Effect of Shear Stress in Flow on Cultured Cell: Using Rotating Disk at Microscope

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ABSTRACT

An experimental system of the Couette type flow with a rotating disk has been designed to apply wall shear stress quantitatively on the cell culture at the microscopic observation *in vitro*. The shear stress on the wall is calculated with an estimated Couette type of the velocity profile between the rotating disk and the culture plate. The constant rotational speed (lower than 400 rpm) produces the wall shear stress lower than 2 Pa. The rotating disk system is mounted on the stage of an inverted phase contrast microscope to observe the behavior of cells adhered on the plate under the shear flow. Two kinds of cells were used in the test: C2C12 (mouse myoblast cell line), and MC3T3-E1 (mouse osteoblast precursor cell line). The experiments show that C2C12 tends to make orientation diagonal to the stream line, and that MC3T3-E1 tends to make orientation parallel to the stream line. Deformation and exfoliation of cells can be observed under controlled wall shear stress by the experimental system.

Keywords: Biomedical Engineering, Cell Culture, Shear Stress, C2C12, MC3T3-E1 and Couette Flow.

1. INTRODUCTION

The cell culture technique has been developed and several methodologies have been clinically applied to the regenerative medicine. The acceleration technique for differentiation and proliferation of cells has been studied to make tissue *in vivo* or *in vitro* [1-3]. The behavior of biological cells depends on electric and magnetic fields [4, 5]. Control methodology for orientation and proliferation of cells would be applied to the regenerative tissue technology.

The mechanical stress is one of the interested points in the environment of cells, because they receive mechanical force *in vivo*. The mechanical stress on cells might induce various responses: deformation, migration, proliferation, and differentiation. Several methods have been designed to apply the mechanical stress to cells [1, 6-15].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold [6]. When fixation between the cell and the scaffold is not enough, the stress is not transmitted to the cell. A flow, on the other hand, can be used to apply a stress field to a specimen [8-15]. The cells directly receive the shear stress in the shear flow.

The high shear flow might deform a cell, peel off a cell from the scaffold, and inhibit proliferation as well as tissue formation. The mild shear flow, on the other hand, might accelerate migration, proliferation, and secretion of materials, which make the extra cellular 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* [9-11]. 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 [12].

In the present study, an experimental system of the Couette type flow with a rotating disk has been used to apply the wall shear stress quantitatively on the cell culture at the microscopic observation *in vitro.*

2. METHODS

Rotating Parallel Disk System

In the present study, a rotating parallel disk system is selected to make Couette type of flow (Figs. 1-3). The fluid is sheared between a rotating disk and a stationary disk. The stationary disk is the bottom of the culture dish. In the system, the shear rate (γ [s^{-1}]) 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 moving wall and the stationary wall (Fig. 3). In the rotating parallel disk system, the shear rate (γ) increases in proportion to the distance (r [m]) from the rotating axis.

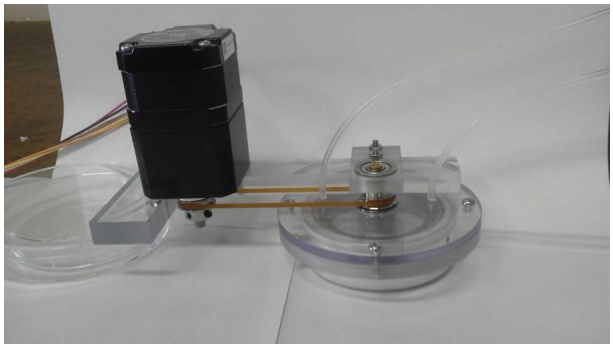

Fig. 1: Disk (right) rotated by motor (left).

