Endothelial Cell Behavior After Stimulation of Shear Flow

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ABSTRACT

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 at the microscopic observation *in vitro*, and the behavior of the cell after stimulation of shear flow has been evaluated. The shear stress on the wall is calculated with an estimated Couette type of the velocity profile between the rotating disk and the stationary culture plate. Variation was made on the wall shear stress of 1.0 Pa and 1.4 Pa adjusted by the rotational speed of the disk. The rotating disk system is mounted on the stage of an inverted phase contrast microscope to observe the behavior of HUVEC (human umbilical vein endothelial cells) adhered on the plate after stimulation of the shear flow. Migration, deformation, and orientation of each cell have been traced at the time-laps image for 24 hours after the stimulation for 24 hours. The experiments show the following results. HUVEC tends to make orientation parallel to the stream line, and that the tendency is maintained for several hours. The migration of each cell is accelerated after stimulation of the wall shear stress of 1.4 Pa. The increase of area of each cell is decelerated by the higher wall shear stress. The Couette type of rotating disk system is effective to detect the behavior of cells after stimulation of the shear flow.

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

1. INTRODUCTION

A biological cell shows passive and active behaviors in an environment. While the shear stress deforms the cell, the cell changes own form to adapt to the shear field. While the strong stimulation excess the threshold damages the cell, the stimulation below the threshold remain in the cell as a memory for the response in the next step.

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* [1-4]. The behavior of biological cells also depends on electric and magnetic fields [5, 6]. 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* [7-19]. 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 [2, 20-30].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold. 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. 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* [23, 24]. When the flow has a free surface, it is difficult to estimate the shear stress in the fluid quantitatively. Between two parallel walls, on the other hand, the velocity profile is easily estimated in the laminar flow [21-23].

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 endothelial cell at the microscopic observation *in vitro*, and the behavior of the cell after stimulation of shear flow has been evaluated.

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) [20]. The fluid is sheared between a rotating disk and a stationary disk. The rotating disk is made of polycarbonate: 44 mm diameter and 10 mm thickness. The stationary disk is the bottom of the culture dish. In the system, the shear rate (γ) is calculated by Eq. 1.

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

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 [s^{-1}]) increases in proportion to the distance (r [m]) from the rotating axis.

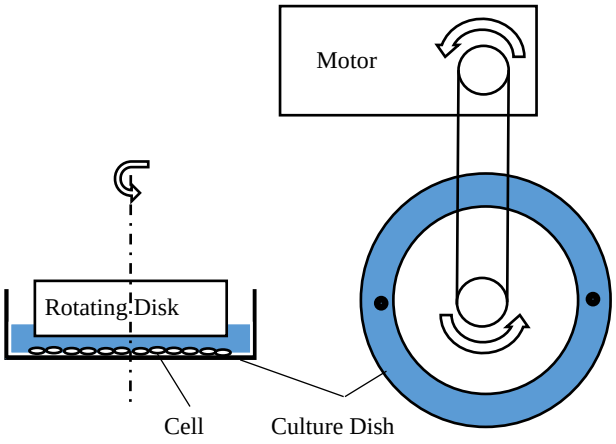

Fig. 1: Cell culture in Couette flow between rotating disk and stationary dish: front view (left), plan view with motor (right).

The rotational movement is transmitted by the belt, which makes connection between the rotating disk and the rotating axis of the stepping motor. The rotating speed (< 400 rpm) is controlled by the motor, which makes variation of angular velocity ω (< 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. The shear rate ($\gamma < 10^3$ s^{-1}) is calculated by these parameters.

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 cell culture in the present study.

The viscosity (η = 0.0014 Pa s) of the medium (Dulbecco's Modified Eagle Medium) is measured by the cone and plate viscometer at 310 K. The shear stress (τ < 1.4 Pa) is calculated by Eq. 1 and Eq. 2.

