Cell Behavior After Stimulation of Excess Gravity

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ABSTRACT

The effects of the excess gravitational field on orientation and deformation of cells have been studied using centrifuge *in vitro*. Two kinds of cells were used in the test: C2C12 (mouse myoblast cell line), and Neuro-2a (mouse neural crest-derived cell line). To apply the mechanical force field (<100 G) to the cells adhered on the glass plate, the plate was set in the tube in a conventional centrifugal machine placed in an incubator (310 K, 5% CO_2). The behavior of cells was traced at the time lapse images for 24 hours, after the excess gravitational stimulation for 1 or 5 hours. The contour of each cell was traced and approximated to ellipse to analyze the angle between the longitudinal axis of the cell and the direction of the excess gravity. The experiment shows following results. The longitudinal axis of C2C12 tends to align to the direction of the excess gravity, and turns to the perpendicular direction after stopping of the excess gravity. C2C12 aligns to the direction perpendicular against the excess gravity at division. The neurite of Neuro-2a extends along the direction of the excess gravity, and tends to extend subsequently apart from the excess gravity. The present study shows that the excess gravitational stimulation affects the behavior of cells after stimulation.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravitational Field, C2C12 and Neuro-2a.

1. INTRODUCTION

A biological cell shows passive and active behaviors in an environment. While the shear stress deforms the cell, the cell deforms by itself to adopt 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 surrounded by mechanical force field *in vivo*. The cell is sensitive to the mechanical stimulation, and shows several responses: deformation, and migration. The response includes passive one and active one. 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 in response to the force. The muscle tissue might decrease in the micro gravitational field [1-3]. The muscle tissue might increase, on the other hand, in the hypergravity [4-13]. The previous study shows that the excess gravitational field thickens the myotubes *in vitro* [5]. The acceleration technique for orientation, proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [5-13]. Control methodology for orientation, proliferation and differentiation of cells would be applied to the regenerative tissue technology. In the present study, the effect of mechanical field on orientation and deformation of cells has been studied by centrifuge *in vitro*, and the behavior of cells has been traced after stopping of the stimulation of excess gravity.

2. METHODS

Hyper Gravitational Force Field

The hyper-gravitational force was applied to cultured cells with the centrifugal force. A glass plate of 38 mm ×13 mm ×1 mm was used for the scaffold for cell culture. The glass 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 1 rad. The variation was made on the direction of the glass plate in the tube. In the group X 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 in the group X. In the group Y, the glass plate was set in the perpendicular position to the former group rotated at the longitudinal axis of the tube. Both the tangential fore (F_t) and the normal force (F_n) at the culture surface are applied by the centrifuge in the group Y (Fig. 1) [6]. In the group X, the variation is made on the position of the glass plate in the tube: at the middle of the tube (X_1), or at the bottom of the tube (X_2). 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_1 (Fig. 3a) and Y. 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.

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

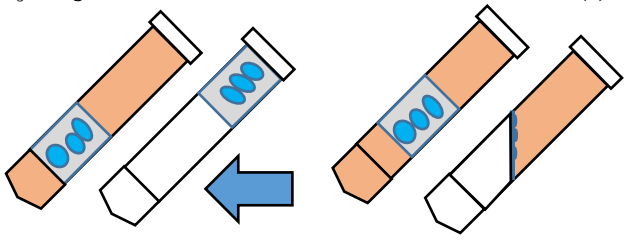

Fig. 1: Tube preparation of group X_2, X_1, X, and Y from left to right: direction of excess gravity from right to left.

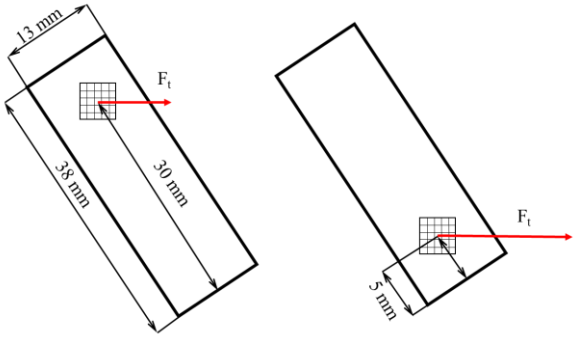

Fig. 2: Position of cell culture observation on glass plate: 50 G (left), and 100 G (right): Square marks, 1 mm × 1 mm.

