Behavior of Cell Cultured on Micro Striped Pattern after Stimulation of Excess Gravity

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ABSTRACT

The behavior of a single cell cultured on the micro striped pattern has been observed after stimulation of the excess gravity *in vitro*. The lines of parallel micro quadrangular ridges (0.7 μm height, 3 μm width, and 3 μm interval) are made on the surface of the plate as the scaffold to make orientation of each cell. Variation is made about the angle between the longitudinal direction of the ridge and the direction of the excess gravity: 0 degree, 45 degrees, and 90 degrees. To apply the excess gravitational force field (50 G, or 100 G) to the cells, the plate was set in the tube in a conventional centrifugal machine placed in an incubator. The contour of each cell at the time lapse images was traced for 24 hours and approximated to ellipse to analyze the angle between the longitudinal axis of the cell and the direction of the excess gravity, after the excess gravitational stimulation for 5 hours. The experiment shows that the longitudinal axis of C2C12 (mouse myoblast cell line) tends to align to the direction of the excess gravity, and returns to the direction of longitudinal direction of micro pattern after stopping of the excess gravity.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravity and C2C12.

1. INTRODUCTION

A biological cell shows passive and active behaviors in an environment. While the shear stress deforms the cell, for example, the cell deforms by itself to adapt to the shear field. 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.

A biological cell is exposed to the mechanical force field *in vivo*. The cell is sensitive to the mechanical stimulation, and shows several responses: deformation, and migration. The cell has compliance, and is deformed by force. The cell deforms, on the other hand, to minimize the intra force. The cell is moved by the force. The cell moves by itself. The muscle tissue might decrease in the micro gravitational field [1]. The muscle tissue might increase, on the other hand, in the hypergravity [2-7]. The previous study shows that the hyper-gravitational field thickens the myotubes *in vitro* [2]. 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.

In the previous study, the longitudinal axis of C2C12 (mouse myoblast cell line) tends to align to the direction of the excess gravity, and turns to the perpendicular direction after stopping of the excess gravity [3]. A single cell migrates at random on the scaffold. The cell tends to align to the longitudinal direction of the micro ridge line [8].

In the present study, the effect of 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 (Fig. 1a). Variation has been made on the angle (θ) between the longitudinal direction of the ridge and the direction of centrifuge: 0, 45, 90 degrees (perpendicular) (Fig. 1b).

Mold for Micro Pattern
The borosilicate glass (Tempax) disk was used for the base of the mold through micromachining process (Fig. 2). 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 pattern for the slit 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 (Fig. 3). 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 cell culture.

Hyper-gravitational Force Field

The hyper-gravitational force was applied to cultured cells with the centrifugal force. The culture plate is inserted in 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 variation is made on the position of the glass plate in the tube: at the middle of the tube (X), or at the bottom of the tube (Y). To stabilize the position of the glass plate in the tube, Polydimethylsiloxane (PDMS) is filled in the tube to fill the vacancy below the glass plate in the group X. The centrifugal force (F_c) is calculated by Eq. 1.

$$F_c = m\, r\, \omega^2 \quad (1)$$

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.

$$F_g = m\, g \quad (2)$$

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

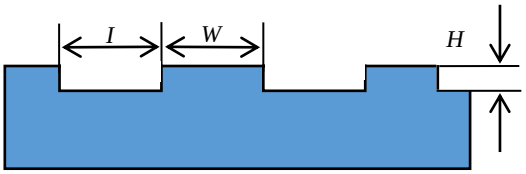

Fig. 1a: Dimension of micro pattern.

