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Fabrication of Micro Patterned Fibronectin for Studying Adhesion and Alignment Behavior of Human Dermal Fibroblasts

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Abstract: The aim of this study was to fabricate a submicro- and micro-patterned fibronectin coated wafer for a cell culture, which allows the positions and dimensions of the attached cells to be controlled. A replica molding was made into silicon via a photomask in quartz, using E-beam lithography, and then fabricated a polydimethylsiloxane stamp using the designed silicon mold. Hexadecanethiol [$HS(CH_2)_{15}CH_3$], adsorbed on the raised plateau of the surface of polydimethylsiloxane stamp, was contact-printed to form self-assembled monolayers (SAMs) of hexadecanethiolate on the surface of an Au-coated glass wafer. In order to form another SAM for control of the surface wafer properties, a hydrophilic hexa (ethylene glycol) terminated alkanethiol [$HS(CH_2)_{11}(OCH_2CH_2)_6OH$] was also synthesized. The structural changes were confirmed using UV and ^{1}H-NMR spectroscopies. A SAM terminated in the hexa(ethylene glycol) groups was subsequently formed on the bare gold remaining on the surface of the Au-coated glass wafer. In order to aid the attachment of cells, fibronectin was adsorbed onto the resulting wafer, with the pattern formed on the gold-coated wafer confirmed using immunofluorescence staining against fibronectin. Fibronectin was adsorbed only onto the SAMs terminated in the methyl groups of the substrate. The hexa (ethylene glycol)-terminated regions resisted the adsorption of protein. Human dermal fibroblasts (P = 4), obtained from newborn foreskin, only attached to the fibronectin-coated, methyl-terminated hydrophobic regions of the patterned SAMs. N-HDFs were more actively adhered, and spread in a pattern spacing below 14 μm, rather than above 17 μm, could easily migrate on the substrate containing spacing of 10 μm or less between the strip lines.

Keywords: submicro-pattern, fibronectin, human dermal fibroblast, self-assembled monolayer, lithography.

Introduction

Tissue engineering is the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals.[1] Tissue is an aggregation of morphologically similar cells and associated intercellular matter acting together to perform one or more specific functions in the body.[2] To provide cells with a microenvironment to control its biological function, such as proliferation, migration, and differentiation of cells, promotes to regenerate and to reconstruct of the tissue or organ. Most mammalian cells grow as attached on the substratum through the focal contact which is the co-localization point of the intracellular cytoskeletal system, integrins at plasma membrane, and extracellular matrix.[3,4]

It has been also reported that the size of focal contact and the spacing between focal contacts may play important roles in determination of the quality and/or quantity of intracellular signal generation at focal contacts and cellular decision making for cell migration and cell survival.[5] Biological function of cell is related to formation of intracellular cytoskeletal filaments and peripheral extracellular matrix; the formation of them has an effect the adhesion of cell to cell or of cell to substrate, which it could control a variety of cellular behavior such as cell shape, cell survival, and their physiological function.[5] Until now, it has been suggested that spacing of obstacles is a critical parameter in cellular attachment and mobility.[6,7]

Therefore, controlling of the adhesion for anchorage dependent cell to biomaterial substrates is one of the key issues in tissue engineering, which rests on the ability

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to direct specific cell types to proliferate, migrate, and express physiological behaviors, in order to yield a cellular architecture and organization performing the functions of the desired tissue.[8,9] In this context, understanding the relationship between cell adhesion and physicochemical properties of biomaterial surfaces appears of the prime importance.[10-13]

With the development of IT industry, the versatility of microfabrication technology in creating precise surface structures makes it an excellent tool for studying biomedical systems.[7] Some studies noted differences in cell behavior with both micrometric and nanometric topographies including grooves, cliffs, pillars and islands.[14,15]

This study is to fabricate defined patterns of extracellular matrix in order to study a cellular behavior, and to evaluate the adhesion and the alignment behavior of a human dermal fibroblast on the substrate. In order to design submicro- and micro patterning substrate with variations in size and spacing, we have employed photolithographic techniques and microcontact printing.[15-17] That is, we made the replica molding into silicon via photomask in quartz by E-beam lithography, and fabricated poly(dimethyl siloxane) (PDMS) stamp using the designed silicon mold. And we also synthesized the hydrophilic hexa (ethylene glycol) terminated alkanethiol derivative, formed two kinds of selective patterned self-assembled monolayers (SAMs) of hexadecanethiolate, methyl-terminated and hexa (ethylene glycol)-terminated on the surface of Au-coated wafer. Because human dermal fibroblasts were used as a model representation of cells a biomaterial may encounter *in vivo*, we evaluated the attachment and proliferation of human dermal fibroblasts from newborn foreskin on submicro- and micro-patterned surface at 4.5 h at culture.

Experimental

Synthesis of Alkanethiol, 1-Mercaptoundec-11-ylhexa (ethylene glycol).

Synthesis of undec-1-en-11-ylhexa (ethylene glycol): A mixture of 3.2 g of hexa (ethylene glycol) and 0.34 mL of 50% aqueous NaOH solution was stirred for about 0.5 h in an oil bath at 100 °C under an atmosphere of nitrogen, and then 1.0 g of 11-bromoundec-1-ene (Algrich) was added. After 24 h, the reaction mixture was cooled down to room temperature and extracted 6 times with n-hexane. Concentration of the combined hexane portions by rotary evaporation at reduced pressure gave yellow oily reaction mixture. Purification of the oil by chromatography on silica gel (particle size: 32-63 μm) gave undec-1-en-11-ylhexa (ethylene glycol) (Fw=434). The eluant for the chromatography was the mixture solution of ethyl acetate and methanol (9:1, v/v): Yield 76%. (^{1}H-NMR (300 MHz, $CDCl_3$), δ: 1.25 (12H), 1.55 (2H), 1.7 (1H), 2.05 (2H), 3.45 (2H), 3.55-3.75 (24H), 4.9-5.05 (2H), 5.75-5.85 (1H)).