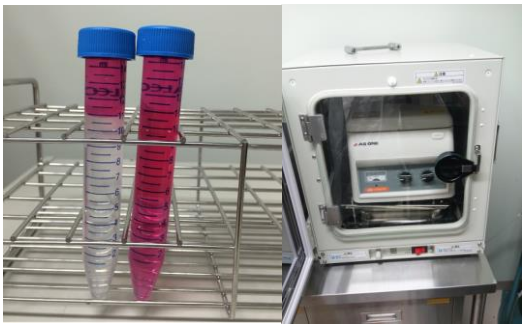

Fig. 3a: Tube for centrifugation: 50 G (left), and 100 G (right).
Fig. 3b: Centrifuge in incubator (right).

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). Perpendicularly crossing lines of grooves, which make 25 squares (5 × 5) of 1 mm × 1 mm, are marked on the rear surface of the glass plate to trace the cell behavior (Fig. 2). One of the directions of the lines corresponds to the direction of the tangential force by the centrifuge (Fig. 2). The grooves (0.016 mm width, 0.002 mm depth) were machined by the ultrashort pulse laser (IFRIT, Cyber Laser Inc., Tokyo, Japan). The glass plate was sterilized in an autoclave before cell seeding. During the stimulation test to cells, the centrifugal machine was placed in an incubator to keep the content of carbon dioxide of 5 % at 310 K (Fig. 3b).

Cell Culture

Two kinds of cells were used in the experiment: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), and Neuro-2a (a mouse neural crest-derived cell line). The cells were seeded on the glass plate at the density of 1000 cells/cm^2.

C2C12 of the passage eight was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. After the cells were cultured for 12 hours in the resting state, excess gravitational stimulation was applied for five hours. In the test with C2C12, the gravity of 50 G (r = 0.045 m, group X_1), and the gravity of 100 G (r = 0.09 m, group X_2) were applied at ω = 104 rad/s.

Neuro-2a 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 for 24 hours in the first step. For differentiation, the medium was changed to D-MEM (Dulbecco's Modified Eagle's Medium) containing 2% decomplemented FBS (fetal bovine serum), 1% penicillin/ streptomycin, and of Retinoic Acid for subsequent 144 hours in the second step. The concentration of Retinoic Acid in the medium is adjusted to 20 μM. After differentiation, the cells were exposed to the excess gravitational field by centrifuge for one hour. In the test with Neuro-2a, the gravity of 5 G (r = 0.085 m) were applied at ω = 24 rad/s.

To apply the excess gravity, the glass plate, on which cells adhered, was set in the medium in the tube in the position. The angle of the glass plate in the tube was adjusted to that of group X or Y (Fig. 1). 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 excess gravitational field. Namely, the centrifugal machine was placed in the incubator during the centrifugation (Fig. 3b). In the control group (normal force of 1 G), the cells were cultured without centrifuge on the glass without collagen coating, which was placed in the polystyrene dish (60 mm diameter) with collagen coating.

Image Analysis

After stimulation of the excess gravity, the glass plate was moved from the centrifugal tube to the 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 ten 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. 4a). As to 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.

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

At the circle, P =1. As the ellipsoid becomes flat, P approaches to zero. The angle (0 degree < θ < 90 degree) between the direction of the centrifuge and the direction of the major axis of each cell was measured at the microscopic image (Fig. 4b). 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. At Neuro-2a, the length of neurite was measured, and the elongation was calculated by Eqs. 4 & 5.

$$\Delta L = L_1 - L_0 \tag{4}$$
$$R = \Delta L / L_0 \tag{5}$$

In Eq. 4, ΔL is elongation, R is elongation ratio, L_0 is length at the stopping of stimulation, L_1 is length at 24 hours after stopping of stimulation. The angle between the direction of neurite and the direction of centrifuge was also measured. To evaluate differentiation of Neuro-2a, the extension of neurite was observed.