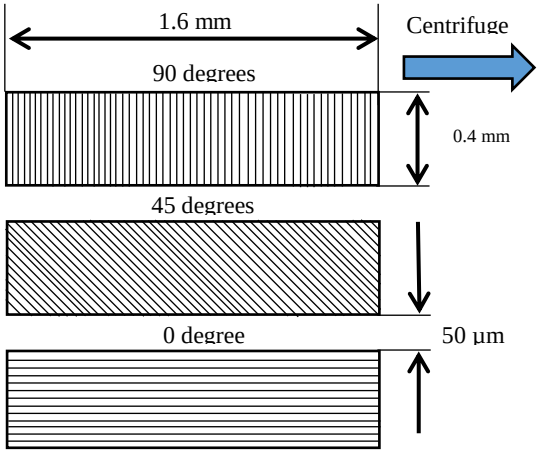

Fig. 1b: Three kinds of micro pattern on PDMS plate.

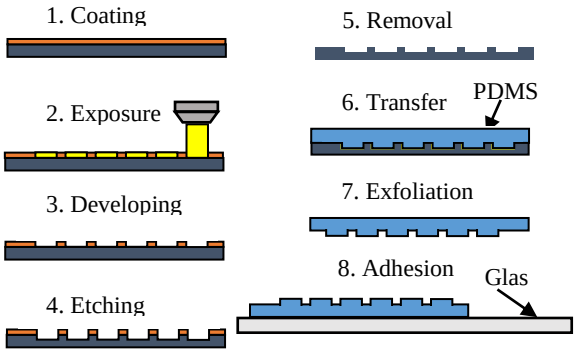

Fig. 2: Micro machining.

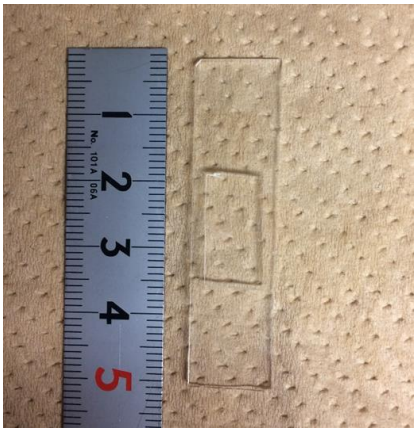

Fig. 3: PDMS plate with micro pattern stacked on glass plate.

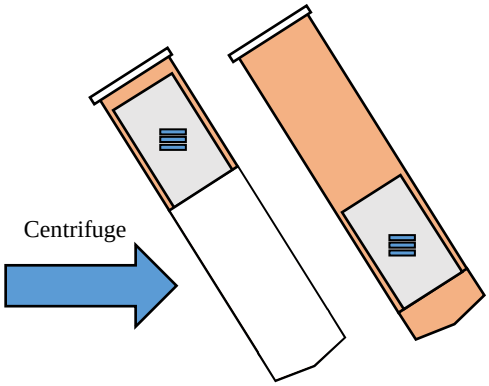

Fig. 4: Plate position in tube: 50 G (left), and 100 G (right).

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the experiment. C2C12 of the passage four was 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, excess gravitational stimulation was applied for five hours. The gravity of 50 G (r = 0.045 m, group X), and the gravity of 100 G (r = 0.09 m, group Y) 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 to cultivate cells of group X and Y, simultaneously. To keep the content of carbon dioxide of 5 % at 310 K, the cells were incubated 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.

Image Analysis

After stimulation of the excess gravity, the glass plate was 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 image 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. 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.

$$P = 1 - b / a \qquad (3)$$

At the circle, P = 0. As the ellipsoid becomes flat, P approaches to unity.

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

In Figs. 5-10, 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.

Fig. 5 shows the direction of the major axis of each cell without centrifuge (control study) on each micro pattern: 0 degree (Fig. 5a), 45 degrees (Fig. 5b), and 90 degrees (Fig. 5c), respectively. Most of cells tend to tilt along the longitudinal direction of the ridge during 24 hours of culture. Fig. 5a shows that data concentrate at zero degree. Fig. 5b shows that data concentrate to 45 degrees. Fig. 5c shows that data concentrate to 90 degrees.

