Effect of Couette Type of Shear Flow by Rotating Disk on Migration of Cell

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ABSTRACT

An experimental system with a rotating disk has been designed to apply wall shear stresses on the cell during incubation in Couette type of flow at the microscopic observation *in vitro*. The rotating disk system is mounted on the stage of an inverted phase contrast microscope to observe cells adhered on the plate under the shear flow (shear stress lower than 1.5 Pa). The migration of cells was evaluated at the time lapse images (every five minutes for 24 hours). The experiments on four kinds of cells show the following results. Under the shear flow (shear stress < 1.0 Pa), C2C12 (mouse myoblast cell line) migrates to the diagonal direction of the stream-line at 1.0 Pa, HUVEC (human umbilical vein endothelial cells) actively migrates at random at 1 Pa, 3T3-L1 (mouse fat precursor cells) migrates to the random direction, and L929 (fibroblast connective tissue) migrates in short distance. At 1.5 Pa, both C2C12 and HUVEC migrate to the downstream. The effect of shear flow on migration of the cell depends on kinds of cells. The designed system is useful to trace cells under the quantitatively controlled wall shear stresses.

Keywords: Biomedical Engineering, Cell Culture, Shear Stress, C2C12, Couette Flow and Migration.

1. INTRODUCTION

The mechanical stimulation is one of the interested points in the environment of cells, because they receive mechanical forces *in vivo*. The mechanical stimulation on cells might induce various responses: deformation, migration, proliferation, and differentiation. Several methods have been designed to apply the mechanical stimulation to cells *in vitro* [1-23].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold [1, 2]. When fixation between the cell and the scaffold is not enough, the stress is not transmitted to the cell. A field, on the other hand, is effective to transmit stimulation to the cell placed in the field: the electric field [24], the magnetic field [25], the gravitational field [3], or the flow field [5-23]. The flow can be used to apply a stress field to a specimen. The cells exposed to the flow directly receive the shear stress in the shear flow.

The high shear flow might deform a cell, peel off a cell from the scaffold [6-10], and inhibit proliferation as well as tissue formation. The mild shear flow with the wall shear stress < 1 Pa, on the other hand, might accelerate migration, proliferation, and secretion of materials, which make the extracellular matrix.

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* [6, 11]. When the flow has an open surface, it is difficult to estimate the shear stress in the fluid.

Between two parallel walls, on the other hand, the velocity profile is easily estimated in the laminar flow. In the previous studies, several preparations were designed to study the effect of mechanical stimulations on biological cells: rhombus [7], cross [8], and rotating disks types [5, 12].

In the present study, an experimental system of the Couette type flow with a rotating disk has been designed to apply wall shear stress quantitatively on the cell during incubation at the microscopic observation *in vitro*, and the effect of the shear flow on the migration of the cell has been studied.

2. METHODS

Rotating Parallel Disk System

In the present study, a rotating parallel disk system is selected to make Couette type of flow (Fig. 1). The fluid is sheared between a rotating disk and a stationary disk. The stationary disk is the bottom of the culture dish (diameter 60 mm, Iwaki, Japan) (Fig. 2).

In the system, the shear rate (γ) is calculated by Eq. 1.

$$\gamma = r \, \omega / d \quad (1)$$

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

The rotating speed is controlled by the stepping motor between 50 rpm and 300 rpm, which makes variation of angular velocity ω between 5.2 rad s^{-1} and 31 rad s^{-1}. The position for the observation has the variation on r (the distance from the rotating axis) between 12 mm and 18 mm.

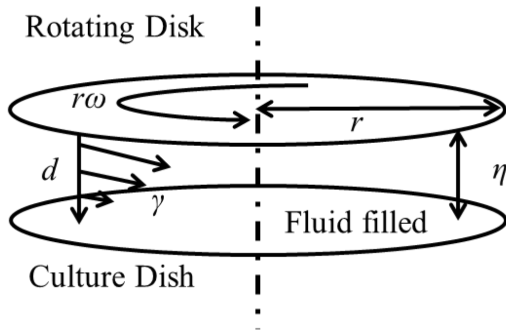

Fig. 1: Couette flow between rotating disk and stationary disk.

