Morphological changes in neurons by self-organized patterned films

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In tissue engineering, micro/nanofabrication is important to modify substrate surfaces for regulating the attachment and growth of cells. In neuroscience, it is significant for neural regeneration; this involves guiding and extending dendrites and axons by a cell culture scaffold which acts as an extra cellular matrix. In this study, we prepared highly regular porous honeycomb-patterned films by a simple casting technique and cultured neurons to investigate their morphologies on the patterned films. The morphologies of neurons were examined by a scanning electron microscope and a confocal laser scanning microscope. The neurons were round and the neurites extended randomly on the flat film. The patterns influenced the morphologies of neurons. The morphologies of neurons were changed by varying the pore size of the honeycomb-patterned films. The neurites spread along the rims of the honeycomb pattern. These results suggest that the self-organized honeycomb-patterned films are useful biomaterials for neural tissue engineering. [DOI: 10.1380/ejssnt.2005.159]

Keywords: Patterning; Neuron; Self-organization; Regenerative medicine; Fabrication; Neural stem cell; Cell differentiation

I. INTRODUCTION

Brain, spinal cord injuries, and neural-degenerative diseases are likely to require the transplantation of neural cells and tissues. The mammalian central nervous system has little capacity for self-repair. The replacement of lost and dysfunctional neurons by tissue transplantation or nerve graft has been investigated [1]. These approaches have been developed as useful tools for restoring function in the damaged central nervous system. In tissue engineering, these approaches are significant for the reconstruction of tissues and organs not only to carry out research on cells or liquid factor but also to develop scaffolds [2, 3]. Scaffolds that have 3-D structures can induce adhesion, proliferation, and differentiation of cells and reorganized tissues and organs. The materials that are used for tissue engineering are made of biodegradable polymers. It has been reported that micro/nano-patterns influenced the morphologies, proliferation, and differentiation of cells [4–6]. It has been found that various micro-patterned substrates fabricated by lithographic techniques can reconstruct artificial neural networks by controlling the morphologies of adhered neurons and outgrowth of neurites [7–13]. These techniques are expected to be applied for neural regeneration in the future. However, these techniques require high energy and involve many processes. In addition, materials for substrates are limited. We have reported that the honeycomb-patterned films are prepared by self-organization [14–16]. The patterned films have regular pores in micro/nano-meter. They can be prepared with ease, low energy, and low cost. Moreover, we can control the pore size of these films. In this study, we cultured neurons on honeycomb-patterned films and investigated the morphological changes in neurons and neurite extension by varying the pore size of the films.

II. EXPERIMENTAL

A. Preparation of self-organized honeycomb-patterned films

Poly (ε-caprolactone) (PCL) and an amphiphilic polymer were mixed together and dissolved in chloroform in a weight ratio of 10:1 (Fig. 1(a)). We cast the polymer mixture onto the glass substrates. Honeycomb-patterned films with regular pores were prepared by blowing humid air on the surface of the polymer solution (Fig. 1(b)).

B. Preparation of the PCL flat film

The polymer solution was dropped onto a cover glass. The cover glass with the polymer layer was spun at 1000 rpm for 30 seconds by using a spin coater (MIKASA, 1H-D7).
FIG. 1: Preparation of honeycomb-patterned films. a) Chemical structures. b) Casting method.

C. Pretreatment of PCL films

The PCL flat film and PCL honeycomb-patterned films were soaked in 1-propanol solution for 5 minutes and then washed with ethanol. The PCL films were then attached to a cover glass, placed in culture dishes, and sterilized by exposure to UV rays. These films were then soaked in poly-L-lysine solution (50 mg/L, 0.1 M Boric acid, pH 8.3) for 1 hour to coat poly-L-lysine on the films.

D. Preparation of neural cells

Neural cells were prepared from the cerebral cortices of embryonic day-14 mice (CLEA Japan, Inc). In brief, the cerebral cortices of embryonic day-14 mice were dissected and the meninges were carefully removed. The tissues were transferred into 15-ml tubes with culture medium containing 55 µM 2-mercaptoethanol and gently triturated with a fire-polished pasteur pipette until most of the tissues were dissociated into single cells. The cell number and viability were determined. Cells were seeded onto the PCL flat film to estimate the population of neural stem cells (NSCs). After incubating the cells for 6 hours, cells were immunocytochemically stained for nestin to identify and estimate their population.

E. Cell culture condition

The neural cells were seeded onto the PCL flat film and PCL honeycomb-patterned films at a density of $2.0 \times 10^4$ cells/cm$^2$. They were cultured in serum medium (Opti-MEM (Invitrogen), 10% fetal bovine serum, 55 µM 2-mercaptoethanol (Invitrogen)) for the first day. After the second day, they were cultured in serum-free medium (Opti-MEM, B27 supplement, 55 µM 2-mercaptoethanol). They were incubated at 37°C under a humidified atmosphere of 5% CO$_2$.

F. Scanning electron microscopic observation

The cultured cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS). They were washed with PBS and water. Subsequently, the samples were dehydrated by washing in increasing ethanol concentrations and then air-dried. The samples were sputtered with platinum and investigated with a scanning electron microscope (Hitachi, S-3500).

