Effect of Mechanical Stimulation on Orientation of Cultured Cell

Haruka HINO, Shigehiro HASHIMOTO, Masashi OCHIAI

Biomedical Engineering, Department of Mechanical Engineering,
Kogakuin University, Tokyo, 163-8677, Japan
shashimoto@cc.kogakuin.ac.jp http://www.mech.kogakuin.ac.jp/labs/bio/

and

Hiromichi FUJIE

Human Mechatronic Systems, Faculty of System Design,
Tokyo Metropolitan University, Tokyo, Japan

ABSTRACT

The effect of mechanical stimulation on orientation of cultured cells has been studied *in vitro*. Cells are exposed to two types of mechanical stimulation: the gravity, and the flow. To apply the excess gravitational force on the cultured cells, the cell culture tube was set in a conventional centrifugal machine. To apply the continuous shear flow on the cultured cells, the cell culture dish with donut shaped canal was contained on the shaker. Three kinds of cells were alternatively used in the test: L929 (fibroblast-like, mouse connective tissue), HUVEC (normal human umbilical vein endothelial cells), or C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse). The shape and the orientation of the cells were observed with a phase contrast microscope during the cell culture. The experimental results show that HUBEC orients to the stream line, although C2C12 tilts from the stream line and from the centrifugal direction.

Keywords: Biomedical Engineering, L929, HUVEC, C2C12, Cell Culture, Flow and Gravitational Force.

1. INTRODUCTION

Cell culture technique has been developed and several methodologies have been clinically applied to regenerative medicine. The acceleration technique for orientation and proliferation of cells has been studied to make tissue *in vivo* or *in vitro* [1-9]. Control methodology for orientation and proliferation of cells would be applied to the regenerative tissue technology.

The mechanical stress is one of the interested points in the environment of cells, because they receive mechanical force *in vivo*. The mechanical stress on cells might induce various responses: deformation, migration, proliferation, and differentiation. Several methods have been designed to apply the mechanical stress to cells [2-10].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold [2-5]. When fixation between the cell and the scaffold is not enough, the stress is not transmitted to the cell. A flow, on the other hand, can be used to apply a stress field to a specimen [7-10]. The specimen directly receives the shear stress in the shear flow.

In the present study, the effect of mechanical stimulation on orientation of cultured cells has been studied *in vitro*.

2. METHODS

Cells are exposed to two types of mechanical stimulation: the gravity, and the flow.

Excess Gravitational Force
The excess gravitational force was applied to cultured cells with the centrifugal force (Fig. 1). Cells were cultured on the lateral wall of a tube, which was placed horizontally in the incubator (Fig. 2). Then, the tube (Fig. 3) was set on a rotor in the centrifugal machine (MX105, Tomy Seiko, Co. Ltd, Tokyo, Japan) (Fig. 4). The rotor was rotated with the speed of 1100 revolutions per minute (rpm), which makes the excess gravitational force of 100 G at the lateral wall of the culture tube. To keep balance of the rotator in the centrifugal machine, two tubes were placed in the counter position each other. On the outer lateral wall of the tube, several lines are marked to trace the same area for the cell culture.

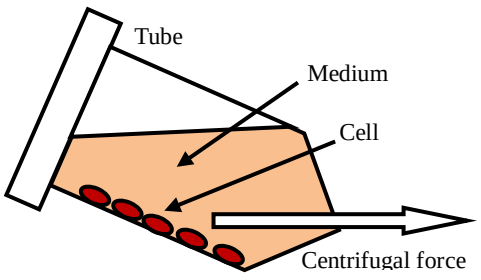

Fig. 1: Centrifugal force (arrow) applied on cell culture in tube.

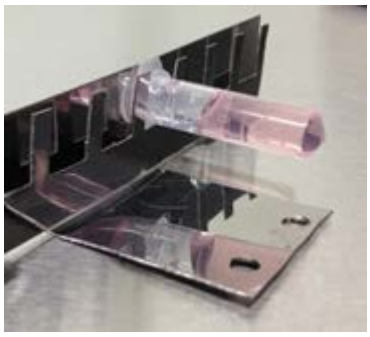

Fig. 2: Keeping horizontal position for cell culture on the inner lateral wall of tube.