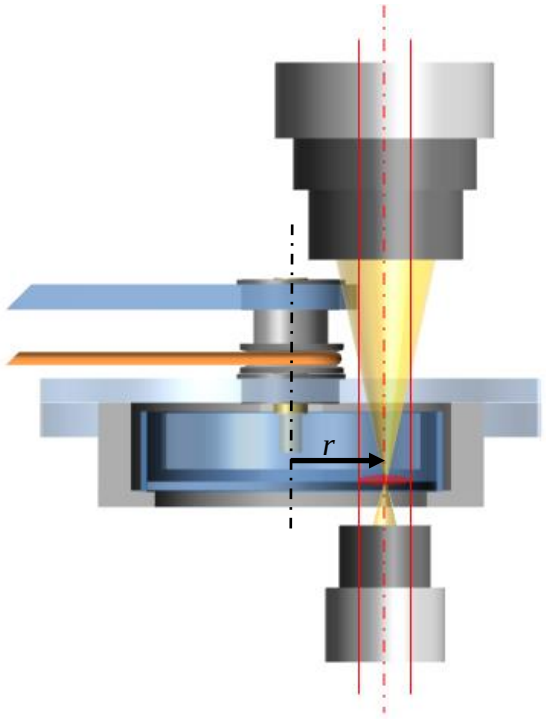

Fig. 2: Observation area by microscope at disk: rotation radius 10 mm < r < 20 mm.

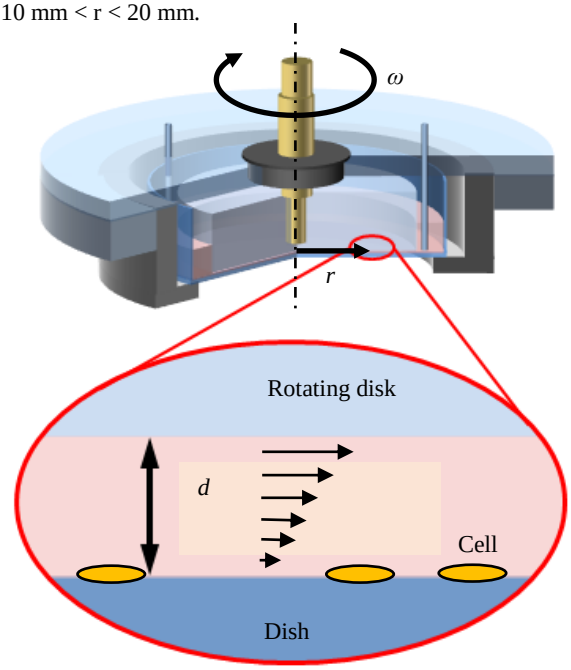

Fig. 3: Couette flow field between rotating disk and stationary disk.

The rotating speed is controlled by the stepping motor between 50 rpm and 400 rpm, which makes variation of angular velocity ω between 5.2 rad s^{-1} and 42 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. The distance d is estimated to 0.8 mm from the positions of the focus of the walls at the microscope. These variations make the shear rates (γ) between 78 s^{-1} and 950 s^{-1} (Eq. 1).

Shear Stress on Cell

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

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

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

When the viscosity of the fluid η is 0.002 Pa s (at 298 K), the shear stress τ varies between 0.15 Pa and 1.9 Pa.

The rotating disk system is mounted on the stage of an inverted phase contrast microscope (IX71, Olympus Co., Ltd., Tokyo) (Fig. 4). The behavior of cells adhered on the stationary wall under the shear stress is observed with the microscope. The system allows observation of cells during exposure to the shear flow.

Cell Culture

Two kinds of cells were used in the test: C2C12 (passage seven and eight, mouse myoblast cell line originated with cross-striated muscle of C3H mouse), and MC3T3 (passage five, an osteoblast precursor cell line derived from Mus musculus (mouse) calvaria) -E1. The cells were seeded on the dish coated with collagen at the density of 1000 cells/cm^2. To make adhesion of cells to the bottom of the dish, the cells were cultured for 24 hours in the incubator. In the incubator, both the temperature and the partial pressure of carbon dioxide are maintained at 310 K and 5 percent, respectively.

After the incubation, the cells were sheared in the rotating disk system for 30 minutes at 298 K out of the incubator. Variation was made in the rotational speed of the disk: 50, 100, 200, and 400 rpm.

