Responses of Cells to Flow in Vitro

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ABSTRACT

The response of cells to a flow has been studied *in vitro*. The response of cells was examined in two types of flow channels: a circumnutating flow in a donut-shaped open channel in a culture dish, and a one-way flow in a parallelepiped rhombus flow channel. Variation was made on the material of the parallelepiped channel to study on adhesion of cells to the plates: glass and polydimethylsiloxane. Behavior of cells on the plate was observed under a flow of a medium with an inverted phase-contrast-microscope. The shear stress on the plate is calculated with an estimated parabolic distribution of the velocity between the parallel plates. The adhesion of cells was evaluated with the cumulated shear, which is a product of the shear stress and the exposure time. The experimental results show that cells are responsive to the flow, which governs orientation, exfoliation, and differentiation. The response depends on the kinds of cells: endothelial cells orient along the stream line, although myocytes orient perpendicular to the stream line. The adhesion depends on the combination between scaffold and cell: myocytes are more adhesive to glass than cartilage cells, and fibroblasts are more adhesive to oxygenated polydimethylsiloxane than glass.

Keywords: Biomedical Engineering, Cell Culture, Flow, Orientation, Adhesion and Shear Stress.

1. INTRODUCTION

Cells are responsive to various environmental factors, such as electric [1, 2], magnetic [3, 4] and mechanical [5-21] fields.

The cell culture technique has been developed and applied to many fields: regenerative medicine, diagnostics, etc. The acceleration technique for orientation and differentiation of cells has been studied to make biological tissue *in vivo* and *in vitro* [1, 5-9]. Control methodology for orientation and differentiation of cells would be applied to regenerative tissue technology.

The mechanical stress is one of the interested points in the environment of cells, because they receive mechanical forces *in vivo*. Several methods have been designed to apply mechanical stress to cells [6-21]. A transmission point of stress to a specimen is important. In the most of 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 can be used, on the other hand, to apply a stress field to a specimen [7-16]. The whole specimen directly receives the shear stress in the shear flow. In the present study, the response of cells to the flow has been studied *in vitro*.

2. METHODS

Donut-Shaped Open Channel

A donut-shaped open channel system for the cell culture in a circumnutating flow has been designed to apply a one-way flow on cells *in vitro* [7-9]. A polystyrene culture dish was used. A silicon rubber disk of 3 mm thick (K-125, Togawa Rubber Co., Ltd., Osaka) is attached on the inner bottom of the culture dish to restrict the space for the flow of the medium (Fig. 1). The silicon rubber disk is stuck on the bottom of the dish with affinity between their surfaces without adhesive.

Two types of donut-shaped channels were prepared. The first one (Fig. 1(a)) is as follows. The silicon rubber disk of 40 mm diameter is attached at the center of the culture dish of 52 mm internal diameter without collagen coating. The second one is similar to the first one, and the diameter of the silicone rubber disk is 30 mm (Fig. 1(b)).

The third one is as follows (Fig. 1(c)). The silicon rubber disk of 30 mm diameter and the silicon rubber ring are attached at the center of the culture dish of 100 mm internal diameter with collagen coating. A silicon rubber ring has the inner hole of 40 mm diameter. The centers of the disk and the ring are adjusted to the center of the dish. Cells are cultured in the donut shape interspace between the silicone disks.

