Behavior of Cell on Vibrating Micro Ridges

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ABSTRACT

The effect of micro ridges on cells cultured at vibrating scaffold has been studied *in vitro*. Several parallel lines of micro ridges have been made on a disk of transparent polydimethylsiloxane for a scaffold. To apply the vibration on the cultured cells, a piezoelectric element was attached on the outside surface of the bottom of the scaffold. The piezoelectric element was vibrated by sinusoidal alternating voltage (Vp-p < 16 V) at 1.0 MHz generated by a function generator. Four kinds of cells were used in the test: L929 (fibroblast connective tissue of C3H mouse), Hepa1-6 (mouse hepatoma), C2C12 (mouse myoblast), 3T3-L1 (mouse fat precursor cells). Cells were seeded on the micro pattern at the density of 2000 cells/cm^2 in the medium containing 10% FBS (fetal bovine serum) and 1% penicillin/ streptomycin. After adhesion of cells in several hours, cells are exposed to the ultrasonic vibration for several hours. The cells were observed with a phase contrast microscope. The experimental results show that the cells adhere, deform and migrate on the scaffold with micro pattern regardless of the ultrasonic vibration. The effects of the vibration and the micro pattern depend on the kind of cells.

Keywords: Biomedical Engineering, Cell Culture, Micro pattern and Vibration.

1. INTRODUCTION

A biological cell adheres, migrates, rotates, and deforms on the scaffold. These behaviors of cell depend on the morphology of the scaffold [1-6]. The previous study show that the orientation of myoblast depends on the height of the micro ridges [1].

The ultrasonic vibration has been applied to human body in several cases: measurements, and lithotrity [7-9]. Cell culture technique has been developed, and cells have been cultured in controlled environment. Effect of vibration on cell culture was studied in previous studies [10-14]. The moderate ultrasonic vibration accelerated proliferation of myoblasts in the previous experiment. The cells might be exfoliated from the ultrasonically vibrating scaffold. The exfoliation depends on affinity between the cell and the scaffold. Specific character of interaction between the cell and the scaffold might be enhanced by vibration. The interaction might depend on the surface micro morphology. The behavior might depend on the kind of cells. The technology can be applied to sorting of cells.

Several methodologies have been clinically applied to regenerative medicine. The acceleration technique for proliferation, orientation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [10, 11, 13, 15]. Control methodology for proliferation and differentiation 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, orientation, and differentiation. Several methods have been designed to apply the mechanical stress to cells [16-18].

In the present study, the effect of micro ridges on cells cultured at vibrating scaffold has been studied *in vitro*.

2. METHODS

Micro Pattern

Several parallel lines of micro ridges have been made at the center on a disk of transparent polydimethylsiloxane (PDMS). The height (*H*), the interval (*I*), and the length (*L*) of the rectangular ridge (Fig. 1) are around 0.001 mm, 0.003 mm, and 0.5 mm, respectively. Variation has been made on the width (*W*) of the ridge: 0.001 mm, 0.003 mm, and 0.005 mm. Each pattern is drawn in the square area of 0.5 mm × 0.5 mm, which is the quarter part of the square area of 1.0 mm × 1.0 mm. One of the quarter areas has a smooth surface, and has no ridges (Figs. 8-11).

A silicon wafer (CZ, Type N, Matsuzaki Seisakusyo, Co., Ltd., Tokyo, Japan) was used for a surface mold for the disk in a photo lithography process [1]. The diameter and the thickness of the wafer are 50 mm and 0.30 mm, respectively. The surface of the wafer was cleaned by a spin-drier (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan). To improve the affinity between the wafer and the photo-resist material (OFPR-800), hydrophobic treatment was applied to the wafer. Hexamethyldisilazane (HMDS) was coated at 3000 rpm for thirty seconds with a spin coater (IH-DX2, Mikasa Co., Ltd., Tokyo, Japan). The photo-resist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd., Tokyo, Japan) of 0.0017 mm thick was coated on the wafer at 7000 rpm (for twenty seconds) and at 3500 rpm (subsequently for five seconds) with the spin coater. The photo-resist was baked on a heated plate at 373 K for ninety seconds. The pattern for the micro grooves was drawn on the wafer with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To save the 0.001 mm

thickness of parylene coating, the dimensions of the width of the three kinds of grooves on the mold are 0.003 mm, 0.005 mm, and 0.007 mm, respectively (Fig. 1). To control the dimension of the pattern on the mold with the laser drawing system, the parameters were selected as follows: the voltage of 3.25 V, the velocity of 0.15 mm/s, the acceleration of 0.375 mm/s^2. The pattern was baked on the heated plate at 393 K for five minutes.

