Effect of Hysteresis of Stimulation of Tangential Force Field on Alignment of 3T3-L1 Cultured on Micro Striped Pattern

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ABSTRACT

The effect of hysteresis of the tangential force field at the surface of the scaffold on the single cell has been studied *in vitro*. The striped pattern (0.7 μm height, 3 μm width, and 3 μm interval) were made on the surface of the scaffold plate to control the orientation of each cell. Variation was made on the angle between the longitudinal direction of the ridge and the direction of the tangential force: 0 degree, 45 degrees, and 90 degrees. 3T3-L1 (mouse fat precursor cells) was used in the experiment. To apply the tangential force field (100 G) to the cells, the scaffold plate was set in the tube in a conventional centrifugal machine placed in an incubator. After the centrifugation for 5 hours, the behavior of each cell at the time-lapse microscope images was traced for 24 hours to analyze the angle between the longitudinal axis of the cell and the direction of the centrifugation. The experimental results show that the tendency of cells to align the longitudinal direction of the striped pattern is strengthened by the hysteresis of the exposure to the tangential force field. The tendency continues before the proliferation of each cell.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravity and 3T3-L1.

1. INTRODUCTION

The recent cell culture technique enables observation of the behavior of each cell *in vitro*. A biological cell adheres on the scaffold and shows several active behaviors: migration, deformation, and proliferation. The cell is deformed by the force, because of its compliance. The cell deforms, on the other hand, to minimize the intra force. The cell is moved by the force. The cell, on the other hand, moves by itself. 3T3-L1 is cell line of mouse fat precursor cells. In the previous study, both the migration and the deformation of 3T3-L1 were restricted by the wall shear stress higher than 1 Pa in flow *in vitro* [1]. Deformability of 3T3-L1 was observed by the slit in the previous study *in vitro* [2, 3]. 3T3-L1 extended along the lines of micro ridges on the scaffold surface [4]. Vibration decelerated adhesion of 3T3-L1 on the micro ridges [5]. 3T3-L1 migrated regardless of the direction of the vibrating micro ridges. The orientation of 3T3-L1 was tried to be measured by the electric impedance *in vitro* [6]. Both differentiation and growth of 3T3-L1 were delayed with electric pulses in the previous study *in vitro* [7]. The floating cells of 3T3-L1 were tried to be sorted by the micro groove on the wall of the flow channel [8].

These behaviors might depend on the history of each cell. While the strong stimulation above the threshold damages the cell, the stimulation below the threshold remains in the cell as a memory for the response in the next step. To trace the hysteresis effect of the mechanical stimulation on the single cell, the time-lapse images are effective. In the previous study, the longitudinal axis of C2C12 (mouse myoblast cell line) tends to align to the direction of the excess gravity. The axis tilts to the perpendicular direction, on the other hand, after stopping of the excess gravity [9]. A single cell migrates at random on the scaffold. The cell tends to align to the longitudinal direction of the micro ridge line [10].

In the present study, centrifugal force has been used to apply the tangential force field on the surface of the scaffold. The hysteresis effect of the force field on the single cell attached on the scaffold has been investigated. To examine the effect of the direction of the force field on the cell at the specific alignment, the orientation of the cell was controlled by the striped pattern on the surface of the scaffold. The effect of the mechanical field on orientation and deformation of a single cell on the micro striped pattern has been studied by centrifuge *in vitro*, and the behavior of each cell has been traced after stopping of the stimulation of the excess gravity.

2. METHODS

Micro-pattern on Scaffold Plate

The micro striped pattern has been made in three partial rectangular areas of 0.4 mm × 1.6 mm on the PDMS (polydimethylsiloxane) plate of the scaffold by photolithography technique. The height (H), the width (W), and the interval (I) of the quadrangular ridges are 0.7 μm, 3 μm, and 3 μm, respectively. Each area has its own specific direction of the striped pattern. Namely, variation has been made on the angle (θ) between the longitudinal direction of the ridge and the direction of centrifuge: 0 degree (parallel), 45 degree, and 90 degree (perpendicular). Three partial area was made on the same surface of the scaffold plate in parallel position, so that the behavior of cells on each area can be compared simultaneously (Fig. 2). The pattern of each area was also used as a marker to trace each cell.