Figs 6 & 7 show the angle between the direction of the major axis of each cell and the tangential force (on the surface of the scaffold by centrifuge) at 50 G, and at 100 G, respectively. Fig. 6 shows the angle between the direction of centrifuge of 50 G and the direction of the major axis of each cell on each micro pattern: 0 degree (Fig. 6a), 45 degrees (Fig. 6b), and 90 degrees (Fig. 6c), respectively. Fig. 7 shows the angle between the direction of centrifuge of 100 G and the direction of the major axis of each cell on each micro pattern: 0 degree (Fig. 7a), 45 degrees (Fig. 7b), and 90 degrees (Fig. 7c), respectively. The tendency of tilting of each cell to the direction of excess gravity is kept for 12 hours after stopping centrifugation. The number of cells, which do not align to the longitudinal direction of ridges, tends to decrease by the hyper-gravitational stimulation. The tendency of the cell to align to the longitudinal direction of ridges is enhanced on the ridge perpendicular to the direction of the excess gravity, after centrifuge (Figs. 6c & 7c).

Figs. 8-10 show the shape index (P). Fig. 8 shows the shape index of each cell without centrifuge (control study) on each micro pattern: 0 degree (Fig. 8a), 45 degrees (Fig. 8b), and 90 degrees (Fig. 8c), respectively. Fig. 9 shows the shape index of each cell at 50 G on each micro pattern: 0 degree (Fig. 9a), 45 degrees (Fig. 9b), and 90 degrees (Fig. 9c), respectively. Fig. 10 shows the shape index of each cell at 100 G on each micro pattern: 0 degree (Fig. 10a), 45 degrees (Fig. 10b), and 90 degrees (Fig. 10c), respectively. The number of rounded cells (small value of P) on the ridges decreases immediately after centrifugation (Fig. 10). Cells tend to elongate to the direction of centrifuge. The tendency is apparent, when the longitudinal direction of the micro ridge is parallel to the direction of centrifuge (Fig. 10a).

4. DISCUSSION

The previous studies showed that a mechanical field governs behavior of cells [2-14]. The effect of shear flow on orientation of cells depends on the kinds of cells [13].

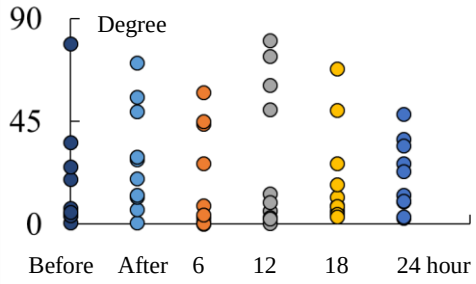

Fig. 5a: Angle of C2C12: control, 0 degree.

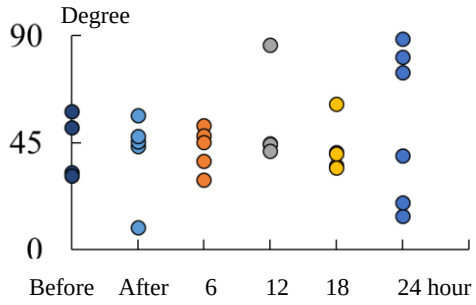

Fig. 5b: Angle of C2C12: control, 45 degrees.

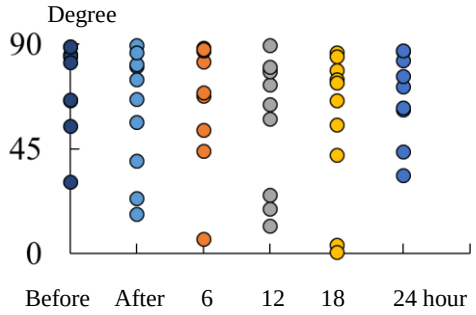

Fig. 5c: Angle of C2C12: control, 90 degrees.