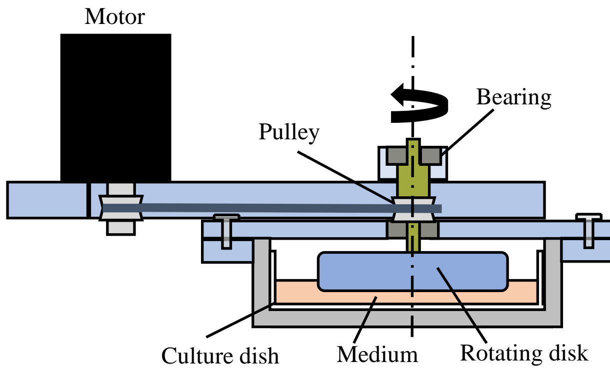

Fig. 2: Cell culture device with rotating disk.

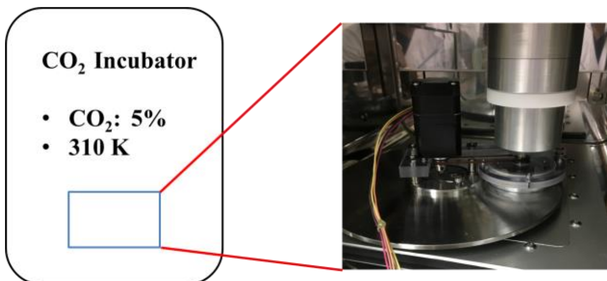

Fig. 3: Couette flow device in incubator.

The distance *d* is estimated to 0.5 mm from the positions of the focus of the walls at the microscope. These variations make the shear rates (γ) between 0.12×10^3 s^{-1} and 1.1×10^3 s^{-1} (see Eq. 1).

The shear rate (γ) generates the shear stress (τ [Pa]) in a viscous fluid.

$$\tau = \eta \gamma \quad (2)$$

In Eq. 2, η is the viscosity of the fluid [Pa s]. The fluid is the medium of cell culture in the present study.

When the viscosity of the fluid η is 0.0015 Pa s (at 310 K), the shear stress τ varies between 0.18 Pa and 1.7 Pa.

The rotating disk system is mounted on the stage of the inverted phase contrast microscope mounted in the incubator (LCV-110SK, Olympus Co., Ltd., Tokyo) (Fig. 3). In the incubator, both the temperature and the partial pressure of carbon dioxide are maintained at 310 K and 5 percent, respectively. The behavior of cells adhered on the stationary wall under shear stress is observed with the microscope. The system allows observation of cells during exposure to the shear flow.

Cell Culture

Four kinds of cells were used in the experiment: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), L929 (fibroblast connective tissue of C3H mouse), 3T3-L1 (mouse fat precursor cells, a cell line derived from cells of mouse 3T3), and HUVEC (human umbilical vein endothelial cells).

C2C12 of the passage between three and nine was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin.

HUVEC of the passage between three and eight was cultured in EBM-2 (Endothelial Cell Basal Medium) containing 2% decomplemented FBS (fetal bovine serum).

3T3-L1 of the fifth passage was used for the cell culture. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% decomplemented FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin was used for the medium.

L929 of the passage between three and nine was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin.

A hemocytometer (Burker-Turk) was used to count number of cells. The cells were seeded on the dish coated with collagen at the density of 2000 cells/cm^2. The dish was placed in the rotating parallel disk system mounted in the incubator (LCV-110SK). To make adhesion of cells to the bottom of the 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 sheared with the rotating disk for 24 hours in the incubator (Fig. 4). Variation was made in the rotational speed of the disk: 100, 200, and 300 rpm.

Microscopic Observation

The several positions are marked by grooves on the outside surface of the culture dish to trace the same position of the scaffold.

The time-lapse image was taken every five minutes during the rotation for 24 hours.

The direction of the flow adjacent to the culture plate is traced by the floating particle in the medium at the video image, and defined as the *x* axis in Figs. 7-10.

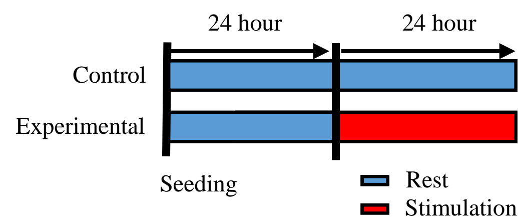

Fig. 4: Experimental protocol.

