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Brief communication

Micropatterning–retinoic acid co-control of neuronal cell morphology and neurite outgrowth

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ABSTRACT

Creating physical–biochemical superposed microenvironments optimal for stimulating neurite outgrowth would be beneficial for neuronal regenerative medicine. We investigated potential co-regulatory effects of cell micropatterning and retinoic acid (RA) soluble factor on neuronal cell morphology and neurite outgrowth. Human neuroblastoma (SH-SY5Y) cell patterning sensitivity could be enhanced by poly-L-lysine-g-polyethylene glycol cell-repellent back-filling, enabling cell confinement in lanes as narrow as 5 μm. Cells patterned on narrow (5 and 10 μm) lanes showed preferred nucleus orientation following the patterning direction. These cells also showed high nucleus aspect ratio but constrained nucleus spreading. On the other hand, cells on wide (20 μm and above) lanes showed random nucleus orientation and cell and nucleus sizes similar to those on unpatterned controls. All these changes were generally maintained with or without RA. Confining cells on narrow (5 and 10 μm) lanes, even without RA, significantly enhanced neurite extension relative to unpatterned control, which was further stimulated by RA. Interestingly, cell patterning on 5 and 10 μm lanes without RA produced longer neurites relative to the RA treatment alone case. Our data on the potential interplay between microscale physical cell confinement and RA-soluble stimulation may provide a new, integrative insight on how to trigger neurite/axon formation for neuronal regenerative medicine.

1. Introduction

Loss of functional circuits and failure in effective neuronal regeneration are typical of various neurological disorders, e.g. Alzheimer's disease, Parkinson's disease, multiple sclerosis, traumatic brain injury, etc. Proper in vitro organization and interconnection of neuronal cells mimicking in vivo neuronal architecture may elucidate the mechanisms behind these diseases and provide new protocols to efficiently regenerate damaged neurons. In this regard, controlling geometric cell organization using neuronal cells and precursor cells via micropatterning has recently been pursued [1–9].

As noted in our previous reviews [10,11], the use of micropatterned substrates for controlling cell function and fate has a strong rationale. Decoding the mechanism of microenvironmental control of cell behavior and the role of cell geometry in this process can be better achieved via micropatterning [12,13]. Confining cells within well-defined extracellular matrix (ECM) protein patterns provides controllable cell morphologies (size, shape and interconnectivity among cells), which can be used to examine the geometric control of cell growth, differentiation and fate in a systematic manner [11]. This approach may also fulfill biomimetic considerations by mimicking in vivo cell architecture and physiology. For neuronal cells, micropatterns have been utilized to control neuronal cell morphology [2,5], organize neuronal networks [3,7] and direct neuronal differentiation of stem cells [8,9].

Various soluble factors, e.g. retinoic acid (RA) [14,15], nerve growth factor [16], brain-derived neurotrophic factor [17] and neuropathiazol [18], have been used to repair/rejuvenate functional neurons. These neurogenic soluble cues and micropatterning-based geometric control may be integrated to induce enhanced neuronal regeneration. However, little has been revealed as regards potential geometric-biochemical synergistic or competitive regulation of neuronal cells. If geometric control renders neuronal cells more responsive to soluble signals, or vice versa, it may be beneficial for successful neuronal regenerative medicine. We patterned SH-SY5Y human neuroblastoma cells on microscale collagen-I lane patterns. The back-filling of collagen-unpatterned region with poly-L-lysine-g-polyethylene glycol (PLL-g-PEG) enabled neuronal cell confinement within lanes as narrow as 5 μm. Patterned cells were grown with or without RA to examine potential interplay between geometric and soluble cues in regulating

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neuronal cell behavior. We reveal the control of neuronal cell morphologies (nucleus orientation and aspect ratio, cell and nucleus sizes) and neurite outgrowth by geometric (micropatterning) and soluble (RA) superposed cues.

2. Materials and methods

2.1. Microcontact printing and PLL-g-PEG back-filling

A microcontact printing, modified from a recent Cold Spring Harbor protocol [19], was utilized. Details of photolithography, microcontact printing of collagen-I and PLL-g-PEG back-filling are explained in the Supplementary data (Fig. S1). Collagen-I and fluorescein isothiocyanate (FITC)-conjugated collagen-I were purchased from Sigma, and PLL-g-PEG and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated PLL-g-PEG from SuSoS (Switzerland). Using dye-conjugated collagen and PLL-g-PEG, proper back-filling was confirmed (Fig. 1). A photomask designed to have arrays of 5, 10, 20, 30 and 40 μm width lanes separated by 50 μm gaps was utilized, each design being within a square of 1 cm^2.

