Effect of Shear Stress on Myoblasts Cultured under Couette Type of Shear Flow between Parallel Disks

Yuji ENDO, Shigehiro HASHIMOTO, Hiroki ERI

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

ABSTRACT

The effect of shear stress on myoblast has been investigated under the uniform shear flow *in vitro*. The culture medium was sandwiched with a constant gap between a lower stationary culture plate and an upper rotating parallel plate to make a Couette type of shear flow. The wall shear stress (τ) on the lower culture disk was controlled by the rotating speed of the upper disk. C2C12 (mouse myoblast cell line) was used in the test. After cultivation without flow for 24 hours for adhesion of cells on the lower plate, τ was continuously applied on cells for 7 days in the incubator. The behavior of each cell was traced at the time-lapse image observed by an inverted phase contrast microscope placed in an incubator. The experimental results show that cells differentiate to myotubes under shear stress < 2 Pa. Both the cell cycle and the cell length tend to scatter in the wider range, and the longitudinal axis of the cell tends to align to the flow direction by the shear stress of 1 Pa. The experimental system is useful to study the quantitative relationships between the shear stress and the cell behaviors: deformation, orientation, and differentiation.

Keywords: Biomedical Engineering, Shear Stress, C2C12, Myotube and Couette Flow.

1. INTRODUCTION

The effect of the shear flow on the endothelial cells, which are exposed to the blood flow on the inner surface of the vessel wall, were investigated in many studies [1–10]. In the previous study with the vortex flow [11] by the swinging plate *in vitro*, C2C12 made orientation perpendicular to the direction of the flow, although HUVEC made orientation along the streamline of the flow [12]. The orientation of each cell in the tissue depends on that of neighbor's cell. To analyze the mechanism of making orientation of cells, the behavior of each cell in the shear flow field should be quantitatively observed *in vitro*.

At the wall shear stress, a cell might show the following responses: elongation [1–3], tilting to the stream line [4, 5], migration [6, 7], deformation to be rounded, proliferation [7, 8, 13], and exfoliation from the wall of the scaffold [14–16].

In the Poiseuille type of flow, the shear rate depends on the distance from the wall: highest at the wall. In the Couette type of flow, on the other hand, the shear rate is constant regardless of the distance from the wall [17–23].

In the present study, an experimental system of the Couette type flow in the constant gap with a rotating disk has been designed to apply the shear stress quantitatively on the cell during incubation at the microscopic observation *in vitro*, and the effect of the shear stress field (< 2 Pa) on the myoblast has been studied about deformation, orientation, and differentiation.

2. METHODS

Couette Type of Shear Flow Device

A Couette type of shear flow device has been designed in the present study: between a rotating disk and a stationary dish (Fig. 1). The medium is sheared between a rotating wall and a stationary wall. The stationary wall is the bottom of the culture dish (diameter 60 mm).

In the devise, the shear rate (γ) in the medium is calculated by Eq. (1).

$$\gamma = r\,\omega / d \tag{1}$$

In Eq. (1), ω is the angular velocity [rad s^{-1}], and d is the distance [m] between the wall of the moving disk and the wall of stationary plate. Between the parallel walls, d is constant. The shear rate (γ [s^{-1}]) in the gap between walls increases in proportion to the distance (r [m]) from the rotating axis.

The angular velocity ω (< 22 rad s^{-1}) was controlled by the stepping motor. In the observation area of the microscope, r varies between 17 mm and 18 mm. The distance d, which was measured by the positions of the focus of the walls at the microscope, was between 0.28 mm and 0.56 mm. Variations on the shear rates (γ) between 0.7×10^3 s^{-1} and 1.4×10^3 s^{-1} are made in the present experiment by adjustment of these parameters.

The shear stress (τ [Pa]) is calculated by the viscosity (η [Pa s]) of the medium.

$$\tau = \eta\,\gamma \tag{2}$$

Using the viscosity of the medium of 1.5×10^{-3} Pa s (measured by a cone and plate viscometer at 310 K), the variations of the shear stress τ have been calculated as the value between 1.0 Pa and 2.0 Pa.