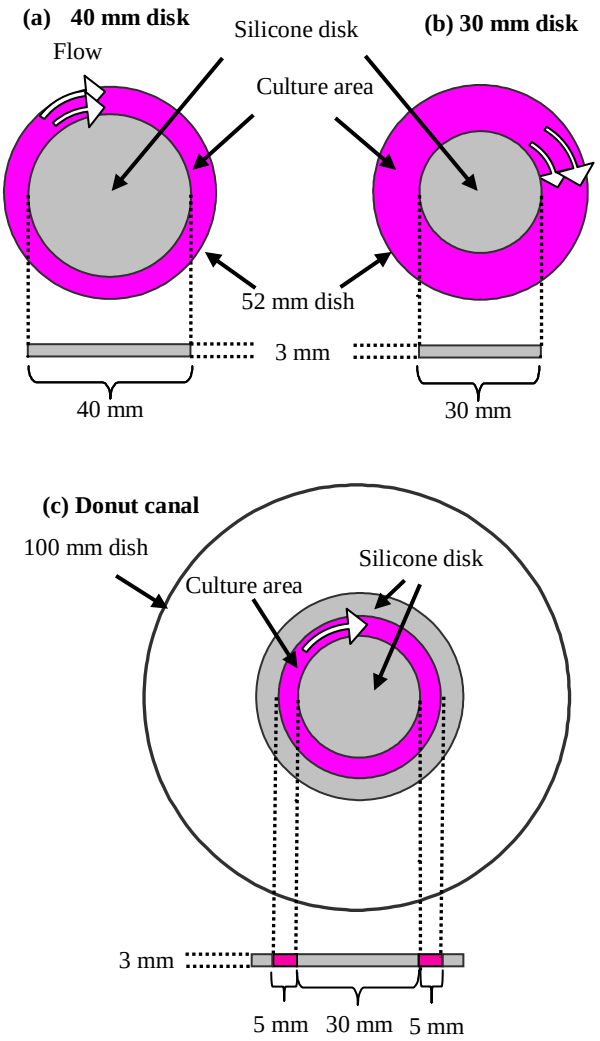

Fig. 1: Culture dish with silicon disk. Three types of donut-shaped area are filled with medium.

The culture dish is placed on a plate, which inclines at 0.1 rad of the horizontal plane (Fig. 2). The plate rotates to generate a swing motion (WAVE-SI, Taitec, Co., Ltd., Koshigaya). The rotating speed of the plate is 20 revolutions per minute (rpm) (2.1 rad/sec). The motion produces a one-way clockwise vortex flow in the medium in the donut-shaped open channel.

The continuously swinging plate is placed in an incubator, where both temperature of 310 K and carbon dioxide partial pressure of 5 percent are maintained.

Parallelepiped Channel

A one-way flow system was designed to observe responses of cells to a fluid shear stress *in vitro*. The system consists of a flow chamber, a syringe pump, tubes and a microscope (Figs. 3&4). TE-331S (Terumo Co., Ltd. Tokyo) or Micro-syringe-pump (ISIS Co., Ltd.) was used for the syringe pump. A plastic tube of 2 mm internal diameter and of 3 mm external diameter was used for the connector to the flow chamber.

Fig. 2: Culture dish on swinging plate in incubator.

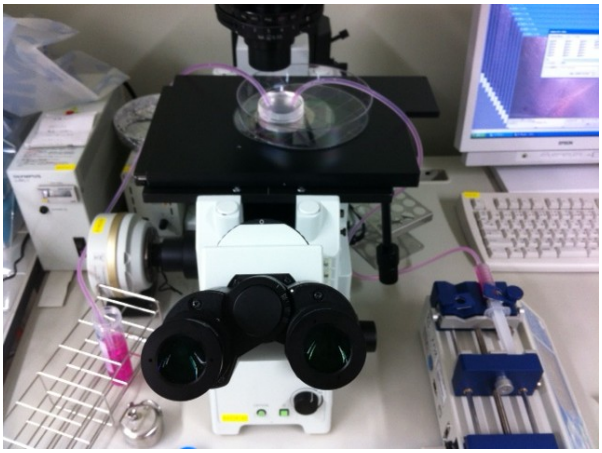

Fig. 3: Parallelepiped flow channel system: flow chamber and microscope (middle), syringe pump (right).

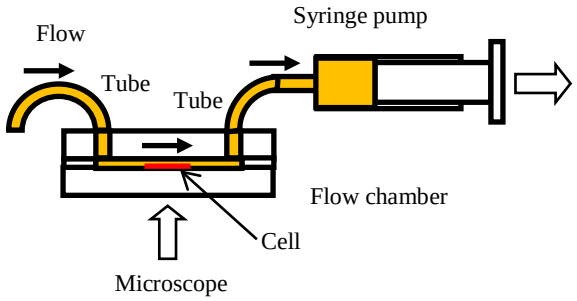

Fig. 4: One-way flow system.

The flow chamber consists of two transparent plates and a thin silicone rubber sheet (Fig. 5). Two kinds of material are alternatively used for the transplant plates: glass or polydimethylsiloxane (PDMS).

A thin sheet (0.1 mm thick) of silicone rubber, which has a void space, is sandwiched between the plates. The void space forms a parallelepiped flow channel.

