Effect of Flow Stimulation on Cultured Osteoblast

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ABSTRACT

An effect of flow stimulation on cultured osteoblast has been studied *in vitro*. To apply the continuous shear flow on cultured cells, a cell culture dish with a donut shaped canal was contained on a shaker. Variation was made on the flow rate with the rotational speed of the shaking plate. MC3T3-E1 (osteoblastic cell line) was incubated for a week under the flow stimulation. To observe the migration of cells, the cells in the targeted area were exfoliated with a tip of a micropipette when the cells proliferated to sub confluent density. Proliferation and migration of the cells were observed with a phase contrast microscope during the cell culture. The experiments show the following results: the osteoblasts proliferate even under the continuous shear flow, and the osteoblasts migrate even to the counter direction along the flow, although osteoblasts do not show special orientation under the flow condition.

Keywords: Biomedical Engineering, Cell Culture, Osteoblast, Proliferation, Migration and Flow.

1. INTRODUCTION

Cell culture technique has been developed and several methodologies have been clinically applied to regenerative medicine. The acceleration technique for orientation and proliferation of cells has been studied to make tissue *in vivo* or *in vitro* [1-9]. 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 [2-10].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold [2-5]. 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 [7-10]. The specimen directly receives the shear stress in the shear flow.

High shear flow might deform cell, peel cells off the scaffold, inhibit proliferation as well as tissue formation. Mild shear flow, on the other hand, might accelerate migration, proliferation, and secretion of materials, which make the extra cellular matrix.

The amount of the bone tissue is controlled with osteoclasts and osteoblasts: osteoblasts form bone, although osteoclasts re-absorb bone. The amount of the bone decreases in the microgravity field.

In the present study, the effect of flow stimulation on cultured osteoblast has been studied *in vitro*.

2. METHODS

Donut-Shaped Open Channel

A donut-shaped open channel system for the cell culture has been designed to apply a shear flow on cells *in vitro* (Fig. 1). A polystyrene culture dish of 50 mm internal diameter (IWAKI 3010-060-MYP) was used. A transparent polydimethylsiloxane (PDMS) disk (30 mm diameter, 3 mm thick) is attached on the inner bottom of the culture dish to restrict the space for the flow of the medium. The PDMS disk is attached at the center of the polystylene culture dish with affinity between their surfaces without adhesive.

A disk of polymethylmethacrylate has been made to adjust the center of the culture dish and to trace the same position in the serial cultivation in the dish (Fig. 2). The disk has a pinhole at the center, and lines in the radial direction. The lines are marked in the middle position of the donut channel for the microscopic observation. The intervals of the lines are 1 mm and 0.3 mm (Fig. 6). The diameter of the disk is 50 mm. A circle of 30 mm diameter is marked on the disk. The circle is used for adjusting the position of the PDMS disk on the bottom of the culture dish, when the PDMS disk is attached on the bottom of the culture dish. Every position in the culture dish is adjusted with the circumferential wall attached on the rim of the disk.

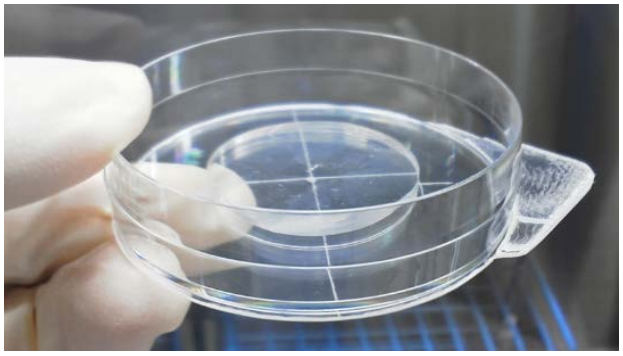

Fig. 1: PDMS disk attached on the inner bottom of the culture dish at the center with the adjuster plate.