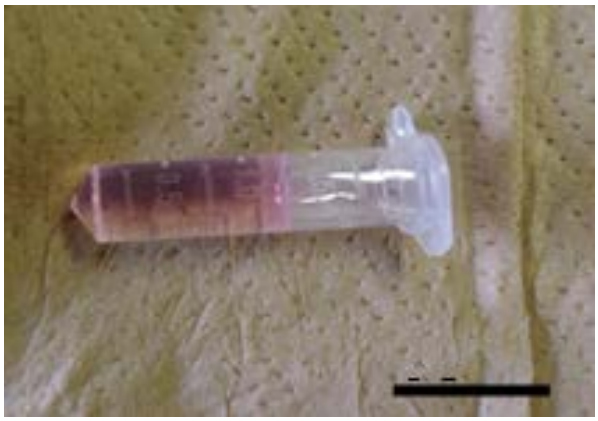

Fig. 3: Cell culture in tube.

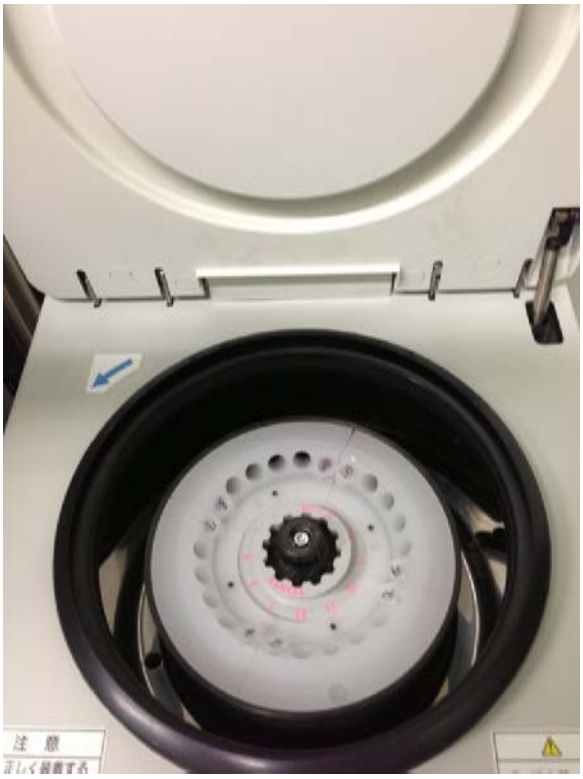

Fig. 4: Rotor in centrifugal machine.

The constant excess gravitational force was applied on the cells at 25 degrees Celsius. After morphological observation, the cells were cultured in the incubator again on the rest part of the day.

Donut-Shaped Open Channel

A donut-shaped open channel system for the cell culture has been designed to apply a vortex flow on cells *in vitro*. A polystyrene culture dish was used. A transparent polydimethylsiloxane (PDMS) disk (30 mm diameter, 3 mm thick) is attached on the inner bottom of the culture dish to restrict the space for the flow of the medium (Fig. 5). The PDMS disk is attached at the center of the culture dish of 52 mm internal diameter with affinity between their surfaces without adhesive.

The culture dish is placed on a plate, which inclines at 0.1 rad of the horizontal plane (Fig. 6). The plate rotates to generate a swing motion (WAVE-SI, Taitec, Co., Ltd., Koshigaya). The rotating speed of the plate is 30 revolutions per minute (rpm) (3.1 rad/sec). The motion produces a one-way counter clockwise vortex flow in the medium through the donut-shaped open channel.

The continuously swinging plate is placed in an incubator (Fig. 7), where both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent are maintained.

