Tracings of Myoblasts Orientation under Shear Flow In Vitro

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ABSTRACT

Myoblast orientation under the flow 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 (2 Pa) 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, the constant shear stress was continuously applied on cells for 7 days in the incubator. The behavior of each cell was traced at the time-lapse images observed by an inverted phase contrast microscope placed in an incubator. The experimental results show that each single cell tends to align parallel to the flow direction under shear stress of 2 Pa. The mean direction of cells in some colonies of C2C12 tends to align perpendicular to the flow direction.

Keywords: Biomedical Engineering, Shear Stress, C2C12 and Orientation.

1. INTRODUCTION

The effects 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 (mouse myoblast cell line) made orientation perpendicular to the direction of the flow, although HUVEC (human umbilical vein endothelial cell) made orientation along the streamline of the flow [12]. The orientation of each cell in the tissue depends on that of neighbor's cell [13]. To study on the orientation of cells, the orientation should be evaluated quantitatively by the parameter. The shape of each C2C12 adhered on the culture plate can be approximated to the ellipsoid. In the present study, the distribution of the direction of the longitudinal axis of each ellipsoid has been measured.

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, 14], and exfoliation from the wall of the scaffold [15–17]. 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 [18–24].

In the present study, an experimental system of the Couette type flow in the constant gap with a rotating disk has been used to apply the shear stress quantitatively on the cell during incubation at the microscopic observation *in vitro*. The orientation of cells in the colony on the culture plate exposed to the continuous constant wall shear stress field has been traced *in vitro*.

2. METHODS

Couette Type of Shear Flow Device

A Couette type of shear flow device has been used 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).

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

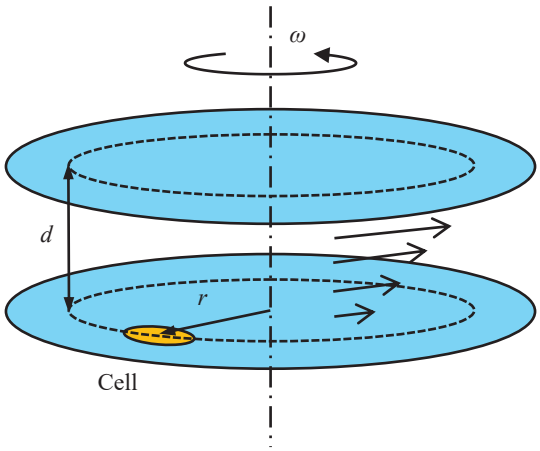

Fig. 1: Couette flow between rotating (angular velocity ω) wall and stationary wall at r (radius): distance (d).

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 0.29 mm. The shear rates (γ) is set at 1.3×10^3 s^{-1} 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.

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

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

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 was approximated to ellipsoid.

The angle (0 degree < θ < 180 degree) between the longitudinal axis of the cell and the flow direction was measured at the microscopic image of each cell (Fig. 2). Four isolated single cells in the sparsely adhered on the bottom surface of the culture dish were selected (Fig. 3). The alignment of each cell was traced for 80 hours under the continuous shear flow stimulation.

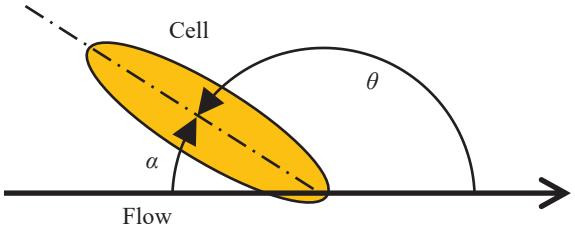

Fig. 2: Angle (θ) between longitudinal axis of cell and flow direction: acute angle (α).

