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Formation and recovery of a cell sheet by a particle monolayer with the surface roughness

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ABSTRACT

We studied the topographical effect of roughness displayed by a closely packed particle monolayer on formation of a cell monolayer (cell sheet). Particle monolayers were prepared by Langmuir–Blodgett deposition using particles, which were 527 nm (SA053) and 1270 nm (SA127) in diameter. Human umbilical vein endothelial cells (HUVECs) were seeded at a high density (2.0×10^5 cells/cm^2) onto particle monolayers. It was found that cells gradually became into contact with adjacent cells on the SA053 monolayer and the formed cell sheet could be readily detached from the particle monolayer by gentle pipetting. On the other hand, cells adhering onto the tissue culture polystyrene (TCPS) and the SA127 particle monolayer were difficult to peel off. At a low cell seeding density (5.0×10^4 cells/cm^2), pre-coating with bovine plasma fibronectin (FN) allowed cell growth on an SA053 particle monolayer, and a confluent monolayer was able to be peeled as a cell sheet from the particle monolayer just by pipetting. By immunostaining of human fibronectin, we found that fibronectin was secreted and concentrated onto the substrate side of a cell sheet. The obtained cell sheet adhered and grew on the TCPS again within 20 min.

1. Introduction

Regeneration of dysfunctional or deficient tissues and organs is currently attracting lots of attention to overcome problems of organ transplantation, such as shortage of organ donors and immunological rejection. In terms of materials science, many approaches using a scaffold to construct tissues and organs from cells and biological entities have been studied as possible solutions to achieve the tissue regeneration [1–4]. Three-dimensional (3D) scaffolds for three-dimensional tissue structures such as heart, liver, pancreas and brain still remain problems including correct spatial arrangement of cells and construction of microenvironments for cell growth and differentiation. On the other hand, thin and two-dimensional (2D) structures such as skin and cartilage are successful examples for tissue engineering. Several techniques have been developed from the viewpoints of surface chemistry because surface properties often influence fundamental cell behavior such as attachment, adhesion, spreading, migration, and proliferation. Surface grafting with temperature-responsive polymers such as poly(*N*-isopropylacrylamide) is a sophisticated method developed by Okano et al. It enables us to recover a cell sheet by changing the wettability in response to ambient temperature, and has been used for reconstruction of cornea, skin, periodontal ligament, and myocardium [5–10]. Degradable polymer scaffolds could also allow peeling the cell sheet from the surface by hydrolysis and enzymatic degradation. For instance, a collagen gel and a fibrin gel have been used as scaffolds to prepare a cell sheet [11,12].

It is known that cell shape and orientation are strongly influenced by the surface topography such as convexes, concaves, pores, ridges, and grooves due to contact guidance [13–16]. The surface topography not only perturbs the plasma membrane such as ruffling membranes but also induces the changes in cytoskeletal systems including stress fiber, filopodia, and lamellipodia formation. Therefore, we focused on a cell–substrate interaction, and fabricated a two-dimensional colloidal array from particles ranging from 0.6 to 1.2 μm in diameter to examine interactions of neutrophil-like cells with a micropatterned surface. We reported that cell stimulation was changed by the pitch between the top of the arrayed particles [17,18], and that adherent cells changed their shapes as if they sensed the surface roughness with subcellular length scales [19]. These indicate that the cell–substrate interaction can be changed by tuning the surface roughness on the particle monolayer. It is thought that a cell–cell interaction plays important roles in cell assembly into a monolayer and cell detachment, which is caused by a constrained force that works among adjacent cells. We suppose that a balance between the cell–substrate interaction

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and the cell–cell interaction is important to peel a cell sheet from the substrate surface. In this study, we aimed to develop an easy and efficient method to fabricate a cell sheet in consideration of relations between the surface roughness and a cell detachment.

Human umbilical vein endothelial cells (HUVECs) were seeded on TCPS, an SA053 monolayer, and an SA127 monolayer at a high cell concentration (2.0×10^5 cells/cm^2) to facilitate the cell–cell interaction from the beginning of the cell culture. On the other hand, cells were seeded at a low cell concentration (5.0×10^4 cells/cm^2) to investigate cell growth on the particle monolayer. The cell culture was also performed on the particle monolayer coated with fibronectin (FN) to study the effect of fibronectin on cell proliferation and detachment. We tried to detach cells as a cell sheet from the substrate surface by pipetting with a micropipette. Cells were observed with an optical microscope to evaluate cell adhesion, shape, and proliferation. Furthermore, double immunostaining of F-actin and β-catenin, which is an intracellular protein that connects cadherin and actin cytoskeleton, was carried out to observe cadherin-mediated cell–cell adhesion with a fluorescence microscope. Localization of cell-secreted extracellular matrix (ECM) proteins was also observed by immunostaining of human fibronectin.

