Cell Culture and Motility Study on a Polymer Surface with a Nanometer-Scaled Stripe Structure

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Received October 14, 2009; Accepted December 3, 2009; Online Publication, March 7, 2010

We developed a large cell culture surface with a nanostripe structure by paving polydimethylsiloxane (PDMS) replicas of a glass mold. The stripe structure has a height of 180 nm and top width of 500 nm with 400-nm intervals between stripes. Human stomach cancer SH-10-TC cells cultured on the surface changed their morphology to elongated shapes parallel to the nanostripes. In addition, cell motility parallel to the stripes was greatly enhanced. These findings strongly suggest that the nanostripe structure affected the cell physiology.

Key words: nanostructure; polydimethylsiloxane; cell morphology; cell migration; cell motility

Cell adhesion plays a critical role in adherent cells. It triggers signal transduction within the cells and affects cell growth and differentiation. Although many biochemical studies of cell adhesion have been reported to date, most of them were molecular-level studies concerning the extracellular matrices (ECMs) and proteins on the cell membrane. While most of these studies were conducted on flat cell-adhesion surfaces, ECMs attached by cells have filamentous structures 10–1,000 nm in diameter. Therefore, the effects of the physical structure are also considerable. The influence of micro- or nano-sized chemical patterns or topographic structures on cell adhesion have also been reported as reviewed in reference 1. In most cases, fibroblasts were elongated and aligned parallel to the stripe structures, 2–100 μm in width. Although cell patterning by chemical modification patterns or topographic structures 2–100 μm in scale, i.e., almost the same size as or larger than the cells, have been well-studied, very few studies have been conducted on structures much smaller than the cell size. Nanopatterned surfaces for cell attachment have been fabricated by colloidal lithography,2 polymer demixing,3 and copolymer formation.4 Although these methods provide surfaces that have a nanometer-scale topography, obtaining precise control of the scale and shape of the patterns is very difficult. In most cases, only dot-shape structures have been realized. An electron-beam lithography process can precisely control the scale and shape of the patterns on silicon or glass substrates. Using these techniques, topographic structures5–7 and chemical patterns8–10 at a scale of about 100 nm have been realized. Fibroblasts on the surface were stimulated by the nanometer-scale topography, and the cells were elongated and aligned along stripe (line-and-space) patterns.10 The morphological change was remarkable in epithelial cells. They had an elongated shape on a stripe structure, while their morphology on a flat surface is very much spread out.7 Because epithelial cells are contacted with and stimulated by fibrillar ECMS, it is important to investigate cells’ response to a nano-scaled topographic stimulus, including cell morphology, motility, growth, and functions. Although morphological studies of the cells cultured on nanopatterns have been reported, no other types of studies have been reported. One of the main reasons for this is the unavailability of cultured surfaces with well-controlled nanostructures, because electron-beam lithography requires a long time and high cost to fabricate even a small area of nanostructure. Moreover, the silicon wafer and quartz glass substrate used in previous research are very hard materials, unlike ECMS. Fibroblasts were mostly used in these experiments, and the responses of other types of cells have not been clarified.

In this study, we focused on the migration and motility of cells cultured on a surface with a nanostripe structure. Because cell migration is an important feature of tumor invasion, SH-10-TC cells from human stomach cancer were chosen for analysis. To realize the experiments, we developed a large surface for cell cultures with a nanostripe structure by paving soft polymer replicas of a glass mold made by electron-beam lithography. Cell imaging and cell migration studies were performed on this structure.

Materials and Methods

Materials, reagents, and cells. Polydimethylsiloxane (PDMS) prepolymer (Silpot 184) was purchased from Dow Corning Toray

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Abbreviations: AFM, atomic force microscope; ECMs, extracellular matrices; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PDMS, polydimethylsiloxane; SEM, scanning electron microscope

10.1271/bbb.90771
Fig. 1. Designs and Images of the Glass Mold and Its PDMS Replica.
(A) Photograph of the glass mold. The nano-fabricated area (1.2 x 1.2 cm square) showed iridescent colors like a diffraction grating. (B) Illustration of the nanostripes on the glass mold, which had 500-nm wide ridges and 250-nm deep grooves on a 900-nm pitch. (C) AFM and (D) SEM images of the PDMS sheet with a nanostructure that had 500-nm wide ridges and 180-nm deep grooves on a 900-nm pitch.

