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Enhanced neuronal differentiation of pheochromocytoma 12 cells on polydopamine-modified surface

Suk Ho Bhang [a,1], Sun-Hyun Kwon [a,1], Seahyoung Lee [a,1], Gui Chul Kim [b], Ah Mi Han [b,c], Yun Hee Kim Kwon [b,c], Byung-Soo Kim [a,d,*]

[a] School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea
[b] Department of Biology, Kyunghee University, Seoul 130-701, Republic of Korea
[c] Department of Life and Nanopharmaceutical Science, Kyung Hee University, Seoul 130-701, Republic of Korea
[d] Bio-MAX Institute, Institute of Chemical Processes, Engineering Research Institute, Seoul National University, Seoul 151-744, Republic of Korea

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ABSTRACT

Since pheochromocytoma 12 (PC12) cells have the ability of neuronal differentiation upon nerve growth factor (NGF) treatment, they are a good model for studying the neuronal differentiation. Establishing a strong adhesion of PC12 cells to the culture substrate may increase neuronal differentiation, and the use of L-3,4-dihydroxyphenylalanine (L-DOPA), which is responsible for the adhesive property of mussel adhesive proteins (MAPs), is a feasible strategy for such strong adhesion. We hypothesized that a polydopamine-modified surface can promote PC12 cell adhesion and subsequent neuronal differentiation. We examined whether polydopamine-modified surface promotes PC12 cell adhesion, and further evaluated the neuronal differentiation of these cells. The polydopamine modification enhanced the cell adhesion and viability, and also promoted the neuronal differentiation of NGF-stimulated PC12 cells, as evidenced by the elongation of neurites and expression of neuronal differentiation markers, by increasing the activation of NGF/Trk-Rho GTPase signal pathway. Our findings will help develop an improved strategy for functionalizing biomaterial substrates for less-adhesive cells including neural cells.

1. Introduction

Pheochromocytoma 12 (PC12) cells, which are derived from a rat pheochromocytoma, have been used for studying neuronal differentiation mechanisms since the discovery of their neuronal differentiation ability upon nerve growth factor (NGF) treatment [1]. In serum-free differentiation medium supplemented with NGF, PC12 cells stop proliferating, sprout neurites, and acquire the properties of sympathetic neurons [2]. The neurite outgrowth, a characteristic of neuronal differentiation, has been suggested to be linked to enhanced cell adhesion [3–5], suggesting cell adhesion to the culture substrate may play an important role in neuronal differentiation of PC12 cells [6,7]. Consequently, establishing a strong adhesion of PC12 cells to the culture substrate may result in the increase of neuronal differentiation.

One of the possible strategies to facilitate the strong adhesion of PC12 cells to the culture substrate is the use of L-3,4-dihydroxyphenylalanine (L-DOPA). The mussel adhesive proteins (MAPs) [8] enable the mussels to adhere firmly to wet marine surfaces. The adhesive property of MAPs is attributed to the presence of DOPA [9]. L-DOPA is known to have cytotoxic effects on neurons [10]. However, a low dose of L-DOPA has been reported to protect PC12 cells from serum depletion-induced cell death [11], and a more recent study demonstrated that the polydopamine surface modification attenuated cytotoxicity of underlying materials [12]. Thus, we hypothesized that polydopamine surface modification can promote PC12 cell adhesion and subsequent neuronal differentiation without any significant cytotoxicity.

We examined whether surface modification with polydopamine promotes the adhesion of PC12 cells in a conventional serum-containing growth medium and also assessed PC12 cell adhesion in a serum-free medium to clarify the effect of polydopamine modification on PC12 cell adhesion without interference from the cell adhesion molecules contained in the serum. Additionally, the neuronal differentiation of these cells were induced by subsequently culturing in an NGF-supplemented, serum-free N2 differentiation medium to evaluate the effect of the polydopamine surface modification on the neural differentiation of the PC12 cells. The spreading, viability, and apoptosis of the PC12 cells cultured on surfaces with different coatings were also examined. The neuronal differentiation of the PC12 cells, which was evaluated by measuring the neurite length and determining the expression levels of neuronal differentiation markers, on the polydopamine-modified surface

* Corresponding author at: School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea. Fax: +82 2 888 1604.
E-mail address: byungskim@snu.ac.kr (B.-S. Kim).
[1] These authors contributed equally.

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was compared to that of the PC12 cells cultured on the gelatin-coated surface [13], which served as a control.

2. Materials and methods

2.1. Gelatin coating and polydopamine modification on culture surface

Culture dishes were coated with gelatin by incubating with 0.1% (v/v in distilled water) gelatin solution overnight at room temperature, followed by washing with sterile phosphate-buffered saline (PBS, Sigma–Aldrich, St. Louis, MO, USA). For the polydopamine modification, L-DOPA solution was prepared by dissolving 2 mg of L-DOPA (D9628, Sigma–Aldrich) in 1 ml of 10 mM Tris buffer base (pH 8.5, Sigma–Aldrich). Under this basic condition, L-DOPA undergoes oxidative polymerization resulting in formation of polydopamine layer on the substrate. The culture dishes were treated with the L-DOPA solution overnight at room temperature, followed by washing with sterile PBS.