The photo-resist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for 60 seconds. The wafer was rinsed two times with the distilled water for three minutes, and dried by the spin-drier. To increase the adhesiveness of the coating, the wafer was baked at 393 K for five minutes. The wafer was etched with the plasma gas using a reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan) to make lines of the micro grooves of 0.001 mm depth. The gas of sulfur hexafluoride (10 cm^3 min^{-1}) with argon (20 cm^3 min^{-1}) and with oxygen (10 cm^3 min^{-1}) was applied on the wafer at 15 Pa for three minutes. To exfoliate the residual photo-resist material from the surface, the wafer was exposed to the oxygen gas of 30 milliliter per minute at power of 100 W for five minutes using a compact etcher (FA-1, Samco Inc., Kyoto): (the oxygen plasma ashing).

The dimensions of the three kinds of the micro grooves of the mold were measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). The morphology along the transverse lines of ridges was traced. The morphology of the micro grooves was observed by a scanning electron microscope (SEM, JSM6360LA, JEOL Ltd., Tokyo, Japan). The surface of the wafer with micro pattern was coated with 0.001 mm thickness of parylene in a parylene coater (PDS-2010, Speciality Coating Systems, Indianapolis, USA) (Fig. 1).

After the wafer was enclosed with a peripheral wall of polyimide, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was poured with the curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning Corp., MI, USA) on the wafer. The volume ratio of PDMS to curing agent is ten to one. After degassing, PDMS was baked at 383 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd). The baked disk of PDMS of 0.25 mm thickness was exfoliated from the mold, and sterilized in an autoclave. The disk with the micro ridges was used for the bottom of the dish. Another disk of PDMS, which has a donut shape (50 mm outer diameter, 5.5 mm thickness) with a hole of 33 mm diameter, was made for the peripheral wall of the dish (Fig. 2). These two disks are contacted with the affinity between them, and make a culture area of 8.5 cm^3 at the bottom. The cap for the dish of PDMS was made of PDMS. The dimension of the diameter and the thickness are 50 mm and the 1 mm, respectively. The dish of PDMS was exposed to the oxygen gas for one minute in a reactive ion etching system (FA-1, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing). The dish was preserved in the ultrapure water to keep the hydrophilic property of the surface, before the cell culture.

The contact angles [1] were measured between the disk of PDMS and the medium (D-MEM) at 298 K by the contact angle analyzer (Phoenix-300, Meiwafosis Co., Ltd., Tokyo, Japan), before and after the oxygen plasma ashing.

Experimental System

The mechanical vibration was applied to cultured cells with the following experimental system. A donut shape (2 mm thick, 20 mm outer diameter, and 10 mm inner diameter) of a piezoelectric element (1Z10x20W-SYX(C-21), Fuji Ceramics Corporation, Tokyo, Japan), which has the resonance frequency of 1 MHz, is used for a vibrator. After the piezoelectric element was placed on the silicon wafer, PDMS was poured with the curing agent over the element to make the holder of the element. The piezoelectric element mounted in the holder (total thickness of 2.2 mm) of PDMS was attached on the outside of the bottom of the dish of PDMS (Figs. 2&3). The contact between the holder and the dish is kept by affinity between them without adhesive. To keep acoustic contact between the outside surface of the bottom of the dish of PDMS and the piezoelectric element, Vaseline is filled between them. The micro pattern is located at the center of donut ring of piezoelectric element to be observed by the microscope.

Measurement of Vibration

The vibration in the medium was measured with a piezo probe [19]. The piezo electric element attached on the bottom of the culture dish is vibrated by a function generator (inverse piezoelectric effect). The vibration propagates through the medium in the dish to a piezo probe, which is dipped in the center of the medium in a well. The piezo probe translates the vibration to the electric oscillation (piezoelectric effect). The oscillation was monitored by an oscilloscope.

