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Combined effects of flow-induced shear stress and micropatterned surface morphology on neuronal differentiation of human mesenchymal stem cells

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This study investigated the combined effects of surface morphology and flow-induced shear stress on the neuronal differentiation of human mesenchymal stem cells. First, to examine the effect of surface morphology, three patterns were fabricated using photolithography and compared to the flat substrate. After selecting the most effective surface pattern, flow-induced shear stresses (0.10 and 0.25 Pa) were engaged parallel to the direction of the grooves. The degrees of alignment and neurite outgrowth were measured using digital image processing techniques for up to 10 days. Functional evaluations were also performed by monitoring the intracellular calcium concentration and the expression of synaptophysin, β-tubulin III, and MAP2. Based on these analyses, the pattern of 5 μm/5 μm/3 μm for groove/ridge/depth, respectively, was selected. Next, shear stresses (0.00, 0.10, 0.25 Pa) were applied to the cells on the selected substrate. The shear stresses affected the expression of those markers. The outgrowth measurements indicated that the shear stresses were effective at day 7. However, the effect of shear stresses tended to decrease at day 10. More cells showed higher calcium concentrations under 0.10 Pa. The alignment was also confirmed. Taken together, these results indicated that a shear stress of 0.10 Pa on the substrate of 5 μm was most effective. Therefore, such combination of mechanical stimuli and surface pattern is expected to promote neuronal differentiation with regard to functional and morphological changes.

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[**Key words:** Mesenchymal stem cells; Neuronal differentiation; Functional differentiation; Micropatterned substrate; Fluid flow-induced shear stress; Calcium imaging]

One of the prominent methods for treating neuronal defects or related diseases is to utilize stem cells. Among the various kinds of stem cells, mesenchymal stem cells (MSCs) are obviously closer to the stage of clinical application than the other stem cells, such as embryonic or induced pluripotent stem cells (1,2). Although various concrete methodologies for the differentiation of mesenchymal stem cells into any specific cell lineages have been studied over the past decades, much remains to be studied or controlled. The starting point of current methodologies relies on the realization of biomimetic environments at the bench (3). The environments that cells experience in the human body can be classified according to biochemical and biophysical factors. The biophysical factors can be addressed by combining the material and mechanical factors. Therefore, it would be natural to combine all of these factors when we try to control the differentiation of stem cells in vitro.

Various methods have been introduced to control the differentiation of stem cells into neuronal cells. Various biochemical reagents, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) (4), cyclic adenosine monophosphate (cAMP), and isobutylmethylxanthine (IBMX) (5,6) have been demonstrated to be effective. Specifically, the differentiation of MSCs into neurons has been reported to occur within 10 h with dimethyl sulfoxide (DMSO), beta-mercaptoethanol (BME), and butyl hydroxyanisole (BHA) based on Western blotting and morphological observations (7,8). However, some investigators have stipulated that the use of DMSO may induce cytoplasm retraction, thereby causing morphological changes to occur (9,10). Therefore, the use of biochemical reagents should be accompanied by functional evaluations.

The morphology of the substrate surface on which cells are cultured is known to be another important factor that regulates differentiation into neuronal cells. Several studies have reported that the micropattern of substrate surfaces consisting of grooves and ridges can have a positive effect on protein expression, neurite outgrowth, and the direction of their growth (11–14).

Meanwhile, the role of mechanical stimulation in regulating stem cell differentiation has been well recognized over the last two decades (15–19). In addition the engagement of flow-induced shear stress has been widely adopted even in other types of cells for elongation, alignments (20–22). However, studies on the effect of mechanical stimulation on neuronal differentiation have been

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limited. The effect of flow-induced shear stress on the neurite outgrowth of PC-12 cells has been described (23), and shear stress was reported to promote neurite outgrowth of Schwann cells (24). Moreover, the neurite outgrowth and its direction are considered as key parameters in evaluating neuronal differentiation. Therefore, we adopted flow-induced shear stress.

Furthermore, information about the combined effects of substrate morphology and mechanical stimuli on neuronal differentiation is limited. In the current study, we investigated the combined effect of flow-induced shear stress on the differentiation of MSCs into neuronal cells and compared the effects of various micropatterns of polydimethylsiloxane (PDMS) substrates utilizing commercially available/proven biochemical reagent kits. For the evaluation of differentiation, we assessed the function of differentiated cells through real-time measurements of intracellular calcium concentration, immunofluorescence staining of related markers, outgrowth measurements, and their alignments.