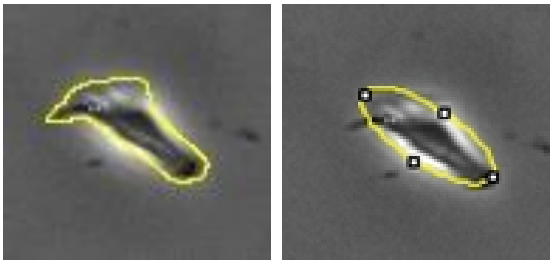

Fig. 4a: Outline of each single cell is traced, and approximated to ellipsoid.

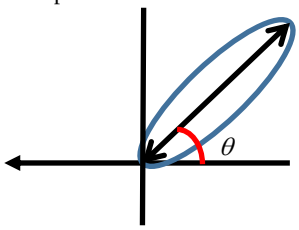

Fig. 4b: Angles θ between direction of centrifuge (horizontal) and direction of major axis of cell.

3. RESULTS

Fig. 5 shows the angle between the direction of the tangential force (on the surface of the scaffold by centrifuge) and the major axis of each cell. In Figs. 5 and 7, data are arranged in the ascending order. When the angles are distributed at random, the data align on the linear line. In the range of dense population, the data align on the gentle inclination line. In the range of sparse population, on the other hand, the data align on the steep inclination line. Fig. 5 shows the following results. In the control study, the angles distribute at random. C2C12 shows tendency to align on the direction to the tangential force of 100 G by centrifuge just after centrifugation. In 18 hours, the distribution shifts from 0 to 90 degrees, which shows the shift to the perpendicular direction against the tangential force. The shift returns to random distribution in 24 hours. At 50 G, C2C12 shows tendency to align on the diagonal (60 degrees) direction to the tangential force by centrifuge in 24 hours (Fig. 5e).

Several cells are divided in 24 hours after stopping of the stimulation of centrifuge for 5 hours. Fig. 6 exemplifies cell division of C2C12 after centrifuge of 100 G. The directions of the major axes of both cells are perpendicular to the direction of the tangential force by the centrifuge (Fig. 6). Fig. 7 shows the distribution of the angle between the direction of the tangential force (by centrifuge) and the major axis of each cell at one hour after cell division. Most of cells distribute in the range of the angle lager than 45 degree after centrifuge of 100 G. In the control group, on the other hand, the angle distribute at random.

Fig. 8a exemplifies the shape index of each cell of C2C12 of each group immediately after stimulation of the centrifuge for five hours. Fig. 8b shows the distribution of the shape index traced every six hours. In Fig. 8b, the column shows the mean value, and the bar shows the range (±) of the standard deviation of 38 samples. Fig. 8 shows that data scatter and do not have statistically significant differences among groups. In the group of 50 G, the value of shape index (*P*) tends to become smaller than the other group after stimulation of the centrifuge. The value of *P* becomes smaller, when the cell is elongated.

Fig. 9 exemplifies the elongation of the neurite of Neuro-2a after stopping of the stimulation of 5 G for one hour. The direction of the tangential force of the centrifuge is from right to left in Fig. 9. Each triangle shows each end of neurite. The neurite extend with time: 11 hours, 11 hours and 45 minutes, 12 hours and 50 minutes, and 15 hours and 40 minutes after centrifuge. The direction of the extension is parallel to that of centrifuge at first, and curves to perpendicular direction against centrifuge with time. Fig. 10 shows the elongation ratio of the neurite of Neuro-2a. Each bar shows mean value of five sample data from each group. The neurite extends in the group of X longer than in the control group. Fig. 11 exemplifies the tracing of orientation of C2C12 at each group by the time lapse image of every ten minutes: control (normal direction of 1G), X_1 (50 G), and X_2 (100 G). Data shows the active random movement of each cell. In the group of 50 G, the fluctuation decrease in the movement of each cell. Fig. 12 exemplifies the tracing of the shape index of C2C12 at each group: control (normal direction of 1G), X_1 (50 G), and X_2 (100 G). Data shows the active random movement of each cell. The movement tends to decrease in the group of X_1.