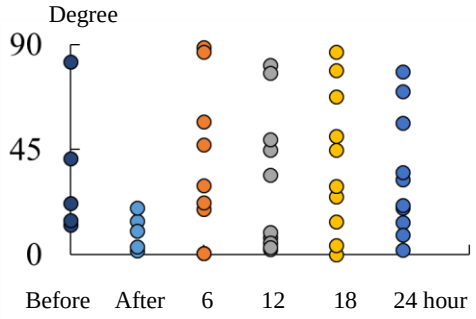

Fig. 6a: Angle of C2C12: 50 G, 0 degree.

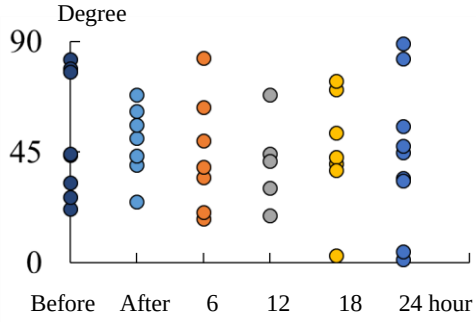

Fig. 6b: Angle of C2C12: 50 G, 45 degrees.

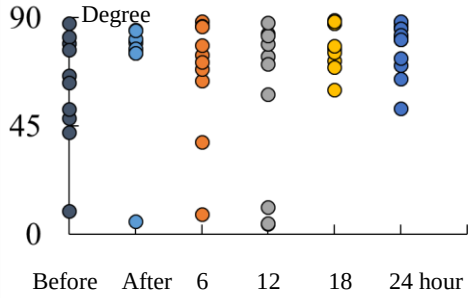

Fig. 6c: Angle of C2C12: 50 G, 90 degrees.

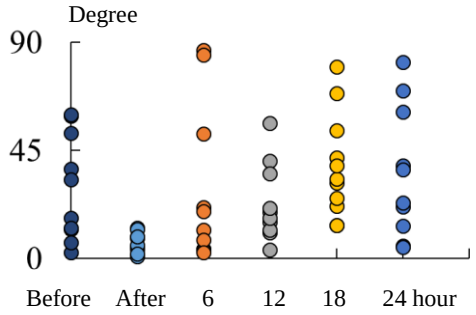

Fig. 7a: Angle of C2C12: 100 G, 0 degree.

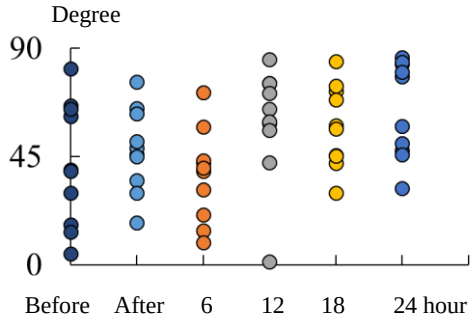

Fig. 7b: Angle of C2C12: 100 G, 45 degrees.

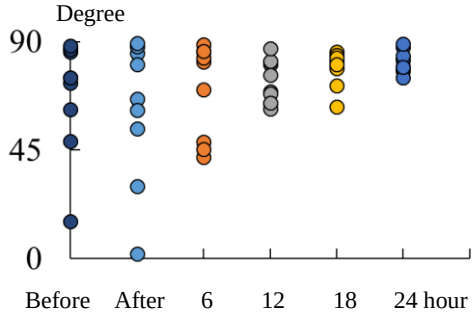

Fig. 7c: Angle of C2C12: 100 G, 90 degrees.

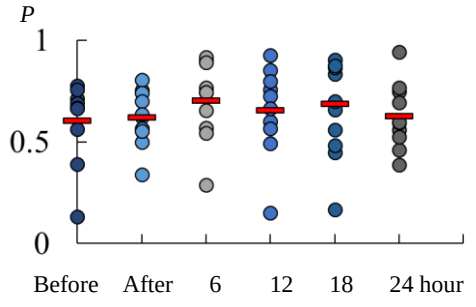

Fig. 8a: Shape Index (*P*) of C2C12: control, 0 degree.