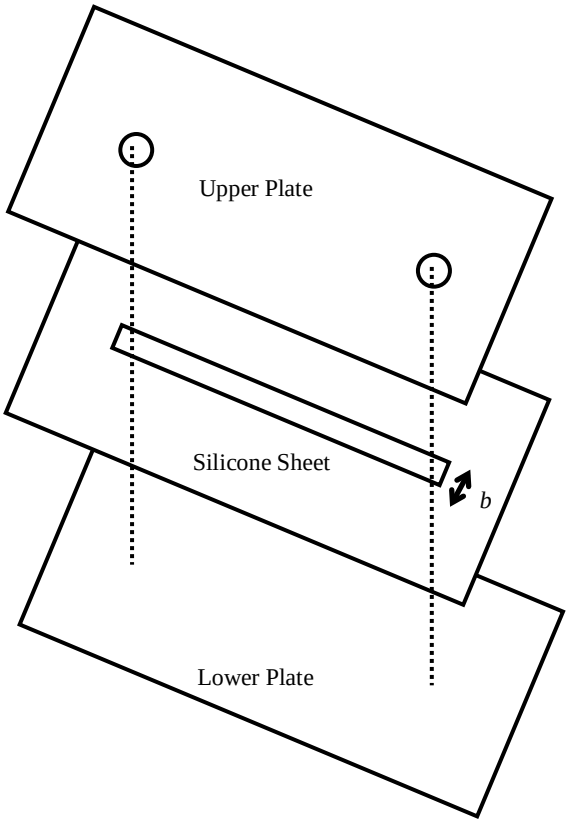

Fig. 5a: Flow chamber of three plates; rectangular void space.

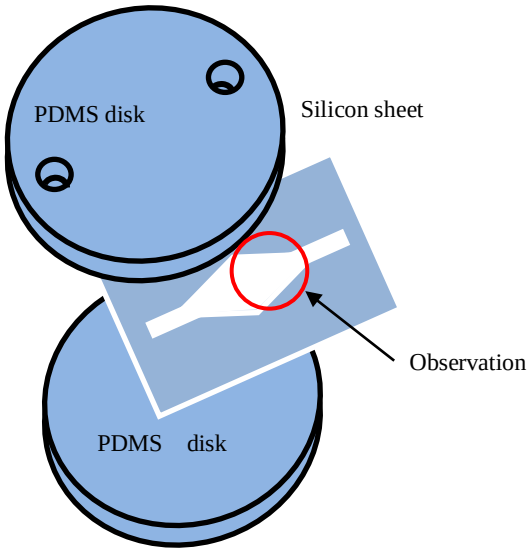

Fig. 5b: Flow chamber of three plates; rhombus void space.

Two types of the void space have been made for the channel. One has a rectangular shape of 2 mm × 52 mm (Fig. 5a), and the other has a rhombus shape of 3 mm × 20 mm (Fig. 5b). The former forms a parallelepiped flow channel of 0.1 mm height with the uniform cross section of 2 mm width, and the latter forms that with the cross section from 1 mm width to 3 mm width.

The three plates stick together with their surface affinity without adhesive. In the case of PDMS disk, the inner surface of PDMS of the chamber was exposed to the oxygen gas in a reactive ion etching system to be characterized as hydrophilic, before assembled.

At the upper plate, two holes were machined to be connected to the plastic tubes. One of the tubes is connected to the plastic syringe pump (Fig. 4). The room temperature was maintained at 298 K. The chamber is placed on the inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo).

Cell

Six kinds of cells were used in the experiment: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), normal cartilage cell (collected from costal cartilage of Sprague Dawley rat, Takara-bio), L6 (rat skeletal muscle cell), A7r5 (rat aortic smooth muscle cell), HUVEC (normal human umbilical vein endothelial cells), and L929 (fibroblast-like, mouse connective tissue, RCB1422, Riken Bio Resource Center, Tsukuba).

Flow Test in Donut-Shaped Open Channel

In the flow test with the donut-shaped open channel, each kind of cells was alternatively suspended in the Dulbecco's Modified Eagle's Medium (D-MEM) with density of 1.0×10^6 cells per cm^3. Fetal bovine serum (FBS) was added to the medium with the volume rate in 10 percent of FBS and in 90 percent of D-MEM. The suspension was poured into the dish and cultured in the incubator for three hours without flow stimulation. After the cultivation for three hours, the cells were cultured with flow stimulation for five days. The continuous rotation of the plate makes a steady flow in the medium through channel.

The volume of the medium is adjusted to cover whole surface of the bottom of the canal in the culture dish, and not to flow over the superior surface of the silicone disk during the swing motion of the plate. The cells were cultured in the vortex flow of the medium, while the plate was continuously rotating at 310 K in the incubator. The medium was refreshed every two days. The directions of cells were observed with the inverted phase-contrast microscope every 24 hours. The results were compared with the control test without flow.

Flow Test in Parallelepiped Channel

In the flow test with the parallelepiped channel, the channel with the suspension was placed in the incubator for several hours (24 hours for the glass, 3 hours for PDMS) to make cells adhere to the plate of the chamber before the flow test.

