Behavior of Cell in Uniform Shear Flow Field between Rotating Cone and Stationary Plate

Shigehiro HASHIMOTO, Hiromi SUGIMOTO, Haruka HINO

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/

ABSTRACT

The behavior of a biological cell in a uniform shear flow between the rotating cone and the stationary plate has been observed *in vitro*. The cone and plate apparatus is mounted on a stage of an inverted phase contrast microscope to observe cells adhered to the plate under the shear flow. Deformation and migration of cells during exposure to the shear flow for 24 hours were traced with the time lapse images. Two kinds of cells were used in the test: C2C12 (mouse myoblast cell line), and HUVEC (human umbilical vein endothelial cell). The experimental results show that HUVEC tends to migrate downstream at the shear stress of 1 Pa. C2C12 tends to tilt vertically in the flow for 18 hours between 1 Pa and 2 Pa. HUVEC tends to be rounded at the shear stress of 2 Pa. The behavior of each isolated cell in the shear flow field has been able to be quantitatively observed by the cone and plate apparatus *in vitro*.

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

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 [1-7], migration [8-17], proliferation [18-19], and differentiation [20]. Several methods have been designed to apply the mechanical stimulation to cells *in vitro* [1-28].

A transmission point of the stress to a specimen is important in vitro test. In many studies, the stress is applied to a scaffold [3, 17, 18]. When immobilization between the cell and the scaffold is not sufficient, the stress is not transmitted to the cell. A field, on the other hand, is effective to transmit stimulation to the cell exposed in the field: the gravitational field [14], or the flow field [1, 2, 4-16, 20-28]. The flow can be used to apply a stress field to a specimen. The cells exposed to the flow are directly subjected to the shear stress in the shear flow.

In the previous study, cells were exposed to the shear flow in a toroidal open channel, and the effect of the flow stimulation on cultured cells has been studied *in vitro* [16]. When the flow has a free 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 (variation of the wall shear rate) [5], cruciform (change in the flow direction) [6], inclination (flow and gravity) [7], and rotating disks types [9, 10, 24, 25]. The cone and plate type [26-28] is one of the useful preparations to make a uniform shear rate field. The Couette type of the uniform shear field is made in the fluid sandwiched between the rotating cone and the stationary plate. In the present study, an experimental system of the Couette type flow between a rotating cone and a stationary scaffold plate has been designed to apply wall shear stress quantitatively to the cell during incubation under the microscopic observation *in vitro*, and the effect of the shear flow on migration of the cell has been studied.

2. METHODS

Cone and Plate Device

In the present study, a cone and plate device was selected to make the uniform Couette type of flow (Fig. 1). The fluid is sheared between a rotating cone and a stationary disk. The stationary disk corresponds to the bottom of the culture dish (diameter 60 mm, Iwaki, Japan) (Fig. 2). In the device, the shear rate (γ [s^{-1}]) is calculated by Eq. 1.

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

In Eq. 1, v is the velocity of the conical wall [m s^{-1}], d is the distance between the moving conical wall and the stationary plane wall [m], r is the distance from the rotating axis [m], ω is the angular velocity [rad s^{-1}], and ψ is the central gap angle [0.023 rad] between the conical wall and the plane wall (Fig. 1). In Eq. 1, d is approximated to "$r\,\psi$", because ψ is very small. In the fluid sheared between a rotating cone and a stationary disk, the shear rate (γ) is constant regardless of r.

The angular velocity (12 rad s^{-1} < ω < and 33 rad s^{-1}) is controlled by the stepping motor (Fig. 2). The distance d is confirmed from the positions of the focal point of the wall at the microscope. These adjustment of parameters makes variations of the shear rate (γ) between 0.5×10^3 s^{-1} and 1.4×10^3 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 for cell culture in the present study.

