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Directing neural differentiation of mesenchymal stem cells by carboxylated multiwalled carbon nanotubes

Yu-Shuan Chen [a], Ging-Ho Hsiue [a,b,*]

[a] Department of Chemical Engineering, National Tsing Hua University, No. 101, Sec. 2, Kuang-Fu Road, Hsinchu 30013, Taiwan, ROC
[b] Department of Chemical Engineering/R&D Center for Membrane Technology, Chung Yuan University, No. 200, Chung Pei Road, Chung Li 32023, Taiwan, ROC

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ABSTRACT

The use of carbon nanotubes (CNTs) to promote neural differentiation is well known. However, most studies have focused on the effect of CNT-derived composites or CNT-based nanopattern substrates on differentiation. Whether or not the intrinsic properties of CNTs themselves can affect neural differentiation and the differentiation mechanism have not been fully investigated. We demonstrated that carboxylated multiwalled carbon nanotubes (MWCNTs) can induce and maintain neural differentiation of human bone marrow mesenchymal stem cells (hBMMSCs) without any exogenous differentiating factors, as evidenced by the protein expression. The low cytotoxicity of carboxylated MWCNTs was also shown by a proliferation assay. Quantitative real-time polymerase chain reaction (Q-PCR) data revealed that neural-associated genes, including growth and transcription factors, were promoted while bone-associated genes were inhibited when the cells were cultured on carboxylated MWCNTs. These up-regulated neural growth factors can also adsorb onto carboxylated MWCNTs. The data suggest that carboxylated MWCNTs play dual roles: promoting hBMMSC neural differentiation, including up-regulating the neural growth factors; and trapping these neural growth factors to create a suitable environment for long-term neural differentiation. Carboxylated MWCNT substrates may provide a method of post-transplantational spontaneous neural differentiation with low cytotoxicity for neuron injury repair.

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1. Introduction

The use of stem cells for the treatment of neural disease in regeneration medicine has become an ongoing trend in recent studies. Human mesenchymal stem cells (hMSCs) are an excellent resource in tissue engineering for cell therapy based on their biological properties, which include easy isolation and expansion and an immunomodulation ability [1,2]. Most importantly, they can be derived from the patient himself to provide autologous transplantation without any ethical concerns [2,3]. hMSCs have been shown to not only have the ability to trans-differentiate into neuroectoderms, expressing neurons, and glial markers but also possess electrophysiological properties in the presence of differentiating factors in vitro [4,5]. Although these studies on hMSCs indicate the potential for stem cell therapy in the treatment of neural injury, introducing exogenous differentiating factors for differentiation of hMSCs under in vivo transplantation is very difficult. Hence, undifferentiated or pre-differentiated hMSCs are mostly used in stem cell therapy studies at present. Undifferentiated hMSCs have been demonstrated to possess immunomodulated ability and secret neuroprotective factors to cure neural diseases [6], but their ability to trans-differentiate for cell replacement is still doubtful [7,8]. Pre-differentiated cells have limited application due to a poor cell survival rate and decreased secretion of soluble factors for cell therapy [9,10]. Therefore, the establishment of substrates directing post-transplantation differentiation without the addition of differentiating factors would be a benefit for cell therapy.

CNTs have performed outstandingly in the field of biomedicine; recent applications include bio-sensors, drug control release, and regeneration medicine [11]. Neuroscience, which is one field that uses regeneration medicine, has many applications using CNTs. CNTs have unique properties including electrical conductivity, nanostructures of hollow graphite cylinders, and non-biodegradability [12–15]. The ability of CNTs—either CNT-derived

* Corresponding author. Department of Chemical Engineering, National Tsing Hua University, No. 101, Sec. 2, Kuang-Fu Road, Hsinchu 30013, Taiwan, ROC. Tel.: +886 3 5719956; fax: +886 3 5726825.
E-mail addresses: ghhsiue@mx.nthu.edu.tw, ghhsiue@che.nthu.edu.tw (G.-H. Hsiue).