MATERIALS AND METHODS

This study consisted of two experiments. First, we investigated the most effective size of micropattern for neuronal differentiation of MSCs. Micropatterns of three different magnitudes were fabricated and compared to determine the pattern most favorable for differentiation. Based on these results, MSCs were cultured on the selected micropatterned substrate under various magnitudes of fluid flow-induced shear stress. In both experiments, the expected functional evaluations were assessed along with quantitative measurements of the morphological changes.

Fabrication of micropatterned PDMS substrates Micropatterned substrates were fabricated based on silicon wafers. Silicon wafers were fabricated using photolithography and deep trench reactive ion etch (DRIE) techniques. The wafer surface patterns contained groove/ridge widths of 5/5, 10/10, or 20/20 μm and the groove depth of 3 μm was the same for all three patterns. Sylgard 184 silicone elastomer and curing agent (PDMS; Dow Corning, Corning, NY, USA) were combined at a ratio of 10:1 and then poured into silicon wafers. After spin coating (500 rpm, 10 s), silicon wafers coated with PDMS were hardened on a hot plate at 125°C for 20 min. The PDMS membranes were peeled off from the silicon wafers, and micropatterned membranes having patterns of the silicon wafer were obtained. Micropatterned PDMS membranes were sterilized using 70% ethanol and UV exposure, and then coated with 15 μg/ml fibronectin (Sigma, St. Louis, MO, USA) to promote cell adhesion on the surface.

Preparation of cells Human MSCs (hMSC, Lonza, Basel, Switzerland) were cultured in Dulbecco's modified Eagle medium-low glucose (DMEM-LG; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Hyclone) in an incubator at 37°C with 5% CO_2. Culture media was replaced every 3 days after seeding. MSCs at the fourth passage were used in the experiments.

Design of the fluid flow system The system was designed to engage fluid flow-induced shear stress. It consisted of a fluid flow chamber for culturing cells, a media reservoir, a gear pump (REGLO-ZS Digital; Ismatec, Glattbrugg-Zurich, Switzerland), and a controller (Fig. 1). The media reservoir was open to the atmosphere. The fluid flow chamber was 5 cm wide, 18 cm long and 1.2 mm high.

Micropatterned PDMS membranes seeded with MSCs were placed inside the fluid flow chamber, and the fluid flow chamber was connected to the gear pump. All equipment, except for the controller, was placed inside the incubator at 37°C with 5% CO_2. The shear stress was calculated using the following equation under the assumption of a Newtonian fluid and fully developed laminar flow:

$$\tau_w = \frac{6\mu Q}{h^2 b} \quad (1)$$

where μ, Q, h, and b are dynamic viscosity, flow rate, height, and width, respectively.

Cell culture with shear stress induced by flow In the first experiment, to observe the effect of the size of the micropattern, substrates containing patterns with groove and ridge widths of 5 μm, 10 μm, or 20 μm were compared against the flat substrate without engaging mechanical stimulation, and the most effective pattern size was selected. Next, the mechanical stimulation, shear stress, was applied to cells on the selected substrate type. MSCs were seeded at 2×10^3 cells/cm^2 on the substrate. To observe the effect of fluid flow-induced shear stress, shear stresses of 0.10 and 0.25 Pa were engaged at days 5 and 6 for 3 h per day for 2 days. In determining the duration of engagement of flow-induced shear stress we found in our preliminary experiment most cells were lost when shear stress was engaged 4 h per day even starting at day 5. We also provided 4 days for the cells to be stabilized on the substrates.