Mold for Micro Pattern

The borosilicate glass (Tempax) disk was used for the base of the mold through micromachining process. The diameter and the thickness of the disk are 50 mm and 1.1 mm, respectively. To remove micro particles on the surface of the glass, the

oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1, Samco Inc., Kyoto, Japan).

To improve affinity to photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the glass plate at 3000 rpm for 30 s with a spin coater. The positive photoresist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the glass with the spin coater (at 3000 rpm for 20 s). The photoresist was baked in the oven (DX401, Yamato Scientific Co., Ltd) at 373 K for ninety seconds.

The micro pattern was drawn on the mold with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). The pattern was baked in the oven at 373 K for five minutes. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for two minute, rinsed with the ultrapure water for three minutes twice, and dried by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan). The glass plate with the photoresist material was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan). For etching, the gas of CF_4 (30 cm^3/min at 1013 hPa) was applied at 100 W at 2 Pa.

To remove the residual OFPR-800LB on the surface of the glass, the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1).

After the mold of the glass disk was enclosed with a peripheral wall of polyimide tape, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was poured with the curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning Corp., MI, USA) on the mold. The volume ratio of PDMS to curing agent is ten to one. After degassing, PDMS was baked at 338 K for one hour in an oven (DX401). The baked plate of PDMS (1 mm thickness) was exfoliated from the mold of the slide glass plate. The PDMS plate was cut to make a plate of 15 mm × 10 mm × 1 mm, and stacked on the glass plate of 50 mm × 13 mm × 1 mm. The surface of the PDMS plate was exposed to the oxygen gas (0.1 Pa, 30 cm^3/min) in the reactive ion etching system (FA-1: oxygen plasma ashing, 50 W) for thirty seconds just before the cell culture.

Tangential Force Field

The tangential force field was applied to the culture surface with the centrifugal force. The culture plate is inserted into the tube, which is contained in the rotor. The angle between the radial direction of the rotation of the rotor and the axial direction of the tube in the rotor is 60 degree. The glass plate was set so that the culture surface is parallel to the radial direction of the rotation. The centrifugal force is applied parallel to the culture surface. The centrifugal force (F_c) is calculated by Eq. (1).

$$F_c = m\, r\, \omega^2 \tag{1}$$

In Eq. (1), m is mass, r is radius of the rotation, and ω is angular velocity. In the gravitational field, gravitational force (F_g) is calculated by Eq. (2), where g is gravitational acceleration.

$$F_g = m\, g \tag{2}$$

In the present study, the centrifugal acceleration of 100 G (1 G is equal to the gravitational acceleration) ((Fc / Fg) = 100) is generated with the centrifugal machine (CN-1040, Matsuuraseisakusyo. Ltd, Tokyo, Japan).

Cell Culture

3T3-L1 (mouse fat precursor cells, a cell line derived from cells of mouse 3T3) of the passage between fourth and tenth was used in the tests. Cells were cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. The cells were seeded on the glass plate at the density of 3000 cells/cm^2. After the cells were cultured for 12 hours in the static state (1 G: normal), excess gravitational stimulation was applied for five hours. The gravity of 100 G (r = 0.09 m) were applied at ω = 104 rad/s.

To apply the excess gravity, the plate, on which cells adhered, was set in the medium in the tube. Several tubes with the glass plate were set in the rotor of the centrifugation to cultivate cells, simultaneously. To keep the content of carbon dioxide of 5 % at 310 K, the cells were cultured in an incubator through the entire experimental term including the term of exposure to the hyper-gravitational field. Namely, the centrifugal machine was placed in the incubator during the centrifugation.

In the control group, the cells were cultured without centrifuge on the plate, which was placed in the polystyrene dish. In the control group, no tangential force field was applied, and only the gravitational force (1 G) normal to the scaffold surface plate was applied.