Synthesis of 1-Methylcarbonylthio-undec-1-en-11-ylhexa (ethylene glycol): Undec-1-en-11-ylhexa (ethylene glycol) (32 g) were thoroughly dissolved in 10 mL of methanol at room temperature under an atmosphere of nitrogen in UV reactor with 450-W medium-pressure mercury lamp (Ace Glass) filtered through Pyrex, and then 0.913 g of thioacetic acid and 10 mg of AIBN were added carefully. After 6 h, the reaction mixture was removed from the reactor. The reaction products were concentrated by rotary evaporation at reduced pressure, and then applied to the chromatography on silica gel (particle size: 32-63 μm) for the purification of 1-methylcarbonylthio-undec-1-en-11-ylhexa (ethylene glycol). The eluant for the chromatography was the mixture solution of ethyl acetate and methanol (9:1, v/v): Yield 78%. (^{1}H-NMR (300 MHz, $CDCl_3$), δ: 1.25 (14H), 1.5 (4H), 2.3 (3H), 2.4 (1H), 2.8 (2H), 3.4 (2H), 3.5-3.7 (24H)).

Synthesis of 1-Mercaptoundec-11-ylhexa (ethylene glycol): Solution of 1-methylcarbonylthio-undec-1-en-11-ylhexa (ethylene glycol) in 0.1 N HCl in methanol was deprotected by stirring at room temperature under an atmosphere of nitrogen. After 3 days, purification with chromatography on silica gel (particle size: 32-63 μm) gave 1-mercaptoundec-11-ylhexa (ethylene glycol) as alkenethiol derivative. The eluant for the chromatography was the mixture solution of ethyl acetate and methanol (4:1, v/v): Yield 90%. (^{1}H-NMR (300 MHz, $CDCl_3$), δ: 1.25 (14H), 1.35 (1H), 1.6 (4H), 2.5 (2H), 2.7 (1H), 3.45 (2H), 3.55-3.75 (24H)).

Fabrication of PDMS Stamp. Master mask patterns to form PDMS stamp were fabricated by using E-beam. Briefly, a quartz wafer with deposited 300 nm Cr layer was spin-coated with a layer (500 nm) of negative photoresist (SAL 601). Designed various shapes of an array of squares and strips were formed a pattern on the substrate upon exposure to E-beam (EBMF 10.5, Leica Co. German). Non-exposed part of spin-coated photoresist on the wafer was removed by treatment with MIF 312 developer for 27 sec. Then, the bare Cr deposit areas of wafer were removed by Cr-7K for 2.5 min. The photoresist coated on Cr-deposited quartz wafer was thoroughly removed by AZ remover 700 at 60 °C for 30 min, and photomask was prepared.

Next, a silicon wafer was spin-coated with a layer (1.52 μm thickness) of positive photoresist (AZ 1512). The spin-coated wafer was exposed to UV Aligner with 350 W mercury lamp (MA-6, Karl-suss Co.) through the photomask. Exposed photoresist on the wafer was removed by treatment with the MIF 300:deionized water (6:1, v/v) mixed developer solution for 70 sec. The wafer was etched by using plasma etcher (SLR-770-10R-B, Plasma Therm Co.), which had a depth of 2 μm. After the rest of photoresist on silicon wafer was removed by AZ remover 700 at 60 °C for 30 min, various submicro- and micro patterned shapes of an array of squares and strips were obtained on master mold (7 mm×7 mm), which had a depth of 2 μm.

PDMS stamps were fabricated by using the prepared mas-

ter mold, as followed. That is, the 10:1 (v/v) mixture of silicone elastomer-184 (Dow corning Co., Midland, MI) and silicone elastomer curing agent-184 (Dow corning Co., Midland, MI) was poured into the master mold. 1 h waited at room temperature under -60 mmHg vacuum until air bubbles in the mixture solution rose and were remove from the master surface. Then the prepolymer was cured at 100 °C in a conventional oven for above 1 h. After the cured PDMS is to be cool a little, the final stamp with a pattern of PDMS at the surface was obtained by detaching the cured PDMS from the mold.

The fabricated pattern on PDMS stamp was confirmed with using scanning electron microscopy (SEM, HITACHI, S520, Japan) and electron microscopy. That is, patterned PDMS stamp was rinsed several times with ethanol, dried thoroughly. Its patterned surface was coated with gold using a sputter coater (EIKO, Japan), and the microscope was operated at 20 kV to image the patterned PDMS stamp surface.

Preparation of Patterned SAMs. PDMS stamp was stored at 4 °C before moving into a small mouse bottle containing with hexadecanethiol (TCI Co.) covered the bottom of bottle. A PDMS stamp was hung in the middle of bottle using small wire at 45 °C for 15 min in order to adsorb alkanethiol molecules onto the surface of the PDMS stamp. Patterned substrates were prepared using a contact printing. The PDMS stamp was placed gently on a gold-coated glass wafer (Au (20 nm)/Cr (5 nm)/Glass wafer) with sufficient pressure to promote conformal contact between the stamp and the wafer. After 10 sec, the PDMS stamp was peeled away from the wafer. The wafer was immersed immediately in a solution of the 2 mM of 1-mercaptoundec-11-ylhexa (ethylene glycol) in ethanol for more than 2 h. The wafer was removed from solution, rinsed with ethanol, and deionized water.