2.2. Cell patterning and growth

Protein-micropatterned substrates were sterilized for 60 min under UV in a cell culture hood before cell seeding. Using growth media composed of Dulbecco's Modified Eagle Medium, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen), SH-SY5Y human neuroblastoma cells were seeded at 15,000 cells cm^{-2} on protein-patterned substrate and incubated at 37 °C and 5% CO_2. After 90 min, unattached cells were removed by washing with phosphate-buffered saline (PBS, Invitrogen). Cells were grown using the growth media for 7 days, with or without RA exposure (10 μg ml^{-1}, Sigma), with media changed every 2 days.

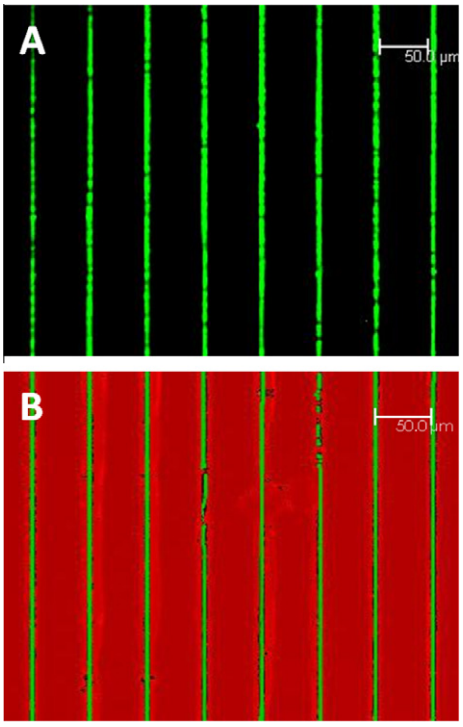

Fig. 1. Protein patterning and cell-repellent back-filling. (A) FITC-conjugated (green) collagen-I lanes patterned to have 5 μm width and 50 μm gap. (B) Collagen-I lane patterns (green) were back-filled with TRITC-conjugated (red) PLL-g-PEG. Scale bar = 50 μm.

2.3. Immunofluorescence

After 24 h of patterning (day 1) and on day 7, patterned cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Actin was stained with 5 μl ml^{-1} rhodamine phalloidin (Invitrogen) in PBS. Nucleus was double-stained with 2 μl ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI, Sigma) in PBS. Cells were observed using a Leica DMI 4000B fluorescence microscope and a Labomed TCM 400 optical microscope.

2.4. Nucleus orientation and aspect ratio measurement

To measure nucleus orientation, the protein patterning direction was referenced as 0° (Fig. S2). From the DAPI-stained image, right (or left) deviation of the axis showing the longest nucleus length from the 0° reference was measured as a nucleus orientation angle. To measure the aspect ratio, the length (width) along the axis perpendicular to the axis showing the longest length was obtained. The aspect ratio was calculated as the ratio of the longest length to the width of the nucleus. For orientation and aspect ratio, at least 100 nuclei for each condition were analyzed from four different imaging spots.

2.5. Cell and nucleus area measurement

We developed a technique to quantify average cell area even for cells adhered to each other (e.g. cells patterned on wide protein lanes). The steps quantifying the cell area using ImageJ are illustrated in the Supplementary data (Fig. S3). Briefly, a total area covered by the entire cells was obtained from the actin image through adjustments of fluorescent color thresholds, and the number of cells was counted from the DAPI image. Cell area was calculated by dividing the area covered by the cells by the total number of nuclei (cells). Ten images from different spots, each containing at least 100 cells, were analyzed for each condition.

2.6. Neurite length measurement

Neurite length was measured by NeuronJ, an ImageJ add-on software, using optical microscopy images. By using NeuronJ, the path of each neurite could be traced out irrespective of whether it is straight or curved (pink trace, Fig. 6C). No established criterion has been reported in the literature as regards where to start the neurite length measurement. We arbitrarily chose in this study that cellular extension would be considered as a neurite when its width becomes less than 3.85 μm (Fig. 6A). When two cells were connected by neurites, one-half of the total traced length was set as one neurite length. A total of at least 100 neurites per condition was analyzed.

2.7. Statistics

In quantifying cell and nucleus morphologies, data from cells grown without back-filling were excluded as these cells were not confined within user-defined geometry. Statistical significance among the data was assessed by one-way analysis of variance followed by Student–Newman–Keuls post hoc tests.

3. Results

3.1. Efficacy of protein patterning and PLL-g-PEG back-filling

Representative fluorescent images of collagen-I (FITC-conjugated, green, 5 μm lane width separated by 50 μm gap) patterned on tissue culture polystyrene (TCPS) substrate are shown in Fig. 1A

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