2.2. PC12 cell culture

PC12 cells (Paragon Biotech, Baltimore, MD, USA) were maintained in Roswell Park Memorial Institute 1640 growth medium (RPMI 1640, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 7.5% (v/v) fetal bovine serum, 7.5% (v/v) horse serum, and 1% (w/v) penicillin/streptomycin. The cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in air, and cells within 10 passages were used for the experiments. The medium was changed every 2 days. For neuronal differentiation, the medium was replaced by the chemically defined N2-supplemented serum-free medium, which was a 2:1 mixture of Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and PBS with 5 mg/ml insulin (Sigma–Aldrich), 100 mg/ml transferrin (Sigma–Aldrich), 20 nM putrescine dihydrochloride (Sigma–Aldrich), and 30 nM sodium selenite (Sigma–Aldrich) [14]. For the induction of neuronal differentiation, nerve growth factor (NGF, 100 ng/ml, Invitrogen, Carlsbad, CA, USA) was added daily for 3 days.

2.3. Contact angle measurements

All of the samples were rinsed 3 times in distilled water, soaked for 30 min to ensure the removal of the residues from the surface, and dried in a vacuum drier. The contact angles were measured using a contact angle analyzer (DSA 100, KRÜSS, Hamburg, Germany).

2.4. Scanning electron microscopy (SEM) imaging

The PC12 cells were cultured on coverslips coated with either gelatin or L-DOPA for 2 h. The cells were fixed in 1% (v/v) glutaraldehyde in distilled water for 1 h. After dehydration in a series of graded ethanol, the coverslips were dried and coated with platinum particles for SEM imaging using the JSM-6701F device (JEOL, Tokyo, Japan).

2.5. Neutral Red assay

The cell viability was evaluated by a rapid colorimetric test based on the uptake of a cationic supravital dye, Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride), into viable cells. Briefly, the cells were rinsed with PBS, a medium containing 50 μg/ml of Neutral Red was replenished, and the cells were incubated for an additional 3 h. After this incubation, the solution was rapidly removed and 0.2 ml of acetic acid (1%,v/v) and ethanol (50%,v/v) was added to each well to extract the dye. After 5 min at room temperature, the absorbance at 540 nm was measured. The intensity of the red color was directly proportional to the number of viable cells.

2.6. Mitochondrial metabolic activity assay

The mitochondrial metabolic activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (2 mg/ml in PBS, Sigma–Aldrich) was added to the culture plates and incubated for 4 h at 37 °C and then replaced with dimethyl sulfoxide (DMSO, 100 ml/well) to dissolve the formazan crystals. The absorbance at 570 nm was measured.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was performed using 5 μg of pure total RNA and SuperScript™ II reverse transcriptase (Invitrogen), followed by PCR amplification of the synthesised cDNA. The PCR product were electrophoresed on a 2% (w/v) agarose gel, visualized using ethidium bromide staining, and analyzed using a gel documentation system (Gel Doc 1000, Bio-Rad, Hercules, CA, USA). β-actin served as an internal control. The results of the RT-PCR were quantified using an Imaging Densitometer (Bio-Rad).

2.8. Western blot

Equal amounts of protein from each sample were mixed with sample loading buffer, loaded, and separated by electrophoresis. The proteins separated by SDS–PAGE were transferred to Immobilon-P membranes (Millipore Corp., Billerica, MA), and the membranes were incubated with primary antibodies overnight at 4 °C and then washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Sigma–Aldrich) for 50 min at room temperature. The blots were developed using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA). The luminescence was recorded on X-ray film (Fuji super RX, Fujifilm Medical Systems, Tokyo, Japan), and the bands were imaged and quantified using an Imaging Densitometer (Bio-Rad).

2.9. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed in PBS. Primary antibodies against β-tubulin (Sigma–Aldrich), caspase-3 (Abcam, Cambridge, MA, USA), and fascin (ECM biosciences, Versailles, KY, USA) were used. The slides were then incubated in PBS containing rhodamine- or FITC-conjugated secondary antibodies (Jackson-Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. For the cell adhesion area measurement, the PC12 cells were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). After washing twice with PBS, the DiI-labeled cells were fixed with 4% paraformaldehyde. The Alexa Fluor 488-labeled phalloidin staining kit (Invitrogen) was used to stain F-actin according to the manufacturer's instructions. The color of F-actin staining was changed from green to red, as a pseudocolor, for clear comparison to FITC-labeled fascin. All of the slides were mounted with mounting solution containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) to stain the nuclei and were photographed using a fluorescent microscope (Olympus, Tokyo, Japan).

2.10. Statistical analysis

All of the quantitative data are expressed as the mean ± standard deviation (SD) of at least 5 independent experiments. For