Image Analysis

After stimulation of the excess gravity, cells on the glass plate were moved from the centrifugal tube to the culture dish. Cells were subsequently cultured in the dish placed in the incubator combined with an inverted phase-contrast microscope (LCV110-SK, Olympus Co., Ltd., Tokyo). The behavior of each cell was analyzed on the time-lapse images captured every five minutes for 24 hours after stimulation of the excess gravity. "Image J" was applied to analyze the behavior of each cell. On the microscopic image, the outline of each cell was traced, and the contour of each cell was approximated to ellipsoid (Fig. 1). On the ellipsoid, the length of the major axis (a), and the minor axis (b) were measured. The ratio of axes is calculated as the shape index (P) by Eq. (3).

$$P = 1 - b / a \tag{3}$$

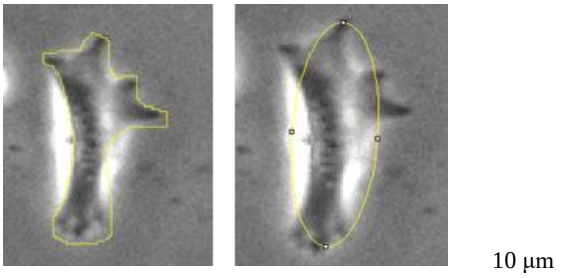

Fig. 1: On microscopic image, outline of each cell was traced (left), and contour of each cell was approximated to ellipsoid (right).

At the circle, P = 0. As the ellipsoid is elongated, P approaches to 1.

The angle (0 degree < θ < 90 degrees) between the direction of the centrifuge and the direction of the major axis of each cell was measured at the microscopic image. When the major axis is parallel to the direction of centrifuge, θ = 0. When the major axis is perpendicular to the direction of centrifuge, θ = 90 degree.

3. RESULTS

By the time-lapse images, continuous activity of each cell was confirmed: migration, deformation, and proliferation (Figs. 2a & 2b). Each cell repetitively extends pseudopods. The centrifuge direction was easily found by the stripe patterns of 0 degree. The behavior of each cell was easily traced with the help of the strip patterns as markers.

In Figs. 3 & 5, each datum point shows the value of each cell at 12 hours, 17 hours, 23 hours, 29 hours, 35 hours, and 41 hours after seeding. The timing of every 6 hours coincides to the centrifugal force stimulation group.

In Figs. 4 & 6, each datum point shows the value of each cell before centrifuge (before), immediately after stopping centrifuge (after), and every 6 hours after stopping centrifuge, respectively: before centrifugal force stimulation, immediately after centrifugal force stimulation for 5 hours, 6 hours after centrifugal force stimulation, 12 hours after centrifugal force stimulation, 18 hours after centrifugal force stimulation, and after 24 hours after centrifugal force stimulation.

Each cell elongates at random orientation on the flat surface with no striped pattern without centrifugation: the angles θ scatters in Fig. 3a. The tendency is the same on the flat surface after centrifugal force stimulation of 100 G (Fig. 4a).

Without centrifugation, each cell not only elongates along the longitudinal direction of the striped pattern, but also tilts to random orientation on the striped pattern: 0 degree in Fig. 3b, 45 degree in Fig. 3c, and 90 degree in Fig. 3d.

Each cell tends to align along the striped pattern by the centrifugal force stimulation of 100 G: 0 degree in Fig. 4b, 45 degree in Fig. 4c, and 90 degree in Fig. 4d. The tendency is relieved in 6 hours after stopping the stimulation, and cells tend to apart from the centrifugal force direction. No cell is in the area of 45 degree at 12 hours, after stopping of the centrifugal force stimulation (Fig. 4c).

On the flat surface with no striped pattern, each cell randomly repeats deformation between elongation and shrinkage without centrifugation: the shape index (P) scatters (Figs. 5a & 6a). On the striped pattern, on the other hand, cells tend to elongate more frequently than on the flat surface in 24 hours. The shape index (P) tends to increase on the striped pattern: 0 degree in Fig. 5b, 45 degree in Fig. 5c, and 90 degree in Fig. 5d. Some cells deform to the round shapes and proliferate after 6 hours from stopping the centrifugal force stimulation on the striped pattern (after 24 hours from seeding): 0 degree in Fig. 6b, 45 degree in Fig. 6c, and 90 degree in Fig. 6d.