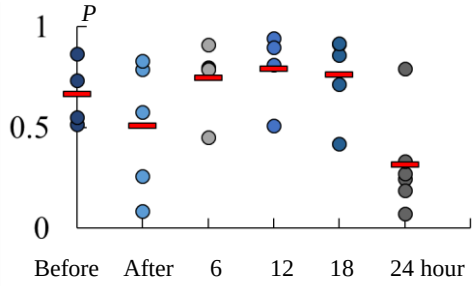

Fig. 8b: Shape Index (*P*) of C2C12: control, 45 degrees.

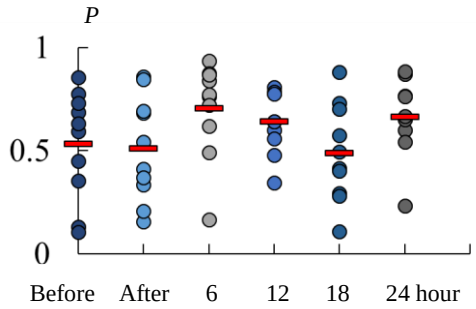

Fig. 8c: Shape Index (*P*) of C2C12: control, 90 degrees.

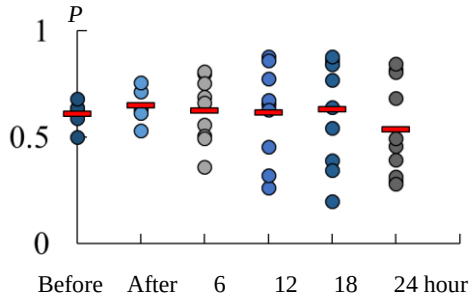

Fig. 9a: Shape Index (*P*) of C2C12: 50 G, 0 degree.

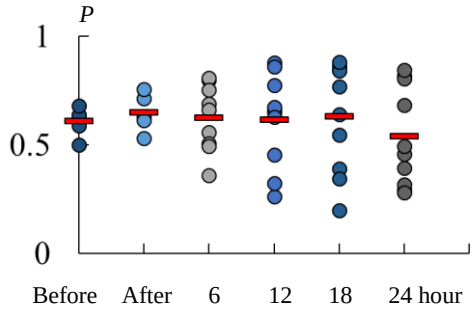

Fig. 9b: Shape Index (*P*) of C2C12: 50 G, 45 degrees.

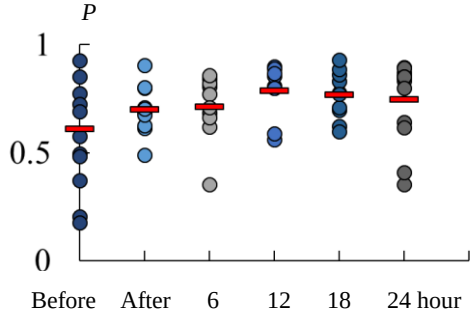

Fig. 9c: Shape Index (*P*) of C2C12: 50 G, 90 degrees.

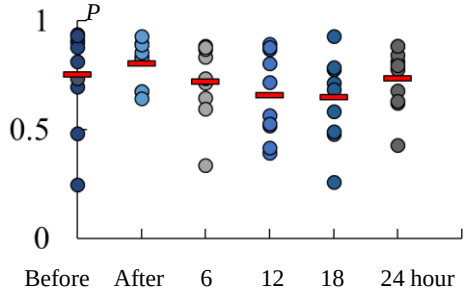

Fig. 10a: Shape Index (*P*) of C2C12: 100 G, 0 degree.

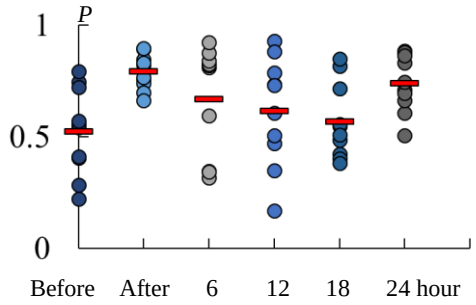

Fig. 10b: Shape Index (*P*) of C2C12: 100 G, 45 degrees.