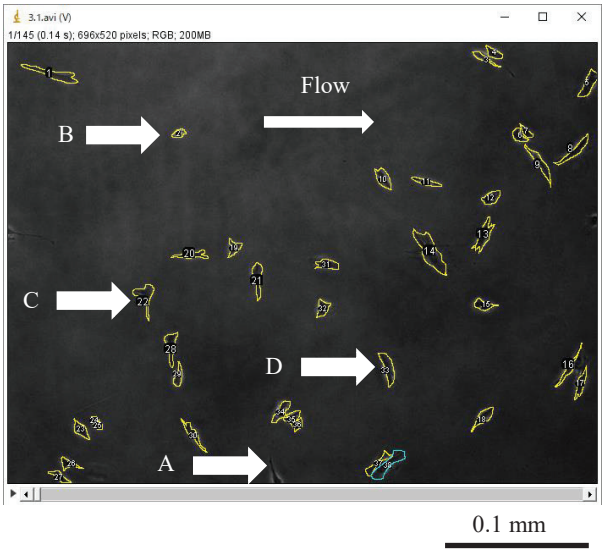

Fig. 3: Traced single cells (A-D).

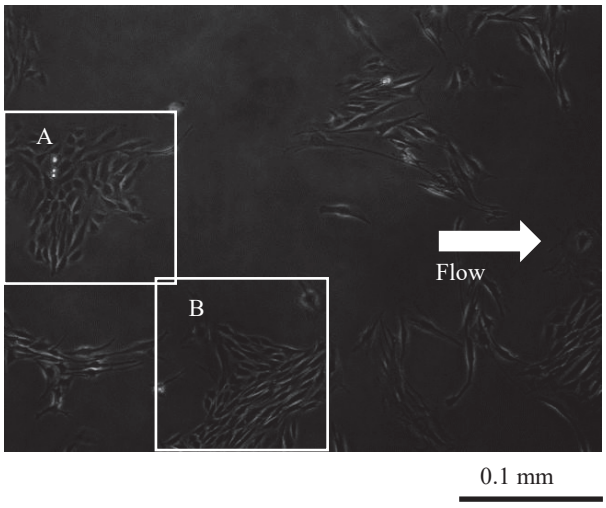

Fig. 4: Analysis of angle (θ) of each cell in colony in areas A and B (0.2 mm × 0.2 mm square each).

Two areas (A and B in Fig. 4) were selected to trace the mean alignment of the cells (the mean value of acute angles (α)) in each colony. The mean value of angles becomes 45 degree, when the alignment of each cell distributes at every direction randomly.

3. RESULTS

The time-lapse tracings show that cells (C2C12) are active on the scaffold surface of the bottom of the culture dish under the continuous constant wall shear stress field of 2 Pa: migration, deformation, proliferation and differentiation.

Fig. 5 shows tracings of the angle of each cell under the wall shear stress of 2 Pa. The alignment of cell perpendicular to the flow direction is stable (θ = 90 degree). The alignment of cell oblique to the flow direction, on the other hand, is changeable (θ around 45 degree, and around 135 degree). The alignment of cell parallel to the flow direction is also stable. The angle (θ) of 0 degree is same alignment as the angle of 180 degree.

Fig. 6 shows the number of cells (N) in area of A (Fig. 4). The number of cells increases in 40 hours, and decreases after 40 hours. The tracings correspond to activities of cells: proliferation, fusion, and exfoliation.

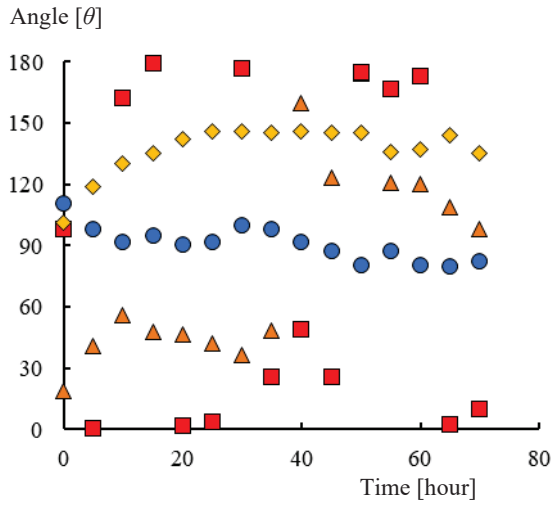

Fig. 5: Tracings of angle of cell (θ): each mark corresponds to each cell.