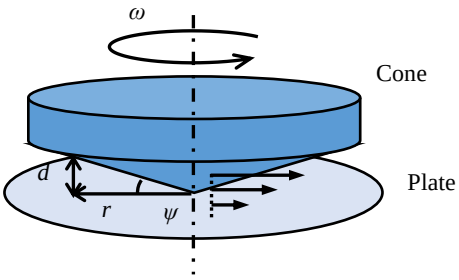
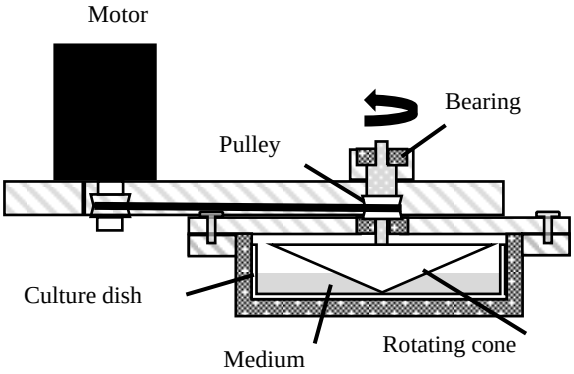


Fig. 1: Couette flow between rotating cone and stationary plate.



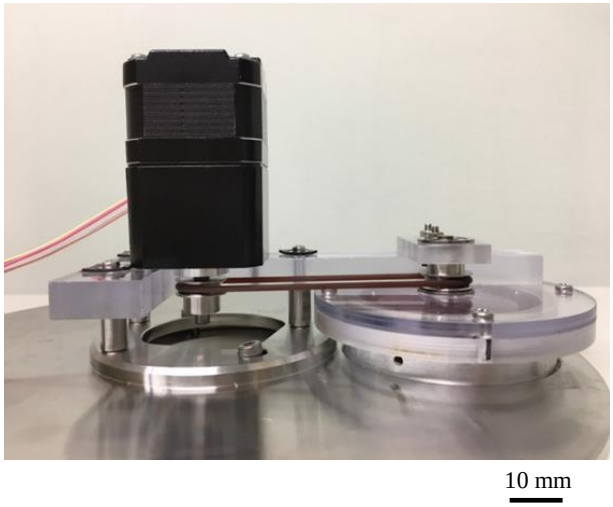


Fig. 2: Manufactured Couette flow device: cross section (upper), and appearance (lower).

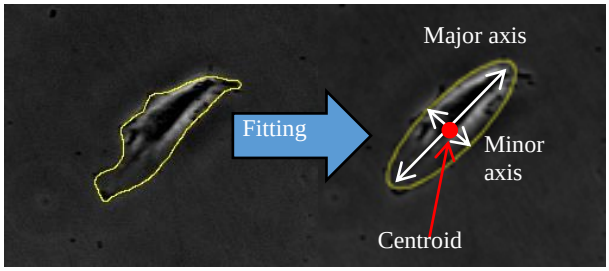


Fig. 3: Contour of cell is approximated to ellipsoid.

The cone and plate device (Fig. 2) is mounted on the stage of the inverted phase contrast microscope places in the incubator (LCV-110SK, Olympus Co., Ltd., Tokyo). 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 adhering to the stationary wall surface under the shear stress is observed with a microscope. The experimental system allows observation of cells during exposure to the shear flow. The flow adjacent to the bottom 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 (Fig. 7).

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 HUVEC (human umbilical vein endothelial cells). C2C12 (passage < ten) was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. HUVEC (passage < four) was cultured in EBM-2 (Endothelial Cell Basal Medium) containing 2% decomplemented FBS (fetal bovine serum).

Cell culture

A hemocytometer (Burker-Turk) was used to count the number of cells. The cells were seeded on the dish coated with collagen at the density of 2000 cells/cm^2.

To make adhesion of cells to the bottom of the dish, the cells were cultured for 24 hours in the incubator without flow stimulation (no rotation of the cone). After the incubation for 24 hours, the cells were sheared with the cone and plate device for 24 hours in the incubator. Variations were made in the rotational speed of the disk.

Microscopic Observation

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 tracked by the moving particles in the medium at the video image, and defined as the *x* axis in Figs. 8&9.

Microscopic observation

At the microscopic image, the contour of each cell was traced (Fig. 3), and the projected area (*S*) of each cell was calculated. The projected area is related to the adhesion area of the cell to the scaffold. The centroid of each cell was used to track the migration of the cell (Fig. 4).

The contour of each cell was approximated to ellipsoid (Fig. 3), and the shape index (*R*) was calculated by Eq. 3.

$$R = 1 - (b / a) \quad (3)$$

In Eq. 3, *b* is the length of the minor axis, and *a* is the length of the major axis of the ellipsoid. *R* is zero at the circle ($a = b$). *R* approaches to unity, when the major axis (*a*) increases as the elongation of the cell (Fig. 5).