Selective Coating with Fibronectin. The Patterned wafer was placed in a solution of fibronectin (human fibronectin, BD, 20 μg/mL in deionized water) at 4 °C. After an incubation for 2 h, the wafer was removed from the solution, carefully rinsed with deionized water to gain the wafer coated with fibronectin.

In order to confirm the formed pattern onto the gold-coated wafer, immunofluorescence staining was performed using an anti-fibronectin antibody (fluorescein anti-mouse IgG, Vector LAB). That is, the adsorbed fibronectin onto the wafer was fixed in 3.7% paraformaldehyde at room temperature for 20 min, rinsed 3 times with PBS. Prior to immunocytochemistry, the adsorbed fibronectin onto the wafer was blocked with 20% normal goat serum in PBS at room temperature for 30 min. Adsorbed fibronectin was incubated with monoclonal antibody against fibronectin (HFN 7.1, ATCC) at room temperature for 60 min. The surfaces were then rinsed 3 times with PBS, and incubated with a secondary antibody against fibronectin (fluorescein anti-mouse IgG, Vector LAB) at room temperature for 60 min. Following rinsing 3 times with PBS, the stained sample was then mounted on a microscope slide containing Vectashield (Vector LAB) and imaged using a fluorescence microscopy.

Cell Culture. Human dermal fibroblasts (N-HDFs) were isolated from newborn foreskins, removed during circumcision as described.[18] Circumcised foreskin samples were kept in F-medium (1 part of Ham's F-12 medium and 3 parts of DMEM, 10% FBS) containing 1% penicillin/streptomycin and 250 ng/mL of fungizone (Gibco-Invitrogen, San Diego, CA, USA) at 4 °C. Cells were isolated within 24 h after surgery. Foreskin samples were washed at least 8-fold in phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin (Gibco-Invitrogen, San Diego, CA, USA). Most subcutaneous tissue was removed from dermis with sterile surgical scissors and the remaining skin was minced to fine pieces less than 1 mm^2. Small pieces of tissues were treated with dispase II solution (2.4 U/mL, Roche, Mannheim, Germany) at 37 °C for 4 h. Dermal layer was separated from epidermal layer, washed, and further incubated in 10 mL of 0.35% collagenase (Roche, Mannheim, Germany) at 37 °C for 2 h. Isolated cells were dissociated into single cell suspension by gentle pipetting, washed twice and inoculated in F-medium at 2×10^4 cells/cm^2 in culture plate. The culture medium was changed two to three-fold a week.

The fibronectin-coated wafer was kept in PBS containing 10% penicillin-streptomycin (Gibco-Invitrogen, San Diego, CA, USA) for above 1 h prior to inoculation of N-HDFs on the surface of wafer. The wafer was copiously rinsed with PBS, was transfer to FGM. Primary cultured N-HDFs in 4 th passages were plated at a density of 800 cells/50 μL/cm^2 on wafer coated with fibronectin, were maintained at 37 °C at 5% CO_2. After desired culture time, the morphologies and physiological properties of adhered N-HDFs onto wafer coated selectively with fibronectin were evaluated by using immunofluorescence staining against fibronectin, β-actin (Dako), $\alpha5\beta1$ integrin (Chemicon), and DAPI (4',6-diamidino-2-phenylindole, Sigma). Cells were observed under fluorescence microscope (Olympus Optical Co., Japan).

Statistical Analysis. All determinations were made 4 times, and the results were expressed as mean±SD. Statistical significance was determined by Student's t-test with $p<0.05$ considerer significant.

Results and Discussion

Synthesis of o-Mercapto-hexa (ethylene glycol), [$HS(CH_2)_{11}(OCH_2\text{-}CH_2)_6OH$]. This step was to synthesize a hydrophilic hexa (ethylene glycol) terminated alkanethiolate that could fabricate a selective patterned SAMs onto gold-coated wafer using contact printing. As shown Figure 1, a hydrophilic hexa (ethylene glycol) terminated alkanethiol derivatives, o-mercaptohexa (ethylene glycol) were pre-

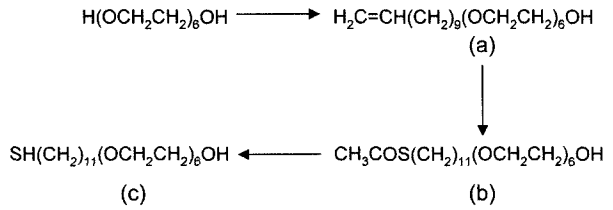

Figure 1. Scheme for synthesis of hydrophilic alkanethiol derivatives for 1-mercaptoundec-11-ylhexa (ethylene glycol) as start material, hexa (ethylene glycol). (a) undec-1-en-11-ylhexa (ethylene glycol), (b) 1-methylcarbonylthio-undec-1-en-11-ylhexa (ethylene glycol), and (c) 1-mercapto undec-11-ylhexa (ethylene glycol).