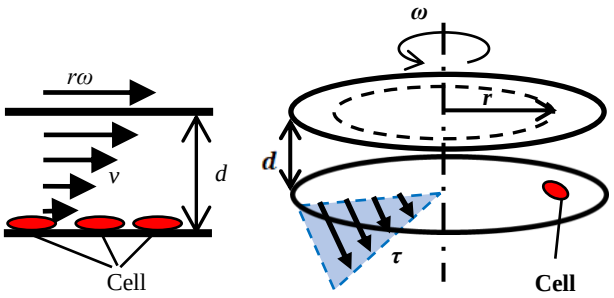

Fig. 1: Couette flow velocity (v) distribution between rotating (angular velocity ω) wall and stationary wall at r (radius) (distance d; left): shear stress (τ) on stationary wall (right).

The rotating disk device is mounted on the stage of the inverted phase contrast microscope placed in the incubator. The device allows the microscopic observation of cells cultured on the stationary wall during exposure to the shear flow.

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse, passage between eight and ten) was used in the test. Cells were cultured in D-MEM (Dulbecco's Modified Eagle's Medium): containing 10% decomplemented FBS (fetal bovine serum), sodium hydrogen carbonate ($NaHCO_3$), and 1% penicillin/ streptomycin.

The cells were seeded on the dish at the density of 3000 cells/cm^2. To make adhesion of cells to the bottom of the culture dish, the cells were cultured for 24 hours in the incubator without flow stimulation (without rotation of the disk).

After the incubation for 24 hours, the cells were continuously sheared with the rotating disk for 7 days in the incubator at the constant rotating speed without the medium exchange. The constant speed was preset for each test to keep the designed shear stress.

Measurement of Cell

The time-lapse microscopic image was taken every thirty minutes during the cultivation.

The contour of each cell adhered on the stationary plate of the scaffold was traced, and the projected two-dimensional area (S) at the image of each cell was calculated. The area ratio occupied by cells was calculated by Eq. (3).

$$Rs = 100 \times (\Sigma S_i) / A \quad (3)$$

In Eq. (3), ΣS_i is accumulated total area of cells, and A is the corresponding scaffold area. In the confluent case, Rs becomes 100 %.

After fusion of cells were observed, the length of each myotube (L) was measured.

The behavior of cells adhering to the stationary wall surface under the shear stress is observed with a microscope. The experimental system allows continuous observation of cells during exposure to the shear flow in the incubator.

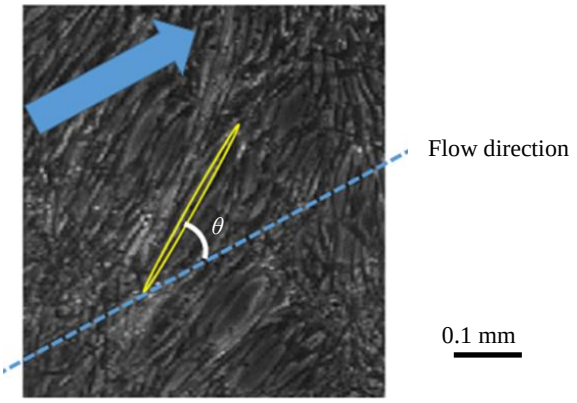

Fig. 2: Angle (θ) between longitudinal axis of myotube (yellow) and flow (arrow) direction.

In the extra test, the flow adjacent to the scaffold surface of the culture dish was traced by the movement of microspheres (borosilicate glass particles: diameter of 10 ± 1.0 μm, and density of 2.4 g / cm^3) recorded by a high speed camera.

The acute angle (−90 degree < θ < 90 degree) between the longitudinal axis of the cell and the flow direction was measured at the microscopic image of each cell (Fig. 2).

3. RESULTS

Fig. 3 shows tracings of the microspheres rolling on the surface of stationary plate in the shear flow field. The tracings show the steady parallel linear movements.

Fig. 4 shows the area ratio occupied by cells after seven days culture under the shear flow in relation to the wall shear stress. Cells proliferate to make the confluent mono layer on the surface of the stationary plate in seven days of culture under shear stress lower than 1 Pa. Under the shear stress higher than 1 Pa, the area ratio occupied by cells is lower than 20 %. Several cells were exfoliated at proliferation, because of the decrease of the contact area to the scaffold at the cell division.