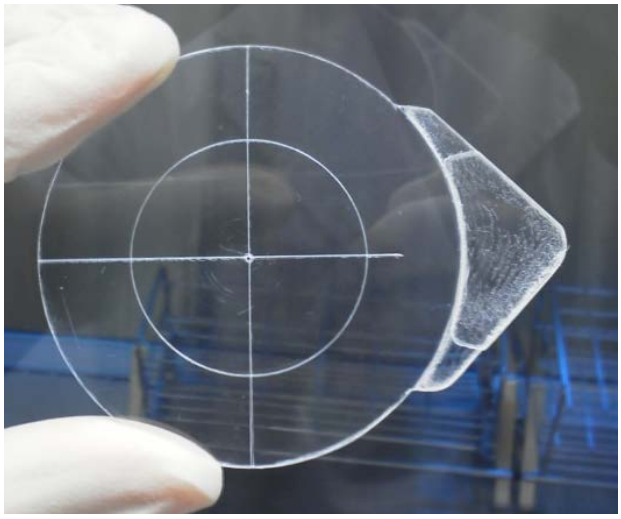

Fig. 2: Adjuster plate of 50 mm diameter has a hole at the center and lines in the radial direction.

The culture dish is placed on a plate, which inclines at 0.1 rad of the horizontal plane (Fig. 3). The plate rotates to generate a swing motion (WAVE-PR, Taitec, Co., Ltd., Koshigaya). Variation was made on the rotating constant speed of the plate: 5, 30, and 40 revolutions per minute (rpm) (0.52, 3.1, 4.2 rad/s). The inclination of the rotating plate was changed to 0.05 rad at 5 rpm. The motion produces a one-way counter clockwise steady vortex flow in the medium through the donut-shaped open channel. The flow speed v (m/s) in the middle part of the donut channel is proportional to the rotating speed N (rad/s).

$$v = r\,N \qquad (1)$$

In Eq. 1, r is radius of the middle part of the channel: 0.04 m. he calculated speeds are 1 cm/s, 6 cm/s, and 8 cm/s, respectively.

The continuously swinging plate is placed in an incubator (Fig. 4), where both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent are maintained.

Cell Culture

MC3T3 (an osteoblast precursor cell line derived from Mus musculus (mouse) calvaria) -E1 was used in the test.

Fig. 3: Culture dish placed on shaker: counter clockwise flow.

Fig. 4: Culture dish on shaker in the incubator.

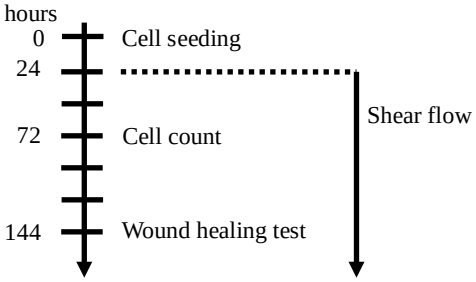

Fig. 5: Protocol of cell culture.

MC3T3-E1 was cultured with the alpha-MEM (alpha modified Eagle's minimal essential medium) containing 10% FBS (fetal bovine serum), which was added after decomplementation. The medium also ontains 2.4% of sodium bicarbonate aqueous solution ($NaHCO_3$, 75 g/L) and 1% of Antibiotic-Antimycotic (penicillin, streptomycin and amphotericin B, Life Technologies). The medium was refreshed every three days during cell culture.

The cells were seeded on the donut canal (Fig. 1) in the density of 1000 cells/cm^2 with 3 cm^3 of medium.

Microscopic Study
Cells were observed with an inverted phase-contrast microscope (IX71, Olympus, Tokyo).

After the cells were cultured for 24 hours without flow in the incubator, the cells were cultured under the continuous flow for several days on the shaker in the incubator. The number of cells was counted in the randomly selected square area of 1 mm^2 in the channel at 72 hours of cultivation.

After the cells were cultured for 144 hours, the cells in the strip area of 0.8 mm width perpendicular to the direction of the flow were exfoliated with a tip of a micropipette, and the migration of cells to the strip area was observed every hour for 12 hours.

The top of the extended area of cells migration is evaluated as the perpendicular distance from the line of exfoliation. The slope of the collinear approximation of the increase of the distance is evaluated as the speed of migration. The speed is evaluated at upper and lower streams of the exfoliated area.

The results with flow stimulation were compared with that of without flow stimulation. Every cell is cultured in the donut channel in the present experiment.

3. RESULTS

Cells were observed by microscope after cultivation for 24 hours (Fig. 6). The figure shows that MC3T3-E1 adheres to the bottom of the dish in 24 hours.

The flow synchronized with the rotational movement of the tilted plate was observed by floating tracers.

Figs. 7-9 exemplify the cells after 72 hours of cultivation under flow compared with that without flow. The figures show that MC3T3-E1 proliferates to sub confluent density in 144 hours even under the continuous shear flow.