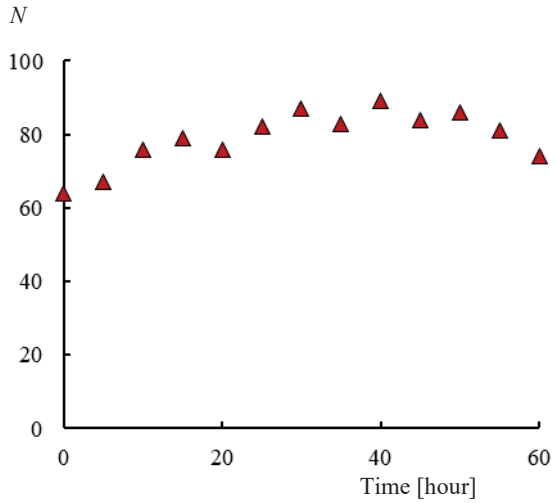

Fig. 6: Number of cells (N) in area A vs. culture time.

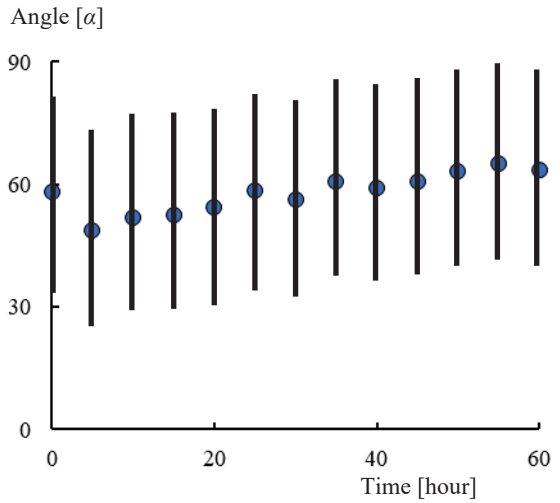

Fig. 7: Mean angle of cells in area A vs. culture time: bar, standard deviation.

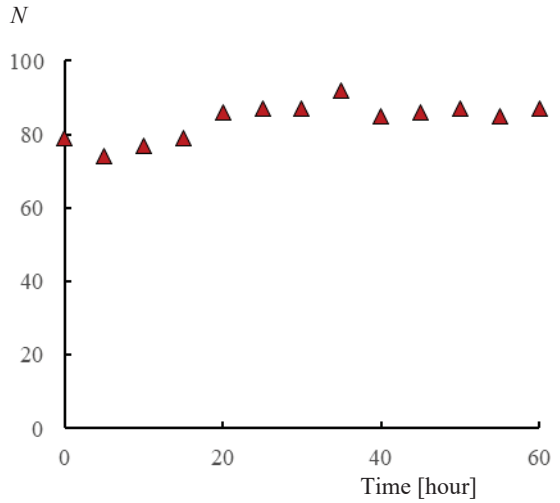

Fig. 8: Number of cells (N) in area B vs. culture time.

Fig. 7 shows the tracing of the mean value of acute angles of cells in area A. The timing of Fig. 7 corresponds to that of Fig. 6. Decrease of the mean value of angles to 45 degrees in five hours correspond to the timing of proliferation of cells. At cell division, the alignment of each divided cell changes its alignment randomly. Although the standard deviation is not small, the mean value of angles tends to increase to 90 degrees: perpendicular to the flow direction.

Fig. 8 shows the number of cells (N) in area of B (Fig. 4). The number of cells increases in 40 hours, and decreases after 40 hours. The tracings correspond to activities of cells: proliferation, fusion, and exfoliation.

Fig. 9 shows the tracing of the mean value of acute angles of cells in area B. The timing of Fig. 9 corresponds to that of Fig. 8. The mean value of angles tends to decrease to 0 degrees: parallel to the flow direction.