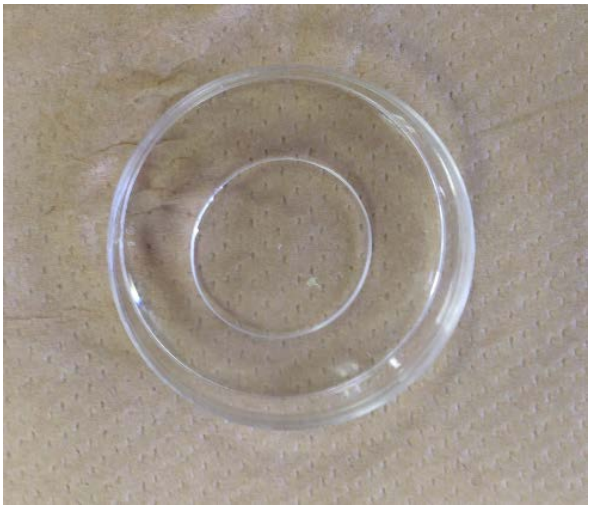

Fig. 5: PDMS disk attached on the bottom of culture dish.

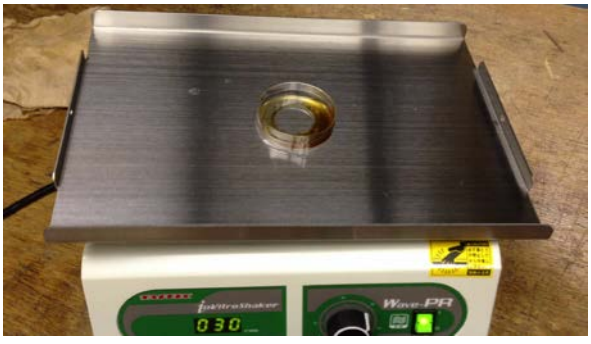

Fig. 6: Culture dish placed on shaker: counter clockwise flow.

Fig. 7: Culture dish on shaker in incubator.

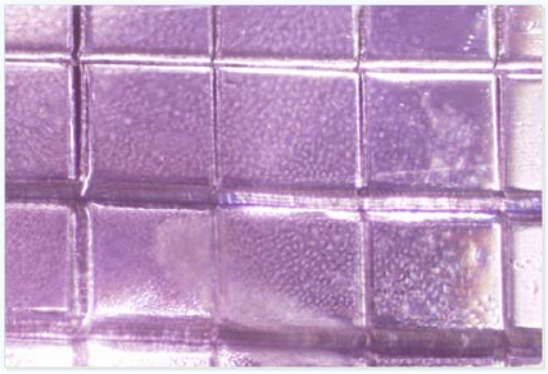

Fig. 8: Mark with interval of 0.5 mm to trace the same area of culture.

After the cells were cultured for 12 hours without flow in the incubator, the cells were cultured under the flow for seven days.

Cell Culture

Three kinds of cells were alternatively used in the test: L929 (fibroblast-like, mouse connective tissue, RCB1422, Riken Bio Resource Center, Tsukuba), HUVEC (normal human umbilical vein endothelial cells), or C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse).

L929 was cultured with the E-MEM (Eagle's minimal essential medium) containing 10% FBS (fetal bovine serum). HUVEC was cultured with the EGM-2 (Endothelial Cell Growth Medium). C2C12 was cultured with the D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS. The medium containing penicillin and streptomycin was refreshed every two days.

Before the centrifugal test, the cells were cultured on the inner lateral wall of the polypropylene tube, keeping the horizontal position in the incubator for 12 hours.

Morphological Study

Morphology of cells was observed with an inverted phase-contrast microscope (IX71, Olympus, Tokyo). To trace the same area in the serial cultivation, crossing lines have been marked on the outside of the wall of culture tubes and dishes (Fig. 8).

3. RESULTS

The experimental results show that cells deform from the round shape to the extended shape, which shows adhesion on the inner lateral wall of the polypropylene tube in 12 hours in the incubator. The appearance of the cell on the tube is similar to that on the culture flask of polystyrene. The microscopic observation shows, on the other hand, that six hours is not enough to adhere on the inner wall of the polypropylene tube.