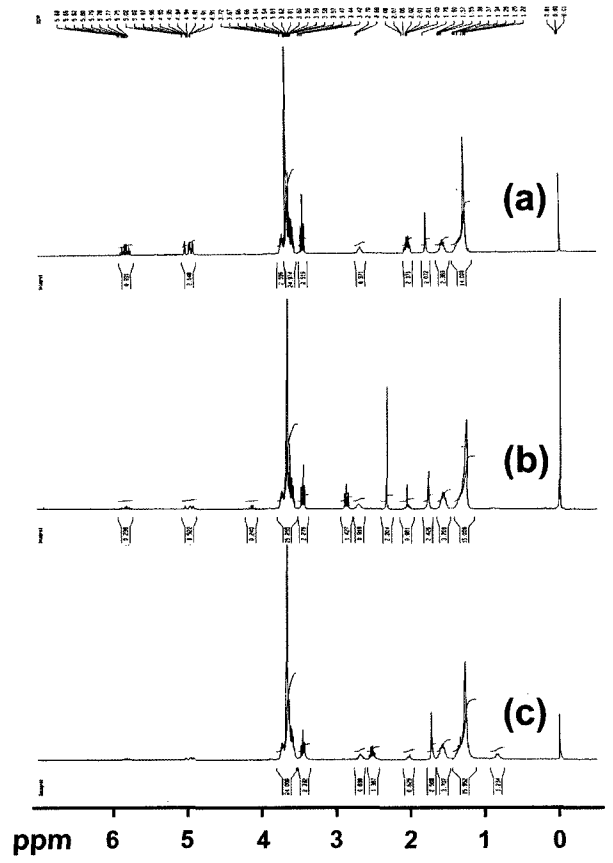

Figure 2. ^{1}H-NMR spectra of (a) undec-1-en-11-ylhexa (ethylene glycol), (b) 1-methylcarbonylthio-undec-1-en-11-ylhexa (ethylene glycol), and (c) 1-mercapto undec-11-ylhexa (ethylene glycol).

pared via a three-step synthesis, as previously described.[19]

^{1}H-NMR (Figure 2) spectra confirmed the structural changes with steps for synthesis of 1-mercaptoundec-11-ylhexa (ethylene glycol).

^{1}H-NMR spectra were measured on a Varian VX 300 using D_2O as a solvent.

The chemical shifts at 4.9-5.05 ppm, and 5.75-5.85 ppm due to alkene groups indicated the introduction of undec-1-en-11-yl groups into ethylene glycol molecules. We con-

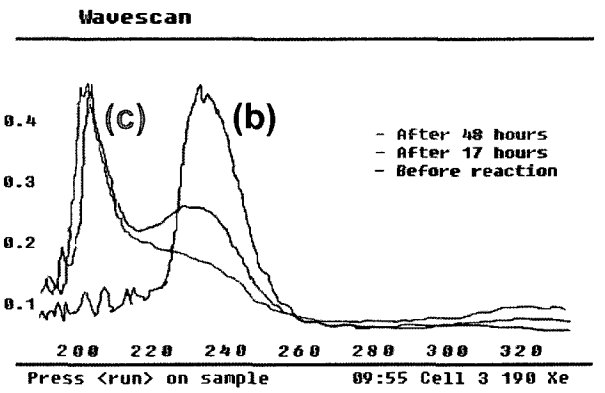

Figure 3. UV spectra of 1-methylcarbonylthio-undec-11-ylhexa (ethylene glycol) and its deacetylated derivatives. After deacetylation for 17 h, UV absorption at 206 nm due to S-H groups was observed. But UV absorption at 235 nm due to acetyl groups was remained. After 48 h, UV absorption at 235 nm was disappeared. (b) 1-methylcarbonylthio-undec-1-en-11-ylhexa (ethylene glycol), and (c) 1-mercapto undec-11-ylhexa (ethylene glycol).

firmed to synthesis a undec-1-en-11-hexa (ethylene glycol) (Figure 1, step 1). For the second reaction mediate, 1-methylcarbonylthio-undec-11-ylhexa (ethylene glycol), the two characteristic peaks of carbonylthiol groups were detected at around 2.30 and 2.80 ppm, due to acetyl groups and sulfide groups, respectively. In addition, the chemical shifts at 4.9-5.05 ppm, and 5.75-5.85 ppm due to alkene groups disappeared. For the final reaction to synthesis for 1-mercaptoundec-11-ylhexa (ethylene glycol), we monitored the deacetylation of 1-methylcarbonylthio-undec-11-ylhexa (ethylene glycol) by using UV spectra (Figure 3). After deacetylation for 17 h, UV absorption at 206 nm due to S-H groups was observed. But UV absorption at 235 nm due to acetyl groups was remained. After 48 h, UV absorption at 235 nm was disappeared. For the final product, 1-mercaptoundec-11-ylhexa (ethylene glycol), two characteristic peaks of carbonylthiol groups were detected at around 2.30 and 2.80 ppm, due to acetyl groups and sulfide groups, respectively, were disappeared. And the chemical shifts at 2.48-2.56 ppm due to the quartet methylene adjacent to sulfur indicated that disulfides were not present. These results indicate that 1-mercaptoundec-11-ylhexa (ethylene glycol) were prepared via a three-step synthesis in good yield without the disulfides. The product was stored under an atmosphere of nitrogen.