At each cell, the angle (θ) between the major axis and the flow direction was measured (Fig. 6). The angle (θ) is zero degree, when the major axis is parallel to the flow direction.

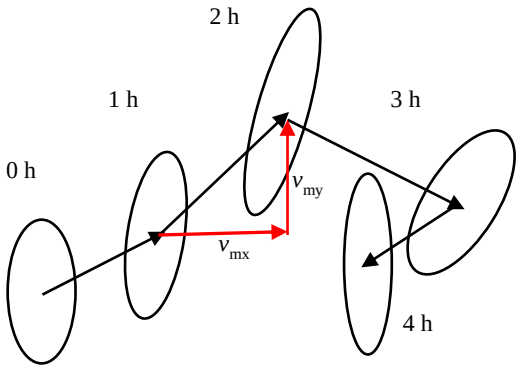


Fig. 4: Migration of cell every hour traced by centroid of approximated ellipsoid.

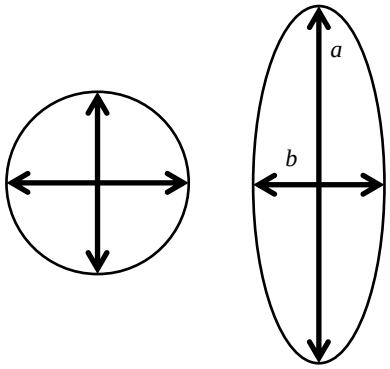


Fig. 5: Shape index (Eq. 3): zero at circle (left), and approach to 1 at elongated ellipsoid (right).

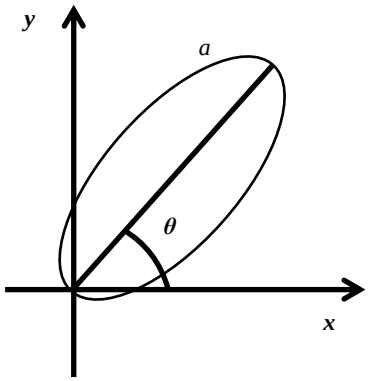


Fig. 6: Angle between major axis (a) of cell and flow direction (x axis).

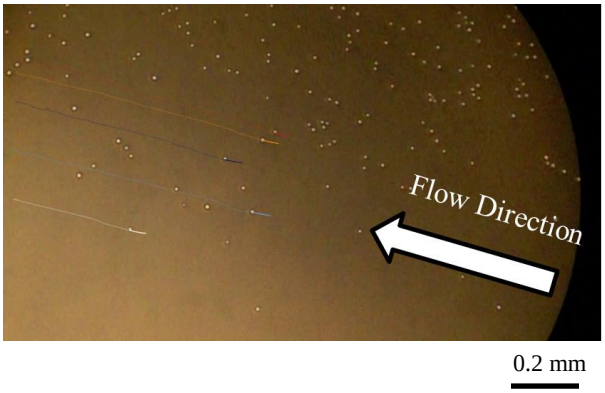


Fig. 7: Tracings of microspheres in flow.

3. RESULTS

At 310 K, the measurement by the cone and plate type of viscometer shows that the viscosities (η) of the D-MEM and EBM-2 are 0.0020 Pa s and 0.0014 Pa s, respectively. The shear stress τ calculated by Eqs. 1&2 varies between 1 Pa and 2 Pa in the experiment. The direction of flow was confirmed by the tracings of the movement of each microsphere on the surface of the scaffold (Fig. 7).

Figs. 8&9 exemplify the tracings of the centroid of each cell under the shear flow for 24 hours. In Figs. 8&9, the direction of the flow is from left to right along the x axis. The y axis corresponds to the direction of the center of the rotation. The coordinate of the origin indicates the original position of each cell. To track the migration speed of each cell, the image of cell every one hour has been selected from the time lapse images. In Figs. 8&9, each polygonal line indicates the connected line segment of the centroid of each cell traced every hour.

Without flow, each cell migrates to the random direction on the culture plate (on the scaffold) (Fig. 8A). At the wall shear stress of 1 Pa, C2C12 migrates to every direction including the counter direction (to the left in Fig. 8B) of the flow. At the wall shear stress of 2 Pa, C2C12 migrates downstream. HUVEC migrates downstream at the wall shear stress of 1 Pa (Fig. 9B).

