Hysteresis Effect of Shear Flow Field on Migration Velocity of Cell: After and Before Division of L929 and 3T3-L1

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ABSTRACT

The effect of shear flow field on the migration of each cell has been investigated under Couette type shear flow field *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 (τ) on the lower culture disk was controlled by the rotating speed of the upper disk. Two types of cells were used in the test: L929 (mouse fibroblast connective tissue), and 3T3-L1 (mouse fat precursor cells). After cultivation without flow for 24 hours for adhesion of cells on the lower plate, $\tau = 1$ Pa was continuously applied on cells for 7 days in the incubator. The behavior of each cell (before and after division) was traced at the time lapse image observed by an inverted phase contrast microscope placed in an incubator. The experimental results show that cells make division under shear stress of 1 Pa. Each cell makes wider variation of adhesion area (before and after division) in L929 than in 3T3-L1. Migration to perpendicular direction is more frequently at L929 than at 3T3-L1. Some cells of 3T3-L1 migrate downstream after division.

Keywords: Biomedical Engineering, Shear Stress, Division, Migration, L929, 3T3-L1 and Couette Flow.

1. INTRODUCTION

A biological cell migrates on the scaffold. In several cases, cells are exposed to the shear stress both *in vivo* and *in vitro*. The direction of the shear stress field might affect the direction of the migration [1]. The hysteresis also might affect the direction of the migration [2]. The behavior of each cell might depend on the initial state. In the most of tests *in vitro*, cells are incubated for several hours for adhesion to the scaffold before flow stimulation. Each cell can make division under the wall shear stress field [3]. Is the effect maintained in each cell after division? The adhesion status of the cell is controlled by itself after division. In the present study, the migration of each cell is traced at the division in the shear stress field of the medium.

The effect 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 [4–8]. In the previous study with the vortex flow by the swinging plate *in vitro*, C2C12 made orientation perpendicular to the direction of the flow, although HUVEC made orientation along the streamline of the flow [9]. The orientation of each cell in the tissue depends on that of neighbor cell [3].

At the wall shear stress, a cell might show the following responses: elongation [6], tilting to the streamline [9], migration [7, 10], deformation to be rounded [10], proliferation [8], and exfoliation from the wall of the scaffold [11].

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 [12, 13].

In the present study, an experimental system of the Couette type flow in the constant gap with a rotating disk has been designed to apply the shear stress quantitatively on the cell during incubation at the microscopic observation *in vitro*, and the effect of the shear stress field (1 Pa) on each cell at division has been studied about migration.

2. METHODS

Couette Type of Shear Flow Device
A Couette type of shear flow device has been used: 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 device, the shear rate (γ) in the medium 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 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 around 0.6 mm. The shear rates (γ) of 6.7×10^{2} s^{-1} are made 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.

$$\tau = \eta\,\gamma \tag{2}$$

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 have been calculated as the value of 1 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

Two types of cells were used in the test: L929 (fibroblast connective tissue of C3H mouse), and 3T3-L1 (mouse fat precursor cells, a cell line derived from cells of mouse 3T3). Cells were cultured in D-MEM (Dulbecco's Modified Eagle's Medium): containing 10% decomplemented FBS (fetal bovine serum), 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 pre-incubation for 24 hours without shear, the cells were continuously sheared with the rotating disk for 24 hours in the incubator at the constant rotating speed. The constant speed was preset for each test to keep the designed shear stress field.

Measurement of Cell

The time-lapse microscopic image was taken every ten minutes during the cultivation. The contour of each cell adhered on the stationary plate of the scaffold was traced, and the projected two-dimensional area (S) at the image of each cell was calculated (Fig. 2). The contour of each cell was approximated to ellipsoid, and the centroid of each cell was used to track the migration of the cell (Fig. 3). The flow direction was defined as x axis. The direction to the rotating axis was defined as y axis.

3. RESULTS

Fig. 4 shows tracings of area (S) before (Fig. 4a) and after (Fig. 4b) division of L929 under shear stress field of 1 Pa. The time zero correspond to the timing of division of the cell. The decrease of the area (S) before division correspond to the rounding of the cell before division. The same kind of line shows the tracings of the same cell in both Fig. 4a and Fig. 4b. The same lines of different color after division show the same origin of cell. After division, the area of each cell tends to return to that of before division.