After the rotation for 30 minutes, the rotation was stopped for 30 minutes (resting). Then the dish was moved from the rotating disk system into the incubator and incubated for 3 hours.

As for the medium, D-MEM (Dulbecco's Modified Eagle Medium) was used for C2C12, and αMEM was used for MC3T3-E1.

Microscopic Observation

The positions are marked by grooves on the outside surface of the culture dish: at 10 mm, 15 mm, 18 mm, and 20 mm.

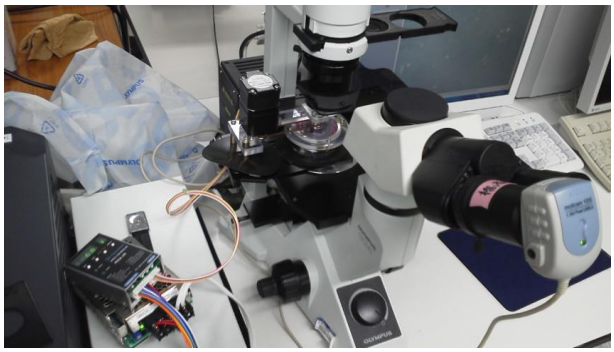

Fig. 4: Observation of cell under Couette flow field between rotating disk and stationary disk.

The static images of cells at area of 1.5 mm × 2 mm around the marked position were taken at the following timings: before the rotation, just after the rotation for 30 minutes, after the stopping for 30 minutes, and after the successive incubation for 3 hours. The time-lapse image was taken every five seconds during the rotation for 30 minutes and during the stopping for 30 minutes.

The microscopic image (Fig. 5a) was binarized (Fig. 5b), and the contour of each cell was approximated to ellipsoid (Fig. 5c). Each cell was selected by the range of area of the ellipsoid (between 0.0005 mm^2 and 0.005 mm^2) at the image (Fig. 5d). The angle between the longitudinal axis of each cell and the circumferential direction of the dish (flow direction) was measured.

Data are arranged in ascending order in Figs. 7 & 8. Data will make flat section at the large frequency. At the random frequency, data will form a simple rising straight line. Every group of data shows the character of "S", which correspond to high frequency of the longitudinal axis of the cell at the flow direction.

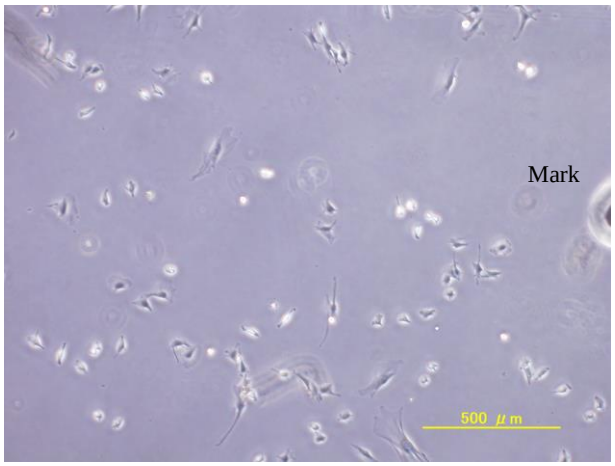

Fig. 5a: Image of C2C12: around mark of r = 18 mm, before flow stimulation. Dimension from left to right is 2 mm.

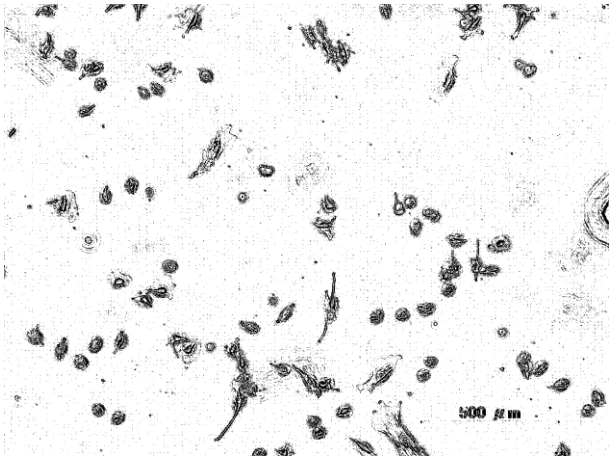

Fig. 5b: Binarized Image of Fig. 5a.