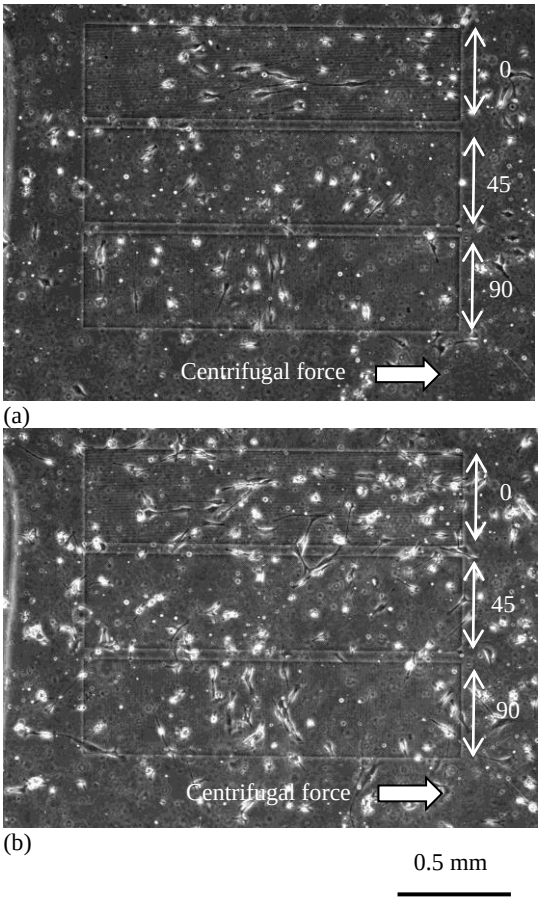

Fig. 2: Cells immediately after stopping centrifuge (a) at 24 hours after stopping centrifuge (b): 0 degree (upper), 45 degree (middle), and 90 degree (lower) in each picture.

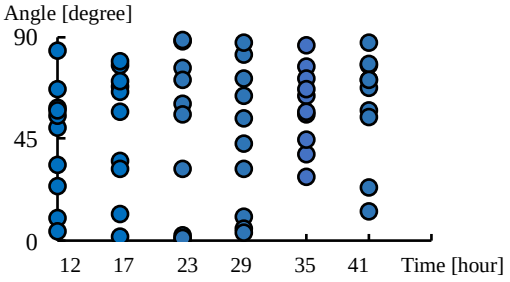

Fig. 3a: Angle of 3T3-L1: control, no pattern.

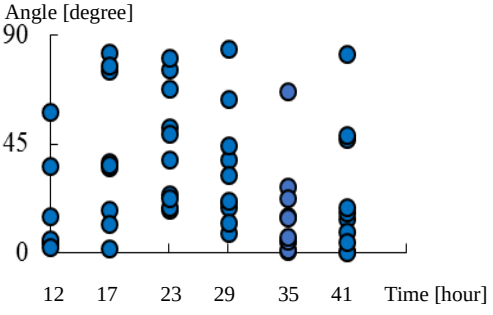

Fig. 3b: Angle of 3T3-L1: control, 0 degrees.

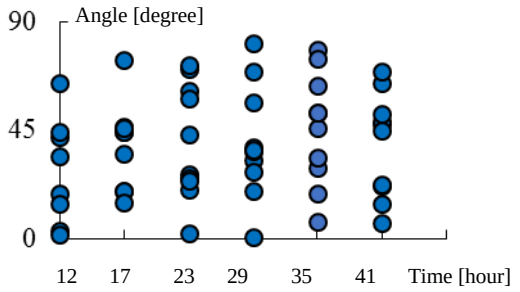

Fig. 3c: Angle of 3T3-L1: control, 45 degrees.

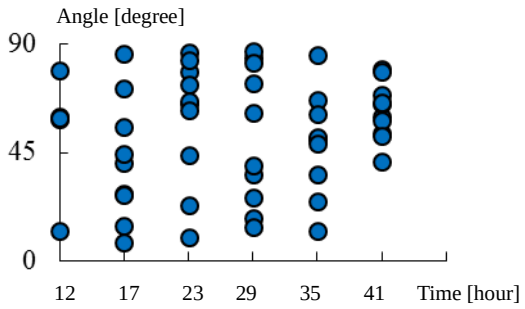

Fig. 3d: Angle of 3T3-L1: control, 90 degrees.