Fabrication of Patterned PDMS Stamp and Micro Contact Printing (μCP). We developed a soft lithographic method for patterning surfaced used in biochemistry and biology via four steps : (1) Preparation of a master pattern in silicon, (2) Fabrication of PDMS stamp using patterned wafer mold, (3) Formation of selectively patterned hydrophobic self assembled monolayer (SAMs) terminated alkanethiol on Au coated wafer by contact printing, and (4)

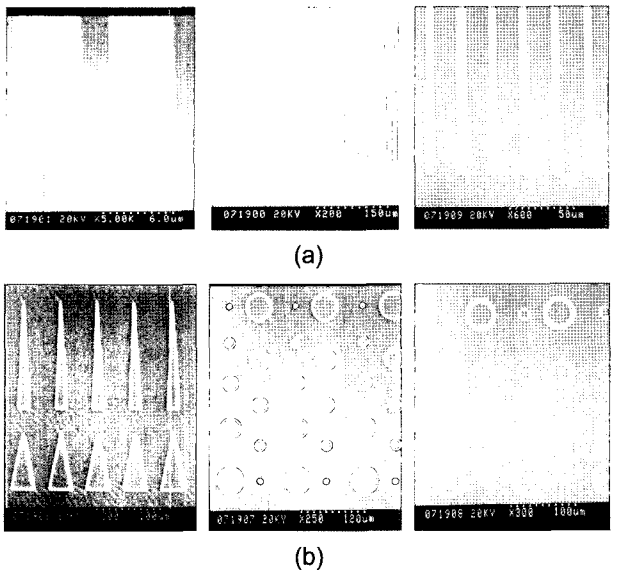

Figure 4. Scanning electron micrographs of (a) the surface of PDMS stamp with an array of 4 μm strips (Magnification: left ×5000, middle ×200, and right ×600) and (b) the surface of PDMS stamp with various shapes of pattern (Magnification: left, and right ×300, and middle ×250).

Formation of patterned hydrophilic SAMs terminated in hexa (ethylene glycol) onto the rest of Au coated wafer.

Master mask patterns to form PDMS stamp were fabricated by using E-beam. PDMS elastic stamp was prepared by casting the fluid prepolymer of an elastomer against a master that has a patterned structure. Figures 4 and 5 show a SEM of surface of this fabricated stamp. It was shown that the stamp has a depth of 2 μm, and various shapes of submicro- and micro pattern and an array of squares and strips were patterned on the substrate (7 mm×7 mm).

Preparation of Patterned SAMs. In order to form the patterned SAMs on gold-coated wafer, we first transferred the hexadecanethiol adsorbed on a PDMS stamp to gold-coated wafer using microcontact printing.[15-17,20] And then, the bare areas of the gold-coated surface of wafer were immersed to a different hydrophilic alkanethiol derivative, 1-mercaptoundec-11-yl hexa (ethylene glycol), to generate a surface patterned with another SAM. The patterned SAMs on gold-coated wafer was placed in a solution of fibronectin to gain the wafer coated with fibronectin. Formation of the patterned fibronectin onto the gold-coated wafer was confirmed by immunofluorescence staining against fibronectin. Figure 6 shows a fluorescent micrograph of adsorbed fibronectin onto the gold-coated wafer. The resulting pattern of adsorbed fibronectin onto the wafer resembled the surface of PDMS stamp with various shapes (see Figure 5) of pattern.

After gold-coated wafer had been coated with fibronectin, they were placed in petri dishes containing defined medium

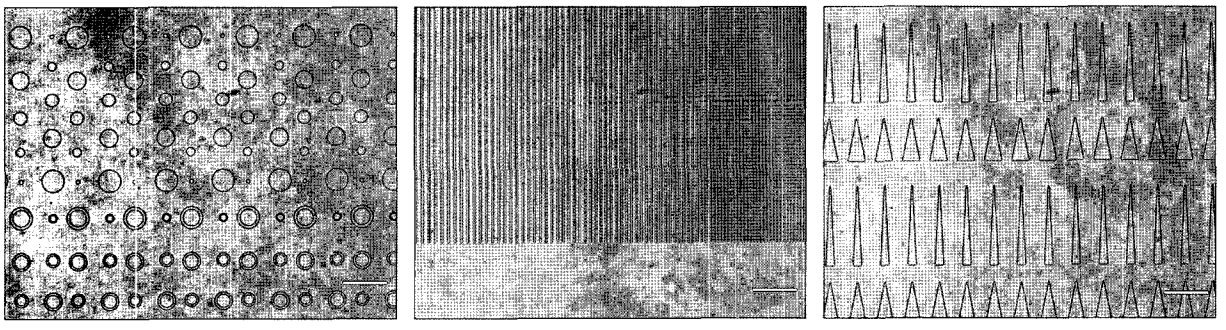

Figure 5. Stereo micrographs of the surface of PDMS stamp with various shapes of pattern (Magnification: left, and right ×200, and middle ×100; bar=100 μm).

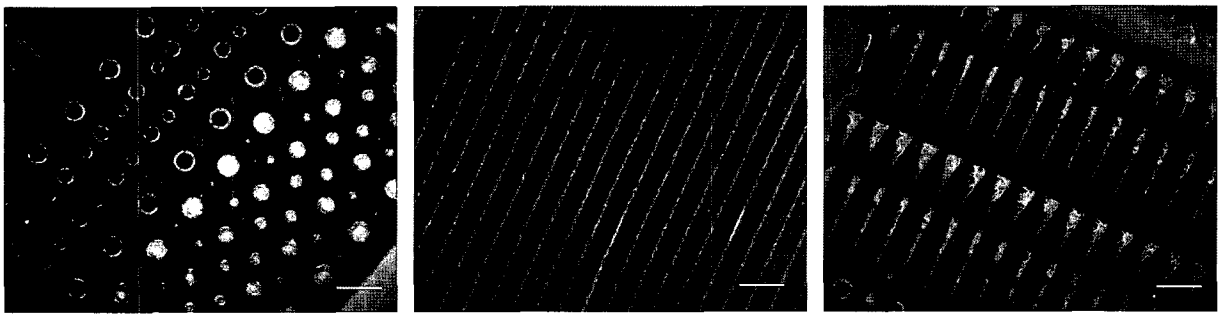

Figure 6. Fluorescent immunostaining of the various shapes of fibronectin coated to patterned alkyl-terminated SAMs with hexadecanethiol molecules (Magnification: left, and right ×200; bar=100 μm, and middle ×400; bar=50 μm).