Figs. 10-12 show the density of cells after 120 hours of cultivation with flow (F) in comparison with that without flow (C). Each point in the vertical line shows the datum of randomly selected square area of 1 mm^2 in the same culture dish. The experimental data show the following results. Proliferation is inhibited with flow higher than 6 cm/s (Fig. 10), although proliferation tends to increase with the flow of 1 cm/s.

Figs. 13 & 14 exemplify cells after 72 hours cultivation. The figures show that the longitudinal axes of the cell distribute in random direction.

Migration of cells to the exfoliated area is exemplified in Fig. 15. The figure shows that cells migrate even to the counter direction along the flow. The speed of migration of cells is calculated from the slope of the tracings of migration with the collinear approximation (Fig. 16). Fig. 17 shows the speed of migration in each flow condition: flow of 1 cm/s (1C-1CL), 6 cm/s (6C-6L), 8 cm/s (8C-8L), without flow (C), upper side of the flow (U), lower side of the flow (L), without flow before exfoliation (CU, CL). The speed of migration of counter direction along the flow decreases with the flow higher than 6 cm/s.

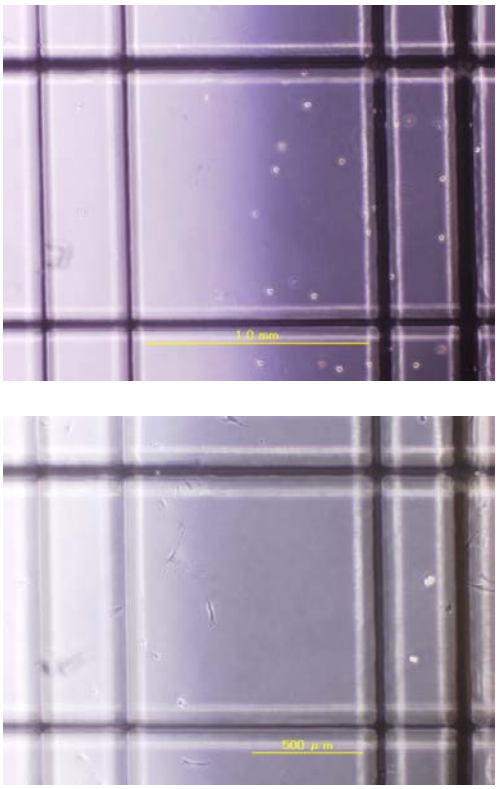

Fig. 6: Immediately after cell seeding (upper), and after 24 hours of cultivation (lower). Dimension from left to right is 2 mm.

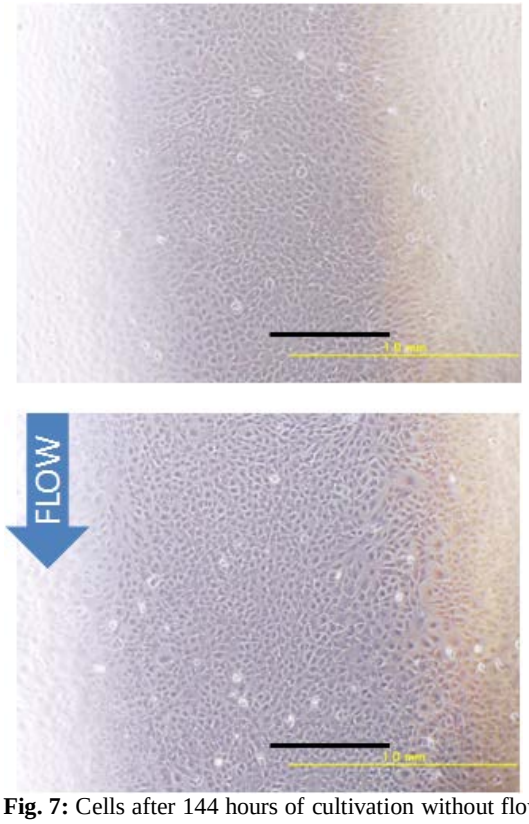

Fig. 7: Cells after 144 hours of cultivation without flow (upper), and with flow of 1 cm/s (lower). The arrow shows direction of flow. Dimension from left to right is 2 mm.