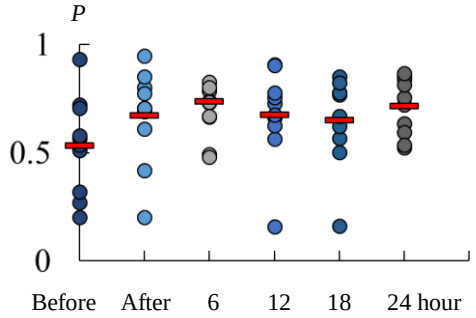

Fig. 10c: Shape Index (*P*) of C2C12: 100 G, 90 degrees.

Although HUVEC (human umbilical vein endothelial cells) orients along the stream lines, C2C12 tilts from the stream lines to make myotubes. When mechanical stimulation is applied to the scaffold, the whole stimulation cannot always be transmitted to the cells. When the tension applied to a scaffold, the deformation of the scaffold generates compression and shear in the different direction simultaneously [10].

To apply continuous uniform mechanical stimulation to the cells, centrifugal force is used in the present study. The effect of mechanical field on orientation and deformation of several kinds of cells was studied using centrifuge *in vitro* in the previous studies [4-6]. The response of biological system to the microgravity field has been studied using a space satellite [1]. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [6].

The variation was made on the position (radius) of cell culture in the same rotor of the centrifuge, to test in the different gravitational environment simultaneously. Cells passively follow the direction in the strong tangential force field. Immediately after the centrifugation, cell might start to show

active response to the mechanical stimulation. The mild tangential force field induces the active reflection of cells to tilt perpendicularly to decrease internal force of the cells. The cell shows adaptation against stimulation. The stimulation leaves hysteresis in the cell. The hyper-gravitational stimulation governs the behavior of the cell after stimulation. C2C12 made perpendicular orientation of myotubes to the flow direction in the previous study [13].

The time lapse image of every ten minutes is effective to trace the movement of each cell. The response of the cell depends on the direction of the hyper-gravitational field. Each cell changes its own direction during migration. The migration depends on the morphology of the scaffold surface [11]. When a cell cannot keep adhesion under stimulation, the shape index (*P*) approaches to "0" to be a sphere.

During centrifugation, the direction of the force field slightly tilts from horizontal direction (normal at the scaffold in the present study) because of the gravity (1 G) of the earth. In the case of centrifuge of 5 G, the shift of the angle is 11 degrees.

The gravity in the fluid is reduced by the buoyancy. From the difference of densities, the effect of the centrifugal force on the cell in the medium is estimated to be 6 G at centrifuge of 100 G (group Y in the present study) [3].

To align the direction 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 low as possible (0.7 μm: just higher than threshold [8]) to allow the effect of centrifuge simultaneously. The change between before and after stimulation can be easily observed with the micro striped pattern on the scaffold.

5. CONCLUSION

The behavior of a cell cultured on the micro striped pattern has been observed after stimulation of the excess gravity *in vitro*. The lines of parallel micro quadrangular ridges are made on the surface of the plate as the scaffold to make orientation of each cell. Variation is made about the angle between the longitudinal direction of the ridge and the direction of the excess gravity: 0 degree, 45 degrees, and 90 degrees. The hyper- gravitational force field (50 G, or 100 G) was applied to C2C12 (mouse myoblast cell line) adhered on the micro pattern by a conventional centrifugal machine placed in an incubator. The behavior of cells was traced at the time lapse images for 24 hours, after the hyper-gravitational stimulation for 5 hours. The longitudinal axis of C2C12 tends to align to the direction of the excess gravity, and returns to the direction of longitudinal direction of micro pattern after stopping of the excess gravity. The present study shows that the hyper-gravitational stimulation affects the behavior of cells after stimulation.

6. ACKNOWLEDGMENT

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