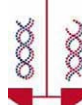
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Directional migration of neuronal PC12 cells in a ratchet wheel shaped microchamber

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Directional migration of neuronal cells over long distances is critical for the developing and regenerating nervous system. A scaffold, which includes radial glia, provides a route between the germinal zone and the destination, with the direction of migration being attributed mainly to chemotaxis. However, the decrease in chemokine concentration with distance complicates cell guidance control over long distances. Our working hypothesis is that neuronal cells migrate directionally on an anisotropic and periodic scaffold. We tested this in a model involving neuronal PC12 cells cultured in a ratchet wheel-shaped microchamber. The microchamber was constructed by printing a patterned, thin film of polydimethylsiloxane (PDMS), to which the cells adhere weakly, onto a collagen-coated dish, to which the cells adhere strongly, using a microcontact printing technique. The cells can attach, extend neurites, and migrate on the anisotropic and periodic collagen-coated area between the ratchet wheel-shaped outer frame and round inner frame of the PDMS. We found that the microchamber geometry affected the direction of migration, even though the mean length of the longest neurite was independent of microchamber geometry. The time-course trace of cell body migration and neurite tips showed that the neurite tips remained around the tips of the ratchet teeth. These results suggested that neuronal cells migrate directionally on a scaffold, even in the absence of chemokine, and reveal a new conceptual framework for neuronal migration.
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[**Key words:** Migration; Neurite outgrowth; Development; Nervous system; Ratchet; Scaffold; PDMS; Microcontact printing; Tissue engineering; Brownian motion]

Long-distance directional migration of neuronal cells is a critical step in the developing and regenerating nervous system. Different germinal zones give rise to different neuronal types, which may migrate up to several millimeters to their respective destinations (1, 2). In the cerebral cortex, excitatory neurons arise in the lateral ventricle and migrate radially toward the cortex surface, while inhibitory neurons originate in the ganglionic eminences and migrate parallel to the cortex surface (3, 4). The cellular mechanisms underlying these migratory activities have been extensively studied (5, 6).

Chemotaxis is the major mechanism thought to direct neuronal migration (5, 6). However, chemokine gradients decrease with distance, making long-distance cell guidance even more challenging. Moreover, the migration of different neurons in different directions generates significant chemokine crosstalk. Neuronal migration is guided by a scaffold comprising various components such as radial glia and blood vessels (7–9). Such a scaffold facilitates long-distance, error-free migration of neurons by providing a definitive pathway from the starting point to the destination. However, it is not fully understood how the scaffold mechanistically determines the direction of neuronal migration. Elucidating this process would not only increase our understanding of how the nervous system is formed, but also facilitate the development of novel tissue engineering techniques for nerve regeneration.

The present study tested the hypothesis that an anisotropic and periodic scaffold structure promotes the directional migration of neuronal cells, using neuronally differentiated PC12 cells and a ratchet wheel-shaped microchamber. The rat adrenal pheochromocytoma PC12 cell line differentiates into sympathetic neuron-like cells with long neurites extended upon the addition of nerve growth factor (10–12). The microchamber was fabricated using a microcontact printing (μCP) technique (13, 14), whereby a patterned thin film of polydimethylsiloxane (PDMS) is deposited on the surfaces of collagen-coated culture dishes. We analyzed the direction of migration of the neuronal cell bodies and the extension of neurites in the microchamber.

MATERIALS AND METHODS

Microchamber fabrication Culture dishes were coated with 30 μg/ml type I collagen (Cellmatrix Type I-A; Nitta Gelatin Inc, Osaka, Japan) in phosphate-buffered saline (PBS) for at least 3 h at room temperature, rinsed twice with deionized distilled water to remove excess type I collagen and ions, and then air-dried. PDMS (Sylgard 184, 10:1 mix; Dow Corning) stamps were made using a mold of stainless steel coated with a

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patterned resistance film (25-μm thickness; Hirai Seimitsu Kogyo, Osaka, Japan). The cured PDMS stamp was immersed in 70% ethanol for 20 min and air-dried for 20 min on a clean bench under UV light to sterilize the stamp. The stamp was inked with non-cured PDMS, which was spread as thinly as possible on another culture dish using a cell scraper. The stamp was placed onto the type I collagen-coated dishes to deposit a thin film of patterned PDMS. The dishes were incubated overnight at 60 °C to cure the printed PDMS, and then sterilized on a clean bench under UV light for 20 min.

Cell culture PC12 cells were obtained from the Riken Cell Bank (Cat. # RCB0009; Ibaragi, Japan). The cells were cultured in a serum-free medium in a humidified incubator with 5% CO_2 at 37 °C, as described previously (12). Briefly, the serum-free medium comprised an equal mixture of RPMI 1640 medium (Invitrogen) and Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with bovine serum albumin (BSA), sodium selenite, ethanolamine, β-mercaptoethanol, apo-transferrin, and insulin. Culture dishes were coated with 30 μg/ml type I collagen. From 10 days before the PC12 cells were plated onto the PDMS-printed dishes, they were cultured in a neurite outgrowth medium comprised 2:2:1 mixture of RPMI 1640 medium, DMEM and 160 mM KCl supplemented with BSA, sodium selenite, ethanolamine, β-mercaptoethanol, apo-transferrin, and 100 ng/ml nerve growth factor, to block proliferation and to prime neurite outgrowth (11, 12). Cells were plated onto the PDMS-printed dish using a cell sorter (ALTRA; Beckman Coulter), as described previously (15), to ensure that individual cells of similar size were plated.