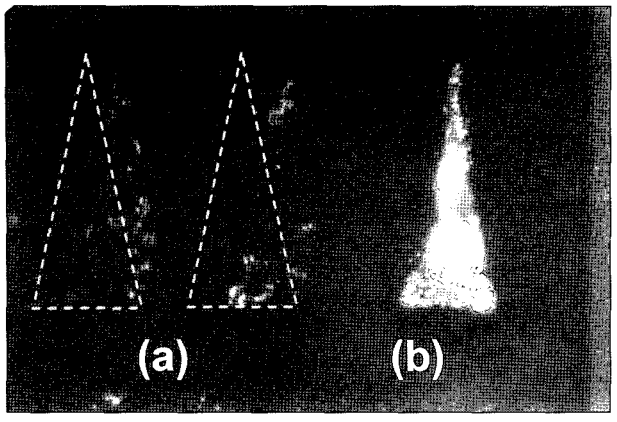

Figure 7. Fluorescent micrograph of fibronectin secreted by attached N-HDFs to each of triangle patterned wafer. (a) Control without N-HDFs on wafer coated with fibronectin and (b) with N-HDFs on wafer coated with fibronectin culture at 6 h. Magnification: ×400.

(10 mL). N-HDFs were added to the slides in defined medium, allowed to adhere. After an incubation for 4 h, defined medium was exchanged with fresh medium containing 10% bovine serum, and maintained in culture for several days. Figure 7 shows a fluorescent micrograph of fibronectin secreted by attached cells to each of patterns. After 6 h, N-HDFs were observed to attach to the patterned substrates only at regions of methyl groups-terminated SAM and immobilized fibronectin. The pattern of N-HDFs in this image conformed well to the pattern of SAMs, which the regions of hexa (ethylene glycol) groups-terminated SAM resisted the attachment of cells. The patterned SAMs of alkanethiolate on the wafer had two different properties, hydrophobicity and hydrophilicity. Alkyl-terminated SAMs were hydrophobic, which allowed proteins to adsorb hydrophobically in these area. However, hexa (ethylene glycol)-terminated SAMs were hydrophilic and resisted essentially the nonspecific adsorption of proteins, which SAMs terminated in hexa (ethylene glycol) groups resisted the attachment of cells-and the spreading of attached cells.[5]

In order to evaluate the behavior of an adherent cell on submicro- and micro-patterned surface, N-HDFs were used as a model representation of cells a biomaterial may encounter *in vivo*. The physiological behavior of N-HDFs from newborn foreskin on the substrate with gradient pattern spacing of 0.9-20 μm has been evaluated at 4.5 h at culture.

Cell adhesion did not significantly depend on the substrate with gradient a pattern spacing of 0.9-20 μm for 4.5 h culture. However, a variety of adherent cell morphologies were observed on the substrate with gradient pattern spacing of 0.9-20 μm. Figure 8 shows a phase contrast morphology of N-HDFs onto substrate with a pattern spacing of 0.9-20 μm. On the whole, N-HDFs onto substrate with a pattern spacing of 0.9-20 μm seemed to be well spread and aligned along the patterned line with having no relation between pattern spacing. However, there was a difference between their morphologies according to the gradient pattern space. Most of N-HDFs are found to spread with needlelike morphology on the pattern spacing of above 17 μm, while they

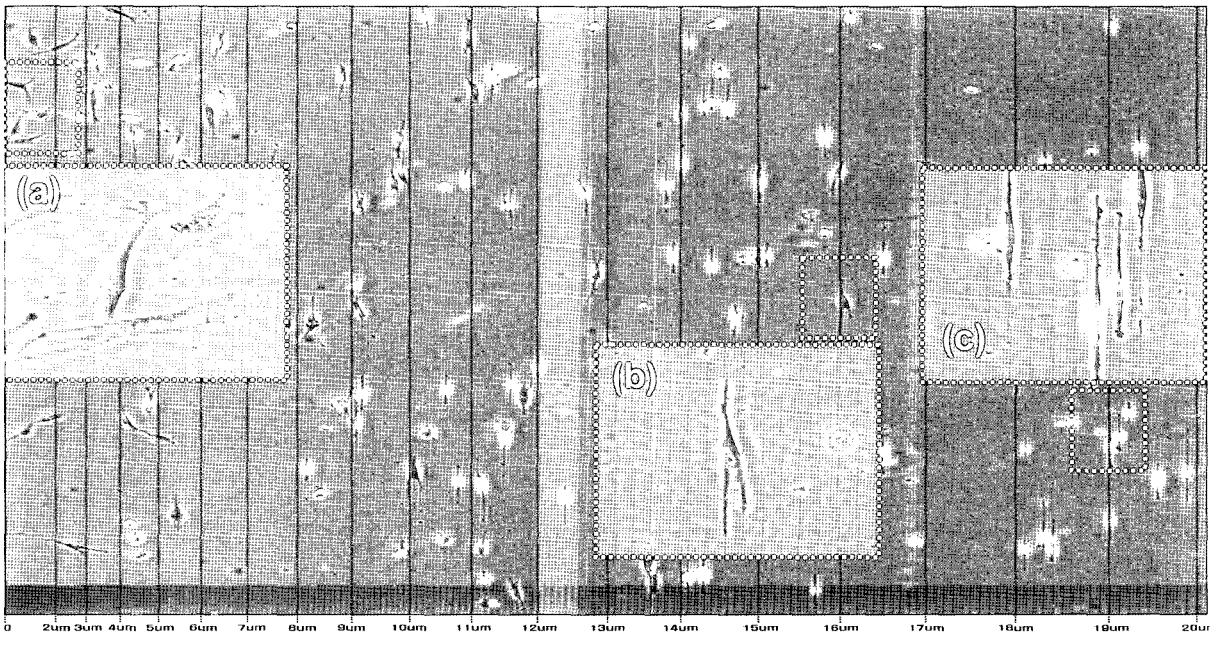

Figure 8. Phase contrast morphology of N-HDFs onto substrate with a pattern spacing of (a) 0.9-2 μm, (b) 15-17 μm, and (c) 18-20 μm. Magnification: ×40 and ×200.