Cell culture. PDMS sheets were cleaned with 70% ethanol in an ultrasonic bath, followed by rinsing with PBS. After surface treatment with a fibronectin solution, a conventional plastic culture dish was paved with the resulting PDMS sheets. SH-10-TC cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a CO2 incubator. For the migration study, a HEPES-modified RPMI-1640 medium supplemented with 10% FBS was used, and cells were incubated at 37 °C in an incubation box without control of the CO2 concentration.

Cell staining. When the culture was grown to 50% confluence, the medium was removed carefully to remove non-adherent cells simultaneously. The attached cells were fixed with 4% paraformaldehyde for 20 min, followed by two washes with 0.05% Tween 20. The cells were permeabilized with 0.1% Triton X-100 for 5 min, washed twice with 0.05% Tween 20, and then incubated for 45 min at room temperature in a solution containing 5 mg/ml of FITC-conjugated goat anti-mouse and 60 ng/ml of rhodamine-conjugated phalloidin. After washing them 3 times with 0.05% Tween 20, the samples were briefly immersed in 0.2 mg/ml of DAPI. The samples were finally washed 3 times with 0.05% Tween 20 and then immersed in PBS. Each sample was photographed using the inverted fluorescence microscope.

Time-lapse motion picture. For analysis of cell migration, an incubation box was fixed on the inverted phase-contrast microscope. In the box, humidified air was maintained at 37 °C without control of the CO2 concentration. The cells were seeded onto PDMS sheets treated with fibronectin, and the dish was placed on the microscope stage in the incubation box. During the experiments, the incubation box was completely covered with a blackout curtain, and the cells were exposed to xenon light at minimum intensity in shooting images. The images were captured every 5 min for 3 d to obtain a movie. To track the cells, 20 cells were chosen from each image frame, the positions of the cells’ nuclei were recorded every 5 h for 50 h, and the positions were connected with straight lines. Position changes after 5 h of culture, i.e., distances and directions from their original position, were recorded for analysis. In the case of the nanostripe PDMS sheets, the angle between the migration line and the direction of the nanostripes was used to define the direction of cell migration.
Results and Discussion

Fabrication and cell culture

PDMS sheets with and without nanostripe structures were fabricated. Figure 1C and D show scanning electron microscope (SEM) and AFM images of the sheet. The cross-sectional view of the AFM image indicates that a stripe had a height of 180 nm and a top width of 500 nm at 400-nm intervals. Although the glass mold had a rectangle cross section, the PDMS structure swelled in the width direction, and hence the bottom width of the nanochannels could not be measured. This

Fig. 2. Photographs of SH-10-TC Cells Cultured on the PDMS Sheets.

Cells were plated at $10^3$ cells/cm$^2$ and cultured for 4 d to 40% confluence. Cells (A, B) on the flat PDMS sheet and (C, D) on the nanostripes. (A, C) Phase-contrast and (B, D) fluorescent images of the cells. To obtain fluorescent images, the cells were fixed and stained with rhodamine phalloidin (red), anti-vinculin (green), and DAPI (blue) to visualize actin filaments, focal adhesions, and nuclei respectively. Arrows indicate the directions of the stripes.

Fig. 3. Results of 5-h Cell Migration Tests.

(A) Position change of the cells after 5 h of static culture; red, on the nanostructure; blue, on the flat surface. Each spot was connected with the coordinate origin and evaluated with two kinds of numerical data, migration distance and migration angle to the stripe. (B) Cell migration distance after 5 h of culture. While most cells on the flat surface migrated below 100 µm, some cells on the nanostructure migrated more than 200 µm. (C) Cell migration angle to the stripes. While the cells on the flat surface migrated in all directions almost equally, the cells on the nanostripes migrated to about $0^\circ$, $180^\circ$, and $360^\circ$ rather than $90^\circ$ and $270^\circ$.

The cells were plated at $10^3$ cells/cm$^2$ and cultured for 12 h before the migration test. The position changes of 20 cells after 5 h of culture in an image flame were recorded by comparing the movie images. The same procedures were repeated 10 times sequentially during 50 h of culture.
inaccurate transcription was probably due to large temperature changes during the fabrication process.