Fig. 5 exemplifies tracings of the angle of the cell without proliferation under the shear stress of 1.5 Pa. In Fig. 5, both +90 degree and −90 degree are the same direction, which is perpendicular to the flow direction. Within 20 hours, each cell changes direction every thirty minutes. One of the cells rotates its longitudinal axis to make perpendicular direction to the flow direction after 20 hours of culture. In eighty hours of culture, the angles of two cells approach to zero degree, which is flow direction. In each case of Fig. 5, the area ratio occupied by cells is lower than 20 %. In densely populated cells, each cell tends to follow the direction of the neighbor cell.

Fig 6 shows the cell cycle of each cell related to the shear stress. The mean value of the cell cycle is 15 hours regardless of the shear stress. At the shear stress of 1 Pa, the cell cycle scatters: the cell cycle extends to a period of 20 hours or longer in some cells.

Fig. 7 exemplifies the tracings of the area of the cell before exfoliation. The area of each cell decreases within six hours. The cell exfoliates regardless of the initial size.

Fig. 8 shows the relationship between the term of differentiation and the shear stress. The term of differentiation tends to increase with the shear stress. Even under the shear stress of 2 Pa, myoblasts differentiate into myotubes within six days of culture without the medium exchange.

Fig. 9 shows the relationship between the length of myotube and the shear stress after seven days of culture. The mean length is 40 μm regardless of the shear stress. At the shear stress of 1 Pa, the length scatters: the length extends longer than 50 μm at one cell.

Figs. 10-12 show the angle of each cell. In Figs. 10-12, every data is plotted in ascending order, to avoid the dependency on the way of segmentation of the parameter in the histogram. Figs 10a, 11a, and 12a show the angle before exposure to the shear flow. Figs 10b, 11b, and 12b show the angle after exposure to the shear flow for three days. Figs 10c, 11c, and 12c show the angle after exposure to the shear flow for seven days. Before exposure to the shear flow, the direction of the cell is random (Figs 10a & 12a), in which data align on the straight line. In Fig. 11a, it is coincidences that many cells tilt from the flow direction with the angle of 60 degree before exposure to the shear flow. Many cells tilt to the flow direction (zero degree) after exposure to the flow (1 Pa and 1.5 Pa) for seven days (Figs. 10c & 11c). At 2 Pa, many cells tilt to the oblique (30 degree) direction from the flow direction after exposure to the flow for seven days (Fig. 12c).

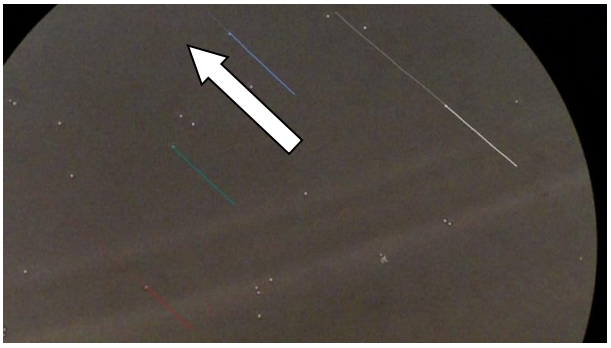

Fig. 3: Tracings of microspheres on surface of stationary plate in shear flow field: flow direction (arrow).

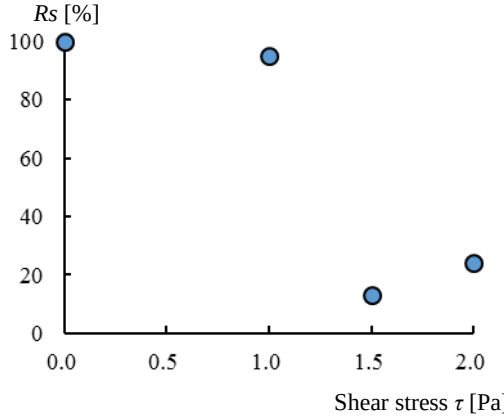

Fig. 4: Area ratio occupied by cells (*Rs* [%]) vs. shear stress (τ [Pa]) after 7 days of cultivation.