Fig. 10 shows the migration speed (v_m [μm/hour]) of C2C12 under the shear flow. Data are presented by the mean value and the range of the standard deviation at each shear stress. C2C12 migrates with the speed in the range of 0.02 mm/hour to every direction. Fig. 11 shows the migration speed (v_m [μm/hour] $< 3\times10^{-8}$ m/s) of HUVEC under the shear flow. At both 1 Pa and 2 Pa, the migration speed of HUVEC is higher to the downstream (Fig. 11 upper) than to the other directions.

Fig. 12 exemplifies the tracings of the projected area of C2C12 under the shear flow for 24 hours. Different from the control study, the projected area tends to increase under the shear flow. Fig. 13 exemplifies the tracings of the projected area of HUVEC under the shear flow for 24 hours. In every case, the projected area tends to increase gradually.

Fig. 14 exemplifies the tracings of the angle of C2C12 under the shear flow for 24 hours. C2C12 tends to tilt to the direction perpendicular to the flow in the first stage for 18 hours under

the flow. After 18 hours of exposure to the shear flow (at 1 Pa and 2 Pa), C2C12 tends to tilt to the flow direction. Fig. 15 exemplifies the tracings of the angle of HUVEC under the shear flow for 24 hours. HUVEC tends to tilt to the flow direction.

Fig. 16 exemplifies the shape index of C2C12 under the shear flow for 24 hours. C2C12 changes the shape at random for 18 hours even under the shear flow. Fig. 17 exemplifies the shape index of HUVEC under the shear flow for 24 hours. HUVEC tends to be rounded at the shear stress of 2.0 Pa.

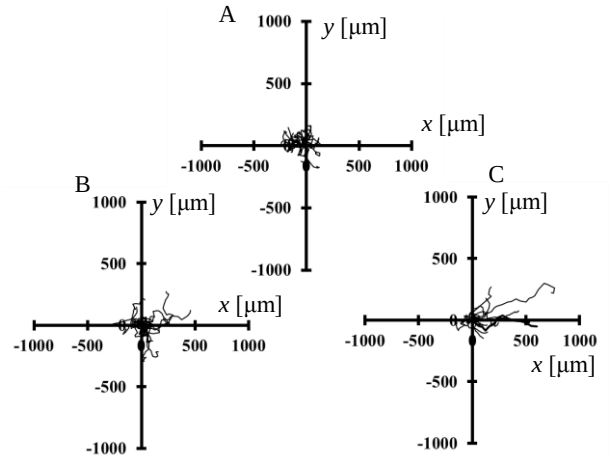


Fig. 8: Migration of ten cells (C2C12): without flow (A), 1 Pa (B), and 2 Pa (C): unit, micrometer.

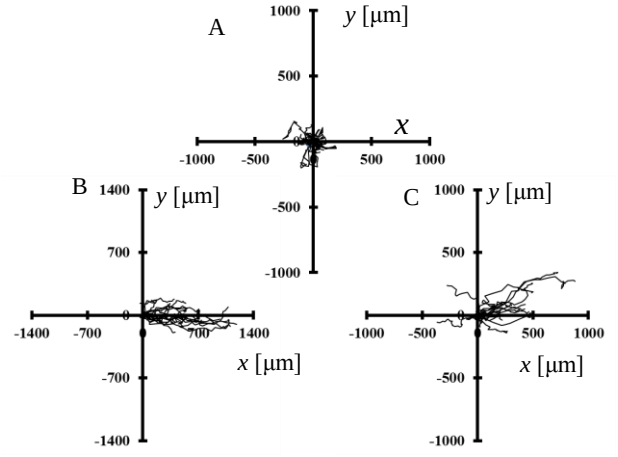


Fig. 9: Migration of ten cells (HUVEC): without flow (A), 1 Pa (B), and 2 Pa (C): unit, micrometer.