After the chamber was set on the microscope out of the incubator, the constant flow of the medium was applied to adhered cells with the syringe pump (Fig. 3). The flow path was carefully examined to avoid mixing of air bubbles, which might stir the medium in the flow chamber and induce exfoliation of cells. The behavior of cells on the plate of the chamber was observed with the microscope. The photos of cells were taken during the flow test for one hour. Variation was made in flow rate between 1×10^{-9} m^3 s^{-1} and 8×10^{-9} m^3 s^{-1}.

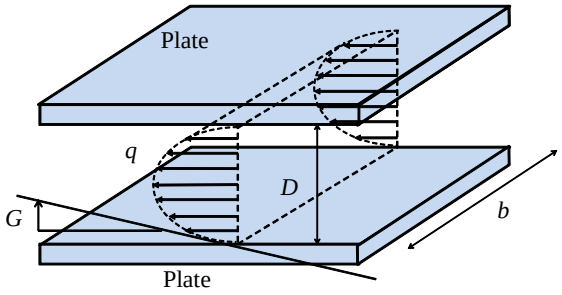

Fig. 6: Parabolic distribution of the velocity between parallel plates.

Shear Stress on Wall of Parallelepiped Channel

The shear rate [G, s^{-1}] on the wall of the plate is calculated by Eq. (1), which is assuming a parabolic distribution of the velocity between parallel plates (Fig. 6).

$$G = 6\, q / (b\, D^2) \quad (1)$$

In Eq. (1), q is the flow rate [$m^3\ s^{-1}$], b is the width of the channel [m] and D is the distance [m] between two parallel walls. In the present study, D is 0.1 mm, and b ranges from 1 mm to 3 mm.

The shear stress T [Pa] is the product of the viscosity N [Pa s] of the fluid and the shear rate G [s^{-1}] of the flow (Eq. (2)).

$$T = N\, G \quad (2)$$

The viscosity of the medium was measured with the cone and plate type of a viscometer (TVE-22L, Toki-Sangyo Co., Ltd. Tokyo).

3. RESULTS

Flow Test in Donut-Shaped Open Channel

The experimental results with C2C12 of 40 mm disk show that cells adhere to the bottom of the culture dish adjacent to the silicon rubber disk in three hours (Fig. 7a). Few adhered cells were observed in the circumferential area in the dish. The cells proliferate in the circumnutating flow of the medium. The array of myotubes grows around the silicon rubber disk day by day, and the alignment curves to the radial direction. The longitudinal axes of cells orient to the direction perpendicular to the flow on the third day of culture (Fig. 7c). This tendency is same as that of 30 mm disk. The results of donut shaped canal show that cells adhere adjacent to the inner circle in the donut shape of the canal between the silicon rubber disks. The orientation develops from the inner circle to the outer circle (Fig. 7). The experimental results show that cells extend to the area, where cells have not adhered yet.

The results with normal cartilage cell (Fig. 8), with L6 (Fig. 9), and with L929 were similar to that with C2C12. They develop orientation perpendicular to the flow. The flow stimulates differentiation of C2C12 to myotubes.

The experiment with HUVEC (normal human umbilical vein endothelial cells) shows that cells adhere to the bottom of the culture dish adjacent to the inner circle of silicone disk in 24 hours. The area of adherence extends to the radial direction. The cells elongate to the spindle shape, of which long axis tilts to the circumferential flow direction in six days of culture (Fig. 10). The results with A7r5 were similar to that with endothelial cells.

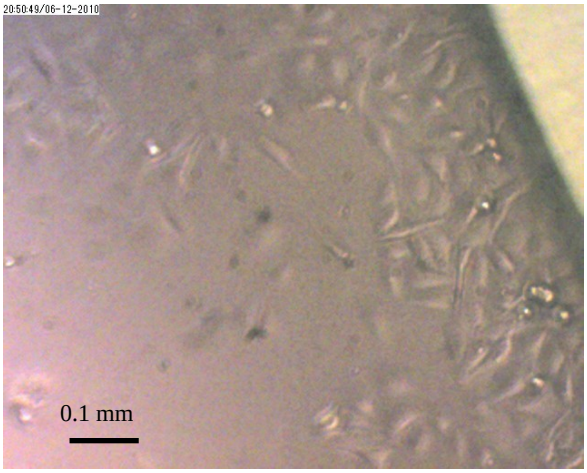

Fig. 7a: C2C12 cultured for 3 hours (near 40 mm silicon disk).