Cell Culture

Four kinds of cells were used in the test. C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), 3T3-L1 (mouse fat precursor cells, cell line of 3T3 mouse), Hepa1-6 (mouse hepatoma cell line of C57L mouse), L929 (fibroblast connective tissue of C3H mouse). The passage between the fourth and the eighth of cell line was used in the experiment with vibration (Table 1). The passage between the third and the eighth of cell line was used in the experiment without vibration (control).

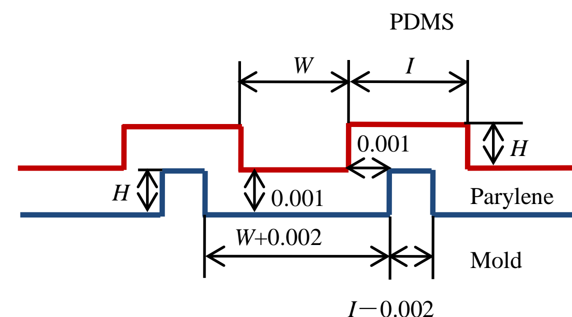

Fig. 1: Dimension for parylene coating on mold for micro ridges on PDMS. Unit of numbers is mm.

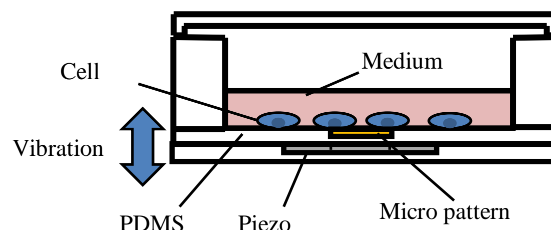

Fig. 2: Cell culture on vibrating scaffold.

Fig. 3: Cell on the vibrating scaffold placed in chamber of incubator at microscope.

Table 1: Passage of cell line.

Cell	Control	Vibration
C2C12	8	6
3T3-L1	7	8
Hepa1-6	3	4
L929	6	5

EMEM (Eagle's Minimum Essential Medium) containing 10% FBS (decomplemented fetal bovine serum) and 1% penicillin/ streptomycin was used for the culture medium of L929. D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (decomplemented fetal bovine serum) and 1% penicillin/ streptomycin was used for the culture medium of C2C12, 3T3-L1, and Hepa1-6.

Cells were seeded on the dish of PDMS at the density of 20 cells/mm^2. The culture dish with the piezoelectric element was kept in the small chamber of the incubator placed on the stage of the microscope to maintain both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent (Fig. 3). The cells were continuously observed with the phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture.

After the culture term for adhesion of cells to the scaffold with the micro pattern, the vibration was continuously applied to the scaffold with micro pattern. The term before vibration was one hour for Hepa1-6. The term before vibration was three hours for 3T3-L1, C2C12, and L929. After the start of vibration, the behavior of cells was successively recorded with the time-lapse microscopic pictures: pseudo extension, migration, exfoliation, and re-adhesion. The interval of the time-lapse was selected to be several minutes: between two and five minutes. The exceptional interval longer than ten minutes was selected for the long time recording for 24 hours.

3. RESULTS

The tracing of the surface morphology across the lines of ridges on the mold measured by the laser microscope is exemplified in Fig. 4. The tracing shows the height of 0.0013 mm, width of 0.0010 mm, and interval of 0.0049 mm.

Table 2 shows the mean values of the dimensions of the three kinds of the grooves on the mold, as the result of the measurement with the laser microscope.

The scanning electron microscope images of the lines of ridges are exemplified in Fig. 5. Fig. 5 shows the border of three kinds of ridges.

Fig. 6 shows the contact angle of D-MEM on PDMS. The angle is 92 degree before the oxygen plasma ashing, and 3.7 degree after the oxygen plasma ashing. The property of the surface changed from hydrophobic to hydrophilic by the oxygen plasma ashing.

Table 2: Dimension of the groove on the mold.

Group	mm		
	Depth	Width	Interval
A	0.0012	0.001	0.0028
B	0.0013	0.001	0.0049
C	0.0013	0.001	0.0069

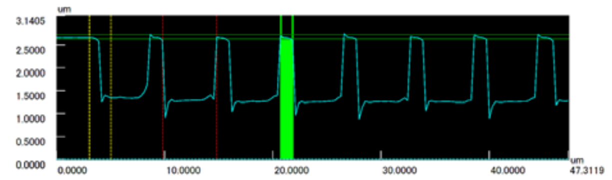

Fig. 4: Surface morphology of mold measured by laser microscope.