Fig. 9 shows L929 cultured for 3 days with 100 G centrifugation for 1 hour a day. The centrifugal force is applied from right to left in Figs. 9-12. The experimental result shows that the longitudinal axes of cells (L in Figs 9, 10(B), 11(B), 12(B)) tend to tilt to the direction perpendicular to the centrifugal force.

Figs. 10(A) and (B) show L929 before and after centrifugation of 100 G for 1 hour, respectively. Figs. 11(A) and (B) show L929 before and after centrifugation of 100 G for 4 hours, respectively. Figs. 10(A) and 11(A) show L929 cultured for 12 hour on the lateral wall of the tube without centrifugation. The figures show migration and deformation of L929.

Figs. 12(A) and (B) show C2C12 before and after centrifugation of 100 G for 3 min, respectively. Fig. 12(A) shows C2C12 cultured for 12 hour on the lateral wall of the tube without centrifugation. The figures show migration and deformation of C2C12.

Fig. 13 shows HUVEC cultured in the donut-shaped open channel for 12 hour without flow. Figs. 14(A) and (B) show HUVEC cultured for 48 hour without and with flow, respectively. The result shows that proliferation decreases with flow. Figs. 15(A) and (B) show HUVEC cultured for 6 days without and with flow, respectively. The experimental result shows that HUBEC orients along the stream lines in 6 days of culture (Fig. 15(B)).

Fig. 16 shows C2C12 cultured in flow for 5 days. The results show that orientation (L in Fig. 16) of C2C12 tilts from the stream line (arrow). Fig. 17 shows C2C12 cultured in flow for 7 days. The results show that C2C12 differentiates to myotubes under flow stimulation (the arrow in Fig. 17).

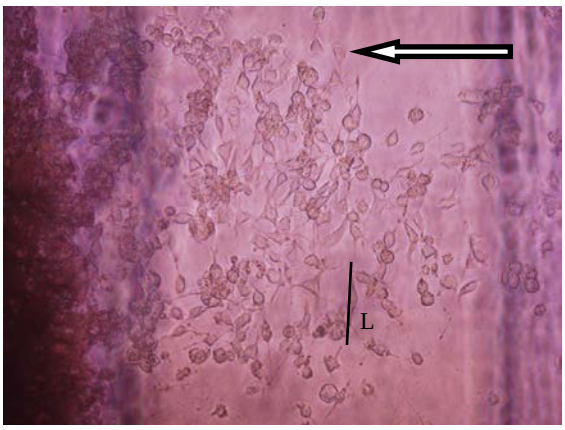

Fig. 9: L929 cultured for 3 days with 100 G centrifugation for 1 hour a day. Centrifugal force: from right to left (arrow). Dimension from left to right is 1 mm.

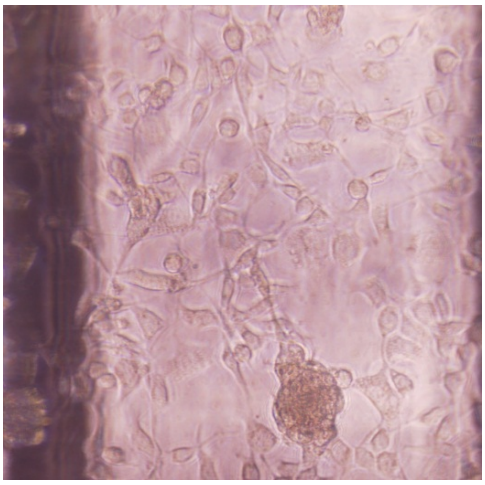

Fig. 10(A): L929 before centrifugation. Dimension from left to right is 0.5 mm.