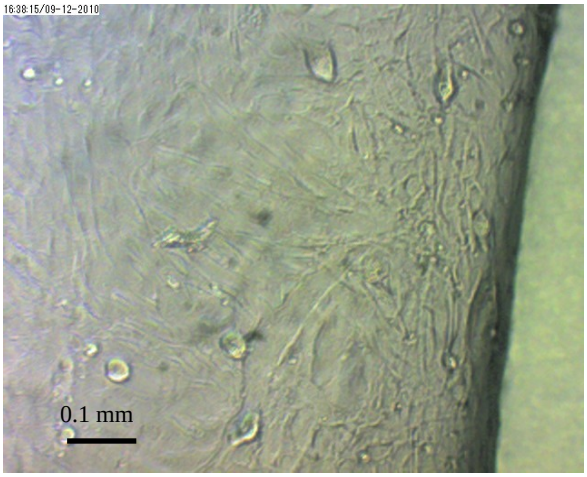

Fig. 7b: C2C12 cultured for 3 days (near 40 mm disk).

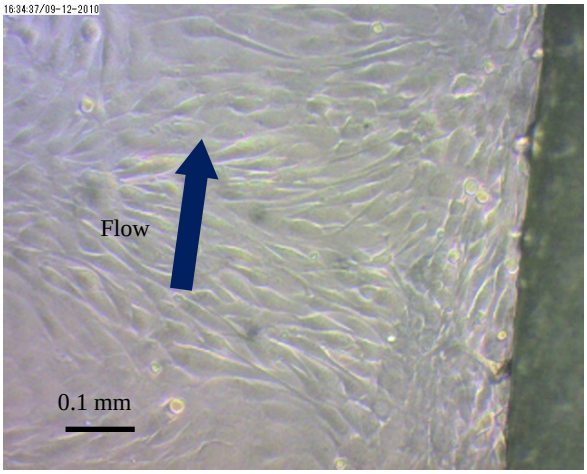

Fig. 7c: C2C12 cultured for 3 days in flow (near 40 mm disk).

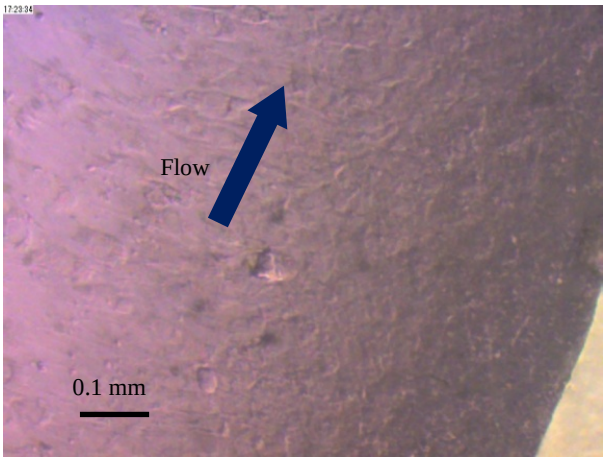

Fig. 8a: cartilage cell cultured for 5 days in flow (near inner circle).

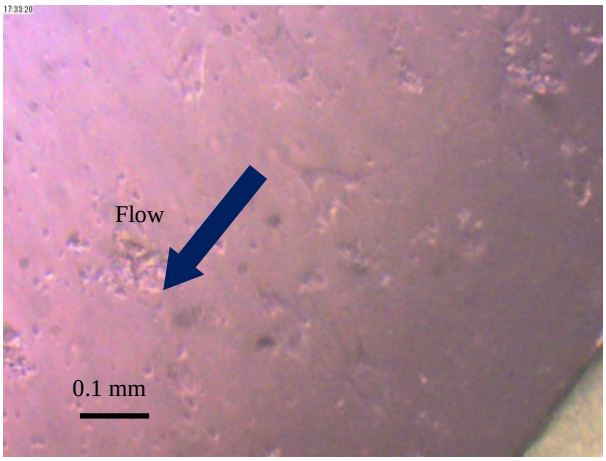

Fig. 8b: cartilage cell cultured for 5 days in flow (near outer circle).

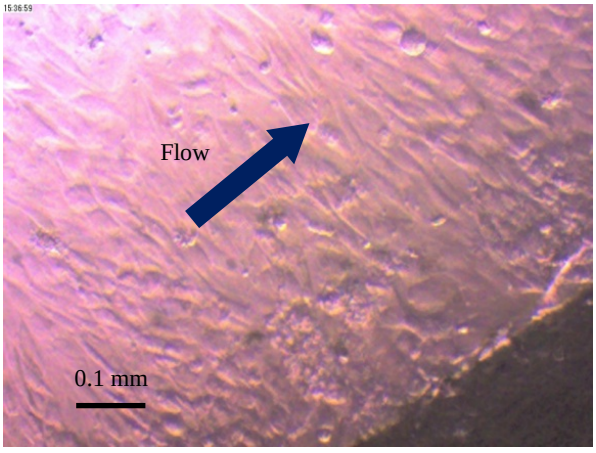

Fig. 9a: L6 cultured for 5 days in flow (near inner circle).