Imaging For immunofluorescence staining of type I collagen, the PDMS-printed culture dishes were incubated for 1 h in a blocking buffer that consisted of 1% fetal bovine serum in PBS. The dishes were incubated for 4 h at room temperature with mouse anti-type I collagen monoclonal antibody (COL-1, 1:500 dilution; Abcam, Cambridge, UK), and then incubated for 4 h at room temperature with Alexa Fluor 488-conjugated donkey anti-mouse IgG(H+L) (1:200 dilution; Invitrogen). The PDMS patterns on the collagen-coated dishes were analyzed using atomic force microscopy (AFM) on a VN-8000 apparatus (Keyence, Osaka, Japan), which was operated in tapping mode with a silicon cantilever. Time-lapse microscopy was used to trace cell movement in a humidified acrylate incubator containing 5% CO_2 at 37 °C and set up on an upright microscope (IX 70; Olympus, Tokyo, Japan). Phase-contrast microscopic images of the cells were acquired using a monochromatic, cooled CCD camera system (Orca ER; Hamamatsu Photonics, Shizuoka, Japan). After importing the images to a computer, an outline of each cell body image and the position of each neurite tip was traced by hand. Acquired images were analyzed using Image J software (NIH, USA).

RESULTS AND DISCUSSION

Trapping and observing PC12 cells in the PDMS-printed microchamber The tracing of neuronal cells in a microchamber for long periods of time is difficult because the cells tend to escape from the chamber. The microchambers used in this study were constructed using the μCP technique (13, 14), as described in Materials and methods. First, to analyze the chemical and physical properties of the microchamber, we made a simple microchamber comprising circular collagen spots surrounded by a thin film of PDMS. Immunofluorescence staining of type I collagen clearly showed the circular collagen spots (Fig. 1A–D), suggesting that type I collagen remains heat-stable during PDMS curing and that PDMS covers the type I collagen on the outside of the spot. A cross-section based on AFM of a typical circular microchamber estimated the thickness of the PDMS film printed on the type I collagen at approximately 0.1 μm (Fig. 1E). Based on AFM imaging, the contact angle of the PDMS was estimated to be 3–6° at the edge of the collagen spot, and the thickness near the edge of collagen spot reached approximately 0.2 μm due to the surface tension of the precured PDMS (Fig. 1E). PC12 cells were then plated onto the microchamber containing neurite outgrowth medium. The cells attached only on collagen spots, showing no neurite extension or cell migration on the PDMS frame for 18 days after plating (Fig. 1F). Cells with long neurites remained in the chamber for more than two months (data not shown). These results certified that the PDMS-printed dish is not toxic for the cells and it chemically and physically restrict cell adhesion over long periods.

Our method has two advantages over conventional μCP techniques, in which an extracellular matrix such as collagen is printed on a nonadherent surface (13). First, few cells escape from the constructed chamber, since the culture dishes are modified both chemically and physically. In addition, the serum-free culture medium developed in our laboratory does not contain extracellular matrix (12), and thus might help to prevent cells escaping over a long time period. Second, it is easy to produce uniform microchambers, and since the surface on which the cells attach is critical for cell movement, the quality should be uniform. Cell adhesion is independent of the quality of the current μCP, with cells attaching to the uniformly coated type I collagen, but not adhering to the printed PDMS surface. Therefore, this μCP technique was used in subsequent experiments to test our working hypothesis.

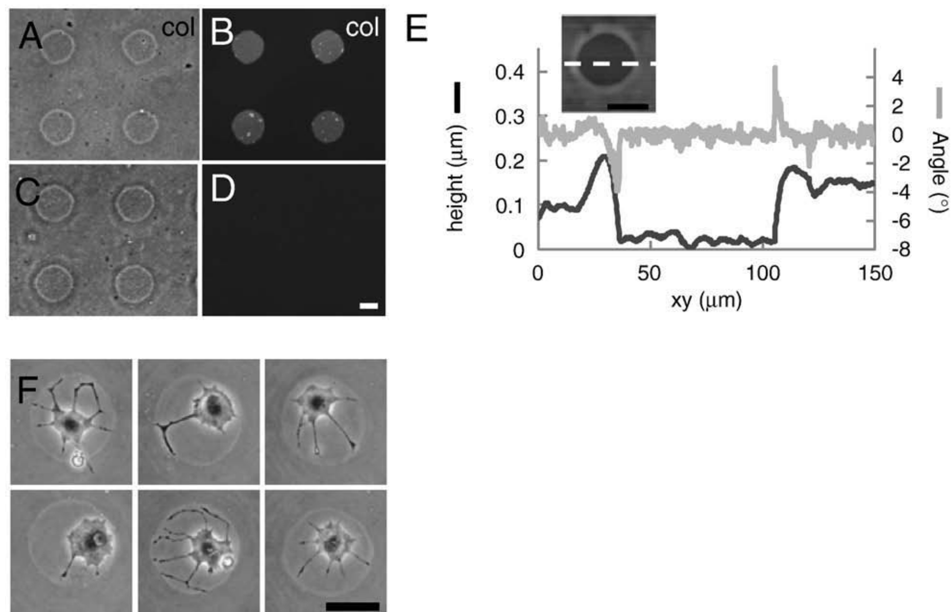

FIG. 1. Physical and chemical properties of the microchambers, which are circular collagen spots surrounded by PDMS film. (A–D) Phase-contrast micrographs (A, C) and immunofluorescence micrographs of anti-type I collagen antibody staining (B, D) of PDMS printed on type I collagen-coated (A, B) and non-coated dishes (C, D). Four circular microchambers are shown. Scale bar = 50 μm. (E) Height (black line, left ordinate) and angle (gray line, right ordinate) profiles of a typical circular collagen spot surrounded by PDMS film at the horizontal dotted line in the AFM image (inset). Scale bar = 50 μm. (F) Phase-contrast micrograph of neurite-extended PC12 cells in a circular microchamber containing neurite outgrowth medium 18 days after plating. Scale bar = 50 μm.

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