The shape and dimensions of the structure, however, were reproducible, and we concluded that the sheets were useful for cell-culture studies. More than 100 PDMS sheets could be made from the glass mold. Smooth PDMS sheets without a stripe structure had 6.5 nm of roughness (data not shown), sufficient for control experiments. Since PDMS is not a suitable material for cell attachment, surface pretreatment is required for cell culture. After optimization experiments, pretreatment procedures were performed as described below. PDMS sheets with and without nanostripes were first washed with 70% ethanol in a sonic bath, followed by washing with PBS. The sheets were dipped in a 5-μg/ml of fibronectin solution for 2 h at 37 °C or overnight at 4 °C. The resulting sheets were washed 3 times with PBS, and then used to pave the entire bottom surface of the conventional plastic cell-culture dish. Cells suspended with the medium were placed in the dish and cultured in a CO₂ incubator for cell attachment, as usual. Whereas the cells on the flat PDMS sheet attached to the surface within 2 h, those on the nanostripes required about 10 h for attachment, but there was no apparent difference in cell growth rate after cell attachment.

**Cell morphology and F-actin alignment**

Figure 2 shows phase-contrast microscopic images and fluorescence microscopic images of the cells. On the smooth PDMS sheet, the cells showed polygonal shapes at random orientation, and most cells appeared in clumps (Fig. 2A). F-actin aligned in all directions. Vinculin, a protein playing a part in focal adhesion, was present at the ends of the F-actin and was found under the cells (Fig. 2B). In contrast, the cells on the nanostructure showed elongated shapes parallel to the nanostructures and many of them were isolated (Fig. 2C). Figure 2D shows a fluorescent microscopic image of the cells cultured on the nanostripes. Because of the optical effects of the nanostripes, it is difficult to take clear images with an inverted microscope. Whereas the images obtained were less clear than those in Fig. 2B, F-actin also parallelly aligned to the nanostripes. Vinculin was present mainly at the edges of the cells, and fewer spots of vinculin were observed at the center of the cells.

Similar cell and F-actin alignment has been found in the case of fibroblasts cultured on microscale stripes in many previous studies and cells on the nanopatterned surface.5–9) The morphological change in the SH-10-TC cells in this study was more remarkable than that of fibroblasts in previous studies of which shape was originally elongated.

**Cell migration**

A time-lapse motion picture of the cells cultured on the nanostripes is shown in Supplemental Movie 1 (see Biosci. Biotechnol. Biochem. Web site). After they were attached to the surface, the cells were stretched to a spindle shape. Many cells walk in a horizontal direction in the movie, parallel to the nanostripes. During culture, small globular cell debris appeared on the nanostripes. They were derived from broken particles of excessively stretched points of the cells. They were left on the attached surface when the cells reversed direction. The cell positions were recorded every 5 h for 3 d to analyze cell migration on the nanostructure. The position changes of the cells after 5 h of culture are plotted in Fig. 3A. The Y-axial movement, i.e., movement perpendicular to the nanostripes, was similar to those of cells on the flat surface. In the case of X-axial movement, many cells on the nanostructures moved a much longer distance (up to 300 μm). Figure 3B shows the distribution of cell migration. The migration distances of the cells on the nanostructures ranged to 300 μm, whereas those on the flat surface were within 100 μm. The migration angles to the stripe are summarized in Fig. 3C. The cells on the nanostructures preferred to migrate to about 0°, 180°, and 360° rather than 90° and 270°. Whereas the cells walked to all directions equally on the flat surface, cell migration on the nanostructures was greatly enhanced, especially in directions parallel to the stripes, and many cells moved a long distance along the stripes. Similar enhancement of cell motility and a guiding effect on cell migration of the nanostructure have not been reported.

The cells have dimensions of several tens of micrometers, which is much larger than the stripe gap (400 nm). Although the 400-nm gap was easily overridden by the cells, they were strongly affected by the gap. These findings carry suggestions as to the mechanism of cell migration and cell attachment. For instance, the following hypothesis might be suggested: If focal adhesion moves with the gliding steps, it is difficult to cross the gap, while the gap has little influence if focal adhesion walks to another point in long inchworm-like strides.

In summary, we developed a large cell-culture surface with a nanostructure surface by paving PDMS replicas of a glass mold. Human stomach cancer SH-10-TC cells cultured on the surface changed their morphology to an elongated shape parallel to the nanostripes. In addition, cell motility parallel to the stripes was greatly enhanced. These findings strongly suggest that the nanostructure structure affected the physiology of the cancer cells.

**Acknowledgment**

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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