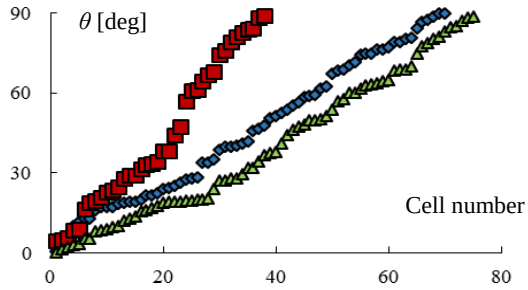

Fig. 5a: Angle of C2C12: immediately after centrifuge: rhombus, control; square, 50 G; triangle, 100 G.

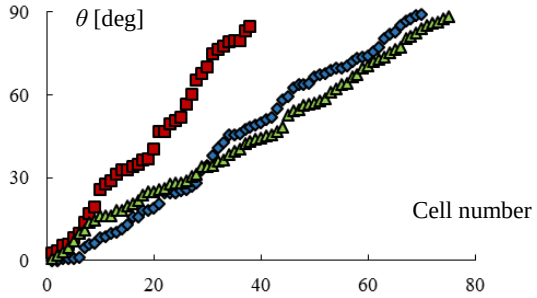

Fig. 5b: Angle of C2C12: 6 hours after centrifuge: rhombus, control; square, 50 G; triangle, 100 G.

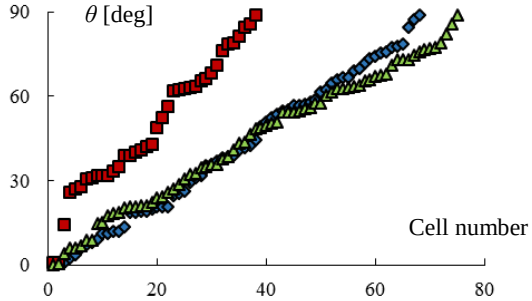

Fig. 5c: Angle of C2C12: 12 hours after centrifuge: rhombus, control; square, 50 G; triangle, 100 G.

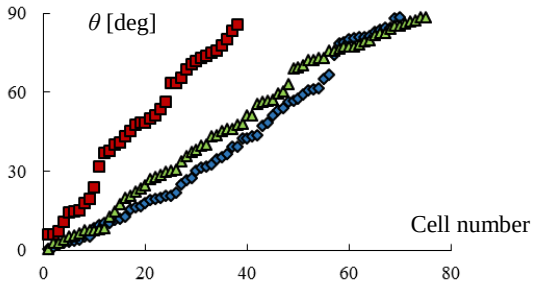

Fig. 5d: Angle of C2C12: 18 hours after centrifuge: rhombus, control; square, 50 G; triangle, 100 G.

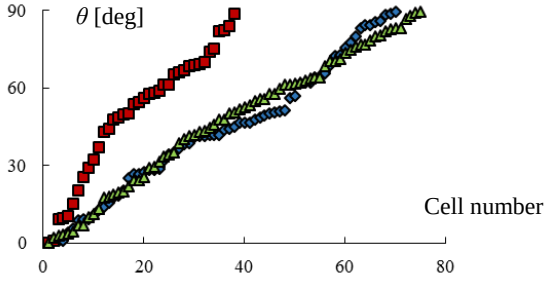

Fig. 5e: Angle of C2C12: 24 hours after centrifuge: rhombus, control; square, 50 G; triangle, 100 G.

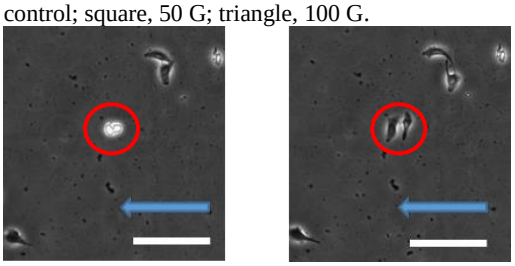

Fig. 6: Cell division (in circle): at 760 min (left) and at 820 min (right) after centrifuge of 100 G: arrow shows centrifuge, and bar shows 0.2 mm.