Flow Test in Parallelepiped Channel

The result of measurement with the viscometer shows that the viscosity of the medium is 0.0010 Pa s at 298 K at the shear rate of 600 s^{-1}. Thus, the calculated wall shear rate of the flow chamber by Eq. (1) varies between 220 s^{-1} and 5000 s^{-1}, when the flow rate varies between 1×10^{-9} m^3 s^{-1} and 8×10^{-9} m^3 s^{-1}. The calculated shear stress, varies between 0.2 Pa and 5 Pa for viscosity of 0.001 Pa s, when the shear rate varies between 220 s^{-1} and 5000 s^{-1}.

Fig. 11 shows cartilage cells in the rectangular channel under the steady flow of 8×10^{-9} m^3 s^{-1}, which generate a wall shear stress of 3 Pa estimated by Eq. (1) & Eq. (2). The cell (A in Fig. 11a) elongates to the downstream (Fig. 11b), and exfoliates in three minutes (Fig. 11c).

Fig. 12 shows L929 in the rhombus channel under the flow of 5×10^{-9} m^3 s^{-1}, which generate a wall shear stress between 2.9 Pa and 3 Pa estimated by Eq. (1) & Eq. (2). The medium flows from left to right in Fig. 12. The distance from left to right is 0.5 mm in Fig. 12. The inlet of the chamber is located near left end of Fig. 12, and the width of the flow path linearly increases from 1.0 mm to 1.1 mm in the figures. As the increase of the width of the flow path, the wall shear stress decreases. The estimated shear stress varies from 3.0 Pa at left-end to 2.9 Pa at right-end. Some cells elongate to the downstream along the streamline of the flow in 40 min (cf. left upper B in Fig. 12). Some cells (cf. center C in Fig. 12) exfoliate, and flow to the downstream in 43 min.

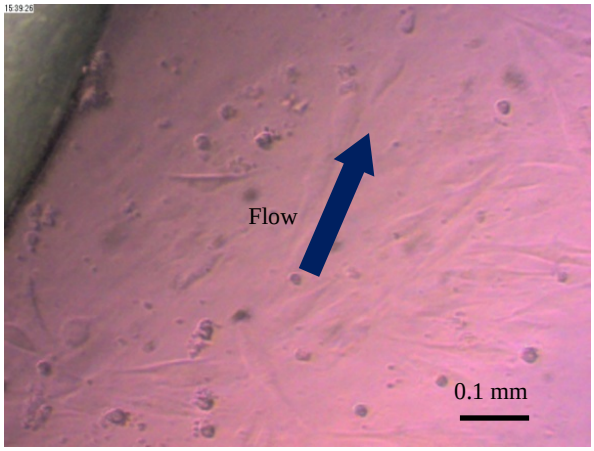

Fig. 9b: L6 cultured for 5 days in flow (near outer circle).

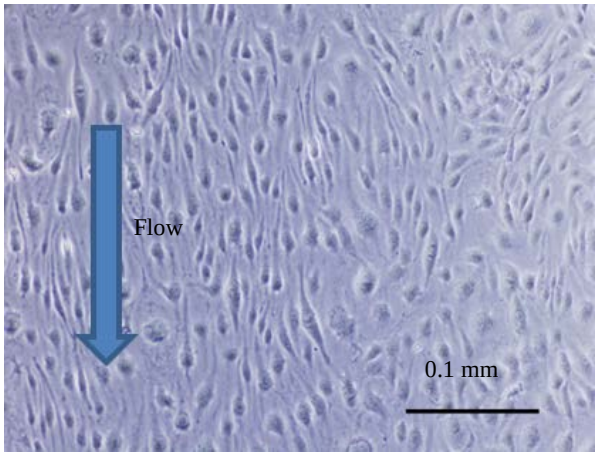

Fig. 10: HUVEC cultured for 6 days in flow.

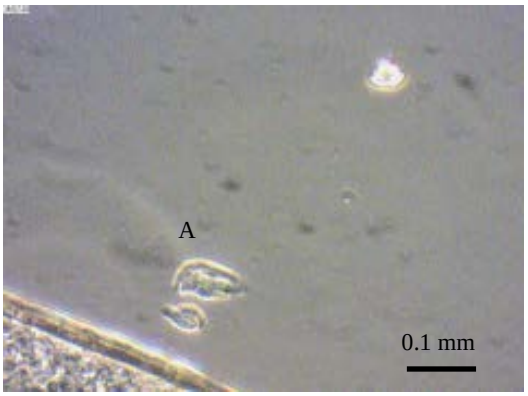

Fig. 11a: Cartilage cells before flow stimulation.