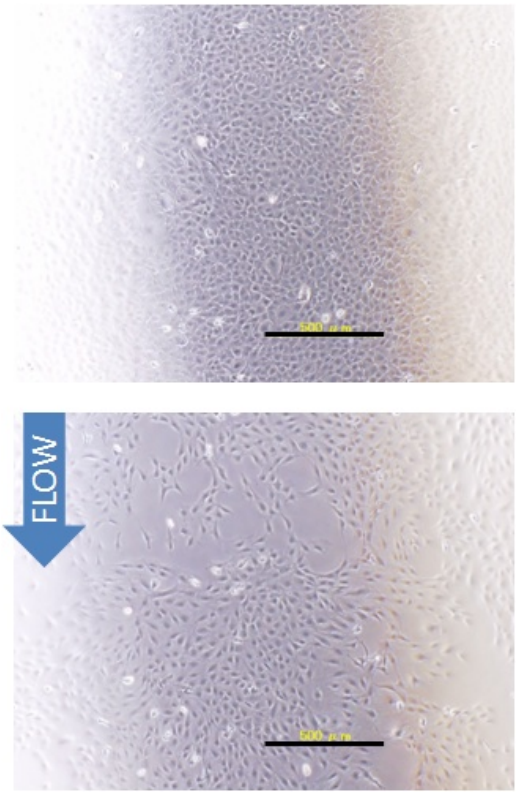

Fig. 8: Cells after 144 hours of cultivation without flow (upper), and with flow of 6 cm/s (lower). The arrow shows direction of flow. Dimension from left to right is 2 mm.

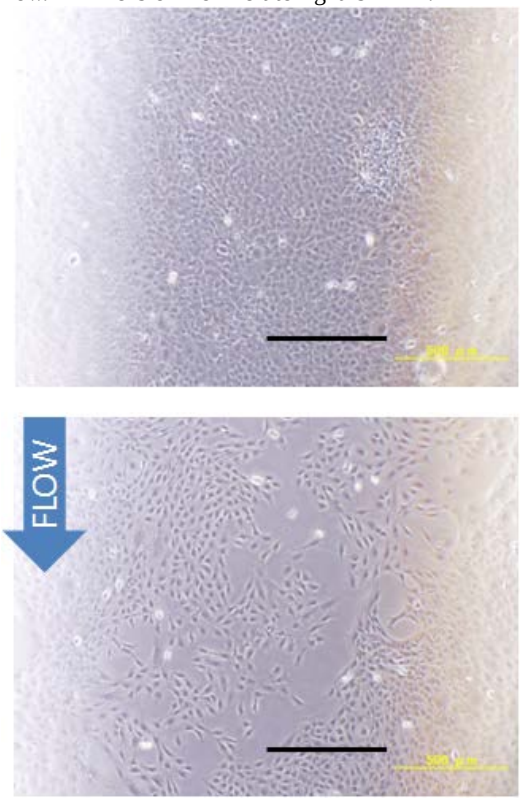

Fig. 9: Cells after 144 hours of cultivation without flow (upper), and with flow of 6 cm/s (lower). The arrow shows direction of flow. Dimension from left to right is 8 mm.

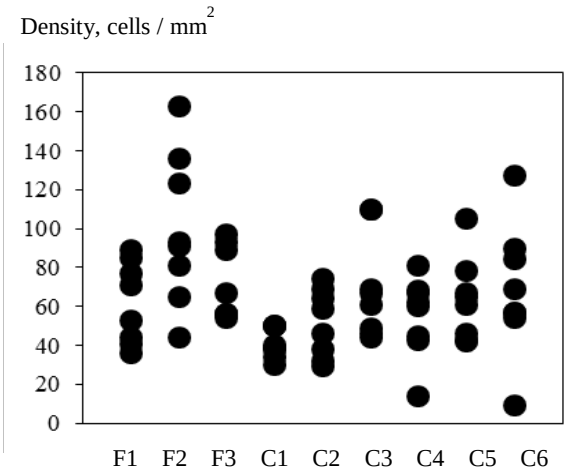

Fig. 10: Density of cells after 72 hours of cultivation with flow of 1 cm/s (F1-F3), and without flow (C1-C6).