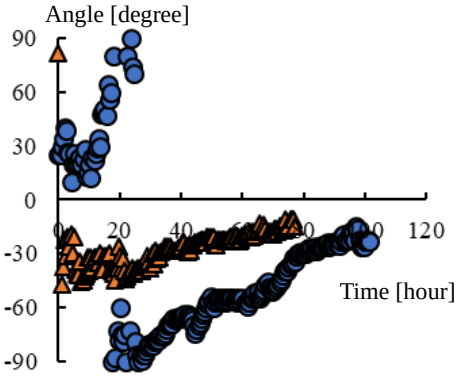

Fig. 5: Tracings of angle of cell [degree]: shear stress 1.5 Pa.

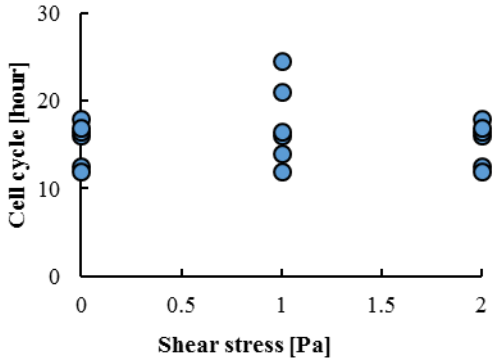

Fig. 6: Cell cycle [hour] vs. shear stress [Pa].

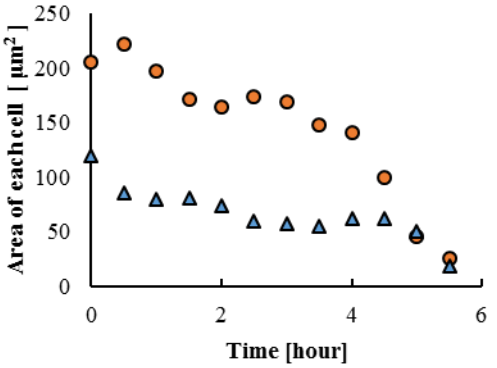

Fig. 7: Area tracings of each cell.

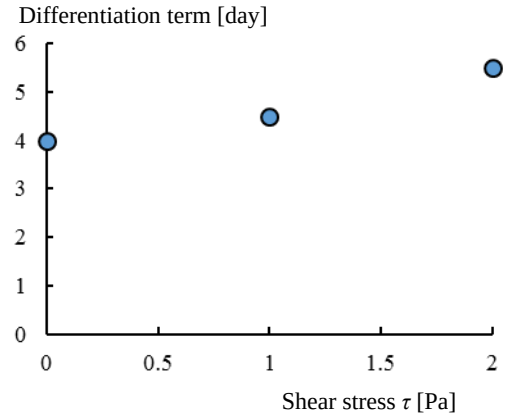

Fig. 8: Differentiation term [day] vs. shear stress [Pa].

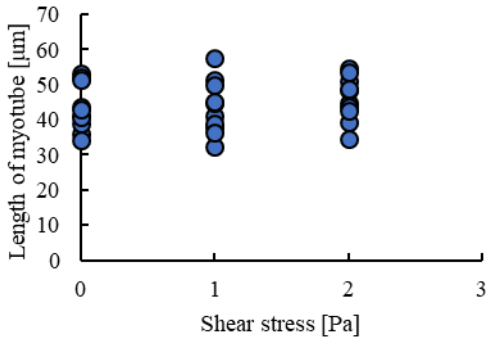

Fig. 9: Length of myotube (L [μm]) vs. shear stress [Pa]: 7 days.

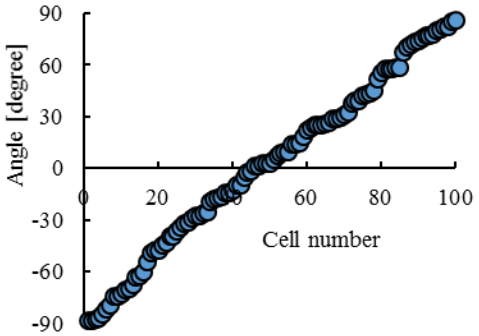

Fig. 10a: Before flow stimulation, angle (θ [degree]) of C2C12: 1 Pa.