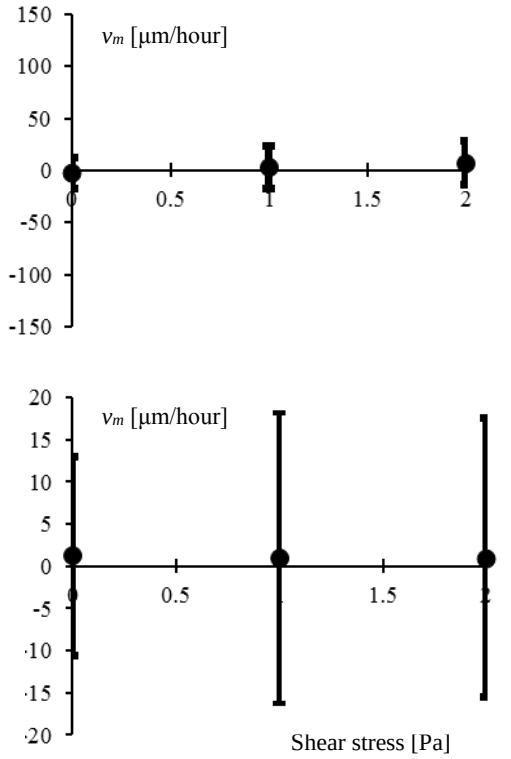


Fig. 10: Migration speed (v_m [μm/hour]) of C2C12 under shear flow: flow direction x (upper), and perpendicular direction y (lower); mean ± standard deviation (N = 20).

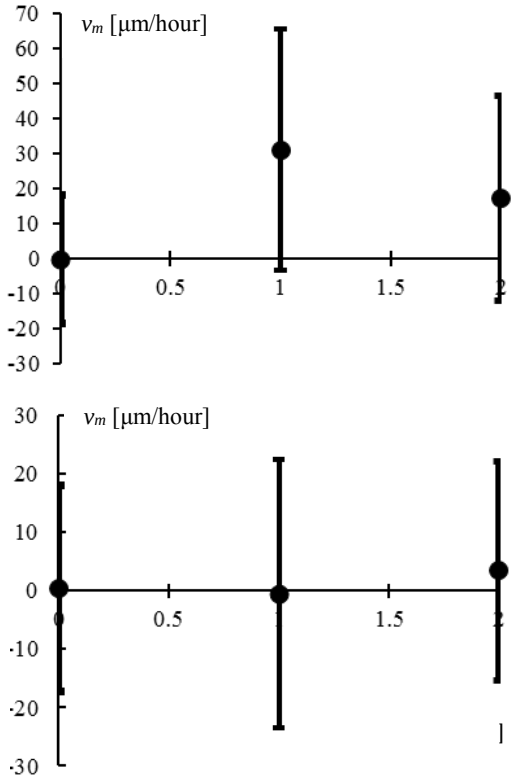


Fig. 11: Migration speed (v_m [μm/hour]) of HUVEC under shear flow: flow direction x (upper), and perpendicular direction y (lower); mean ± standard deviation (N = 20).

4. DISCUSSION

In the previous study, myotubes make orientation perpendicular to the streamline in the donut-shaped flow system [16]. The toroidal open channel is convenient to study the effect of flow direction on the cell culture, 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 useful for estimating the shear stress on the wall using the parabolic velocity distribution for Newtonian fluid. Typical preparations for Hagen-Poiseulle type of flow are the flow in the cylindrical pipe, and the flow between the parallel plates [4-7].

The Couette type of flow is also useful for estimating the shear stress in the flow at a 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 [9, 10, 24, 25].

The cone and plate type device has a uniform shear field throughout the space between the rotating cone and the stationary plate. The uniform shear field was confirmed by the same velocity of each microsphere on the surface of the scaffold independent of the distance from the rotating axis in the present study.

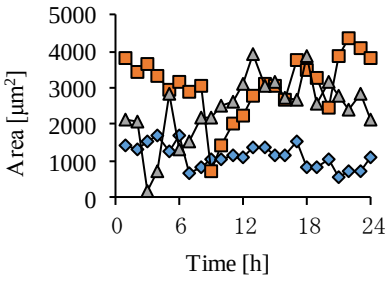


Fig. 12: Tracings of projected area of C2C12: without flow (control, diamond), 1 Pa (square), and 2 Pa (triangle).

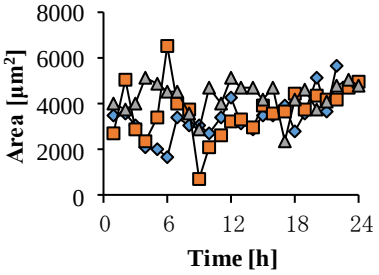


Fig. 13: Tracings of projected area of HUVEC: without flow (control, diamond), 1 Pa (square), and 2 Pa (triangle).