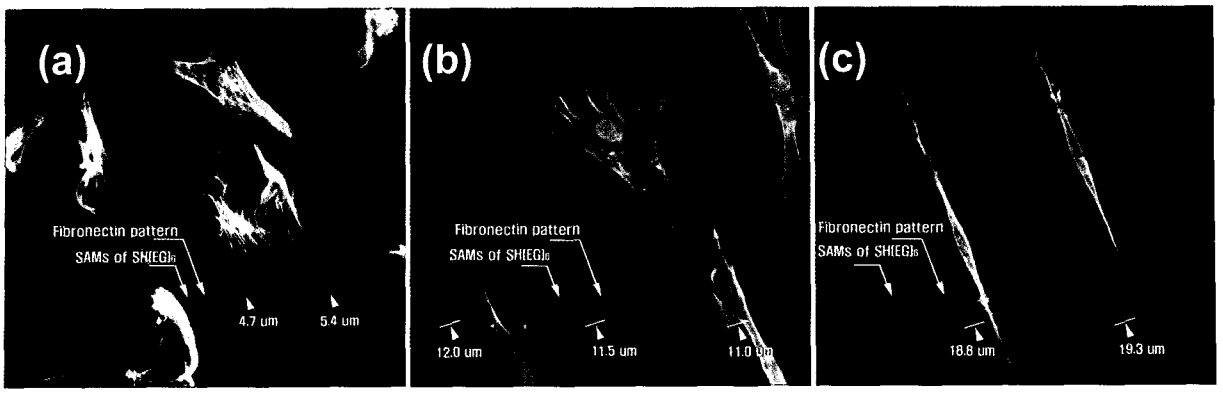

Figure 9. Pictures of immunohistochemical staining of N-HDFs at 4.5 h of culture onto substrate with pattern spacing of (a) 4-6 μm, (b) 10-12 μm, and (c) 18-20 μm. Red: β-actin; Green: $\alpha5\beta1$; DAPI: Blue. Representative fields were photographed. Magnification: ×400.

exhibit flattened morphology representative of well spread cells longitudinal along the fibronectin-coated micropatterns strips covered by two or three strips on the pattern spacing of below 14 μm. The cell alignment in needle type could not be observed at the pattern spacing of below about 10 μm. The adhesion and spreading of N-HDFs were more stable onto the hydrophobic regions in the pattern spacing of below 14 μm than those of above 17 μm.

To further evaluate the morphological characteristics of adhered N-HDFs onto wafer coated selectively with fibronectin after 4.5 h culture, the N-HDF was stained with immunofluorescence staining against β-actin, $\alpha5\beta1$ fibronectin binding integrin, cultured N-HDFs secreted, and DAPI for nuclear staining.

Figure 9 shows the pictures of immunohistochemical staining of N-HDFs onto fibronectin-coated wafer with pattern spacing at 4.5 h of culture. The immunofluorescence image of $\alpha5\beta1$ in green was overlapped with that of β-actin in red. The distribution of β-actin, one of cytoskeletal proteins, of N-HDFs onto substrate with a pattern spacing of 4-6 μm were freely spread up, and random over the area of substrate (Figure 9(a)). But the morphology of N-HDFs onto substrate with a pattern spacing of 10-12 μm is longitudinal along the fibronectin micropatterns covered by two or three strips (Figure 9(b)). Expression of β-actin at the gap larger than 17 μm was denser without any spreading, which a N-HDF alignment at this area was longitudinal along the fibronectin micropatterns covered by just one strip (Figure 9(c)), the nuclei was also elongated and aligned along the fibronectin micropatterns.

The elongations of cells and nucleus have been correlated with changes in gene expression profile and cell differentiation in other studies.[21,22] The nucleus is mechanically integrated with the physical entity of the cell. Forces are transferred to the nucleus through actin-intermediate filament system during changes in cell shape,[21,23] affecting the cellular structure and phenotype in N-HDFs.