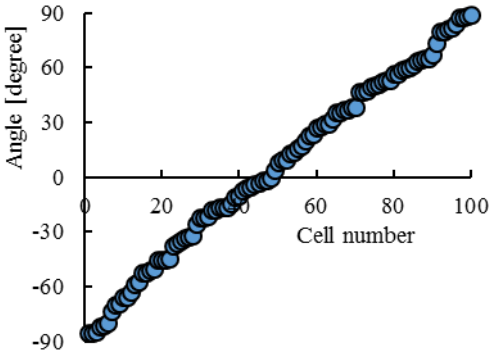

Fig. 10b: After three days of continuous flow stimulation, angle (θ [degree]) of C2C12: 1 Pa.

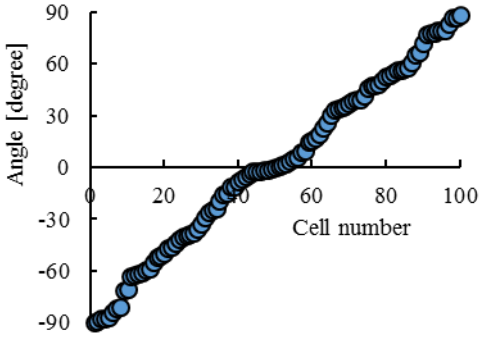

Fig. 10c: After seven days of continuous flow stimulation, angle (θ [degree]) of C2C12: 1 Pa.

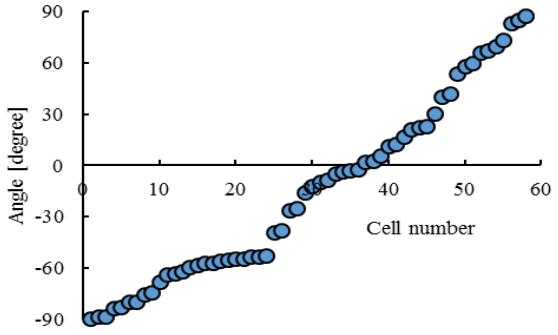

Fig. 11a: Before flow stimulation, angle (θ [degree]) of C2C12: 1.5 Pa.

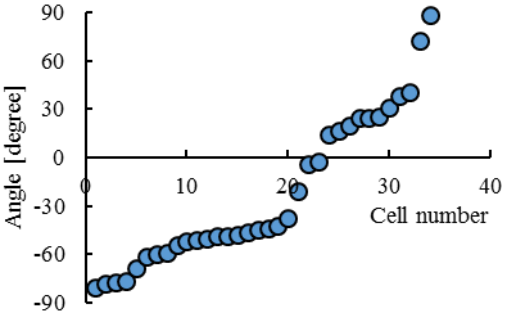

Fig. 11b: After three days of continuous flow stimulation, angle (θ [degree]) of C2C12: 1.5 Pa.

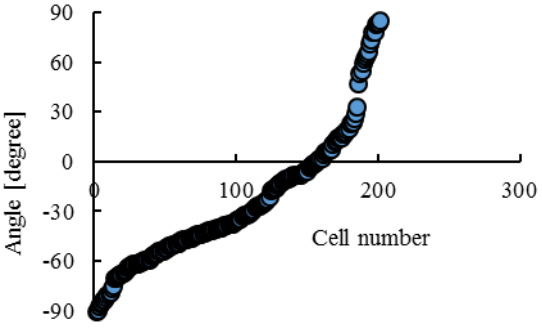

Fig. 11c: After seven days of continuous flow stimulation, angle (θ [degree]) of C2C12: 1.5 Pa.

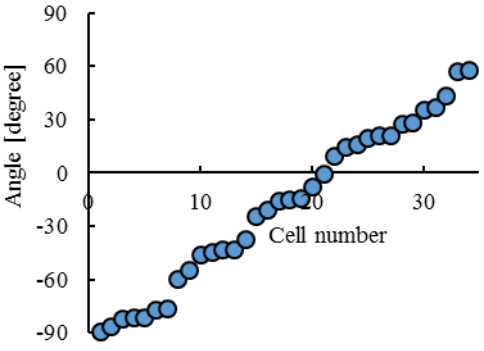

Fig. 12a: Before flow stimulation, angle (θ [degree]) of C2C12: 2 Pa.