Fig. 5 shows tracings of area (S) before (Fig. 5a) and after (Fig. 5b) division of 3T3-L1 under shear stress field of 1 Pa. The decrease of the area of 3T3-L1 before division is slower than that of L929. The increase of the area of 3T3-L1 before division is also slower than that of L929.

Fig. 6 shows the velocity of the migration before (Fig. 6a) and after (Fig. 6b) division of L929 under shear stress field of 1 Pa. Several cells migrate upstream. Some cells migrate to the direction of the rotating axis, where the wall shear stress is lower (Fig. 3). The migration velocity is lower after division (data concentrate to origin) than before division. The distribution of migration direction is random (symmetrical distribution of data) after division.

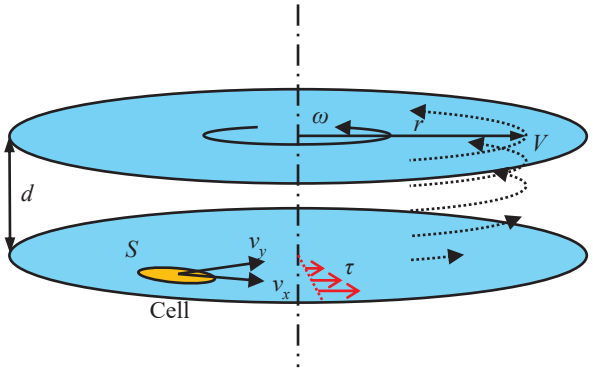

Fig. 1: Couette flow (velocity (V) distribution) between rotating (angular velocity ω) wall and stationary wall at r (radius) (distance d): migration velocity (v_x, v_y) of cell on stationary wall: wall shear stress (τ).

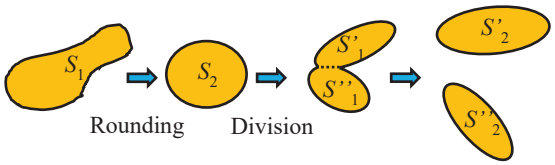

Fig. 2: Area (S) change of cell before (from S_1 to S_2) and after division (from S'_1 to S'_2, and from S''_1 to S''_2).

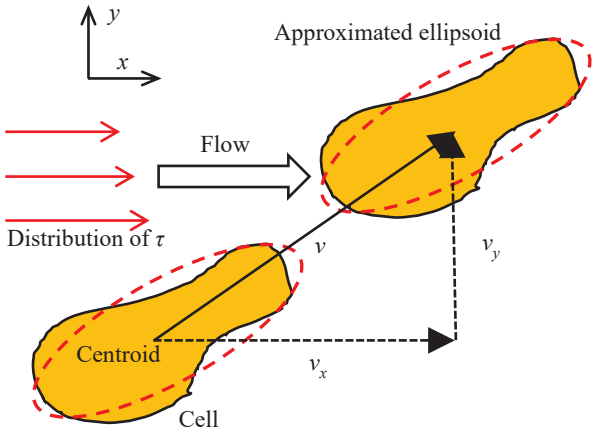

Fig. 3: Centroid of approximated ellipsoid of cell is traced: migration velocity (v_x, v_y) of cell: wall shear stress (τ) distribution; x, parallel to flow; y, perpendicular to flow.

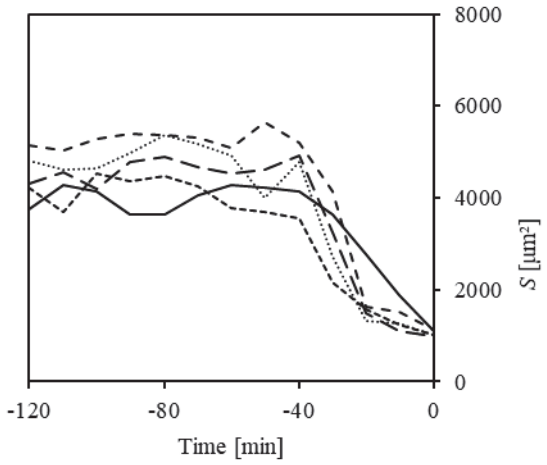

Fig. 4a: Tracings of area (*S*) before division of L929 under shear stress field of 1 Pa.