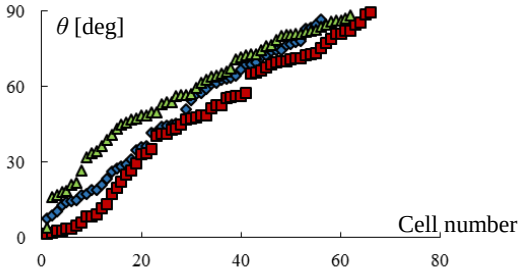

Fig. 7: Angle of C2C12: one hour after division of cell, stimulated by centrifuge: rhombus, control; square, 50 G; triangle, 100 G.

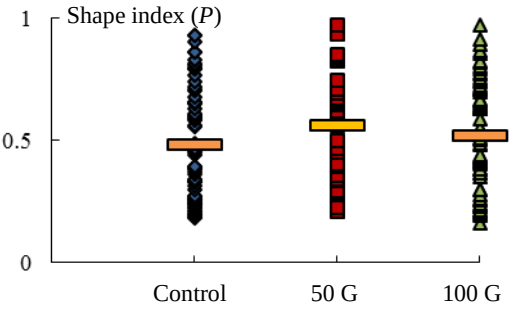

Fig. 8a: Shape index of C2C12 immediately after stimulation of centrifuge: horizontal bar shows mean value.

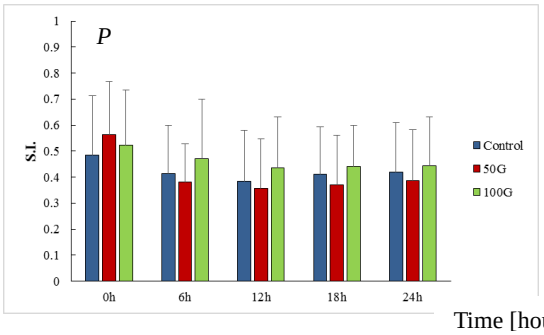

Fig. 8b: Shape index of C2C12 every six hours: from left, control, 50 G, 100 G: mean (column) ± S.D. (bar): n=38.

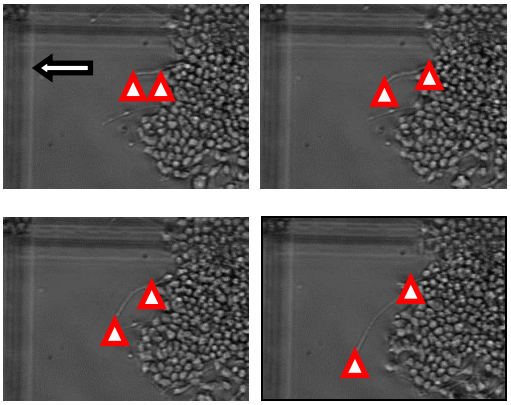

Fig. 9: Elongation of neurite of Neuro-2a: 11 h after centrifuge (upper left), 11 h 45 m (upper right), 12 h 50 m (lower left), and 15 h 40 m (lower right): centrifuge from right to left; dimension from left to right of each figure is 1 mm. Each triangle shows each end of neurite.

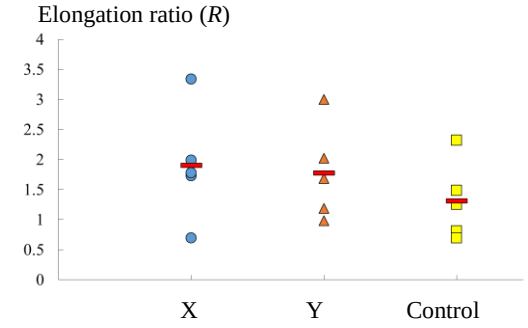

Fig. 10: Elongation ratio of neurite of Neuro-2a: mean (bar): n = 5.

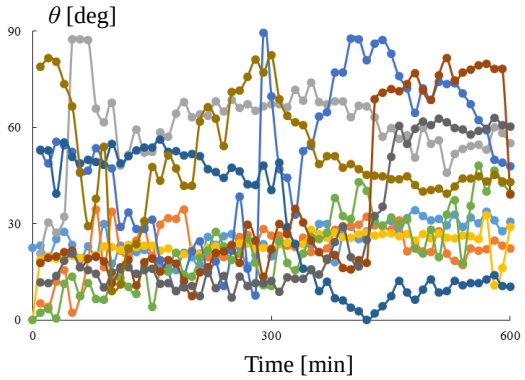

Fig. 11a: Orientation tracing of C2C12 at control.