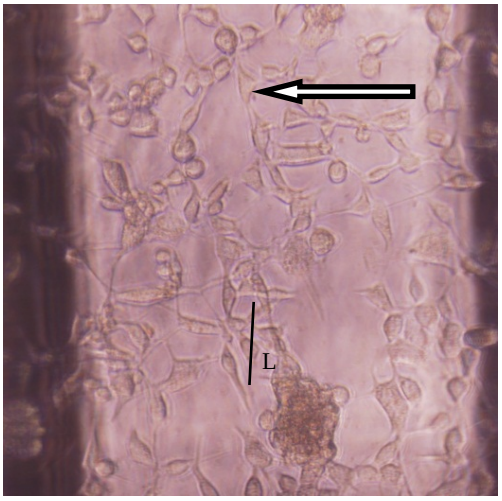

Fig. 10(B): L929 after centrifugation: 100 G, 1 hour. Dimension from left to right is 0.5 mm.

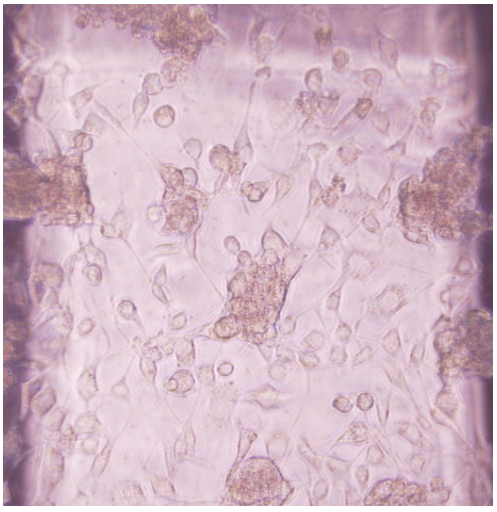

Fig. 11(A): L929 before centrifugation. Dimension from left to right is 0.5 mm.

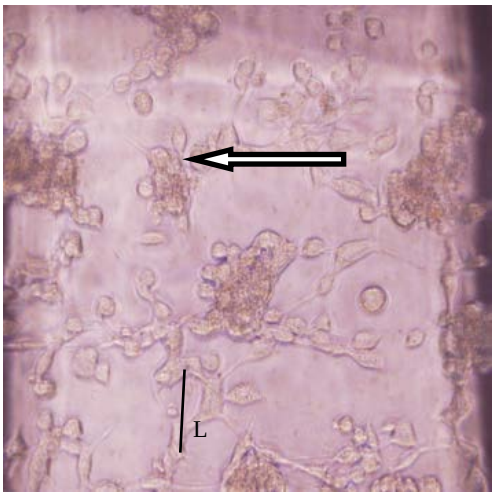

Fig. 11(B): L929 after centrifugation: 100 G, 4 hour. Dimension from left to right is 0.5 mm.

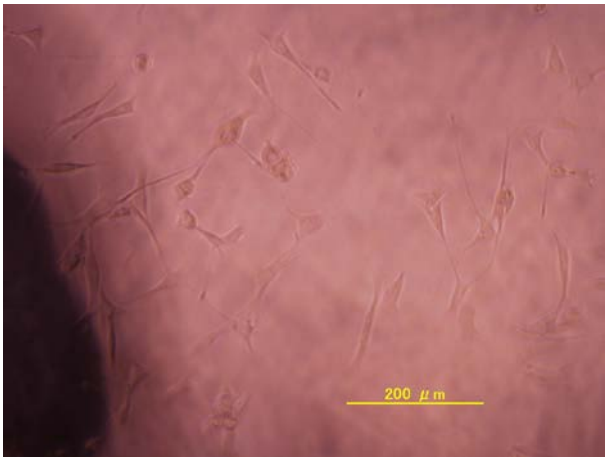

Fig. 12(A): C2C12 before centrifugation.

