, NCAM, FAK (Cell Signaling, USA), β3 tubulin (TUJ-I), microtubule-associated protein 2 (MAP2) (Santa Cruz, USA) for 1 h at room temperature. In order to detect cells bound by primary antibodies, the cells were incubated with either alexa488-conjugated anti-mouse or alexa555-conjugated anti-rabbit secondary antibodies (Cell Signaling). DAPI was used for counterstaining nuclei. Fluorescent images were captured by confocal microscopy and analyzed by ImageJ program (National Institutes of Health, USA).

2.7. RT-PCR analysis

RNA was isolated using RNeasy Mini Kit (Qiagen, USA) as per manufacturer's protocol. cDNA was synthesized from 0.5 μg of total RNA with TOYOBO ReverTra Ace® qPCR kit (TOYOBO, Japan) according to the manufacturer's instruction. To amplify cDNA, 35 cycles of PCR was performed by using 2x KAPA2G Fast HotStart Ready Mix (KAPA Biosystens, USA). PCR products were separated by 1% agarose gel electrophoresis and visualized by UV light. The intensity of fluorescent DNA bands was quantified by using ImageQuant software (Molecular Dynamics, USA).

2.8. Western blot analysis

Western blot was performed as standard protocol. All antibodies were purchased from Santa Cruz. Mouse anti-fibronectin and anti-β-actin (an internal control) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody was used as secondary antibody.

2.9. Microarray analysis

Global gene expression analysis was carried out by using Affymetrix GeneChip® Human Gene 2.0 ST oligonucleotide arrays (Affymetrix, USA). Total RNA samples were isolated and processed as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetrix, Inc.). The chips were stained and washed in a Genechip Fluidics Station 450 (Affymetrix, USA) and scanned by using a Genechip Array scanner 3000 7G (Affymetrix). Scanned image data were extracted through Affymetrix Command Console software v 1.1, and the expression data were generated by Affymetrix Expression Console software v 1.1. For the normalization, RMA (Robust Multi-Average) algorithm implemented in Affymetrix Expression Console software was used. In

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