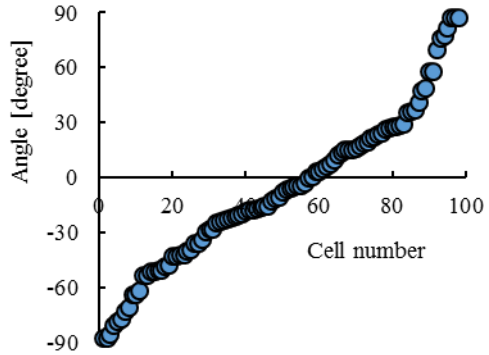

Fig. 12b: After three days of continuous flow stimulation, angle (θ [degree]) of C2C12: 2 Pa.

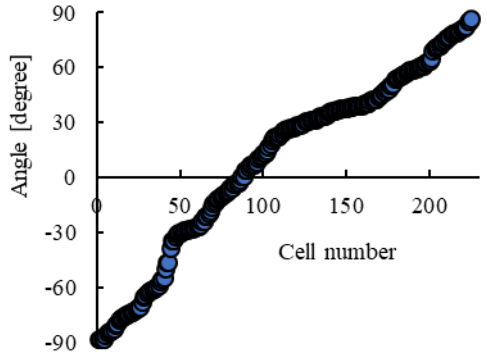

Fig. 12c: After seven days of continuous flow stimulation, angle (θ [degree]) of C2C12: 2 Pa.

4. DISCUSSION

Endothelial cells are exposed to the shear flow in the blood vessels *in vivo*. The effect of shear flow on endothelial cells was investigated in the previous studies [1−10]. Cells are exfoliated under the shear flow at the wall shear stress higher than 2 Pa [14−16]. A biological cell shows passive and active responses in an environment [6]. While the flow enhances the cell migration to the downstream, a cell migrates 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 [5, 9]. The hysteresis effect governs the active response of the cell.

In the previous study, cells were exposed to the shear flow in a donut-shaped open channel, and the effect of flow stimulation on cultured cells has been studied *in vitro* [11, 12]. When the flow has an open surface, it is difficult to estimate the shear stress value in the fluid. Between two parallel walls, on the other hand, the velocity profile is estimated to be parabolic in the laminar flow. In the previous studies, several preparations were designed to study the effect of mechanical flow stimulations on biological cells: the tilting disk channel [14], the rhombus channel [15], the cross flow channel [16], and the rotating disk type [17].

The Couette type of flow is convenient to estimate the shear stress in the flow with the constant shear rate between the moving wall and the stationary wall, which is also available to non-Newtonian fluid. Several kinds of the devices of Couette type flow were designed for quantitative experiments of biological fluid in the previous studies [17−23]. The cone-and -plate type device has the uniform shear field in the entire space between the rotating cone and the stationary plate [18−21]. The shear stress is constant independent of the distance from the rotating axis. The clot formation was quantitatively studied between the rotating cone and the stationary plate [19], and between the rotating concave cone and the stationary convex cone [20]. The erythrocyte destruction was studied between the rotating concave cone and the stationary convex cone [21].

A parallel disks system between rotating disk and the stationary disk, on the other hand, has several advantages: stability of the rotating motion of the disk, stability of the optical path for the microscopic observation, morphologic preciseness of the plane of the disks, and simultaneous observation over the range of variation of the shear rate proportional to the radius from the rotational axis. The floating erythrocyte deformation was observed between counter rotating parallel discs [22, 23].