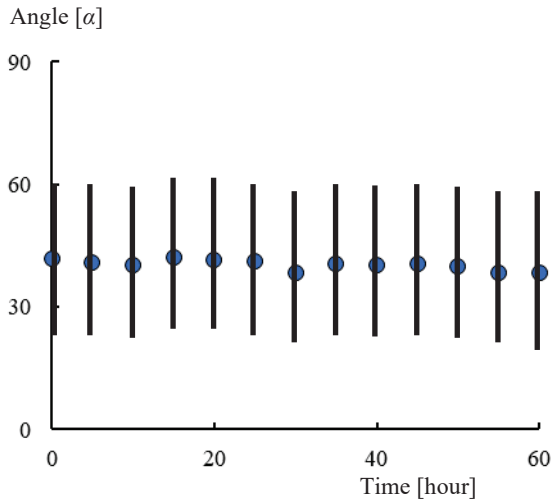

Fig. 9: Mean angle of cells in area B vs. culture time.

4. DISCUSSION

In the tissue technology, orientation of cells was controlled by the design of the scaffold *in vitro*. Alignment of fibers of the scaffold was controlled by the electrospinning technique [25]. 3D-printing technology was applied to make orientation of cells [26]. The Micro-robotic technique was applied to the cell manipulation to control orientation of each cell [27].

Endothelial cells are exposed to the shear flow in the blood vessels *in vivo*. The shear flow affects vessel wall [28, 29] and clot formation [30]. The effects of shear flow on endothelial cells were investigated in the previous studies [1−10]. Cells are exfoliated under the shear flow at the wall shear stress higher than 2 Pa [15−17]. 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 [15], the rhombus channel [16], the cross flow channel [17], and the rotating disk type [18].

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 [18−24]. The cone-and -plate type device has the uniform shear field in the entire space between the rotating cone and the stationary plate [19−22]. 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 [20], and between the rotating concave cone and the stationary convex cone [21]. The erythrocyte destruction was studied between the rotating concave cone and the stationary convex cone [22].

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 [23, 24].

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. (3).

$$Re = \rho\, v\, d / \eta = \rho\, r\, \omega\, d / \eta \tag{3}$$

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 1.5×10^2, when ρ, r, ω, d, and η are 1×10^3 kg m^{-3}, 18 mm, 22 rad s^{-1}, 0.56 mm, 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 interaction between cells governs the behavior of each cell. The orientation of each cell depends on the orientation of the neighbor cell. Myoblasts tend to migrate to the oblique direction under the shear stress field of 1.5 Pa [31]. The effect of shear flow on cells depends on the cell types. The dependency might be applied to the cell sorting technology [32, 33]. 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 on cells cultured under the shear stress field (2 Pa) *in vitro*, myoblasts are active on the scaffold. The myoblast proliferates regardless of the shear flow stimulation. The cell cycle does not vary under the shear flow [34]. Cells make colonies 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.

In the previous studies, several methods were applied to evaluate orientation of cells in the tissue. Orientation of cells was measured by the light scattering [35]. The nucleus was used to evaluate the orientation of each cell [36]. Pixel intensity data at the image of cells was converted to orientation data [37].

In the present study, the time-lapse microscopic image is taken every thirty minutes during the cultivation. The contour of each cell adhered on the stationary plate of the scaffold is traced, and is approximated to ellipsoid. Orientation of cells in the colony is evaluated by distribution of major axes of cells.

5. CONCLUSION

Myoblast orientation under the uniform shear flow has been investigated using the Couette type flow device of parallel disks *in vitro*. After cultivation without flow for 24 hours for adhesion of cells (C2C12) on the lower plate, the constant shear stress 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 each single cell tends to align parallel to the flow direction under shear stress of 2 Pa. The mean direction of cells in some colonies of cells tends to align perpendicular to the flow direction.

ACKNOWLEDGMENT

The authors thank to Mr. Hiroki Eri for his assistance of the experiment.

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