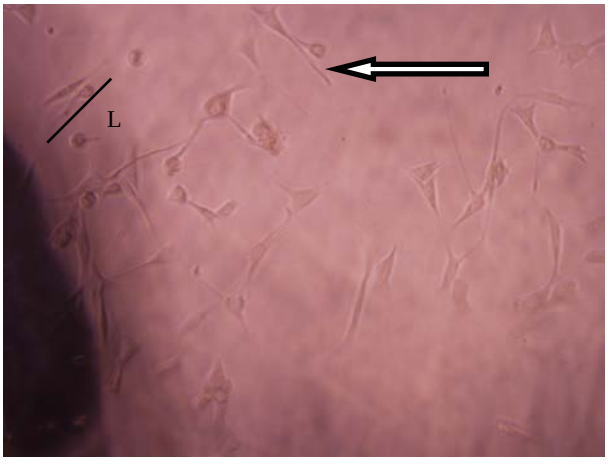

Fig. 12(B): C2C12, after centrifugation: 100 G, 3 min. Dimension from left to right is 1 mm.

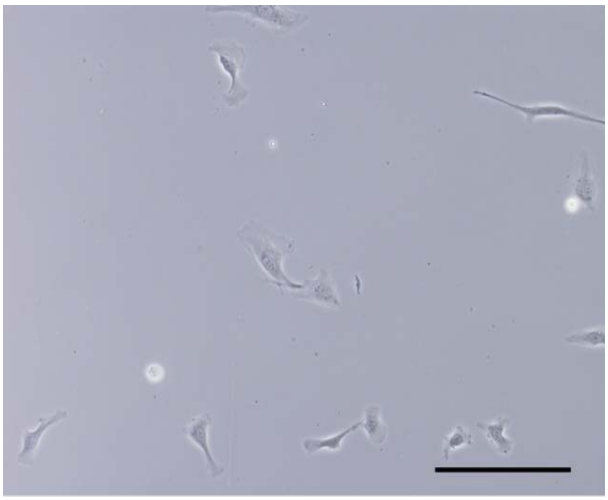

Fig. 13: HUVEC cultured for 12 hour. The bar shows 0.1 mm.

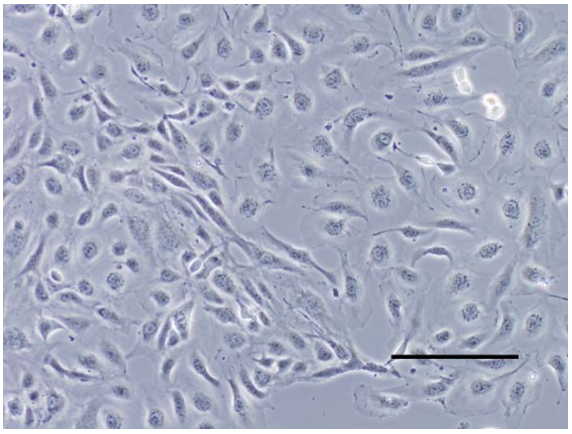

Fig. 14(A): HUVEC cultured for 48 hour without flow. The bar shows 0.1 mm.

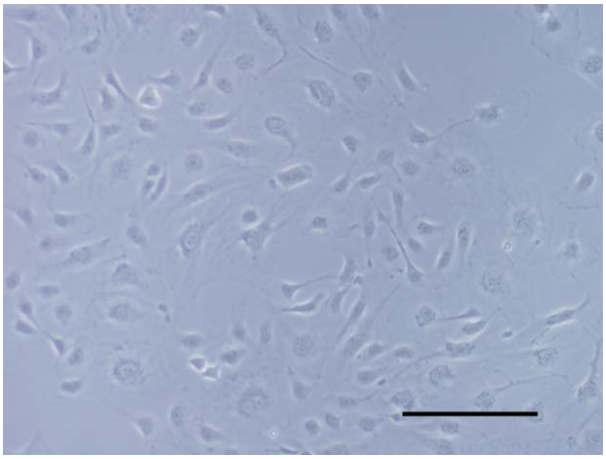

Fig. 14(B): HUVEC cultured in flow for 48 hour: medium flows from top to bottom. The bar shows 0.1 mm.