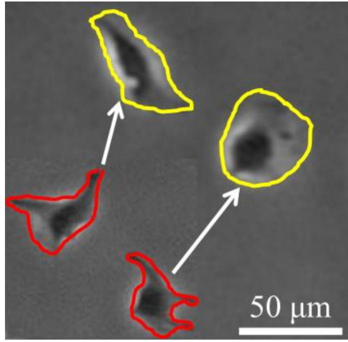

Fig. 5: Migration of C2C12: cell contour of red, shear for 3 hours; cell contour of yellow, shear for 12 hours.

At the microscopic image, the contour of each cell was approximated to ellipsoid. The centroid of each cell was used to trace the migration of the cell (Fig. 5).

3. RESULTS

In Figs. 6-9, the direction of the flow is from left to right along the *x* axis. The *y* axis shows the direction of the center of the rotation. The wall shear stress is proportional to the wall shear rate, so that the wall shear stress decreases with *y* cordinate. The coordinate of the origin shows the original position of each cell. To tarce the migration of each cell, the image of cell every one hour has been selected from the time lapse image every five minutes. Each polygonal line in Figs. 6-9 shows the connected line segments of the centroid of each cell traced every hour.

Each cell migrates on the cuture plate (on the scaffold) to random direction without flow (Fig. 6a). At the wall shear stress of 0.5 Pa, C2C12 migrates to every direction including the counter direction (to the left in Fig. 6b) of the flow. At the wall shear stress of 1 Pa, C2C12 migrates to the oblique direction: along the flow direction, and to the center (to the lower wall shear stress area). At the wall shear stress of 1.5 Pa, C2C12 migrates to the down stream.

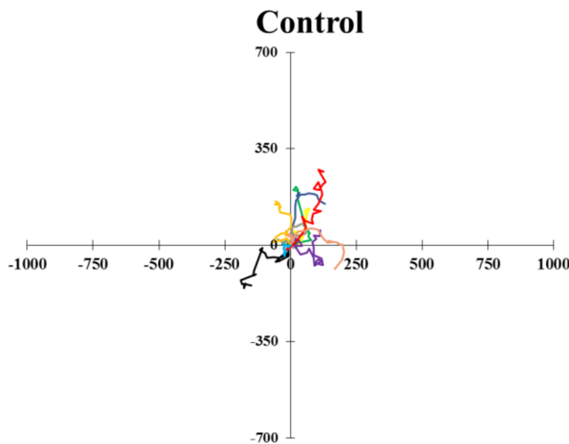

Fig. 6a: Migration of ten cells (C2C12) without flow. Micrometer.

Both at 0.5 Pa and at 1 Pa, HUVEC actively migrates to every direction including the perpendicular and the counter direction of the flow (Figs. 7b & 7c). HUVEC migrates to the down stream at the wall shear stress of 1.5 Pa (Fig. 7d). 3T3-L1 migrates to the random direction at the wall shear stress of 1 Pa (Fig. 8). L929 migrates within the short distance to perpendicular direction of the stream line at the wall shear stress of 1 Pa (Fig. 9).

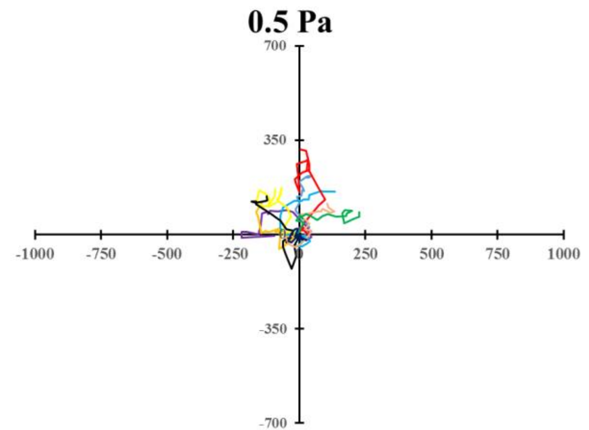

Fig. 6b: Migration of ten cells (C2C12) under shear flow: wall shear stress of 0.5 Pa. Micrometer.