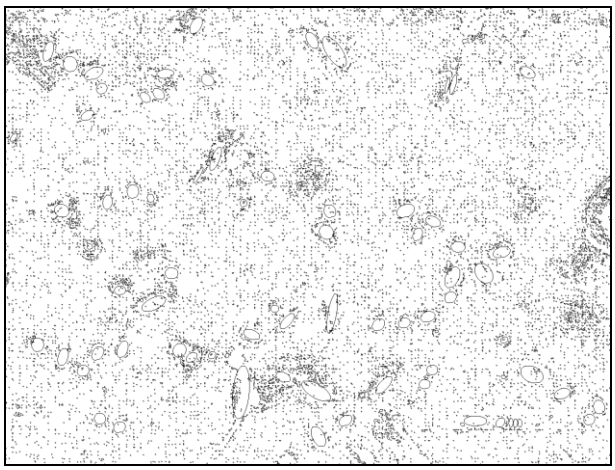

Fig. 5c: Contour of each cell was approximated to ellipsoid at Fig. 5b.

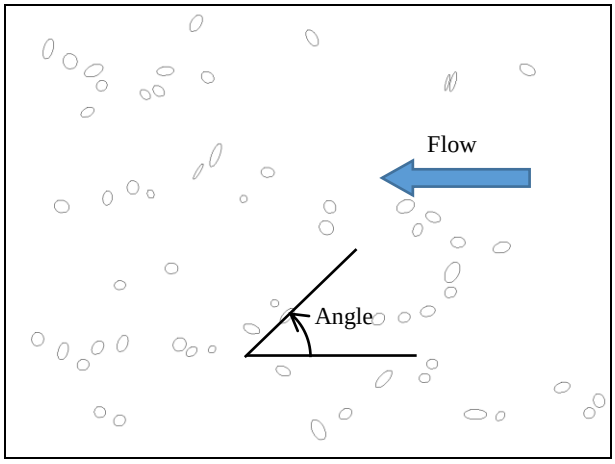

Fig. 5d: Each cell was selected by area of ellipsoid (between 0.0005 mm^2 and 0.005 mm^2) at Fig. 5c.

3. RESULTS

At the following figure, the direction of the flow is from right to left (Fig. 6).

Some of cells are extended along the stream line and exfoliated at 400 rpm. Some of cells are rounded and exfoliated at 200 rpm.

During exposure to the shear flow, C2C12 exfoliates within 30 minutes at the wall shear stress higher than 0.2 Pa. During exposure to the shear flow, MC3T3-E1 extends pseudopodium, contracts and changes own direction.

The slope change in Fig. 7 shows that the number of C2C12 cells decreases at 90 degrees. The number of C2C12, on the other hand, increases between 40 and 70 degrees and between 110 and 150 degrees. The results shows that C2C12 tilts to diagonal direction of the stream line in 30 minutes after exposure to the shear stress of 0.3 Pa < τ < 0.4 Pa for 30 minutes (Figs. 7b & 7c).

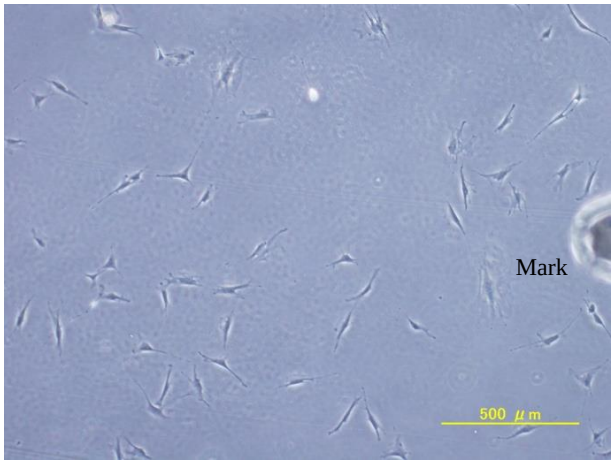

Fig. 6a: MC3T3-E1 before flow simulation around mark of r = 18 mm. Dimension from left to right is 2 mm.