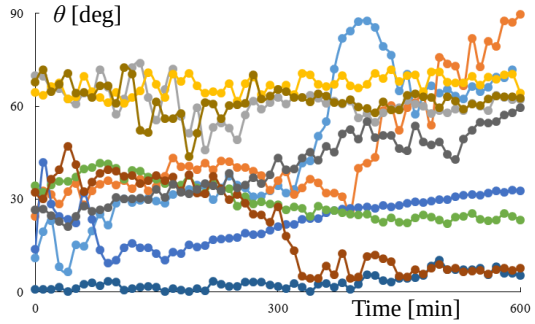

Fig. 11b: Orientation tracing of C2C12 at 50 G.

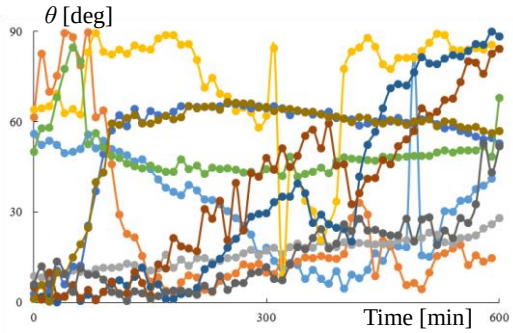

Fig. 11c: Orientation tracing of C2C12 at 100 G.

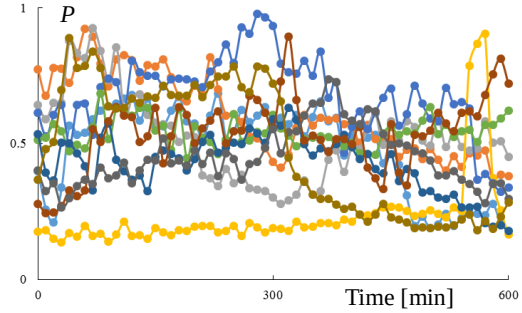

Fig. 12a: Shape index tracing of C2C12 at control.

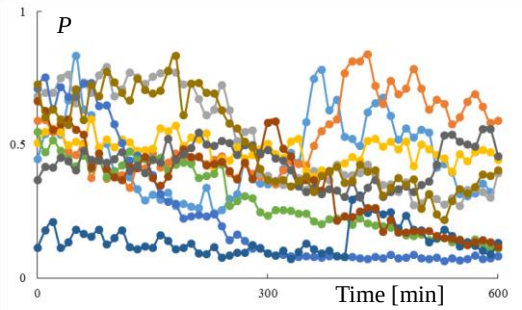

Fig. 12b: Shape index tracing of C2C12 at 50 G.

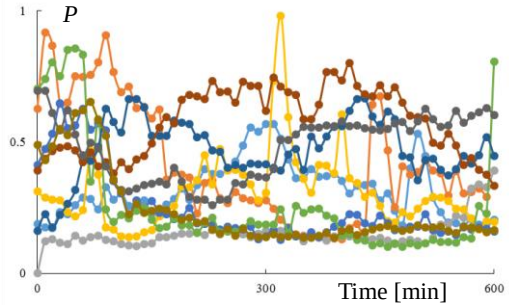

Fig. 12c: Shape index tracing of C2C12 at 100 G.

4. DISCUSSION

The previous studies showed that a mechanical field governs behavior of cells [3-21]. The direction of the mechanical field affects fibroblasts [15]. The effect of shear flow on orientation of cells depends on the kinds of cells [20]. 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 [15]. 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 [5-8]. The differentiation of cells was accelerated by the mechanical stimulation by centrifuge in the previous study [13]. The response of biological system to the microgravity field has been studied using a space satellite. The cell cycle might extend in the space [1, 2]. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [7].

The object is exposed to the force at the surface with two kinds of directions: normal, and tangential. The object is pressed by the normal force. The object is, on the other hand, sheared on the surface with the tangential force. 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. 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 gravitational stimulation governs the behavior of the cell after stimulation. The cells might change the orientation at the division and at differentiation. C2C12 made perpendicular orientation of myotubes to the flow direction in the previous study [20]. The orientation of C2C12 at diagonal direction of flow might be preparation to make perpendicular orientation of myotubes in the successive cultivation. C2C12 might differentiate earlier on polystyrene dish than glass plate [5].