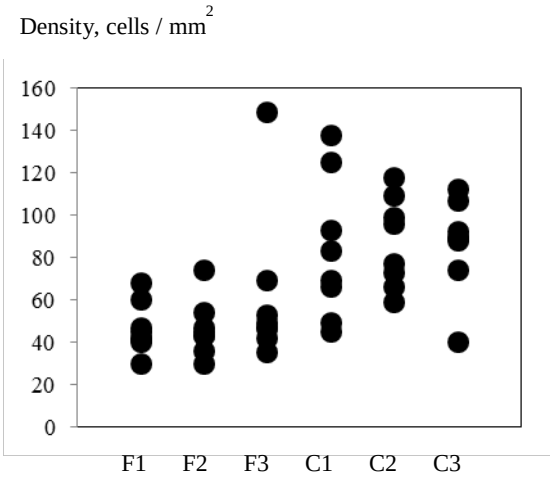

Fig. 11: Density of cells after 72 hours of cultivation with flow of 6 cm/s (F1-F3), and without flow (C1-C3).

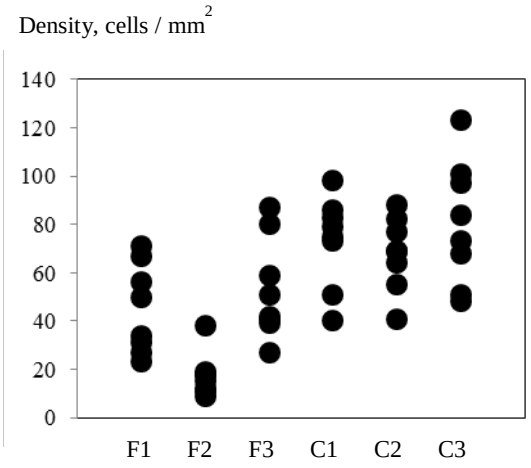

Fig. 12: Density of cells after 72 hours of cultivation with flow of 8 cm/s (F1-F3), and without flow (C1-C6).

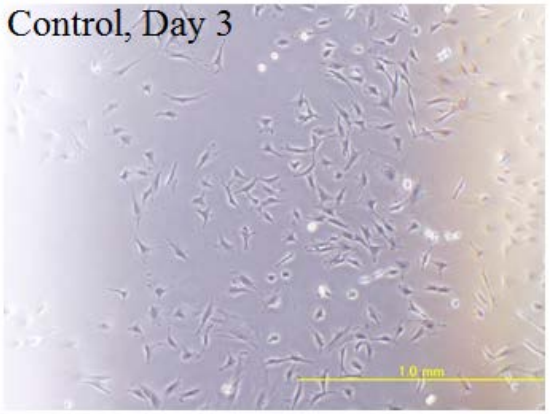

Fig. 13: Cells after 72 hours of cultivation without flow. Dimension from left to right is 2 mm.

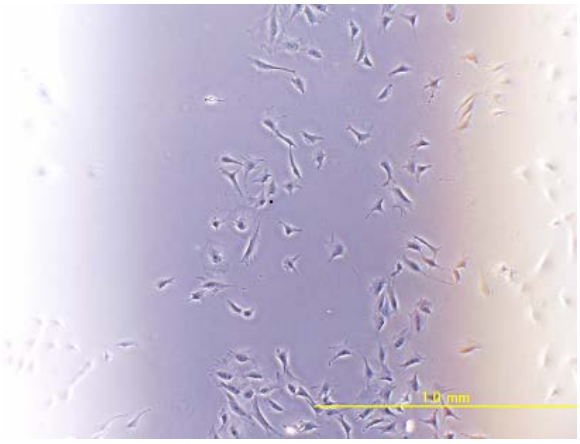

Fig. 14: Cells after 72 hours of cultivation with flow. Direction of flow is downward. Dimension from left to right is 2 mm.

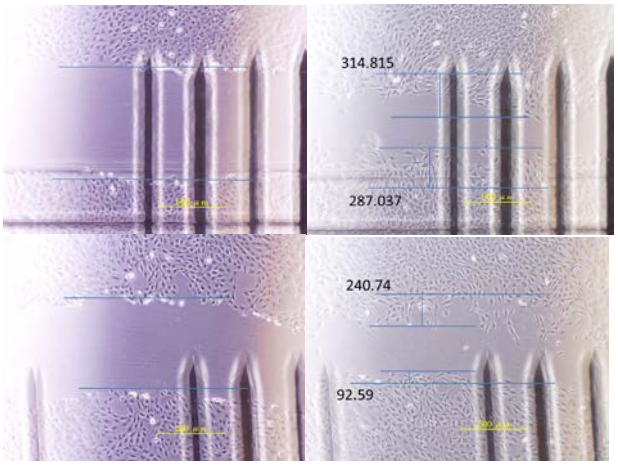

Fig. 15: Immediate after exfoliation (left); after 12 hours cultivation without flow (upper right) with flow (from top to bottom) of 6 cm/s (lower right). Dimension from left to right is 2 mm, respectively.