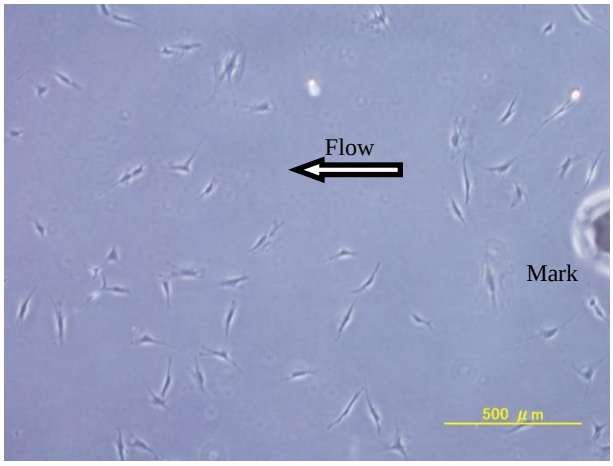

Fig. 6b: MC3T3-E1 just after shear flow simulation (1.9 Pa) for 30 minutes. Dimension from left to right is 2 mm.

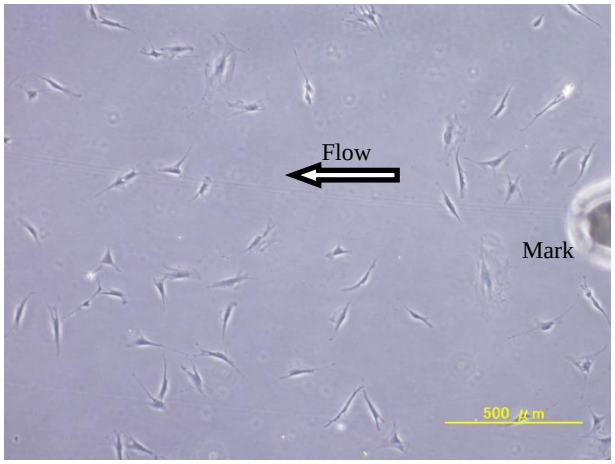

Fig. 6c: MC3T3-E1 at 30 minutes after shear flow simulation (1.9 Pa). Dimension from left to right is 2 mm.

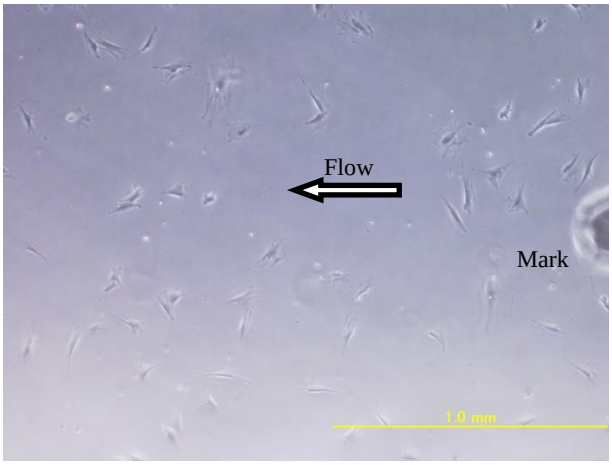

Fig. 6d: MC3T3-E1 at incubation of 3 hours after shear flow simulation (1.9 Pa). Dimension from left to right is 2 mm.

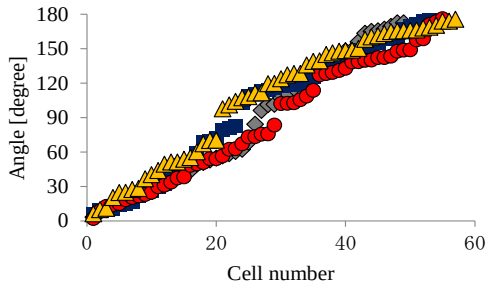

Fig. 7a: Angles of C2C12 at 50 rpm, r = 12 mm, 0.16 Pa. 0 degree is direction of flow: rhombus, before flow stimulation; square, after flow stimulation for 30 minutes; circle, after resting for 30 minutes; triangle, after incubation for 3 hours.