In the present study, the rotating parallel disk system is selected to make Couette type of flow instead of the cone and plate system. At the constant angular velocity of 22 rad s^{-1} (d = 0.0003 m), the shear rate (γ) increases from 0.88×10^3 s^{-1} to 1.3×10^3 s^{-1}, when the distance from the axis (r) increases from 0.012 m to 0.018 m in the observation area (Eq. (1)). The range of the shear rate enables the simultaneous observation of the behavior of cells related to variation of the shear stress between 1.3 Pa and 2.0 Pa [17]. The rotating flow might induce the secondary flow by the centrifugal effect. The rotational speed of the disk is smaller than 0.4 m s^{-1} in the present system. The microscopic video image of the flowing cells between the rotating disk and the stationary disk shows the steady flow in the present experiment. Reynolds number (Re) is calculated by Eq. (4).

$$Re = \rho\, v\, d / \eta = \rho\, r\, \omega\, d / \eta \tag{4}$$

In Eq. (4), ρ is density of the fluid [kg m^{-3}], v is the circumferential velocity [m s^{-1}], ω is the angular velocity [rad s^{-1}], r is the distance [m] from the rotating axis, d is the distance [m] between the moving wall and the stationary wall, and η is the viscosity of the fluid [Pa s]. Re is 1.5×10^2, when ρ, r, ω, d, and η are 1×10^3 kg m^{-3}, 0.018 m, 22 rad s^{-1}, 0.00056 m, and 0.0015 Pa s, respectively. The turbulent flow may not occur in the flow of small value of Reynolds number. The steady actual flow direction adjacent to the scaffold surface of cell culture has been confirmed by the streamline traced by the direction of exfoliation of the cell and of the moving particle adjacent to the surface. The flow velocity, which increases in proportional to the distance from the rotating axis, has also been confirmed by tracings of the moving particle adjacent to the surface.

The interaction between cells also governs the behavior of each cell. The orientation of each cell depends also on the orientation of the neighbor cell. The most of myoblasts tend to migrate to the oblique direction of the lower shear stress field at 1 Pa. The effect of shear flow on cells depends on the cell types. The dependency might be applied to the cell sorting technology. The quantitative relationships between the shear stress and the cell orientation might be applied to tissue technology to control of cells *in vitro*.

In the present experiment *in vitro*, the myoblast tends to be

exfoliated, when the area of the attachment to the scaffold decreases during proliferation. The myoblast proliferates regardless of the shear flow stimulation. The cell cycle does not vary under the shear flow. When myoblasts are cultured in the continuous steady shear flow without change of the medium between the plates, myoblasts differentiate to myotubes. Myotubes do not proliferate, so that the contact area did not decrease. The movement of each cell is able to be traced by the time-lapse image with the interval of thirty minutes in the present experiment.

5. CONCLUSION

The effect of shear stress on myoblast has been investigated under the uniform shear flow *in vitro*. The culture medium was sandwiched with a constant gap between a lower stationary culture plate and an upper rotating parallel plate to make a Couette type of shear flow. The behavior of each cell (C2C12) was traced at the time-lapse image observed by an inverted phase contrast microscope placed in an incubator for 7 days. The experimental results show that cells differentiate to myotubes under shear stress < 2 Pa. Both the cell cycle and the cell length tend to scatter in the wider range, and the longitudinal axis of the cell tends to align to the flow direction by the shear stress of 1 Pa. The experimental system is useful to study the quantitative relationships between the shear stress and the cell behaviors: deformation, orientation, and differentiation.

REFERENCES

[1] N. Sakamoto and N. Saito, "Effect of Spatial Gradient in Fluid Shear Stress on Morphological Changes in Endothelial Cells in Response to Flow", **Biochemical and Biophysical Research Communications**, Vol. 395, 2010, pp. 264−269.

[2] N. Kataoka, S. Ujita and M.Sato, "Effect of Flow Direction on the Morphological Responses of Cultured Bovine Aortic Endothelial Cells", **Medical & Biological Engineering & Computing**, Vol. 36, 1998, pp. 122−128.

[3] N. DePaola, M.A. Gimbrone Jr., P.F. Davies and C.F. Dewey Jr., "Vascular Endothelium Responds to Fluid Shear Stress Gradients", **Arteriosclerosis Thrombosis and Vascular Biology**, Vol 12, No 11 November 1992, pp. 1254−1257.

[4] M.J. Levesque and R.M. Nerem, "The Elongation and Orientation of Cultured Endothelial Cells in Response to Shear Stress", **Journal of Biomechanical Engineering**, Vol. 107, No. 4, 1985, pp. 341−347.