The rotating disk system is mounted on the stage of an inverted phase contrast microscope (LCV110-SK, Olympus Co., Ltd., Tokyo). The system allows observation of cells during exposure to the shear flow.

Cell Culture

HUVEC (human umbilical vein endothelial cells) was used in the test. EGM+BulletKit was used for the medium. 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 24 hours in the incubator microscope (LCV110-SK). After stopping the rotation of the disk, the cells were successively cultured in the incubator microscope (LCV110-SK) for 24 hours. The behavior of each cell was analyzed at the microscopic time-laps (every five minutes) images.

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.

On the microscopic image, the outline of each cell was traced, and the contour of each cell was approximated to ellipsoid "by Image J" (Fig. 2) The irregular shape of cell is not included in the analysis of approximation to elipsoid. As to the ellipsoid, the area, the coordinates of the centroid, the length of the major axis (a), and the minor axis (b) were measured. The ratio of axes is calculated as the shape index (*S.I.*) by Eq. 3.

$$S.I. = b / a \qquad (3)$$

At the circle, *S.I.* = 1. As the ellipsoid becomes flat, *S.I.* approaches to zero.

The angles (θ) between the direction of the flow and the direction of the major axis of each cell were also measured at the microscopic image. The direction of the flow was confirmed by the flowing particle at the microscopic image. To trace the migration of each cell by the coordinates, the x axis is corrected to the direction of the flow.

3. RESULTS

The cells were able to be continuously exposed to the Couette type shear flow by the rotating disk for twenty-four hours in the incubator. Fig. 3 shows the microscopic images of cells after the shear flow stimulation for twenty-four hours at each constant shear stress: 0 Pa, 1.0 Pa, and 1.4 Pa. The flow direction is from left to right in Fig. 3. Each red circle is marked to trace the same cell in Fig. 3. The control condition of 0 Pa shows cells cultured in static medium without shear flow. Several cells migrate with long distance at 1.4 Pa. Several cells tilt to the direction of flow at the end of shear stress stimulation of 1.0 Pa and 1.4 Pa. The tendency of tilt decreases with time.

In the following figure, proliferating cells do not included. Ten cells were selected to be traced. The data show the behavior of each cell every hour.

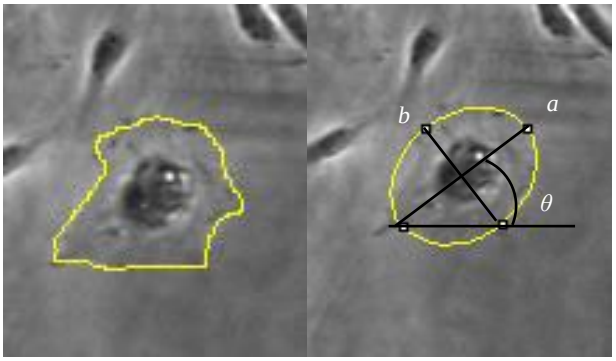

Fig. 2: Tracing of contour of each cell (left), contour of each cell approximated to ellipsoid (right).

Fig. 4 shows the trace of position of each cell on the X-Y plane of the scaffold. The flow direction is parallel to the X axis from minus to plus. Migrations of ten cells at every hour are exemplified in Fig. 4. Many cells migrate with the long distance after the shear stress stimulation of 1.4 Pa. The migration distances are short after the shear stress stimulation of 0 Pa and 1.0 Pa.

Fig. 5 shows the migration distance of the center of each cell per every hour after stopping of the shear flow stimulation for 24 hours. In each group, the line shows the mean migration distance of ten traced cells. Each same color of the filled circles shows the traced same cell. In every group, the migration speed around 0.02 mm/hour at each cell is maintained for twenty-four hours. Several cells migrates 0.05 mm/hour after the shear stress stimulation of 1.0 Pa and of 1.4 Pa. The most active part is in two hours after the shear stress stimulation of 1.0 Pa.