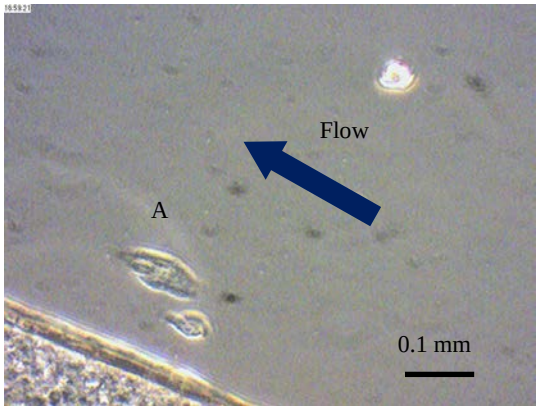

Fig. 11b: Cartilage cells in flow of 8×10^{-9} m^3 s^{-1} for 2 min.

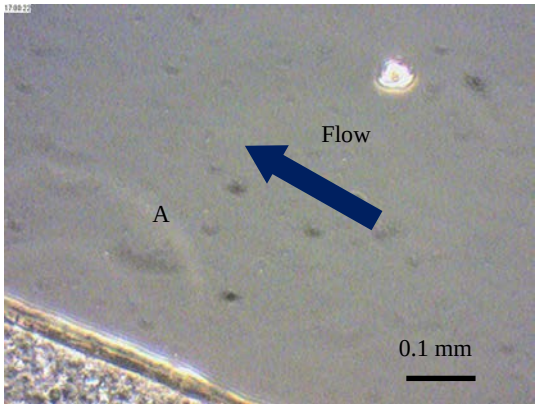

Fig. 11c: Cartilage cells in flow of 8×10^{-9} m^3 s^{-1} for 3 min.

Table 1: Shear stress of exfoliation (on glass)

Cell	Shear stress [Pa]	Cumulated shear [Pa min]
C2C12	6	18
Cartilage Cell	3	9
L6	0.4	8
A7r5	0.3	4.5
L929	*	*
L929**	3	129

* exfoliated immediately at the flow rate of 1×10^{-9} m^3 s^{-1}.
**on polydimethylsiloxane.

The critical shear stress, at which the cells are exfoliated, is summarized in Table 1 with the cumulated shear. The cumulated shear is the integral of the shear stress during the exposure time. The results show that C2C12 is more adhesive than the other cells to the glass surface after the incubation for 24 hours.

The experimental results show that L929 is easily exfoliated from the glass plate. Adhesion between fibroblasts and PDMS (oxygenated), on the other hand, is much stronger. Three hours is enough to make adhesion of L929 to PDMS (oxygenated), although 24 hours is not enough to make adhesion of L929 to glass.

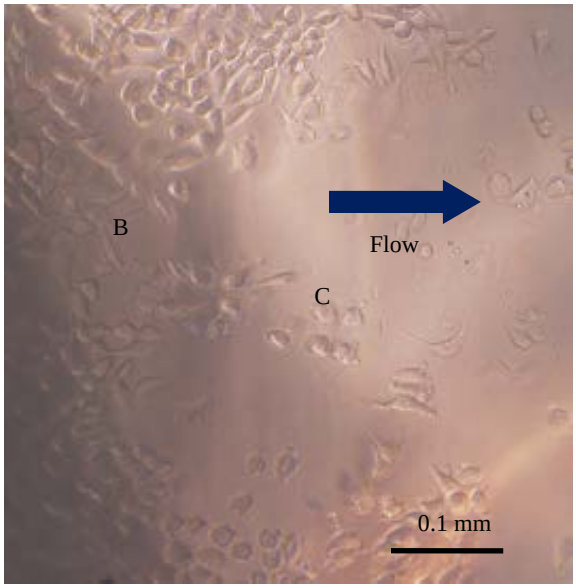

Fig. 12a: L929 after flow stimulation of 5×10^{-9} m^3 s^{-1} for 10 min.