[5] R. Steward Jr, D. Tambe, C.C. Hardin, R. Krishnan and J.J. Fredberg, "Fluid Shear, Intercellular Stress, and Endothelial Cell Alignment", **American Journal of Physiology–Cell Physiology**, Vol. 308, 2015, C657–C664.

[6] M.A. Ostrowski, N.F. Huang, T.W. Walker, T. Verwijlen, C. Poplawski, A.S. Khoo, J.P. Cooke, G.G. Fuller and A.R. Dunn, "Microvascular Endothelial Cells Migrate Upstream and Align Against the Shear Stress Field Created by Impinging Flow", **Biophysical Journal**, Vol. 106, No. 2, 2014, pp. 366–374.

[7] Y. Tardy, N. Resnick, T. Nagel, M.A. Gimbrone Jr and C.F. Dewey Jr, "Shear Stress Gradients Remodel Endothelial Monolayers in Vitro via a Cell Proliferation-Migration-Loss Cycle", **Arteriosclerosis Thrombosis and Vascular Biology**, Vol. 17, No. 11, 1997, pp. 3102–3106.

[8] C.R. White, M. Haidekker, X. Bao and J.A. Frangos, "Temporal Gradients in Shear, but Not Spatial Gradients, Stimulate Endothelial Cell Proliferation", **Circulation**, Vol. 103, 2001, pp. 2508−2513.

[9] T. Nagel, N. Resnick, W.J. Atkinson, C.F. Dewey Jr and M. A. Gimbrone Jr, "Shear Stress Selectively Upregulates Intercellular Adhesion Molecule-1 Expression in Cultured Human Vascular Endothelial Cells", **The Journal of Clinical Investigation**, Vol. 94, No. 2, 1994, pp. 885–891.

[10] R.H.W. Lam, Y. Sun, W. Chen and J. Fu, "Elastomeric Microposts Integrated into Microfluidics for Flow-mediated Endothelial Mechanotransduction Analysis", **Lab on Chip**, Vol. 12, No. 10, 2012, pp. 1865−1873.

[11] M. Ochiai, S. Hashimoto and Y. Takahashi, "Effect of Flow Stimulation on Cultured Osteoblast", **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 156−161.

[12] 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.

[13] W. Yu, H. Qu, G. Hu, Q. Zhang, K. Song, H. Guan, T. Liu and J. Qin, "A Microfluidic-based Multi-shear Device for Investigating the Effects of Low Fluid-induced Stresses on Osteoblasts", **PLoS ONE**, Vol. 9, No. 2, 2014, pp. 1−7.

[14] 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.

[15] F. Sato, S. Hashimoto, T. Yasuda and H. Fujie, "Observation of Biological Cells in Rhombus Parallelepiped Flow Channel", **Proc. 17th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 1, 2013, pp. 25−30.

[16] H. Hino, S. Hashimoto, Y. Takahashi and S. Nakano, "Design of Cross Type of Flow Channel to Control Orientation of Cell", **Proc. 20th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2016, pp. 117−122.

[17] 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.

[18] S. Hashimoto, H. Sugimoto and H. Hino, "Behavior of Cell in Uniform Shear Flow Field between Rotating Cone and Stationary Plate", **Journal of Systemics Cybernetics and Informatics**, Vol. 16, No. 2, 2018, pp. 1−7.

[19] S. Hashimoto, H. Maeda and T. Sasada, "Effect of Shear Rate on Clot Growth at Foreign Surfaces", **Artificial Organs**, Vol. 9, No. 4, 1985, pp. 345−350.

[20] S. Hashimoto, "Clot Growth under Periodically Fluctuating Shear Rate", **Biorheology**, Vol. 31, No. 5, 1994, pp. 521−532.

[21] S. Hashimoto, "Erythrocyte Destruction under Periodically Fluctuating Shear Rate; Comparative Study with Constant Shear Rate", **Artificial Organs**, Vol. 13, No. 5, 1989, pp. 458−463.

[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.

[23] S. Hashimoto, "Detect of Sublethal Damage with Cyclic Deformation of Erythrocyte in Shear Flow", **Journal of Systemics Cybernetics and Informatics**, Vol. 12, No. 3, 2014, pp. 41−46.