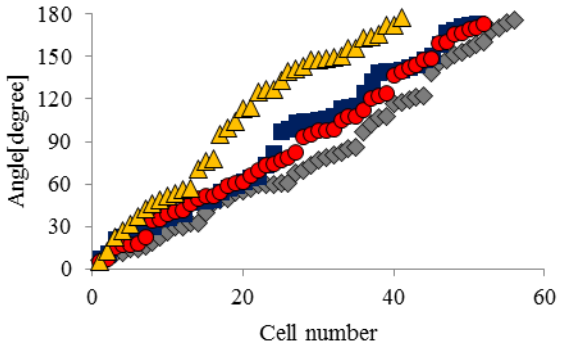

Fig. 7b: Angles of C2C12 at 100 rpm, r = 12 mm, 0.32 Pa. 0 degree is direction of flow: rhombus, before flow stimulation; square, after flow stimulation for 30 minutes; circle, after resting for 30 minutes; triangle, after incubation for 3 hours.

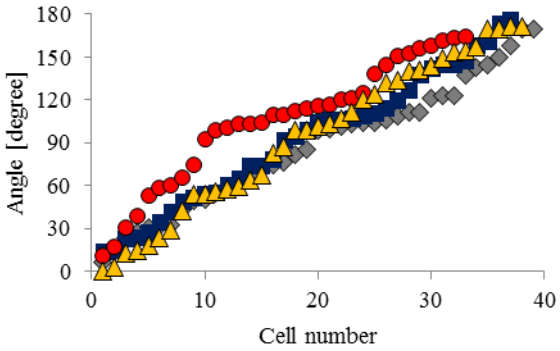

Fig. 7c: Angles of C2C12 at 100 rpm, r = 16 mm, 0.42 Pa. 0 degree is direction of flow: rhombus, before flow stimulation; square, after flow stimulation for 30 minutes; circle, after resting for 30 minutes; triangle, after incubation for 3 hours.

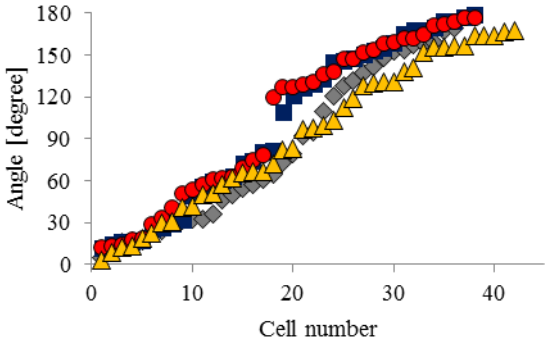

Fig. 8a: Angles of MC3T3-E1 at 100 rpm, r = 16 mm, 0.42 Pa, 0 degree is direction of flow: rhombus, before flow stimulation; square, after flow stimulation for 30 minutes; circle, after resting for 30 minutes; triangle, after incubation for 3 hours.

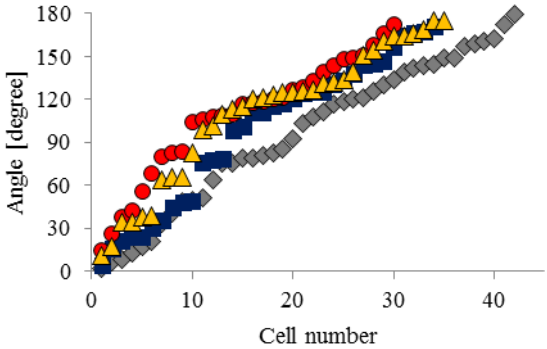

Fig. 8b: Angles of MC3T3-E1 at 400 rpm, r = 12 mm, 1.3 Pa, 0 degree is direction of flow: rhombus, before flow stimulation; square, after flow stimulation for 30 minutes; circle, after resting for 30 minutes; triangle, after incubation for 3 hours.