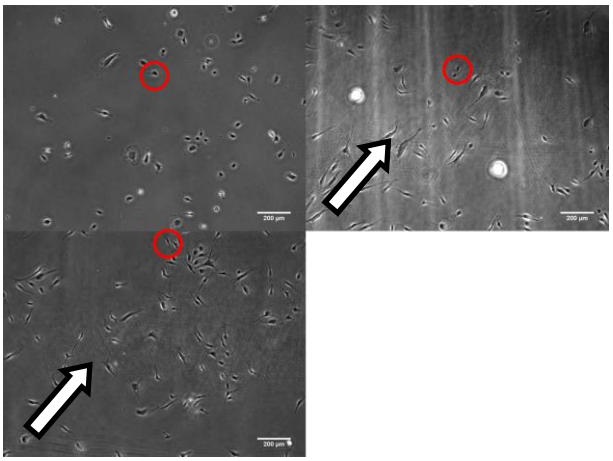

Fig. 3a: Cells at the end of shear flow stimulation for 24 hour: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left): arrow shows flow direction: bar shows 0.2 mm.

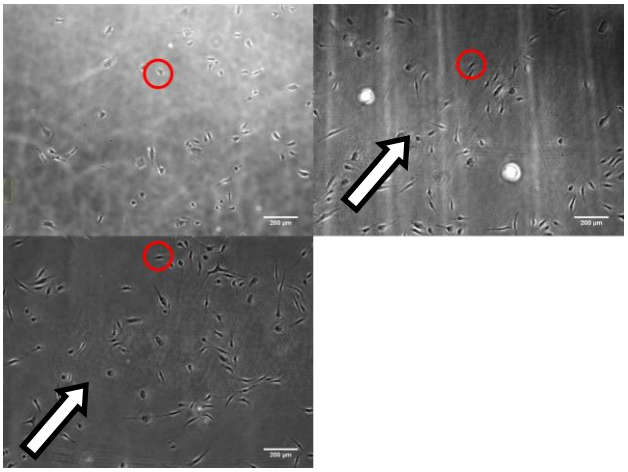

Fig. 3b: Cells at 6 hour after shear flow stimulation for 24 hour: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left): arrow shows flow direction: bar shows 0.2 mm.

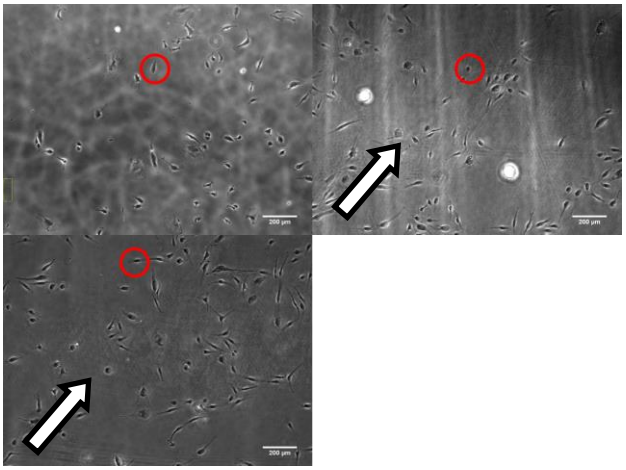

Fig. 3c: Cells at 12 hour after shear flow stimulation for 24 hour: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left): arrow shows flow direction: bar shows 0.2 mm.