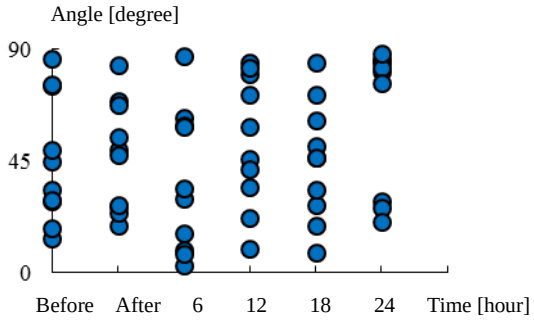

Fig. 4a: Angle of 3T3-L1: 100 G, no pattern.

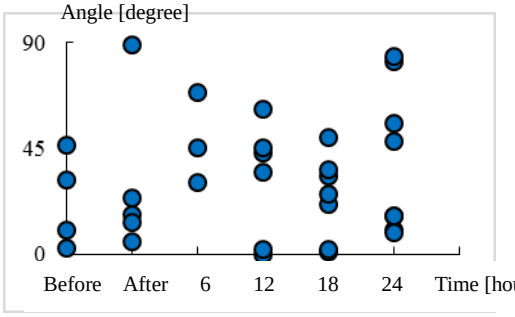

Fig. 4b: Angle of 3T3-L1: 100 G, 0 degree.

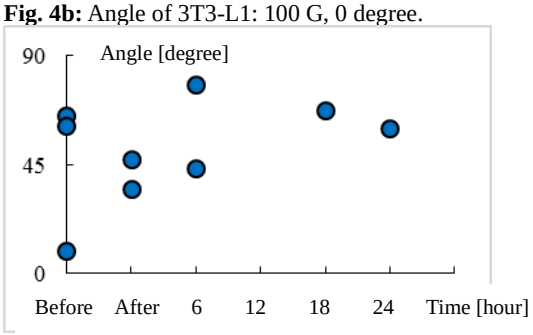

Fig. 4c: Angle of 3T3-L1: 100 G, 45 degrees.

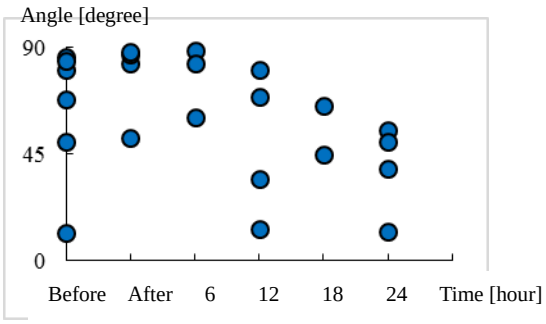

Fig. 4d: Angle of 3T3-L1: 100 G, 90 degrees.

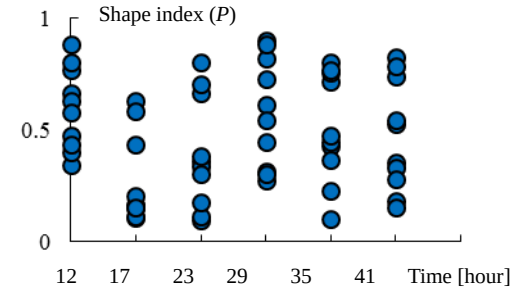

Fig. 5a: Shape Index (*P*) of 3T3-L1: control, no pattern.

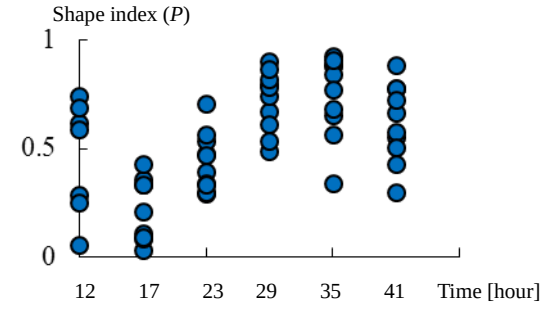

Fig. 5b: Shape Index (*P*) of 3T3-L1: control, 0 degree.