G. Immunocytochemistry

1. Immunostaining for β-tubulin III

The cultured cells were fixed with 10% formalin in PBS for 30 minutes at room temperature. The samples were washed with PBS 3 times for 5 minutes. They were then incubated in blocking solution (5% goat serum, 0.2% Triton X-100 in PBS) for 1 hour. Then, the samples were incubated with mouse monoclonal anti-β-tubulin III (1:500) in PBS for 2 hours. After washing with PBS, the samples were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:200) for 2 hours. After washing with PBS and water, the samples were air-dried.
FIG. 2: SEM images of honeycomb patterned films. a) Pore size, b) Rim size, c) Porosity. Bar=10 µm

<table>
<thead>
<tr>
<th>a) Pore size (µm)</th>
<th>b) Rim size (µm)</th>
<th>c) Porosity (%)</th>
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</thead>
<tbody>
<tr>
<td>3.2 (± 0.09)</td>
<td>1.1 (± 0.04)</td>
<td>49.7 (± 2.54)</td>
</tr>
<tr>
<td>5.1 (± 0.05)</td>
<td>1.3 (± 0.07)</td>
<td>49.8 (± 1.93)</td>
</tr>
<tr>
<td>8.4 (± 0.03)</td>
<td>2.8 (± 0.17)</td>
<td>51.3 (± 3.87)</td>
</tr>
<tr>
<td>10.2 (± 0.10)</td>
<td>3.8 (± 0.09)</td>
<td>52.6 (± 2.96)</td>
</tr>
</tbody>
</table>

FIG. 3: Population of neural stem cells on PCL flat film after 6 hours culture. (a) Staining with phalloidin and nestin. (b) Staining with phalloidin. (c) Staining with nestin. Bar=100 µm

and then mounted with mounting media for focal microscopic observation.

2. Immunostaining for nestin

The cultured cells were fixed with 10% formalin in PBS for 30 minutes at room temperature. The samples were washed with PBS 3 times for 5 minutes. They were then incubated in blocking solution (5% goat serum, 0.2% Triton X-100 in PBS) for 1 hour. Then, they were incubated with mouse monoclonal anti-nestin (1:1000) in PBS for 2 hours. After washing with PBS, the cells were incubated with biotinylated anti-mouse IgG (1:10000) in PBS for 2 hours. Then, they were incubated with Alexa 488-conjugated avidin (1:2000) and Texas-red conjugated phalloidin (1:50) for 2 hours at 37°C. After washing with PBS and water, the samples were air-dried and then mounted with mounting media for confocal microscopic observation.

III. RESULTS AND DISCUSSION

A. Preparation of self-organized honeycomb-patterned films

We could prepare self-organized honeycomb-patterned films by casting a polymer solution. The pore size could be controlled in the range from 3 to 10 µm by changing the casting volume [16]. We prepared PCL flat films and PCL patterned films (3 µm, 5 µm, 8 µm, and 10 µm in diameter) for culturing neural cells. The rims of the honeycomb-patterned films widened with increasing pore size of the patterned films. The porosity of each film was about 50% (Fig. 2).

B. Cell preparation from cerebral cortices of embryonic mice

The viability of neural cells prepared from the cerebral cortices of 4 mice (embryonic day-14) was 90~95%. We
investigated the population of neural NSCs in the prepared cells by staining with nestin. We found that the cell mixture contained 90\textasciitilde95% of NSCs (Fig. 3).

C. Morphologies of adhered neural cells and neural extension

After 5 days of culture, we investigated the morphologies of adhered neural cells and the extension of neurites on both PCL flat films and PCL honeycomb-patterned films. The neurons were stained for $\beta$-tubulin III, a neuron-specific tubulin for their identification and for the observation of neural networks on the films (Figs. 4 (a1)-(f1)).

The neurons possessed highly branched, multi-polar neurites and rounded cell bodies (Fig. 4(a)). On the other hand, several neurons aggregated near each other and adhered with lamella structure around the cell body on the patterned film (pattern pore size: $\phi 3 \, \mu m$) (Fig. 4(b)). Single neurons adhered with lamella structure on the patterned film (pattern pore size: $\phi 5 \, \mu m$) (Fig. 4(c)). The neurons were round with no lamella structure on the patterned film (pattern pore size: $\phi 8 \, \mu m$) (Fig. 4(d)). The neurites extended along the honeycomb-pattern rims and neurons formed network structures on the patterned...
FIG. 5: The number of neurites on flat film and patterned films.

TABLE I: Morphological changes of adhered neurons on PCL film and PCL honeycomb-patterned films.

<table>
<thead>
<tr>
<th>Pore size (µm)</th>
<th>Width of the pattern rim (µm)</th>
<th>Number of neurites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>1.3</td>
<td>5</td>
</tr>
<tr>
<td>8.4</td>
<td>2.8</td>
<td>4</td>
</tr>
<tr>
<td>10.2</td>
<td>3.8</td>
<td>3</td>
</tr>
</tbody>
</table>

Morphology: see Fig. 4.

We could prepare biodegradable polymer patterned films and control the pore size of the patterned films. Neurons formed neural networks on the patterned films, and the morphologies of cells could be changed by varying pore size of the patterned films. The self-organized honeycomb patterned films are useful biomaterials for neural tissue engineering.

Acknowledgments

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