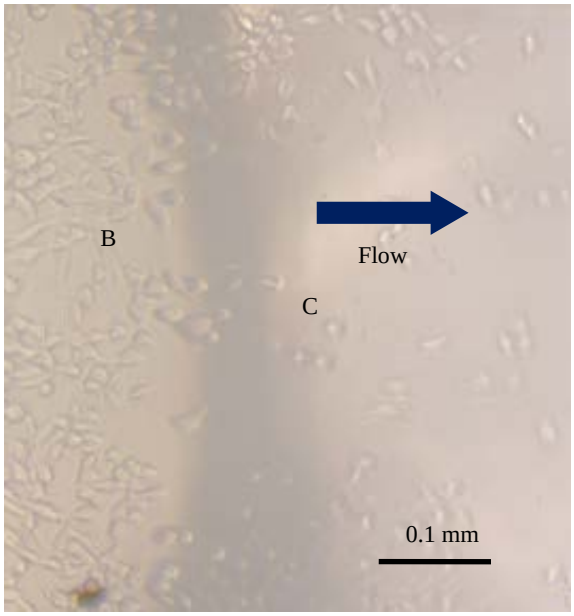

Fig. 12b: L929 after flow stimulation of 5×10^{-9} m^3 s^{-1} for 43 min.

4. DISCUSSION

Several movements occur on adhered cells in the flow: deformation, tilting to downstream, elongation along the streamline, deformation to be rounded, exfoliation, rolling to downstream [10-12].

It is not easy to estimate the wall shear stress in the medium flow at the bottom of the donut-shaped open channel, which has free surface to the air [7-9]. It is possible, on the other hand, to estimate the wall shear stress in the medium flow in the parallelepiped flow channel [12-14].

To estimate the wall shear rate in the channel, a parabolic velocity profile is hypothesized as a laminar flow in the present experiment. Reynolds number (*Re*) is a useful index for estimation of the laminar flow.

$$Re = d\, D\, v / N \qquad (3)$$

In Eq. 3, d is density [kg m^{-3}] of the fluid, D is distance [m] between two parallel walls, N is viscosity [Pa s] of the fluid, and v is the mean velocity of the flow [m s^{-1}]. The mean velocity is calculated by Eq. 4.

$$v = q / (b\, D) \qquad (4)$$

In Eq. 4, q is the flow rate [m^3 s^{-1}], and b is the width [m] of the channel.

Re is in the range between 0.3 and 8, calculated with d (10^3 kg m^{-3}), N (10^{-3} Pa s), D (10^{-4} m), q (8×10^{-9} m^3 s^{-1}), and b (ranges from 1 mm to 3 mm) in the present experiment. The number is small enough to estimate the laminar flow.

The parallelepiped rhombus chamber designed in the present study realizes the wall shear stress field, where the shear stress varies linearly along the stream line. The chamber is convenient to observe the response of cells simultaneously with the variation of the wall shear stress, while the wall shear stress is constant in the parallelepiped rectangular chamber [12].

The similar response of the cell to the medium flow in the present study shows that the shear stress is in the same level between tests of two types of flow channel: the donut-shaped open channel, and the parallelepiped channel.

The inner surface of the chamber was exposed to the oxygen gas in a reactive ion etching system (RIE-10NR, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing), before assembling. The oxygenized surface of PDMS might make strong affinity to L929.

Micromachining technique might extend the fabrication of the flow channel for biological cells [16]. Flowing cells show various responses around micro morphology: a slit and a trap [22, 23].

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on cultured biological tissue. The previous study shows that electric stimulation enhances differentiation of muscle cells [1]. Another study shows that mechanical stimulation improves a tissue-engineered human skeletal muscle [5]. Another previous study shows that muscle cells can adhere and proliferate under electric stimulation with periodical pulses, and that adhesion of muscle cells can be controlled with the amplitude of the pulse [1].

The previous studies show that a mechanical field, on the other hand, affects cells' behavior. Erythrocytes are very flexible, and are rolled and deformed in the shear flow [13]. The shear flow also affects on the orientation of endothelial cells [7, 10, 11, 17, 18]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [19, 20]. The direction of the mechanical field affects orientation of fibroblasts [6]. The flow *in vivo* might affect remodeling of the bone [24].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [7, 21]. The mechanical stimulation decreases proliferation of cells [8]. The mechanical stress also exfoliates several cells, which makes vacancy around the adhesive cell. The differentiation might be optimization of cells to changing environment. In the present study, the differentiation was confirmed that the developed myotubes show synchronous contraction with electric pulses, which applied to the culture medium.

The channels which have been used in the present study are useful to investigate the response of cells to the flow, because of the uniform direction of the stream line. The parallelepiped flow channel is available to quantify the effect of shear stress on the cell.

5. CONCLUSIONS

Response of cells to the flow has been studied *in vitro*. The experimental results show that cells are responsive to the flow, which governs orientation, exfoliation, and differentiation. The response depends on the kinds of cells: endothelial cells orient along the stream line, although myocytes orient perpendicular to the stream line. The adhesion depends on the combination between scaffold and cell: myocytes are more adhesive to glass than cartilage cells, and fibroblasts are more adhesive to oxygenated polydimethylsiloxane than glass.

6. ACKNOWLEDGMENT

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