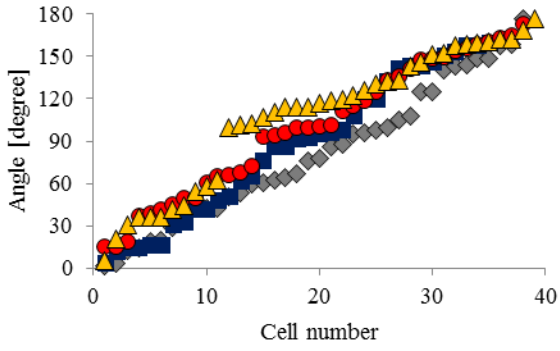

Fig. 8c: Angles of MC3T3-E1 at 400 rpm, r = 16 mm, 1.7 Pa, 0 degree is direction of flow: rhombus, before flow stimulation; square, after flow stimulation for 30 minutes; circle, after resting for 30 minutes; triangle, after incubation for 3 hours.

The slope change in Fig. 8 shows that the number of MC3T3-E1 cells decreases at 90 degrees. The results shows that cells tilt to the stream line in 30 minutes after exposure to the shear stress between 0.4 Pa and 1.7 Pa (Figs. 8a-8c).

4. DISCUSSION

At the constant angular velocity of 10 rad s^{-1} (d = 0.8 mm), the shear rate (γ) increases from 150 s^{-1} to 230 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 enables the simultaneous observation of the behavior of cells related to variation of the shear stress in the same view [15].

The donut-shaped open channel is convenient to study the effect of flow direction on the cell culture [9-11], but it is not easy to estimate quantitatively the shear stress in the fluid because of the free surface.

Many kinds of the devices of Couette type flow were designed for quantitative experiments of biological fluid in the previous studies. The clot formation was quantitatively studied between a rotating cone and a stationary plate [16], and between a rotating concave cone and a stationary convex cone [17]. The erythrocyte destruction was studied between a rotating concave cone and a stationary convex cone [18]. The erythrocyte deformation was observed between counter rotating parallel discs [19, 20].

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 decrease of the rotational speed. The rotational speed of the disk is smaller than 0.8 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, and v is the circumferential velocity. Re is 300, when ρ, r, ω, d, and η are 1000 kg m^{-3}, 0.018 m, 42 rad s^{-1}, 0.0008 m, and 0.002 Pa s, respectively. The turbulent flow may not occur in the flow of small value of Reynolds number.

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 [21-26].

In the present study, C2C12 tends to tilt along the stream line under the wall shear stress lower than 0.21 Pa, shortly after seeding. The tendency decreases under the wall shear stress higher than 0.23 Pa. MC3T3-E1 tends to deform and make orientation at a certain time after exposure to the shear flow. MC3T3-E1 tends to tilt along the stream line of flow stimulation.

In the present study, the tilting of cell is mainly observed at 30 minutes after flow stimulation on MC3T3-E1, although the tilting of cell is mainly observed during flow stimulation on C2C12. C2C12 makes orientation diagonal to the stream line under the shear stress lower than 0.3 Pa. C2C12 recovers from orientation in 3 hours after exposure to the shear stress higher than 0.3 Pa. The previous study shows that the tendency of orientation of cells decreases under the shear stress higher than 0.36 Pa [2].

Cells show both the passive behavior and active behavior. Cells are passively tilted along the stream line. Cells actively tilt perpendicular to the stream line, to minimize the internal stress. The hysteresis effect also governs the behavior of the cell. Relatively low density is selected in the present experiment to trace the image of each cell.

5. CONCLUSION

An experimental system with a rotating disk has been designed to apply wall shear stresses on the cell culture in Couette type of flow at the microscopic observation *in vitro*. The experiments show the following results. C2C12 makes orientation diagonal to the stream line under the shear stress. MC3T3-E1 makes orientation along to the stream line after the stimulation of the wall shear stress. The designed system is useful to observe cells under the quantitatively controlled wall shear stress.

6. ACKNOWLEDGMENT

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