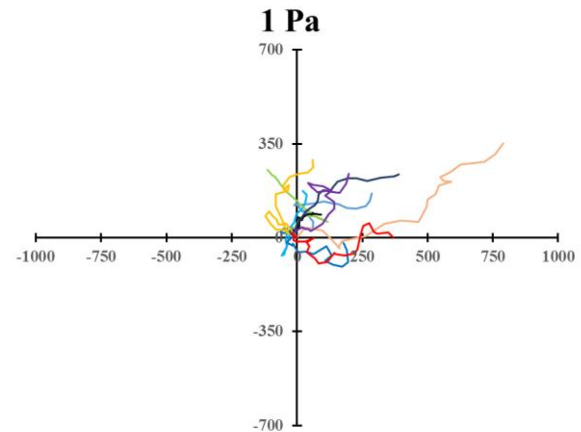

Fig. 6c: Migration of ten cells (C2C12) under shear flow: wall shear stress of 1 Pa. Micrometer.

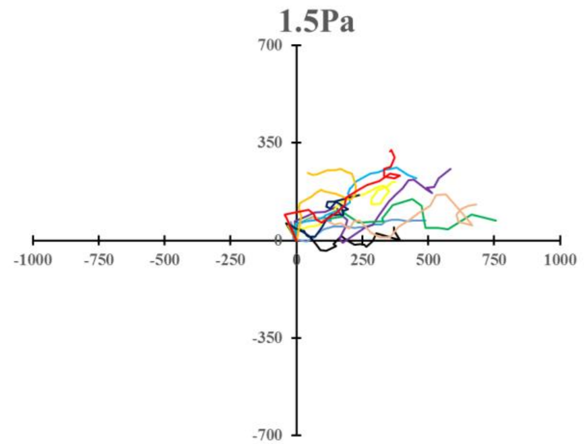

Fig. 6d: Migration of ten cells (C2C12) under shear flow: wall shear stress of 1.5 Pa. Micrometer.

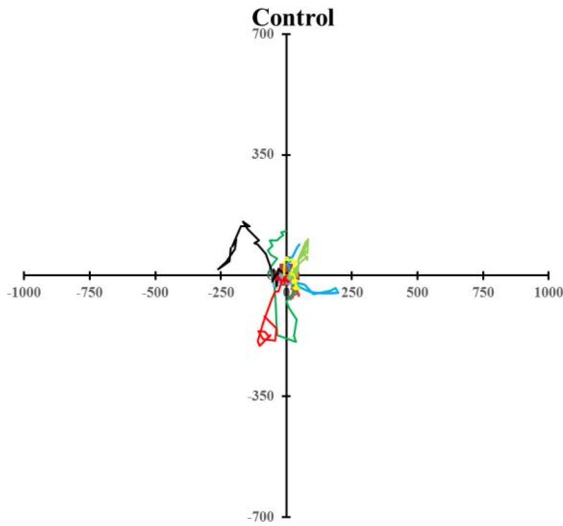

Fig. 7a: Migration of ten cells (HUVEC) without flow

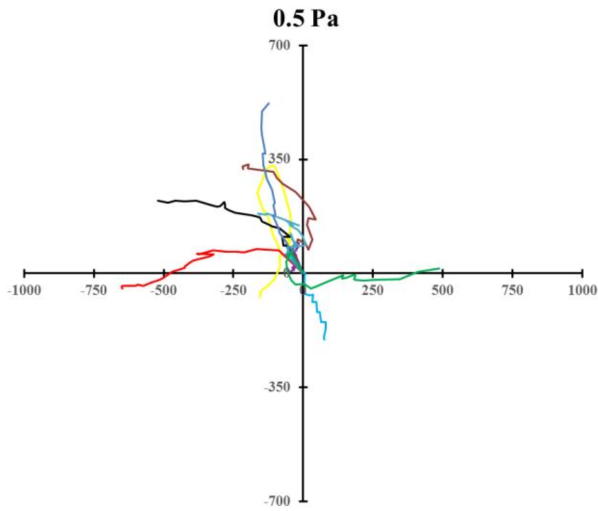

Fig. 7b: Migration of ten cells (HUVEC) under shear flow: wall shear stress of 0.5 Pa. Micrometer.