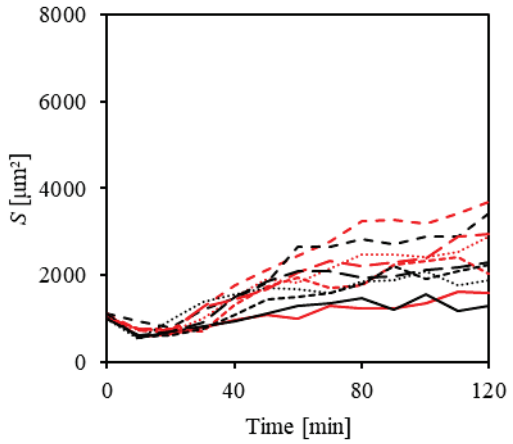

Fig. 4b: Tracings of area (*S*) after division of L929 under shear stress field of 1 Pa.

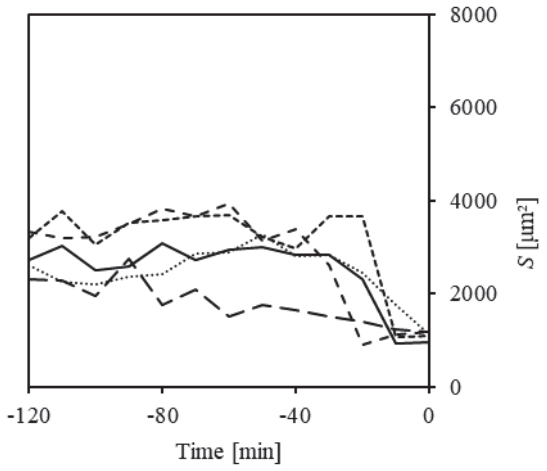

Fig. 5a: Tracings of area (*S*) before division of 3T3-L1 under shear stress field of 1 Pa.

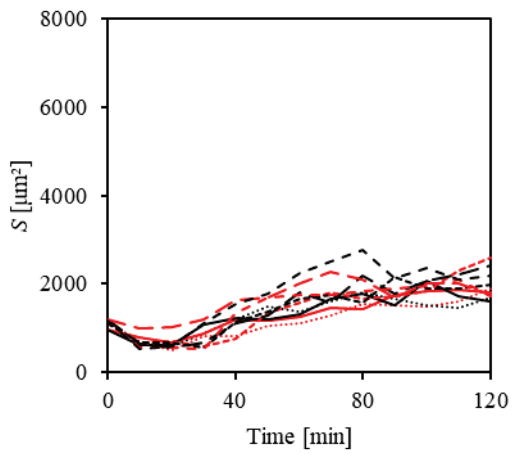

Fig. 5b: Tracings of area (*S*) after division of 3T3-L1 under shear stress field of 1 Pa.

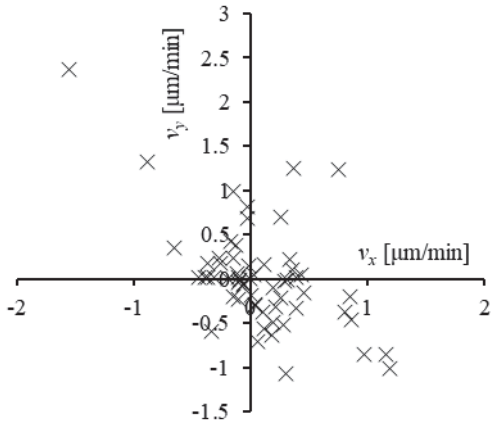

Fig. 6a: Velocity of the migration before division of L929 under shear stress field of 1 Pa.

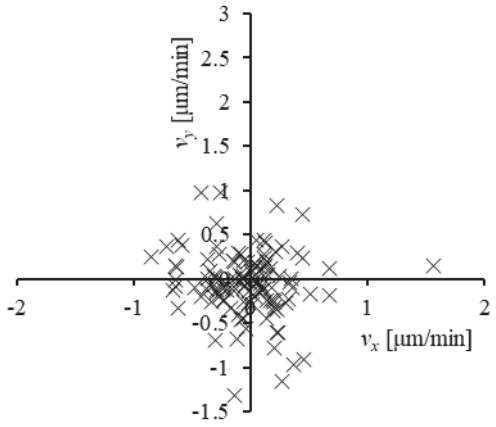

Fig. 6b: Velocity of the migration after division of L929 under shear stress field of 1 Pa.