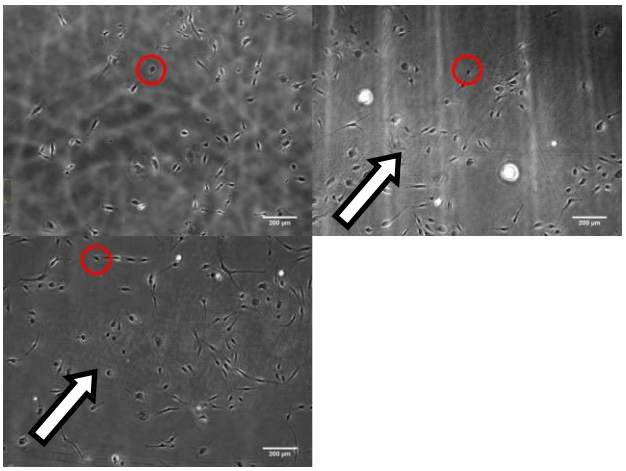

Fig. 3d: Cells at 18 hour after shear flow stimulation for 24 hour: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left): arrow shows flow direction: bar shows 0.2 mm.

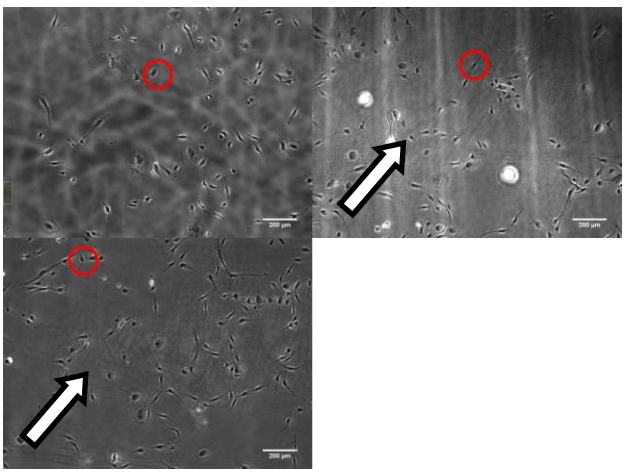

Fig. 3e: Cells at 24 hour after shear flow stimulation for 24 hour: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left): arrow shows flow direction: bar shows 0.2 mm.

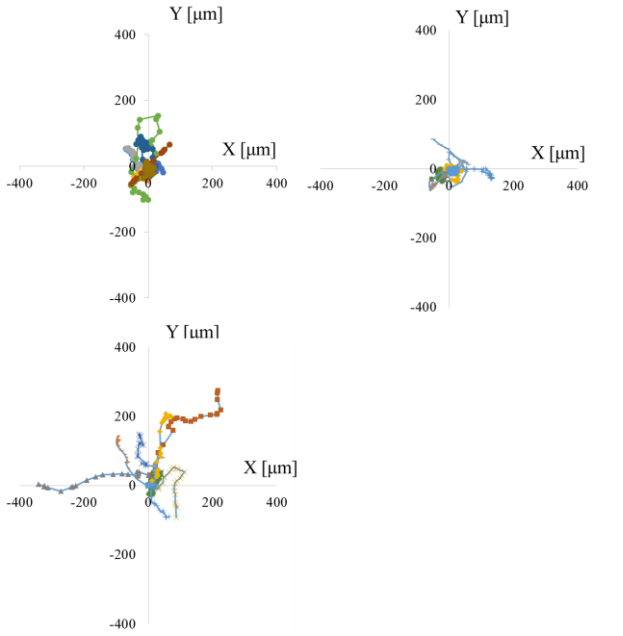

Fig. 4: Migration of center of each cell for 24 hour after stopping of shear flow stimulation for 24 hour: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left): n = 10.

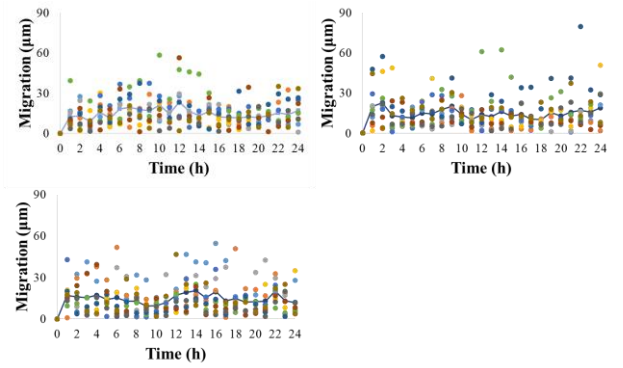

Fig. 5: Migration distance of center of each cell per every hour after stopping of shear flow stimulation for 24 hours: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left).