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composites or CNT-based nanopattern substrates—to promote neural differentiation of neural stem cells (NSCs) and hMSCs has been widely investigated [16–20]. However, it is unclear whether the intrinsic properties of CNT itself affect neural differentiation. Current studies have revealed little information on the differentiation mechanism for CNTs.

In this study, carboxylated MWCNTs films deposited onto a collagen (CA)-coated polystyrene dish were prepared for hBMMSC culturing. Immunofluorescence staining with neural markers was used to determine whether carboxylated MWNCTs in a basal medium can direct hBMMSC differentiation without exogenous differentiating factors. The cytotoxicity of the carboxylated MWCNTs was verified by cell morphology observation and proliferation assay. Q-PCR was used to analyze the regulation of growth factors, transcription factors, and neural lineage markers to resolve the intracellular pathway in hBMMSCs. We then further examined the relation between the regulated growth factors and carboxylated MWCNTs through an experiment on protein adsorption to determine the effect of carboxylated MWCNTs on neural differentiation.

2. Materials and methods

2.1. Characterization of MWCNTs

MWCNTs were received from Carbon Nanotube Co., Ltd., Incheon, Korea. MWCNTs were carboxylated by refluxing 65% HNO_3 for 1 d and then washed several times with distilled water by filtration until the pH reached 7.0. The samples were characterized by high-resolution transmission electron microscopy (HR-TEM, JEOL JEM-2010) and Fourier transform infrared (FT-IR, Perkin–Elmer). The samples observed with HR-TEM were suspended in ddH_2O for sonication for 4 h and then dripped onto Formvar stabilized with carbon grids (Ted Pella). The samples prepared for FT-IR analysis were mixed with potassium bromide at the ratio of 1:100 and detected by the Perkin–Elmer spectrum over 32 scans to obtain reliable data.

2.2. Preparation of carboxylated MWCNT-coated substrates

The carboxylated MWCNT solution was prepared by the addition of 0.5 mg/ml carboxylated MWCNT in distilled water (pH 7.2); this was then sterilized at 120 °C for 15 min by autoclaving. The sterilized solution was sonicated for at least 4 h by sonicator (Delta) and then centrifuged at 5000 rpm for 10 min. The centrifuged carboxylated MWCNT solution was homogenously deposited onto the collagen (CA) type I (Sigma, from rat tail) pre-coated culture dishes or cover slips for 3 h at room temperature; the solution was then discarded. The substrates were then washed several times with distilled water until almost all of the suspended and unattached carboxylated MWCNTs were removed. The carboxylated MWCNT-coated substrates were dried overnight in a laminar hood for hBMMSC cultivation.

2.3. hBMMSC viability and proliferation assay

The hBMMSCs (ScienCell Research Laboratories) were regularly expanded by following the manufacturer's instructions. In brief, regular expansion of the cells was cultured in a mesenchymal stem cell medium (MSCM, ScienCell Research Laboratories) on a poly-L-lysine (PLL, ScienCell Research Laboratories) coated cultured flask. After cell expansion, the spontaneous differentiation condition was induced on control or MWCNT substrates in the basal medium. The seeding density for spontaneous differentiation of the cells was 1×10^4 cells/cm^2. During the culture period of spontaneous differentiation, fresh basal medium was changed every 2 days until the indicated day. The basal medium contained high-glucose Dulbecco's Modified Eagles media (Invitrogen), 5% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acid, and 1% of penicillin/streptomycin mixture. The growth curve was applied with an MTS reagent (Promega), and the assay protocol was performed following the manufacturer's instructions.