Migration of a cell on the scaffold plate was observed in previous studies. 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 gravitational field. Each cell changes its own direction during migration. The migration depends on the morphology of the scaffold surface [17]. The movement of the cell is observed on the flat surface of plate in the present study. When a cell cannot keep adhesion under stimulation, the shape index approaches to "1" to be a sphere. Cells are exposed to 5 G for one hour in the present test of Neuro-2a, because Neuro-2a was not able to survive under centrifugation for one hour at 100 G. During centrifugation, the direction of the force field tilts from horizontal direction because of the gravity (1 G) of the earth. In the case of centrifuge of 5 G, the shift of the angle is 11 degree. The gravity in the fluid is reduced by the buoyancy. The density of the cell of C2C12 is 1.065×10^{3} kg m^{-3} measured by the centrifugal method: each separator adjusted to various

density of the fluid of phthalate-ester is compared with the density of the cell in the glass capillary by centrifuge [22]. The density of medium of DMEM, on the other hand, is 1.0055×10^3 kg m^{-3}. The difference between two densities is 0.0595×10^3 kg m^{-3}. The effect of the centrifugal force on the cell in the medium is 6 G at centrifuge of 100 G (group X_2 in the present study).

5. CONCLUSION

The effects of excess gravitational field on orientation and extension of cells have been studied using centrifuge *in vitro*. Two kinds of cells were used in the test: C2C12 (mouse myoblast cell line), and Neuro-2a (mouse neural crest-derived cell line). The behavior of cells was traced at the time lapse images for 24 hours, after excess gravitational stimulation (<100 G) for 1 or 5 hours. The experiment shows following results. The longitudinal axis of C2C12 tends to turn to the direction perpendicular direction. C2C12 aligns to the direction perpendicular against the excess gravity at division. The neurite of Neuro-2a tends to extend apart from the excess gravity. The present study shows that the excess gravitational stimulation affects the behavior of cells after stimulation.

6. ACKNOWLEDGMENT

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REFERENCES

[1] G.R. Adams, V.J. Caiozzo and K.M. Baldwin, "Skeletal Muscle Unweighting: Spaceflight and Ground-based Models", **Journal of Applied Physiology**, Vol. 95, No. 6, 2003, pp. 2185–2201.

[2] A.P. Le Traon, M. Heer, M.V. Narici, J. Rittweger and J. Vernikos, "From Space to Earth: Advances in Human Physiology from 20 Years of Bed Rest Studies (1986–2006)", **European Journal of Applied Physiology**, Vol. 101, No. 2, 2007, pp. 143–194.

[3] T.B. Damm, A. Franco-Obregón and M. Egli, "Gravitational Force Modulates G2/M Phase Exit in Mechanically Unloaded Myoblasts", **Cell Cycle**, Vol. 12, No. 18, 2013, pp. 3001-3012.

[4] T.B. Damm, I. Walther, S.L. Wüest, J. Sekler and M. Egli, "Cell Cultivation Under Different Gravitational Loads Using a Novel Random Positioning Incubator", **Biotechnology and Bioengineering**, Vol. 111, No. 6, 2014, pp. 1180-1190.

[5] S. Hashimoto, H. Hino and T. Iwagawa, "Effect of Excess Gravitational Force on Cultured Myotubes in Vitro", **Journal of Systemics, Cybernetics and Informatics**, Vol. 11, No. 3, 2013, pp. 50-57.

[6] H. Hino, H. Sugimoto, Y. Takahashi, S. Hashimoto and S. Miura, "Behavior of Cells in Excess Gravitational Field: Using Centrifuge", **Proc. 20th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2016, pp. 123-128.

[7] H. Hino, S. Hashimoto and T. Yasuda, "Effect of Centrifugal Force on Cell Culture", **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 132-137.