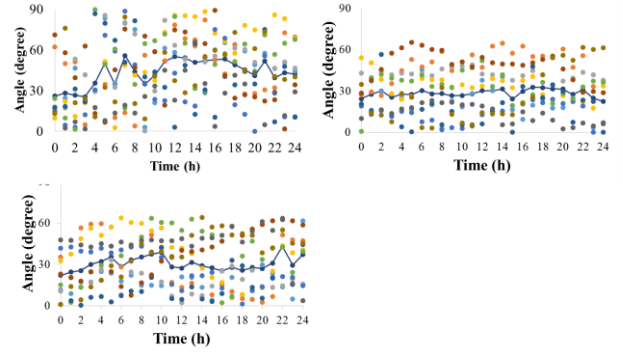

Fig. 6: Angle [degree] of each cell every hour after stopping of shear flow stimulation for 24 hours: 0 Pa (upper left, control), 1.0 Pa (lower left), 1.4 Pa (upper right).

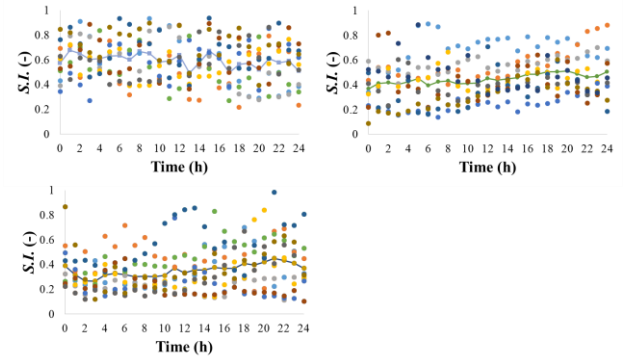

Fig. 7: Shape index (*S.I.*) of each cell every hour after stopping of shear flow stimulation for 24 hours: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left).

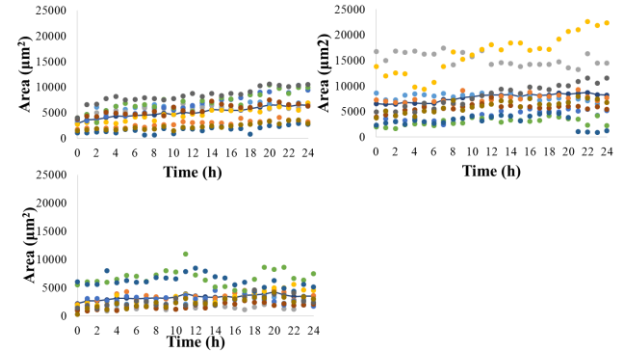

Fig. 8: Area of each cell every hour after stopping of shear flow stimulation for 24 hours: 0 Pa (upper left, control), 1.0 Pa (upper right), 1.4 Pa (lower left).

Fig. 6 shows the angle between the longitudinal axis of the cell and the direction of the X axis, which is parallel to the flow. The zero degree shows the direction of the flow. The 90 degree shows the direction perpendicular to the flow. In each group, the line shows the mean angle of ten traced cells. Each same color of the filled circles shows the traced same cell. The angle distributes between zero and 90 degree in the control group without flow stimulation. The mean value is around 45 degree. The angle distributes, on the other hand, between zero and 60 degree in the group after flow stimulation of 1.0 Pa and of 1.4 Pa. The mean value is around 30 degree, which shows the cell keeps tilt to the flow direction for twenty-four hours after flow stimulation.