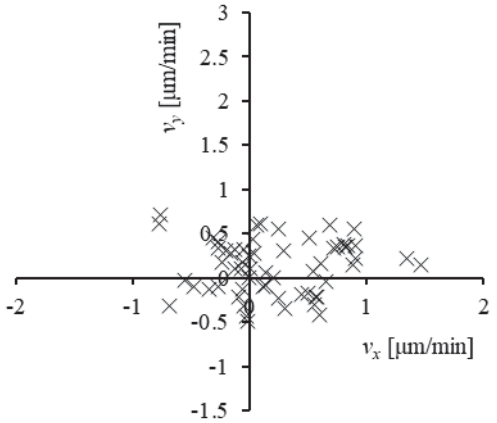

Fig. 7a: Velocity of the migration before division of 3T3-L1 under shear stress field of 1 Pa.

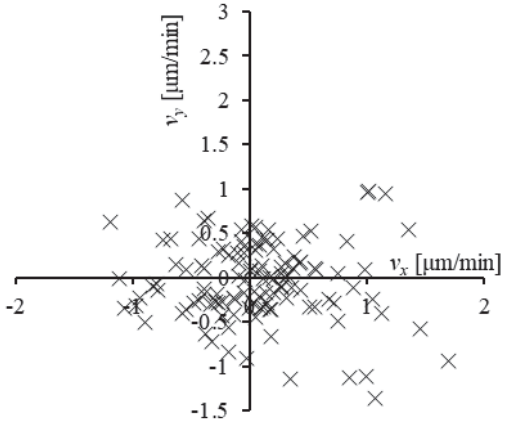

Fig. 7b: Velocity of the migration after division of 3T3-L1 under shear stress field of 1 Pa.

Fig. 7 shows the velocity of the migration (Fig. 3) before (Fig. 7a) and after (Fig. 7b) division of 3T3-L1 under shear stress field of 1 Pa. 3T3-L1 migrates to the parallel direction to the flow frequently than to the perpendicular direction to the flow. Cells tend to migrate downstream. Several cells migrate downstream at higher velocity (data in 3rd quadrant) after division.

4. DISCUSSION

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 [4−8]. Cells are exfoliated under the shear flow at the wall shear stress higher than 2 Pa [11]. A biological cell shows passive and active responses in an environment [1]. 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 [14]. 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* [9]. 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 [11], the rhombus channel [15], the cross flow channel [16], and the rotating disk type [12].

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 [12, 13, 17−19]. The cone-and-plate type device has the uniform shear field in the entire space between the rotating cone and the stationary plate [13]. The shear stress is constant independent of the distance from the rotating axis. The erythrocyte destruction was studied between the rotating concave cone and the stationary convex cone [20].

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 [1]. The floating erythrocyte deformation was observed between counter rotating parallel discs [21].

In the present study, the rotating parallel disk system is selected to make Couette type of flow instead of the cone and plate system. The shear rate (γ) increases from 0.88×10^3 s^{-1} to 1.3×10^3 s^{-1} (Eq. (1)), when the distance from the axis (r) increases from 12 mm to 18 mm in the observation area at the constant angular velocity of 22 rad s^{-1} (d = 0.3 mm). The range of the shear rate enables the simultaneous observation of the behavior of cells related to variation of the shear stress between 1.3 Pa and 2.0 Pa [12]. 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 [4]. The flow velocity, which increases in proportional to the distance from the rotating axis, has also been confirmed by tracings of the moving particle adjacent to the surface.

The most of myoblasts tend to migrate to the oblique direction of the lower shear stress field at 1 Pa [10]. The effect of shear flow on cells depends on the cell types. The dependency might be applied to the cell sorting technology [17]. After division, cells tend to migrate counter direction each other. The tendency makes symmetrical distribution of the migration velocity of cells in Figs. 6b and 7b. To trace the cell after division is convenient to study on the initial behavior of the cell.

The cells proliferate regardless of the shear flow stimulation. The cell cycle does not vary under the shear flow [3]. The movement of each cell can be traced by the time lapse images with the interval of ten minutes in the present experiment.

The effect of shear flow on cells was investigated in many previous studies. The shear flow affects adhesion of each cell [22]. Adhesion of cells can be controlled with design of the scaffold [23]. The behavior of cells in the shear flow was simulated in the previous study [24]. The effect of fluid induced shear stresses on osteoblasts was studied in the previous study [25].

5. CONCLUSION

The effect of shear flow field on the migration of each cell has been investigated under the Couette type of the shear flow field *in vitro*. The area of each cell adhere to the scaffold decreases before division. The decrease of the area of 3T3-L1 before division is slower than that of L929. After division, the area of each cell returns to that of before division. Some cells of L929 migrate to the direction of the rotating axis, where the wall shear stress is lower. 3T3-L1 tends to migrate downstream.

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