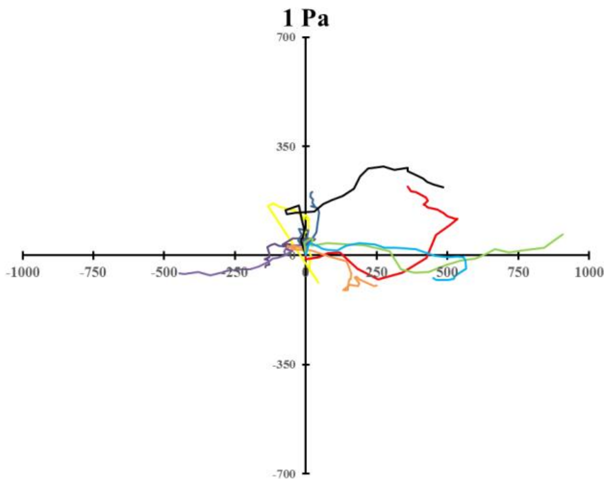

Fig. 7c: Migration of ten cells (HUVEC) under shear flow: wall shear stress of 1 Pa. Micrometer.

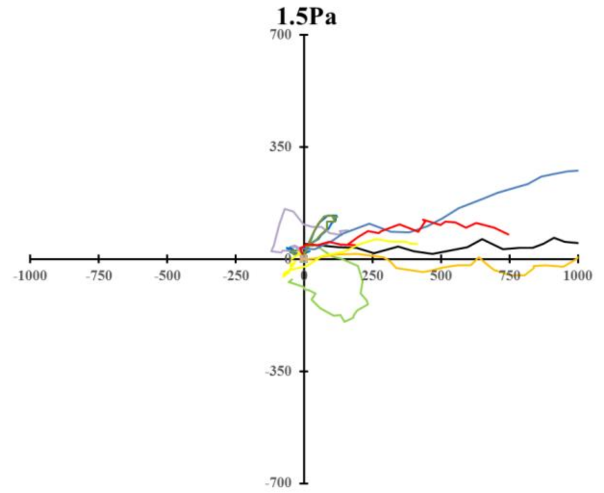

Fig. 7d: Migration of ten cells (HUVEC) under shear flow: wall shear stress of 1.5 Pa. Micrometer.

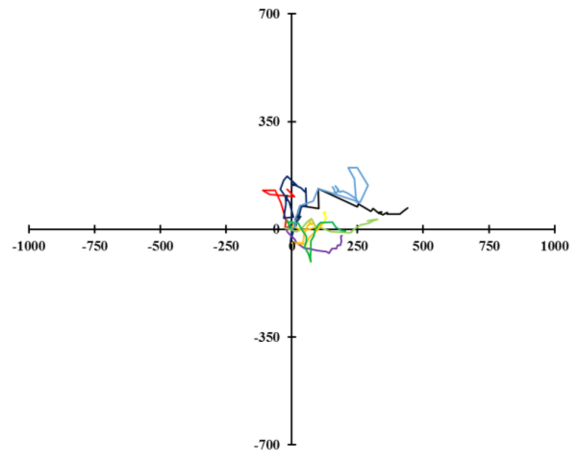

Fig. 8: Migration of ten cells (3T3-L1) under shear flow: wall shear stress of 1 Pa.

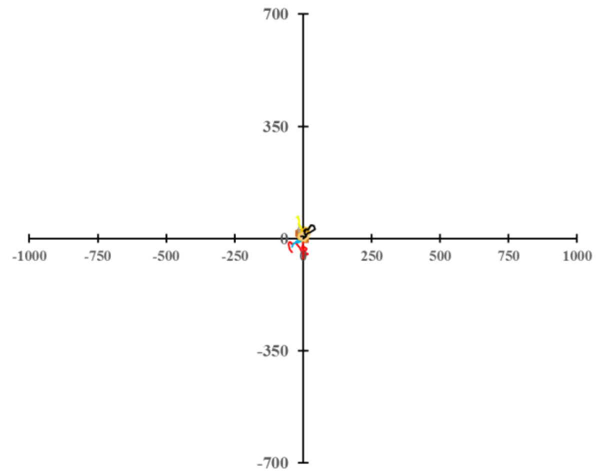

Fig. 9: Migration of ten cells (L929) under shear flow: wall shear stress of 1 Pa.