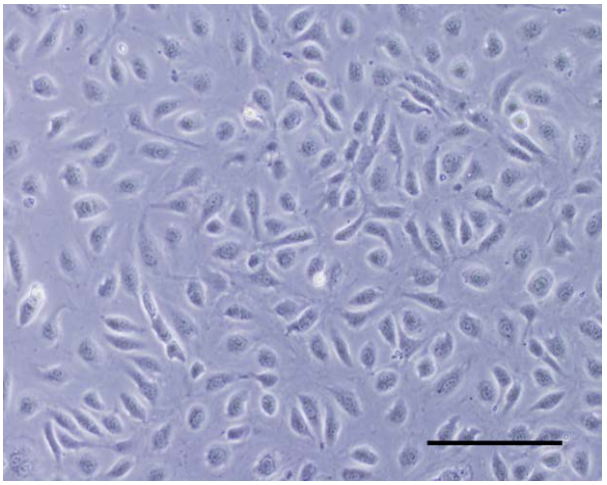

Fig. 15(A): HUVEC cultured for 6 day without flow. The bar shows 0.1 mm.

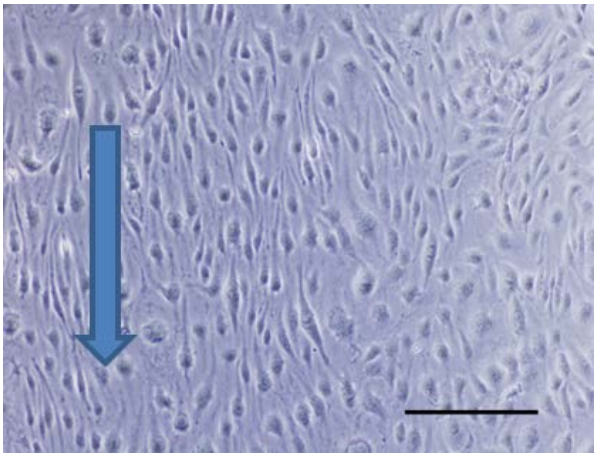

Fig. 15(B): HUVEC cultured in flow for 6 day: arrow shows direction of flow. The bar shows 0.1 mm.

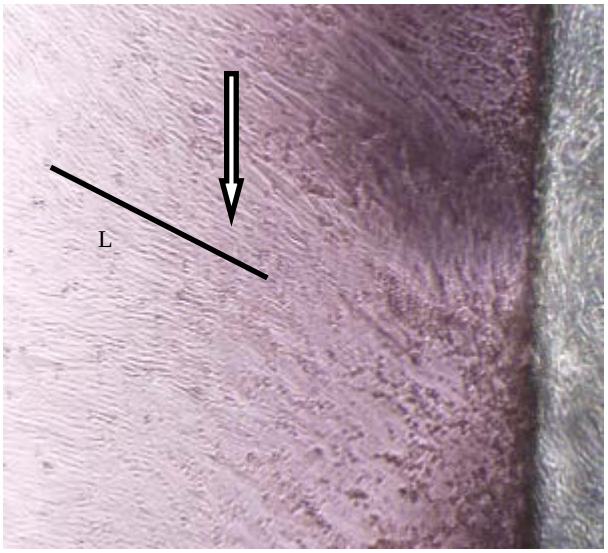

Fig. 16: C2C12 cultured in flow for 5 days: medium flows from top to bottom. Dimension from left to right is 1 mm.

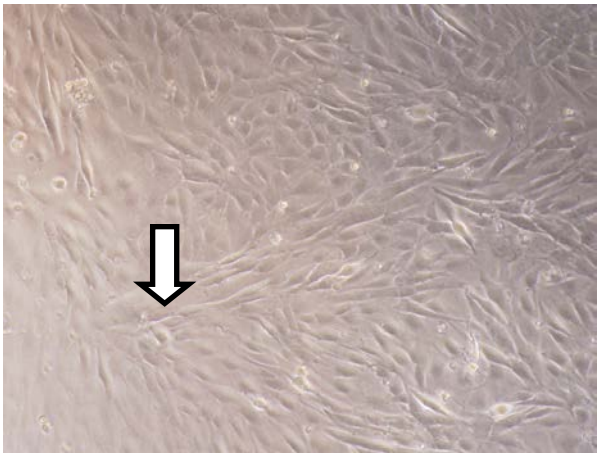

Fig. 17: C2C12 cultured for 7 days in flow: medium flows from top to bottom. Dimension from left to right is 0.5 mm.