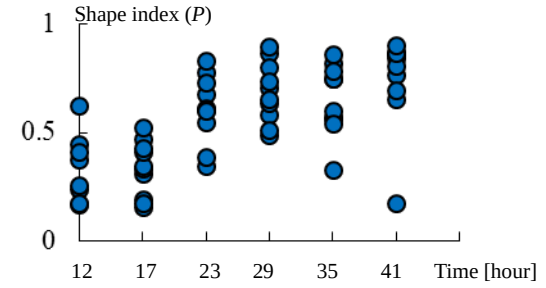

Fig. 5c: Shape Index (*P*) of 3T3-L1: control, 45 degrees.

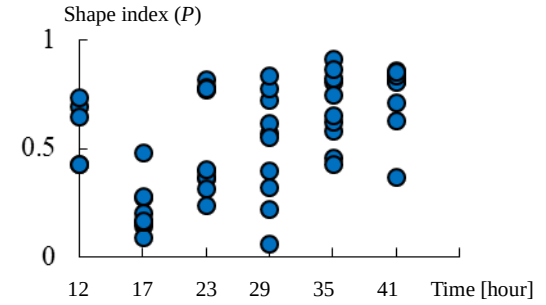

Fig. 5d: Shape Index (*P*) of 3T3-L1: control, 90 degrees.

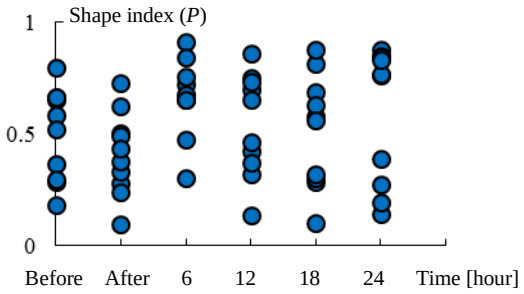

Fig. 6a: Shape Index (*P*) of 3T3-L1: 100 G, no pattern.

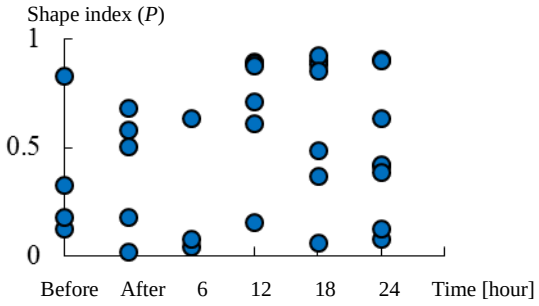

Fig. 6b: Shape Index (*P*) of 3T3-L1: 100 G, 0 degree.

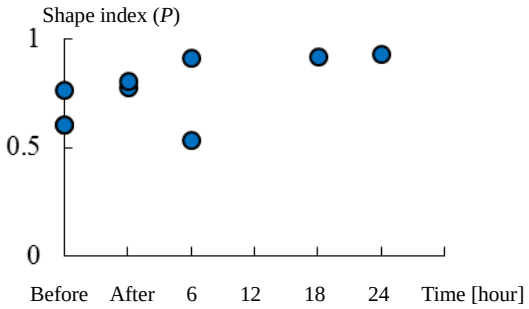

Fig. 6c: Shape Index (*P*) of 3T3-L1: 100 G, 45 degrees.

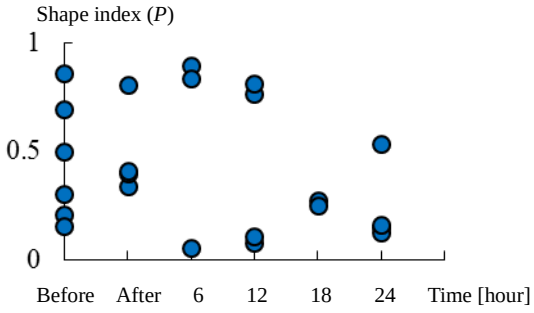

Fig. 6d: Shape Index (*P*) of 3T3-L1: 100 G, 90 degrees.

4. DISCUSSION

The hysteresis of centrifugation might make each cell to increase the contact area with the surface of the scaffold. Each cell might adapt to the tangential force field by maximization of the contact area between the cell and the scaffold.