2.4. Q-PCR

Two groups of cells were cultured under the spontaneous differentiation protocol for 8 days; the total RNA was then obtained using an RNeasy Mini Kit (QIAGEN). Q-PCR analysis was then performed using MB (Mission Biotech) following the two-step RT-PCR procedure. The forward and reverse primers are in Table S1 of Supporting Information. In brief, the RNA samples were reverse-transcribed for 120 min at 37 °C with a high-capacity cDNA reverse transcription kit (Applied Biosystems). Q-PCR was performed under the following conditions: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems), and 200 nM of forward and reverse primers. Data were obtained as average *CT* values and normalized with GAPDH as ΔCT. Expression changes in the gene transcripts for carboxylated MWCNTs were calculated as fold changes with respect to the cells cultured on the control group using $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT$ of MWCNT group$-\Delta CT$ of control group).

2.5. Immunofluorescence analysis

Two groups of the cells with spontaneous differentiation at 8 and 14 days were fixed by paraformaldehyde (Alfa Aesar) for 20 min. The cells were permeabilized with 0.2% triton-X in PBS for 5 min and then blocked with 1% BSA in PBS for 1 h. The blocking reagent was removed, and the cells were incubated with primary antibodies including mouse anti-MAP2 (1:200, Millipore), rabbit anti-GFAP (1:500, Abcam), and rabbit anti-synaptophysin (1:100, Abcam) overnight at 4 °C. The cells were then incubated with secondary antibodies, including anti-rabbit (1:800, Abcam) and anti-mouse (1:500, Invitrogen). After 60 min, the samples were washed with PBST and then stained with DAPI (Invitrogen) for the nucleus. The positive cells were visualized by confocal fluorescence microscopy (Zeiss) for at least 10 fields captured at 200× magnification.

2.6. Quantity of protein adsorption

A suspended carboxylated MWCNT solution was homogenously prepared at a concentration of 30 μg/ml in ddH_2O by sonication. Proteins including BDNF, IGF1, NGF, and GDNF (all from PEPROTECH) were dissolved in PBS to obtain a concentration of 30 μg/ml. Equal volumes of carboxylated MWCNTs and each protein were gently mixed and then rotated at 10 rpm for 4 h under ambient temperature. After the adsorption procedure, the mixed solution was centrifugated at 15,000 rpm for 60 min at 4 °C to separate the un-adsorbed protein (supernatant) and carboxylated MWCNTs (pellet). The protein concentrations were detected with the micro BCA™ protein assay kit (PIERCE). The adsorbed proteins were calculated by deducting the un-adsorbed protein from the total proteins.

2.7. Statistical analysis

Data for the growth curve, Q-PCR, immunofluorescence, and protein adsorption were presented as means ± standard deviation (SD). The statistical significance between compared groups with regard to Q-PCR (ΔCT of MWCNT and control) and immunofluorescence was determined using the paired Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Characterization of carboxylated MWCNTs

To remove metallic and carbonaceous impurities for cell culturing available, HNO_3 was used to prepare carboxylated MWCNTs. The structures of carboxylated MWCNTs were characterized by HR-TEM and FT-IR. Fig. 1A shows the diameter of carboxylated MWCNTs (20–35 nm) and their multi-walled structure. FT-IR characterization confirmed that the absorption of the carboxylic acid groups of carboxylated MWCNTs at 3413, 1627, and 1463 cm^{-1} was significantly increased compared with that of unmodified MWCNTs (Fig. 1B).

3.2. Cellular behavior on MWCNT group

To prepare substrates coated with carboxylated MWCNTs for hBMMSCs culturing, CA is a required pre-coating reagent to avoid the MWCNTs peeling off and floating away from the cultured substrates after the addition of the culture medium [21]. Thus, the CA-coated dishes were defined as the control group in the experiments. The carboxylated MWCNTs deposited on CA-coated dishes were defined as the MWCNT group. Fig. 2A shows the schematic for the preparation of the two groups. The distribution of carboxylated MWCNTs on the MWCNT group was observed using a phase-contrast light microscope. The data showed that the carboxylated MWCNTs were deposited homogeneously on the CA-coated dish; they did not peel off but formed a thin film on the dish after the medium was added (Fig. S1).

hBMMSCs were used in this study. Data on the flow cytometry and morphology images confirmed that the hBMMSCs were very

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