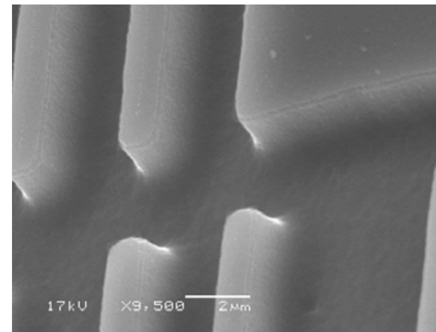

Fig. 5: Scanning electron microscope image of the lines of grooves on mold before parylene coating. Perspective view.

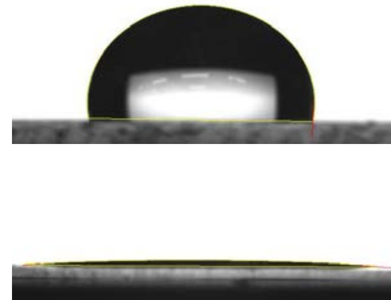

Fig. 6: Contact angle of D-MEM on PDMS before (upper) and after (lower) oxygen plasma ashing.

The vibration in the medium measured with the piezo probe is exemplified in Fig. 7. The wave pattern shows the frequency of 1 MHz.

The behavior of cells on the vibrating ridges is exemplified in Figs. 8-11. The longitudinal direction of ridge is vertical. The upper left square area of 0.5 mm edge has a smooth surface, and has no ridges. The width of ridges in each quarter area among 1.0 mm edge is 0.001 mm (upper right), 0.003 mm (lower right), and 0.005 mm (lower left), respectively. Dimension from left to right is 1.3 mm in Figs 8-11.

The behavior of C2C12 on the vibrating ridges is exemplified in Fig. 8. The cells keep adhesion on the top of the ridge and migrate along the longitudinal direction of the ridges, although some cells exfoliate from the smooth surface without ridges. Migration is faster at the ridges of the narrow ridges (0.001 mm and 0.003 mm width) than at the ridges of the wide ridges (0.005 mm width). Vibration accelerates migration and extension of cells, especially on the ridges of 0.001 mm width. The repetitive elongation along the longitudinal direction of the ridges is observed on the vibrating scaffold. The elongation occurs most frequently on the ridges of 0.003 mm width.

Fig. 9 shows the behavior of 3T3-L1 on the ridge. The number of cells adhered on the scaffold is smaller than that of the other kinds of cells. Cells adhere faster on the smooth surface than on the ridges. Cells extend pseudo to every direction regardless to the longitudinal direction of the ridges. On the ridges of 0.001 mm and of 0.003 mm width, cells make orientation along the longitudinal direction of the ridges in 24 hours of incubation, after the stop of the vibration for three hours.

Fig. 10 shows the behavior of Hepa1-6 on the ridge. The cell has round shape, so that it is difficult to distinguish deformation by adhesion. A few cells migrate along the longitudinal direction of the ridges of 0.001 mm and 0.003 mm without vibration. The vibration was started, when the cells extend pseudo. The number of adhesion of cell decreases on the wider ridges with vibration. The cell repeats extension and shrinkage of pseudo on the vibrating scaffold regardless of the longitudinal direction of ridges. Vibration decelerates migration of cells. A few cells make orientation along the longitudinal direction of the ridges in 24 hours of vibration. The exfoliated cells do not re-adhere to the scaffold during vibration. Cells make orientation along the longitudinal direction of the ridges of 0.001 mm width in 24 hours of incubation, after the stop of the vibration for 24 hours.

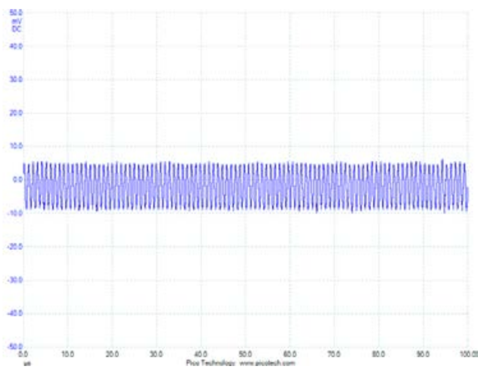

Fig. 7: Vibration in the medium. From left to right is 0.1 s.