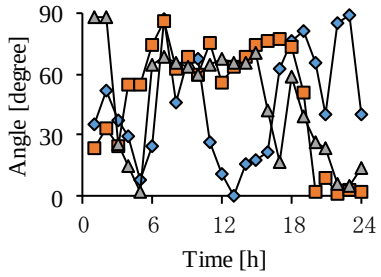


Fig. 14: Tracings of angle of C2C12: without flow (control, diamond), 1 Pa (square), and 2 Pa (triangle).

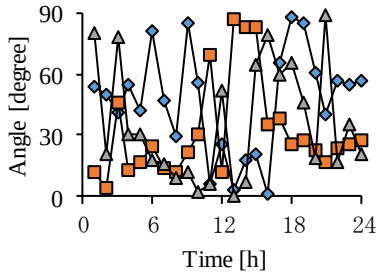


Fig. 15: Tracings of angle of HUVEC: without flow (control, diamond), 1 Pa (square), and 2 Pa (triangle).

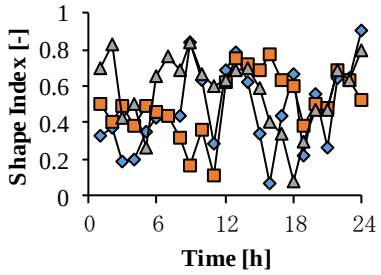


Fig. 16: Tracings of shape index of C2C12: without flow (control, diamond), 1 Pa (square), and 2 Pa (triangle).

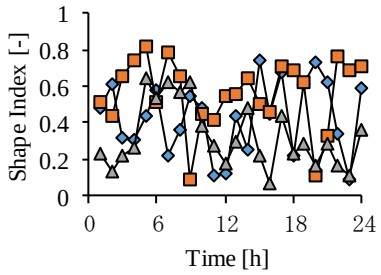


Fig. 17: Tracings of shape index of HUVEC: without flow (control, diamond), 1 Pa (square), and 2 Pa (triangle).

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 [28].

The rotating flow induces a 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 flow velocity is smaller than 0.7 m s^{-1} in the present system. The microscopic image of the cells flowing between the rotating cone and the stationary disk does not show turbulent motion. Reynolds number (*Re*) is calculated by Eq. 4.

$$Re = \rho\, v\, d / \eta = \rho\, r\, \omega\, d / \eta \quad (4)$$

In Eq. 4, ρ is the density of the fluid [kg m^{-3}], v is the circumferential velocity [m s^{-1}], r is the distance [m] from the rotating axis, ω is the angular velocity [rad s^{-1}], 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}, 1.8×10^{-2} mm, 33 rad s^{-1}, 5×10^{-4} mm, and 2×10^{-3} Pa s, respectively.

Turbulence is hardly generated in a flow with a small Reynolds number. The steady actual flow direction adjacent to the scaffold surface of the cell culture has been confirmed by the streamlines tracked by the direction of cell detachment and of the moving microspheres adjacent to the surface in the present study.

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, 8, 13, 16, 21-23]. 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 [4-7].

A biological cell shows passive or active behaviors depending on the surrounding environment. While the flow promotes downstream cell migration, a cell migrates to adapt to the shear field. While a strong stimulus exceeding the threshold damages the cell, a stimulus below the threshold remains in the cell as a memory for the response at the next step. The hysteresis effect governs the active behavior of the cell.

The interaction between cells also governs the behavior of each cell. The seeding density is chosen low to track the image of each cell in the present study. The migration of the cell might also depends on the morphology and the property of the scaffold [3, 29]. The temperature in the medium after stimulation of the shear flow for 24 hours was measured with a thermocouple. The result shows that the temperature is at the same level as 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 including the counter direction of the flow. The effect of shear flow on migration of the cell depends on the type of cells, which might be applied to the cell sorting technology.

5. CONCLUSION

The cone and plate device placed on the stage of an inverted phase contrast microscope mounted in the incubator has been designed to observe cells adhered on the plate under the shear flow *in vitro*. The experimental results indicate that HUVEC (human umbilical vein endothelial cells) tends to migrate downstream at the shear stress of 1 Pa. C2C12 (mouse myoblast cell line) tends to tilt to the perpendicular direction of the flow for 18 hours between 1 Pa and 2 Pa. HUVEC tends to be rounded at the shear stress of 2 Pa. The effect of the shear field on the response of the isolated cell depends on the cell type and on the value of the shear stress.

6. ACKNOWLEDGMENT

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