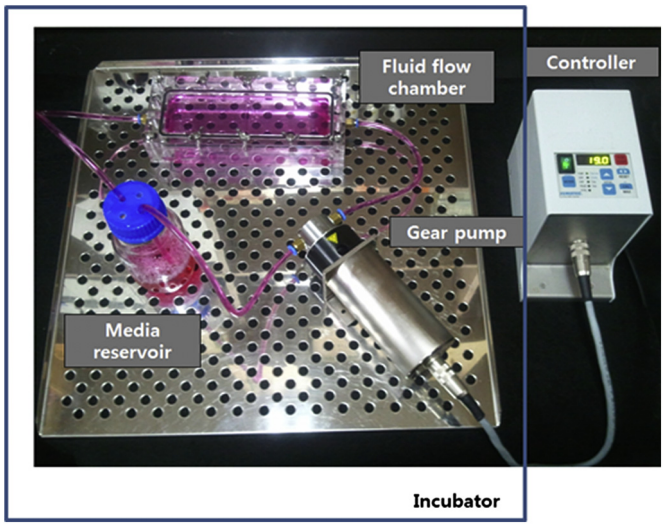

FIG. 1. Fluid flow system for shear stress. The system consisted of a flow chamber, a gear pump, a controller, and a media reservoir.

To induce neuronal differentiation after seeding, the media was changed to NPBM media (neural progenitor cell basal medium; Lonza) containing 5 μM cAMP, 5 μM IBMX, 25 ng/ml NGF, and 2.5 μg/ml insulin (Sigma).

Immunofluorescence staining To identify neuronal differentiation, neuronal-specific markers such as β-tubulin III, synaptophysin, and microtubule-associated protein 2 (MAP2) were observed by immunofluorescence staining.

Cells fixed with 10% formalin (neutral buffered; Sigma) were permeabilized in 0.2% Triton-X 100 (USB, Cleveland, OH, USA) and then blocked with 1% bovine serum albumin (BSA; Sigma) for 30 min. The cells in each group were incubated with anti-β-tubulin III (1:200; Sigma), monoclonal anti-synaptophysin (1:200; Sigma), and MAP2 antibody (1:100; Cell Signaling Technology, Inc., Beverly, MA, USA) for 1 h. Following incubation with the primary antibody, the cells were incubated with secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (H + L), Alexa Fluor 488 goat anti-mouse IgG (H + L), and Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1:200; Molecular Probes, Eugene, OR, USA) for 1 h. Finally, the nucleus was stained using 4,6-diaminidino-2-phenylin-dole (VectaShield-DAPI; Vector Laboratories Inc., Burlingame, CA, USA). Images were obtained using a confocal laser microscope (LSM 510 META; Carl Zeiss, Jena, Germany).

Neurite analyses and cell alignment Neurite outgrowth and cell orientation were measured using fluorescence images of β-tubulin III (25). Any outgrowth longer than 10 μm detected by staining of β-tubulin III was considered to be neurite. A module of the NeuronJ plugin provided by ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to measure neurite length. We defined the length as the distance from the center of a nucleus stained with DAPI to the end of a selected neurite stained by β-tubulin III. Cell orientation was also evaluated using a module of the OrientationJ plugin of Image J. The direction parallel to that of the micropattern, which was also parallel to the flow direction, was referred as 0°. The orientation in degrees was classified from 0° to 90° in intervals of 10°.

Calcium imaging Calcium imaging was used to evaluate the expected functional differentiation of hMSCs using a calcium indicator dye at day 10. Cells were loaded with Hank's balanced salt solution containing 5 μM fluo-4/acetoxymethylester (Molecular Probes) and 0.05% Pluronic F-127 (Molecular Probes), and incubated for 30 min in the dark. Changes in the intracellular calcium concentration were recorded using a confocal laser microscope at a wavelength of 488 nm for later analyses. The frame rate was 2 s.

To investigate the formation of voltage-gated ion channels and neurotransmitter receptors, 133 mM KCl (Junsei Chemical Co., Ltd., Tokyo, Japan), 500 μM acetylcholine iodide, L-glutamic acid, and dopamine hydrochloride (Sigma) were added to each sample.

The calcium response of each cell was measured using region of interest (ROI) and the time-series analyzer of ImageJ from recorded images. The intensity of images was normalized by the first 10 values obtained prior to the addition of neuronal activators. The cells were considered to be responsive to a given neurotransmitter when the normalized intensity increased by more than 10% within 60 s after the addition of neuronal activators. For this more than 40 cells as a total were observed from 3 different images. Then the percentage of the responded cells was calculated.

Statistical analysis Statistical analyses were performed using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Fisher' least significant difference (LSD) method were used for multiple comparisons. Differences were considered statistically significant at confidence level of 95 % ($p < 0.05$).

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