[8] H. Hino, H. Sato, S. Hashimoto and Y. Takahashi, "Effect of Excess Gravitational Force and Electric Pulse Field on Myoblast", **Proc. 19th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2015, pp. 258-263.

[9] A. Tschopp and A. Cogoli, "Hypergravity promotes cell proliferation", **Experientia**, Vol. 39, No. 12, 1983, pp. 1323-1438.

[10] M. Monici, N. Marziliano, V. Basile, G. Romano, A. Conti, S. Pezzatini and L. Morbidelli "Hypergravity affects morphology and function in microvascular endothelial cells", **Microgravity Science and Technology**, Vol. 18, No. 3, 2006, pp. 234–238.

[11] J. Gebken, B. Liiders, H. Notbohm, H.H. Klein, J. Brinckmann, P.K. Muller and B. Batge, "Hypergravity Stimulates Collagen Synthesis in Human Osteoblast-Like Cells: Evidence for the Involvement of p44/42 MAP-Kinases (ERK 1/2)", **The Journal of Biochemistry**, Vol. 126, No. 4, 1999, pp.676-682.

[12] G. Ciofani, L. Ricotti, J. Rigosa, A. Menciassi, V. Mattoli and M. Monici, "Hypergravity Effects on Myoblast Proliferation and Differentiation," **Journal of Bioscience and Bioengineering**, Vol. 113, No. 2, 2012, pp. 258-261.

[13] G.G. Genchi, F. Cialdai, M. Monici, B. Mazzolai, V. Mattoli and G. Ciofani, "Hypergravity Stimulation Enhances PC12 Neuron-Like Cell Differentiation", **BioMed Research International**, Vol. 2015, 2015, Hindawi. 748121, pp. 1-10.

[14] H. Hino, S. Hashimoto, Y. Takahashi and H. Nakajima, "Effect of Ultrasonic Vibration on Proliferation and Differentiation of Cells", **Journal of Systemics, Cybernetics and Informatics**, Vol. 14, No. 6, 2016, pp. 1-7.

[15] J.H.-C. Wang, G. Yang, Z. Li and W. Shen, "Fibroblast Responses to Cyclic Mechanical Stretching Depend on Cell Orientation to the Stretching Direction", **Journal of Biomechanics**, Vol. 37, 2004, pp. 573-576.

[16] Y. Sugaya, N. Sakamoto, T. Ohashi and M. Sato, "Elongation and Random Orientation of Bovine Endothelial Cells in Response to Hydrostatic Pressure: Comparison with Response to Shear Stress", **JSME International Journal**, Series C, Vol. 46, No. 4, 2003, pp. 1248-1255.

[17] P. Uttayarat, M. Chen, M. Li, F.D. Allen, R.J. Composto and P.I. Lelkes, "Microtopography and Flow Modulate the Direction of Endothelial Cell Migration", **Am. J. Physiol. Heart Circ. Physiol.**, Vol. 294, 2008, pp. H1027-H1035.

[18] N. Azuma, S. A. Duzgun, M. Ikeda, H. Kito, N. Akasaka, T. Sasajima and B. E. Sumpio, "Endothelial Cell Response to Different Mechanical Forces", **Journal of Vascular Surgery**, Vol. 32, No. 4, 2000, pp. 789-794.

[19] S. Hashimoto, F. Sato, H. Hino, H. Fujie, H. Iwata and Y. Sakatani, "Responses of Cells to Flow in Vitro", **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 5, 2013, pp. 20-27.

[20] S. Hashimoto and M. Okada, "Orientation of Cells Cultured in Vortex Flow with Swinging Plate in Vitro", **Journal of Systemics Cybernetics and Informatics**, Vol. 9, No. 3, 2011, pp. 1-7.

[21] H. Hino, S. Hashimoto, Y. Takahashi and M. Ochiai, "Effect of Shear Stress in Flow on Cultured Cell: Using Rotating Disk at Microscope", **Journal of Systemics, Cybernetics and Informatics**, Vol. 14, No. 4, 2016, pp. 6-12.

[22] S. Hashimoto, et al., "Effect of Aging on Deformability of Erythrocytes in Shear Flow", **Journal of Systemics, Cybernetics and Informatics**, Vol. 3, No. 1, 2005, pp.90-93.