2. Materials and methods

2.1. Materials

Styrene (St) was purified by distillation under reduced pressure before use. Acrylamide (AAm) and potassium persulfate (KPS) were purified by recrystallization from benzene and water, respectively, and then dried in a vacuum. *N*,*N*-Dimethylformamide (DMF) was used without further purification. These reagents were all purchased from Wako Pure Chemicals (Osaka, Japan). The following reagents were used without further purification. Sodium chloride, ethanol and paraformaldehyde were purchased from Junsei Chemical (Tokyo, Japan). Rhodamine–phalloidin and bovine plasma fibronectin were purchased from Invitrogen Co. (CA, USA) and Koken Co. Ltd. (Tokyo, Japan), respectively. Mouse monoclonal antibodies against human β-catenin and human fibronectin were purchased from Sigma–Aldrich Inc. (MO, USA) and Takara Bio Inc. (Shiga, Japan), respectively. Fluorescein-conjugated goat anti-mouse IgG antibody was purchased from Chemicon (CA, USA). Polystyrene standard particles (474 ± 7 nm) were purchased from Nissin EM Co. Ltd. (Tokyo, Japan).

2.2. Preparation of particles and particle monolayers

Soap-free emulsion copolymerization of St was performed as follows. Ten grams of St, 120 g of distilled water, 0.025 g of sodium chloride, were added into a four-necked flask and then degassed by bubbling with nitrogen for 60 min under stirring at a speed of 300 rpm. The polymerization and purification of PS particles were carried out in a same manner as the preparation of SA particles. The obtained PS particle (532 ± 22 nm in diameter) was referred to as PS053. Poly(styrene-co-acrylamide) (SA) particles used to obtain particle monolayers were prepared according to a method described previously [19]. Soap-free emulsion copolymerization of St and AAm was performed as follows: St, AAm, distilled water, sodium chloride, and DMF were added into a four-necked flask and then deoxygenated by bubbling with nitrogen for 60 min under stirring at a speed of 300 rpm. The reaction temperature was raised to 70 °C and an initiator solution was then injected rapidly into the reaction mixture to start the polymerization. The polymerization proceeded for approximately 24 h under a nitrogen atmosphere. The resulting particles were purified by centrifugation, decantation and resuspension. The dried particle diameters were measured using a transmission electron microscope (HU-12AF, Hitachi, Tokyo, Japan). The zeta-potential of particles was measured in 150 mM phosphate-buffered saline (Zeecom, Microtec Co. Ltd., Chiba, Japan). A monolayer of PS particles was prepared by spin-coating method. One milliliter of 2.5 wt% PS053 latex (in ethanol) was added into a PS dish. After 7-h incubation, sample was spun at 2000 rpm for 60 s by spin-coater (K-359S-1, Kyowariken Inc., Tokyo, Japan). PS particle monolayer on a PS dish was cut square (10 mm × 10 mm on a side) and used in following experiments. Two kinds of SA particles with different sizes (527 ± 8 nm and 1270 ± 20 nm in diameter) were used in this study and were referred to as SA053 and SA127. A monolayer of particles was prepared by the Langmuir–Blodgett (L–B) technique [20]. The suspension of SA particles in ethanol was gently added drop-wise on the surface of the aqueous solution containing 0.008 M KCl in a Teflon trough (75 mm × 320 mm) using a micropipette. The spread particles were allowed to stand on the water surface for 30 min, and then surface compression was carried out at a speed of 4 mm/min by a moving barrier of L–B film deposition apparatus (NL-LB 240N-MWC, Nippon Laser and Electronics Lab., Nagoya, Japan). The monolayer of particles was transferred onto a circular cover glass (15 mm in diameter) at a surface pressure of 18 mN/m and dipping speed of 4 mm/min. After the particle monolayers were dried in air, heat treatment at 100 °C above the glass transition temperature (T_g) was carried out for 30 min to fuse particles. The obtained surfaces were observed using a field-emission scanning electron microscope (FE-SEM S-4700, Hitachi, Tokyo, Japan) and a scanning probe microscope (SPM-9500J3, Shimadzu, Kyoto, Japan). Static contact angles against water were measured using the inverted bubble method. Five measurements on different parts of the dish were averaged. Water was purified by deionization after double distillation.

2.3. Cell culture

Human umbilical vein endothelial cells and endothelial growth medium (EGM-2 Bullet Kit) were purchased from Cambrex Co. (NY, USA). Endothelial growth medium contains 2% fetal bovine serum (FBS), human epidermal growth factor (hEGF), heparin, vascular endothelial growth factor (VEGF), human fibroblast growth factor-β (hFGF-β), ascorbic acid, R3-insulin-like growth factor-1 (IGF-1), hydrocortisone, and geneticin/amphotericin. Cells were cultured in the endothelial growth medium at 37 °C in an atmosphere of humidified air containing 5% CO_2. When cells were grown to 80–90% confluence, cells were passaged and used for experiments. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) without Ca^{2+} and Mg^{2+} and then removed from the dish with Hank's balanced salt solution (HBSS) containing 0.1% trypsin and 4 mM EDTA for 3 min. The cell suspension was transferred to a centrifuge tube, and centrifuged at 1000 rpm for 5 min at room temperature. After centrifugation, the supernatant was removed by decantation and a cell pellet was suspended again with a fresh medium. The cell number was counted with a hemocytometer and the cell suspension was then diluted with a medium to adjust the cell seeding density.

2.4. Cell detachment and adhesion of a cell sheet

The monolayers of PS053, SA053 and SA127 particles were transferred to a circular glass cover to investigate the effect of surface roughness on cell proliferation and detachment. A square-shaped slip (10 mm × 10 mm on a side) fabricated by cutting a TCPS dish