4. DISCUSSION

In the previous study, myotubes make orientation perpendicular to the stream line in the donut-shaped flow system. The donut-shaped open channel is convenient to study the effect of flow direction on the cell culture [6, 11], but it is not easy to estimate quantitatively the shear stress in the fluid because of the free surface.

Hagen-Poiseulle type of flow is convenient to estimate the shear stress on the wall with the parabolic velocity distribution for Newtonian fluid. The flow in the cylindrical pipe, and the flow between the parallel plates are typical preparations for Hagen-Poiseulle flow. The rhombus (variation of wall shear rate) [7], the cross (variation of flow direction) [8], and the tilting (flow and gravity) [9] types are variations to study the effect of mechanical stimulations on biological cells.

The Couette type of flow is also convenient to estimate the shear stress in the flow with the uniform shear rate between the moving wall and the stationary wall, which is also available to non-Newtonian fluid. Many kinds of the devices of Couette type flow were designed for quantitative experiments of biological fluid in the previous studies [5, 12]. The cone and plate type device has the uniform shear field in the entire space between the rotating cone and the stationary plate. The clot formation was quantitatively studied between the rotating cone and the stationary plate [26], and between the rotating concave cone and the stationary convex cone [27]. The erythrocyte destruction was studied between the rotating concave cone and the stationary convex cone [17].

A parallel disks system does not have the uniform shear field in the entire space between rotating disk and the stationary disk. The parallel disks system, 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 shear rate proportional to the radius from the rotational axis. The erythrocyte deformation was observed between counter rotating parallel discs [18, 19]. 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 31 rad s^{-1} (d = 0.5 mm), the shear rate (γ) increases from 0.73×10^3 s^{-1} to 1.12×10^3 s^{-1}, when the distance from the axis (r) increases from 12 mm to 18 mm in the observation area (Eq. 1). The gradient of shear stress (0.6 Pa / 0.006 m = 10^2 Pa m^{-1}) enables the simultaneous observation of the behavior of cells related to variation of the shear stress (1.1 Pa - 1.7 Pa) in the same view [5, 12]. The migration speed is slower than 1 mm / 24 hour = 10^{-8} m/s.

The rotating flow induces the secondary flow by the centrifugal effect. The effect is smaller in the system with the rotation of outer concave cone than with that of inner convex cone. The effect decreases with the decrease of the rotational speed. The rotational speed of the disk is smaller than 0.7 m s^{-1} in the present system. The microscopic image of the flowing cells between the rotating disk and the stationary disk does not show turbulent flow. Reynolds number (Re) is calculated by Eq. 3.

$$Re = \rho\, v\, d / \eta = \rho\, r\, \omega\, d / \eta \qquad (3)$$

In Eq. 3, 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 1.4×10^2, when ρ, r, ω, d, and η are 1×10^3 kg m^{-3}, 0.018 m, 300 rpm=31 rad s^{-1}, 0.0005 m, and 0.002 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 stream line traced by the direction of exfoliation of the cell and of the moving particle adjacent to the surface.

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 [11, 20-23]. Cells are not exfoliated under the shear flow at the wall shear stress lower than 1.5 Pa. Cells were exfoliated at the higher wall shear stress in the previous studies [6-10].

A biological cell shows passive and active behaviors in an environment. While the flow might enhance the cell migration to the downstream, a cell migrates to adapt 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. The hysteresis effect governs the active behavior of the cell. The interaction between cells also governs the behaviou of each cell. The seeding density is selected not so high to trace the image of each cell in the present study. The migration of the cell might also depends on the morphology and the mechanical property of the scaffold [28-30]. At the cell division, migration of two segments to the counter direction each other is observed in the present study. The temperature in the medium after stimulation of the shear flow for 24 hours was measured by the thermocouple. The result shows the temperature is the same level of 310 K and is not elevated by the heating effect of the shear flow.

In the present study, each cell migrates independently to every direction includes the counter direction of the flow. 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 migration of the cell depends on the kind of cells, which might be applied to the cell sorting technology.

5. CONCLUSION

An experimental system with a rotating disk has been designed to apply shear stresses on the cell during incubation in Couette type of flow at the microscopic observation *in vitro*. The experiments show the following results. Under the shear flow at the constant shear stress of 1 Pa for 24 hours, C2C12 migrates to the diagonal direction of the stream-line, HUVEC migrates along the stream-line, 3T3-L1 migrates to the random direction, and L929 migrates in short distance. The effect of shear flow on migration of the cell depends on kinds of cells. The designed system is useful to trace cells under the quantitatively controlled wall shear stresses.