Fig. 7 shows the shape index (*S.I.*) of the cell. *S.I.* is unity at the circle at the image. When the ellipse is extended to the longitudinal direction, *S.I.* approaches to zero. In each group, the line shows the mean angle of ten traced cells. Each same color of the filled circles shows the traced same cell. *S.I.* at the end of flow stimulation is smaller than *S.I.* without flow stimulation. *SI* after the flow stimulation of 1.0 Pa gradually approaches to the value without flow stimulation with time.

Fig. 8 shows the projected area of the cell. In each group, the line shows the mean angle of ten traced cells. Each same color of the filled circles shows the traced same cell. Some cells have the large area after flow stimulation of 1.0 Pa. The area of each cell gradually increases without the flow stimulation.

The area of each cell tends to increase after the flow stimulation of 1.0 Pa. The area of each cell, on the other hand, keeps small value after the flow stimulation of 1.4 Pa.

4. DISCUSSION

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 [31], and between a rotating concave cone and a stationary convex cone [32]. The erythrocyte destruction was studied between a rotating concave cone and a stationary convex cone [26]. The erythrocyte deformation was observed between counter rotating parallel discs [27, 28].

The Couette flow system of the cone and plate type realizes the uniform shear field independent of the distance from the rotational axis [31]. To keep easily the preciseness of the dimension, parallel disk system has an advantage [27, 28]. The precisely even surface can be fabricated easier at the plain surface than at the cone surface. To keep the stable movement, the parallel disk system has an advantage than the cone and plate system. The parallel position between disks can be self-regulated by balance in the uniform distance between disks. The machining of the surface is easier to make the plane than the cone to minimize the roughness of the surface. These conditions make the optical observation easier.

The microscopic image at the Couette type flow system with the rotating disk includes the wall shear stress distribution within the observation frame according to the distance from the rotating axis. The wall shear stress increases in proportion to the distance from the rotating axis. This situation has advantage to observe the migration direction of each cell according to the distribution of the wall shear stress [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 in the present experiment.

Reynolds number is calculated by Eq. 4.

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

In Eq. 4, ρ is density of the fluid, and v is the circumferential velocity. Re is 400, when ρ, r, ω, d, and η are 10^3 kg m s^{-3}, 0.018 m, 42 rad s s^{-1}, 0.0008 m, and 0.0014 Pa s, respectively. The turbulent flow may not occur in the flow of small value of Reynolds number. The actual flow direction is confirmed by the movement of medium adjacent to the culture plate on the video image 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 [3, 14-19, 24, 29, 30].

Too high shear stress might damage cells. Extension of the projected area of each adhered cell might decrease after the sub-lethal damage [28] by the higher shear stress. The optimum shear stress might accelerate adaptation of the cell to the environmental stimulation. The wall shear stress around 1 Pa in the flow might be effective to accelerate orientation of HUVEC.

In the present study, every parameter has been measured only on sparsely populated cells to prevent interaction between cells. The active behavior is measured every five minutes by the present experimental system. The proliferating cells are not included in the data in Figs. 4-8. In the confluent state, on the other hand, cells reveal different responses. The angle between the flow direction and the longitudinal axis of the cell scatters within 60 degree in the present experiment. The density of adhered cells might be too low to make the regular orientation of cells.

5. CONCLUSION

An experimental system of the Couette type of flow with a rotating disk has been used to apply the wall shear stress (1.0 Pa, 1.4 Pa) quantitatively on the HUVEC (human umbilical vein endothelial cell) at the microscopic observation *in vitro*, and the behavior of the cell after stimulation of shear flow has been evaluated. Migration, deformation, and orientation of each cell have been traced at the time-laps image for 24 hours after the stimulation for 24 hours. The experiments show that cells tend to make orientation parallel to the stream line, and that the tendency is maintained for several hours. The migration of each cell is enhanced after stimulation of the wall shear stress of 1.4 Pa. The increase of area of each cell is decelerated by the higher wall shear stress. The Couette type of rotating disk system is effective to detect the behavior of cells after stimulation of the shear flow.

6. ACKNOWLEDGMENT

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