To control the alignment of the single cell on the scaffold before stimulation, the micro stripe pattern has been used in the present study. The height of the ridge is designed as 0.7 μm, which is slightly higher than threshold to make orientation of cells [10]. The average diameter of the floating cell in the medium is 20 μm. The low ridge and the narrow width were selected so as not to disturb cell migration. Both deformation and migration of 3T3-L1 are at random over the striped pattern in the present study. Immediately after the tangential force stimulation of 100 G, 3T3-L1 frequently tilts to the striped pattern in the present study (Figs. 4b–d). C2C12, on the other hand, tended to elongate and align along the striped pattern even in the case without the tangential force stimulation in the previous study [9]. The tilting tendency of C2C12 was enhanced by the tangential force of 100 G. C2C12 tends to elongate during the tangential force stimulation. The mild tendency was observed on MC3T3-E1 in the previous study [11]. The behavior of each cell can be easily traced with the aid of the micro striped pattern on the scaffold as the marker. The tracking ability both to the tangential force and to surface morphology was higher in C2C12 than in MC3T3-E1 in the previous study. The tracking ability of 3T3-L1 is much lower than that of MC3T3-E1. On the striped pattern parallel to the tangential force (0 degree), every type of cell tracked along the striped pattern. The tracking ability on another direction of striped pattern depends on the cell types.

Most of cells do not peel off, but keep adhesion on the scaffold and randomly migrate on the scaffold. 3T3-L1 tends to migrate from the area of 45 degree, and to refrain from migration to the area of 45 degree in 12 hours after hysteresis of the tangential force stimulation of 100 G (Figs. 4c & 6c). C2C12, on the other hand, tended to stay on the micro striped pattern regardless of the angle between the longitudinal direction of the concave-convex striped pattern and the tangential force. In the previous study, C2C12 was most frequently elongated on the striped pattern of 45 degree between the tangential force direction and the longitudinal direction of the stripe [9]. The elongated direction of the pseudopods of the cell aligns to the longitudinal direction of the pattern on the scaffold. The cell elongates more frequently on the striped pattern than on the flat surface.

The gravity in the fluid is reduced by the buoyancy. Measurement of the density of cells by Phthalate ester method shows that the mean density of each cell is 1.07×10^3 kg/m^3. When the cells floating in the medium of the density of 1.00×10^3 kg/m^3, the effective centrifugal force ratio calculated from the difference of two density is 7 G at centrifuge of 100 G.

The previous studies showed that the mechanical [12] and the electrical [13–15] fields governs behavior of cells. The effect of shear flow on orientation of cells depends on the kinds of cells [16, 17]. The response of biological system to the microgravity field has been studied using a space satellite. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [18, 19]. The previous study shows that the hyper-gravitational field thickens the myotubes *in vitro* [20]. The hypergravity promotes proliferation and differentiation of cells [21, 22]. The acceleration technique for orientation, proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro*. Control methodology for orientation, proliferation and differentiation of cells would be applied to the regenerative tissue technology.

The present experimental results show following results. 3T3-L1 shows migration, deformation, and proliferation, even after the tangential centrifugal force stimulation of 100 G.

Without the tangential centrifugal force stimulation, each cell not only elongates along the longitudinal direction of the striped pattern, but also tilts to random orientation on the striped pattern. The tendency of cells to align the longitudinal direction of the striped pattern is strengthened by the hysteresis of the exposure to the tangential force field. The tendency of each cell continues before its proliferation.

5. CONCLUSION

The effect of hysteresis of the tangential force field at the surface of the scaffold on the single cell has been studied *in vitro*. The striped pattern (0.7 μm height, 3 μm width, and 3 μm interval) were made on the surface of the scaffold plate to control the orientation of each cell. Variation was made on the angle between the longitudinal direction of the ridge and the direction of the tangential force: 0 degree, 45 degrees, and 90 degrees. The experimental results show that the tendency of 3T3-L1 (mouse fat precursor cells) to align to the longitudinal direction of the striped pattern is strengthened by the hysteresis of the exposure for 5 hours to the tangential centrifugal force field (100 G). The tendency continues before the proliferation of each cell. The average cell cycle of proliferation is 24 hours after seeding.

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