Quantitative analysis of single cell attachment on sub-

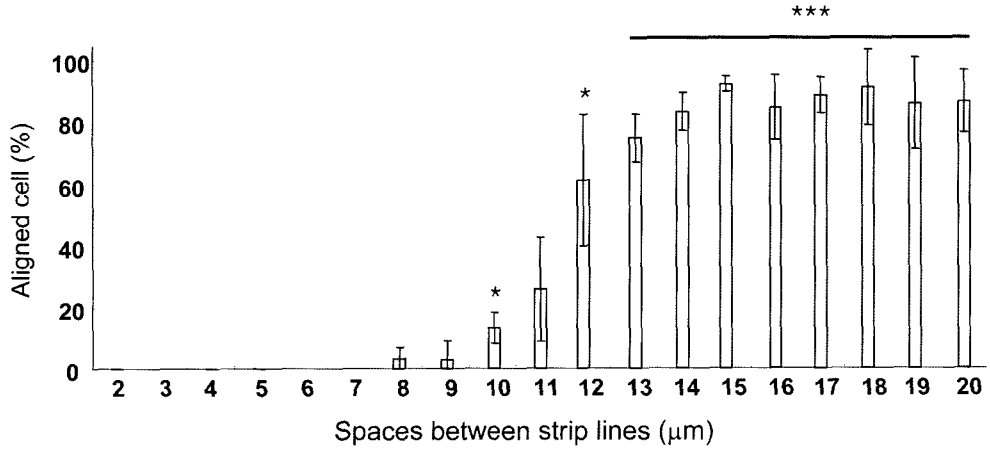

Figure 10. Quantitative analysis of single N-HDF attachment property on substrate with pattern spacing of 0.9-20 μm was measured as the ratio of needle type cells to total adherent cells along the patterned line at each spacing strip line from phase contrast image in Figure 8. At 4.5 h of culture. Error bars indicate standard error of the mean, n=4, *; p<0.05, ***; p<0.0005.

strate with pattern spacing of 0.9-20 μm was statistically measured as the ratio of needle type cells to total adherent cells along the patterned line at each spacing strip line from phase contrast image in Figure 8, according to the following equation (n=4).

$$\text{Aligned N-HDF (\%)} = A/B \times 100$$

where, A and B represent the number of adhering needle type cells at each spacing strip line, and total cells at each spacing strip line, respectively.

Figure 10 shows the adhesive property of N-HDF on substrate, measured according to above quantitative analysis equation. Quantitative analysis of single N-HDF attachment properties at 4.5 h of culture on substrate with pattern spacing of 0.9-20 μm was measured as the ratio of needle type cells to total adherent cells along the patterned line at each spacing strip line from phase contrast image in Figure 8. After initial adhesion for 4.5 h, N-HDFs on substrate were freely spread up to 7 μm of patterned spacing between strip lines. For at 8 and 9 μm of patterned spacing between strip lines, N-HDFs above 96.7±3.8 and 96.9±6.3%, respectively, on substrate were maintained their freely spreading morphologies. But, above 10 μm of patterned spacing between strip lines was to markedly reduce the N-HDF maintained as it freely spread on substrate. The N-HDF attachment was gradually decreased in 86.7±5.1, 74.1± 16.9, 38.3±21.2, and 25.2±7.6% as patterned spacing between strip lines was 10, 11, 12, and 13 μm, respectively.

These results show there are optimal pattern spacing between strip for well adhesion and migration of N-HDF. That is, the critical pattern spacing between strips was about 10 μm for migrating N-HDF to next to fibronectin-coated strip.

The size of fibroblast varies according to a microenvironment around cell, such as physical property of substrate, cell morphology, passage number, and aging etc. The size of a healthy N-HDF was generally known about 20 and 12 μm of length and width, respectively, when it has flattened morphology on culture dish.[24] Therefore, N-HDFs could not easily adhere and migrate across on the pattern spacing of above the size of them at the initial adhesion stage for 4.5 h of culture, could easily migrate on the substrate containing 10 μm or less spacing between strips. This discrepancy in cell and nuclei alignment may be reflected by filopodia protrusion, which may be critically limited by the adhesion-gap size over 10 μm. The findings demonstrated that fibroblasts are sensitive to a topography with regard to the gap of the strips, in a way that influences motility, and possibly also cell proliferation. This cell patterning may be applicable in various fields including neurobioscience and cell biology. Also, the patterning would be useful in evaluating the various cellular responses, such as focal contact and stress fiber formation, onto biomaterials surface. A further study on the mechanism of cell adhesion, and proliferation onto patterned SAMs is now in progress.

Conclusions

In this study, we developed a soft lithographic method for patterning surfaced used in biochemistry and biology via four steps: (1) Preparation of a master pattern in silicon, (2) Fabrication of PDMS stamp using patterned wafer mold, (3) Formation of selectively patterned hydrophobic SAM terminated alkanethiol on Au coated wafer by contact printing, and (4) Formation of patterned hydrophilic SAMs terminated in hexa (ethylene glycol) onto the rest of Au coated wafer.

1) We made the replica molding into silicon via photomask in quartz by E-beam lithography, and fabricated PDMS stamp using the designed silicon mold.

2) In order to form a hydrophilic SAM terminated alkanethiol, we synthesized the hydrophilic hexa (ethylene glycol) terminated alkanethiol [$HS(CH_2)_{11}(OCH_2CH_2)_6OH$] via a three-step synthesis. Its structural changes were confirmed by using UV, and ^{1}H-NMR.

3) Formation of patterned two kinds of SAMs onto the gold-coated wafer was confirmed by immunofluorescence staining against fibronectin.

4) Fibronectin was adsorbed only on SAMs terminated in the methyl groups of the substrate. N-HDFs (P=4) from newborn foreskin attached only to the fibronectin-coated, methyl-terminated hydrophobic regions of the patterned SAMs. The hexa (ethylene glycol)-terminated hydrophilic regions resisted the adsorption of protein and cells.

5) The initial adhesion and spreading of N-HDFs were more stable onto the hydrophobic regions in the pattern spacing of below 14 μm than those of above 17 μm.

6) N-HDFs alignment at the gap smaller than 10 μm is random but that at the gap larger than 10 μm is longitudinal along the fibronectin micropatterns covered by two or three strips, that at the gap larger than 17 μm is longitudinal along the fibronectin micropatterns covered by just one strip. This discrepancy in cell alignment may be reflected by filopodia protrusion, which may be critically limited by the adhesion-gap size over 10 μm.

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