4. DISCUSSION

It is not easy to estimate the shear stress value on the wall in the present experiment, because the medium has the free surface. The parallel piped chamber is convenient to observe the response of cells under controlled shear stress [8].

When mechanical stimulation is applied to the scaffold, the whole stimulation cannot always be transmitted to the cells. To apply mechanical stimulation to the cells, centrifugal force or shear flow is used alternatively in the present study.

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. The previous study shows that electric stimulation enhances differentiation of muscle cells [1]. Another study shows that mechanical stimulation improves a tissue-engineered human skeletal muscle [2].

The previous studies show that a mechanical field, on the other hand, governs behavior of cells. The shear flow governs the orientation of endothelial cells [7, 9]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [3]. The direction of the mechanical field affects fibroblasts [5].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [6]. The mechanical stimulation can decrease proliferation of cells [6]. The mechanical stress also exfoliates several cells, which makes vacancy around the adhesive cell. The differentiation might be optimization of cells to the changing environment. The mechanical stress can accelerate differentiation of C2C12 to make myotubes (Fig. 17).

The effect of shear flow on orientation of cells depends on the kinds of cells [7]. Although HUBEC orients along the stream lines, C2C12 tilts from the stream lines to make myotubes. The previous study shows orientation of cells perpendicular to the stretch direction [4].

The results of the study will contribute to acceleration technique in regenerative medicine

5. CONCLUSION

The effect of gravitational force and flow on orientation of cell has been studied *in vitro*. To apply excess gravitational force on cells, the cell culture tube was set in a conventional centrifugal machine. To apply continuous shear flow on cells, the cell culture dish with the donut shaped canal was contained on the shaker. The experimental results show that HUBEC orients to the stream line, although C2C12 tilts from the stream line and from the centrifugal force line.

6. ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Strategic Research Foundation at Private Universities from the Japanese Ministry of Education, Culture, Sports and Technology.

REFERENCES

[1] S. Hashimoto, F. Sato, R. Uemura and A. Nakajima, “Effect of Pulsatile Electric Field on Cultured Muscle Cells in Vitro”, **Journal of Systemics Cybernetics and Informatics**, Vol. 10, No. 1, 2012, pp. 1-6.

[2] Y. Akiyama, R. Terada, M. Hashimoto, T. Hoshino, Y. Furukawa and K. Morishima, “Rod-shaped Tissue Engineered Skeletal Muscle with Artificial Anchors to Utilize as a Bio-Actuator”, **Journal of Biomechanical Science and Engineering**, Vol. 5, No. 3, 2010, pp. 236-244.

[3] K. Nakagawa, N. Morishima and T. Matsumoto, “Effect of Three-Dimensional Culture and Cyclic Stretch Stimulation on Expression of Contractile Protein in Freshly Isolated Rat Aortic Smooth Muscle Cells”, **Journal of Biomechanical Science and Engineering**, Vol. 4, No. 2, 2009, pp. 286-297.

[4] L. Terracio, B. Miller and T. Borg, “Effects of Cyclic Mechanical Stimulation of the Cellular Components of the Heart: in Vitro”, **In Vitro Cellular & Developmental Biology**, Vol. 24, No. 1, 1988, pp. 53-58.

[5] 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.

[6] S. Motoda, S. Hashimoto, T. Iwagawa and A. Nakajima, “Effect of Excess Gravitational Force on Cultured Myotubes in Vitro”, **Proc. 15th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2011, pp. 118-123.

[7] 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.

[8] S. Hashimoto, F. Sato, K. Oya, H. Fujie, H. Iwata and Y. Sakatani, “Responses of Cells to Flow in Vitro”, **Proc. 16th World Multi-conference on Systemics Cybernetics and Informatics**, Vol. 2, 2012, pp. 91-96.

[9] 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.

[10] 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.