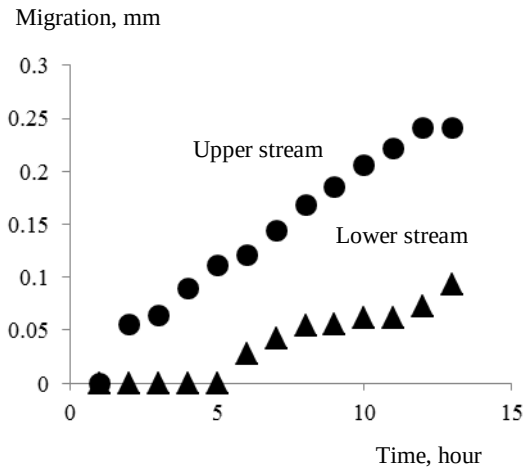

Fig. 16: Migration of cells vs. time with flow of 6 cm/s.

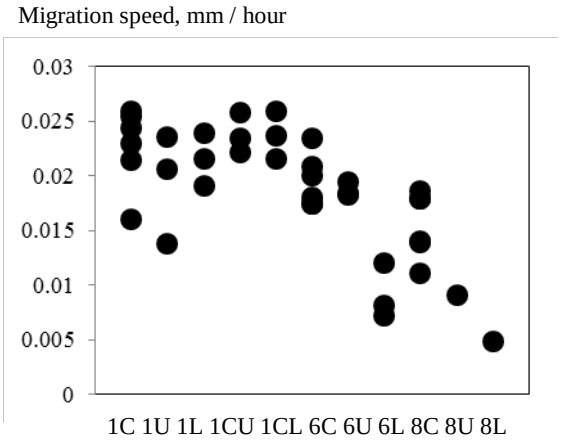

Fig. 17: Migration speed of cells in 12 hours.

4. DISCUSSION

The murine-derived MC3T3-E1 cell line provided by the American Type Culture Collection (ATCC) is a well-known osteogenic cell culture model system to test materials *in vitro*. The osteoblastic cell line MC3T3-E1 has been established from a C57BL/6 mouse calvaria and selected on the basis of high alkaline phosphatase (ALP) activity in the resting state. Cells have the capacity to differentiate into osteoblasts and osteocytes *in vitro*.

It is not easy to estimate the shear stress value on the wall in the present experiment, because the medium has the free surface. The parallel piped chamber is convenient to observe the response of cells under controlled shear stress [8]. The shear stress on the bottom of the donut canal of the dish in the present study is estimated as several pascal with the behavior of endothelial cells in the same experimental flow system [7, 8].

When mechanical stimulation is applied to the scaffold, the whole stimulation cannot always be transmitted to the cells. To apply mechanical stimulation to the cells, centrifugal force [6] or shear flow is used alternatively in the present study.

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. The behavior of biological cells depends on electric and magnetic fields [1, 11]. Another study shows that mechanical stimulation improves a tissue-engineered human skeletal muscle [2].

The previous studies show that a mechanical field, on the other hand, governs behavior of cells [12]. The shear flow governs the orientation of endothelial cells [7, 9]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [3]. The direction of the mechanical field affects fibroblasts [5].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [6]. The mechanical stimulation can decrease proliferation of cells [6]. The mechanical stress also exfoliates several cells, which makes vacancy around the adhesive cell [13-16]. The differentiation might be optimization of cells to the changing environment.

The effect of shear flow on orientation of cells depends on the kinds of cells [7]. Although HUBEC orients along the stream lines, C2C12 tilts from the stream lines to make myotubes. The previous study shows orientation of cells perpendicular to the stretch direction [4].

5. CONCLUSION

The effect of flow on behavior of osteoblasts has been studied *in vitro*. To apply continuous shear flow on cells, the cell culture dish with the donut shaped canal was contained on the shaker. The experimental results show that the osteoblasts migrate even to the counter direction along the flow, and that the osteoblasts proliferate even under the continuous shear flow without orientation to the flow direction.

6. ACKNOWLEDGMENT

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