6. ACKNOWLEDGMENT

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REFERENCES

[1] J.H.-C. Wang, E.S. Grood, J. Florer and R. Wenstrup, "Alignment and Proliferation of MC3T3-E1 Osteoblasts in Microgrooved Silicone Substrata Subjected to Cyclic Stretching", **Journal of Biomechanics**, Vol. 33, No. 6, 2000, pp.729-735.

[2] G. Yourek, S.M. McCormick, J.J Mao and G.C. Reilly, "Shear Stress Induces Osteogenic Differentiation of Human Mesenchymal Stem Cells", **Regenerative Medicine**, Vol. 5, No. 5, 2010, pp. 713-724.

[3] 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.

[4] 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, pp. 1-7, 2016.

[5] 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.

[6] 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.

[7] 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.

[8] 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.

[9] H. Iwata, S. Hashimoto, S. Okuda and H. Nakaoka, "Effect of Medium Flow on Cultured Cells", **Proc. 14th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2010, pp. 265-268.

[10] H. Hino, M. Ochiai, S. Hashimoto, K. Kimura, Y. Takahashi and T. Yasuda, "Effect of Wall Shear Stress in Flow on Myoblast", **Proc. 19th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2015, pp. 246-251.

[11] 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.

[12] M. Ochiai, H. Hino, S. Hashimoto and Y. Takahashi, "Rotating Disk to Apply Wall Shear Stress on Cell Culture at Microscopic Observation", **Proc. 19th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2015, pp. 288-291.

[13] M.L.C. Albuquerque, C.M. Waters, U. Savla, H.W. Schnaper and S.A. Flozak, "Shear Stress Enhances Human Endothelial Cell Wound Closure in Vitro", **American Journal of Physiology - Heart and Circulatory Physiology**, Vol. 279, No. 1, 2000, pp. H293-H302.

[14] 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.

[15] S.D. Tan, T.J. deVries, A.M. Kuijpers-Jagtman, C.M. Semeins, V. Everts and J. Klein-Nulend, "Osteocytes Subjected to Fluid Flow Inhibit Osteoclast Formation and Bone Resorption", **Bone**, Vol. 41, No. 5, 2007, pp. 745-751.

[16] 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.

[17] 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.

[18] 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.

[19] 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.

[20] 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.

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

[22] M. Gouverneur, B. Van den Berg, M. Nieuwdorp, E. Stroes and H.Vink, "Vasculoprotective Properties of the Endothelial Glycocalyx: Effects of Fluid Shear Stress", **Journal of Internal Medicine**, Vol. 259, No. 4, 2006, pp. 393-400.

[23] T.D. Oblak, P. Root and D.M. Spence, "Fluorescence Monitoring of ATP-Stimulated, Endothelium-Derived Nitric Oxide Production in Channels of a Poly(dimethylsiloxane)-Based Microfluidic Device", **Analytical Chemistry**, Vol. 78, No. 9, 2006, pp. 3193-3197.

[24] 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.

[25] S. Hashimoto and K. Tachibana, "Effect of Magnetic Field on Adhesion of Muscle Cells to Culture Plate", **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 4, 2013, pp. 7-12.

[26] 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.

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

[28] H. Hino, S. Hashimoto and F. Sato, "Effect of Micro Ridges on Orientation of Cultured Cell", **Journal of Systemics Cybernetics and Informatics**, Vol. 12, No. 3, 2014, pp. 47-53.

[29] B.C. Isenberg, P.A. DiMilla, M. Walker, S. Kim and J.Y. Wong, "Vascular Smooth Muscle Cell Durotaxis Depends on Substrate Stiffness Gradient Strength", **Biophysical Journal**, Vol. 97, No. 5, 2009, pp. 1313-1322.

[30] C.M. Lo, H.B. Wang, M. Dembo and Y.I. Wang, "Cell Movement Is Guided by the Rigidity of the Substrate", **Biophysical Journal**, Vol. 79, No. 1, 2000, pp. 144-152.