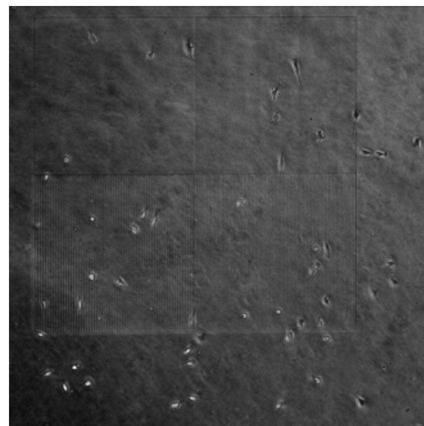

Fig. 8: C2C12 cultured for 3 hour with vibration.

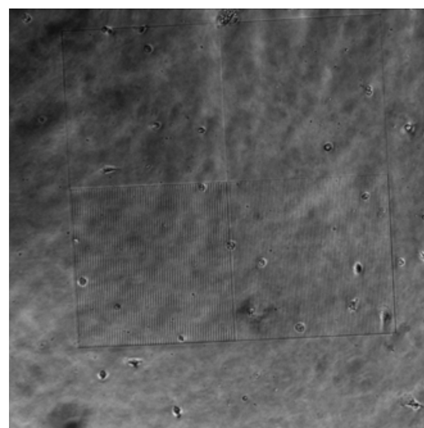

Fig. 9: 3T3-L1 cultured for 3hour with vibration.

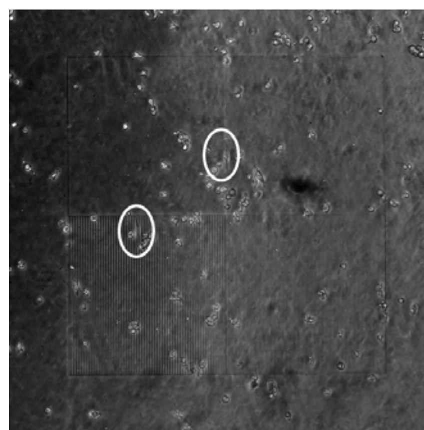

Fig. 10: Hepa1-6 cultured for 24 hour with vibration. Circles show oriented cells.

Fig. 11 shows the behavior of L929 on the ridge. The cells adhere to the scaffold in one hour. The cells slightly extend to the longitudinal direction of the ridges, but do not migrate along the ridges. Cells adhere to the scaffold more stable than the other kinds of cells, so that every cell keeps adhesion at the same place for three hours under vibration. Fig. 12 exemplifies migration of C2C12 on the ridges of 0.003 mm width for three hours with vibration. Data shows the position

of every ten minutes from original position (origin). The coordinates of y and x are longitudinal and perpendicular axis of the ridge, respectively. C2C12 migrates along the ridge. Fig. 13 shows migration of cells on the ridges of 0.003 mm width for three hours with vibration. Data shows the ratio of Δy per Δx, where Δy and Δx are migration for every ten minutes. The ratio higher than one corresponds to the migration along the ridge. The ratio is high at C2C12. Table 3 shows the number of cells of four kinds oriented along the longitudinal direction of micro ridge, and adhered on the scaffold at each timing. The number was counted in each square area of 0.5 mm edge. When the angle between the longitudinal axis of the cell and the longitudinal axis of the ridge is smaller than 0.17 rad, the cell is counted as the oriented cell.

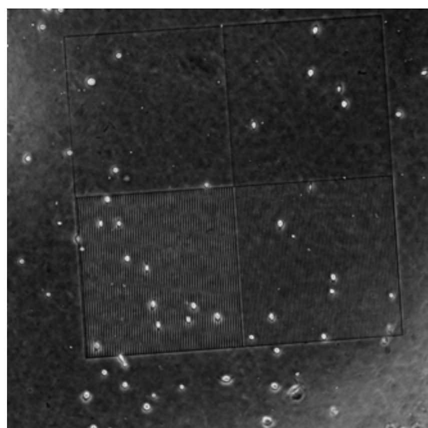

Fig. 11: L929 cultured for 3 hour with vibration.

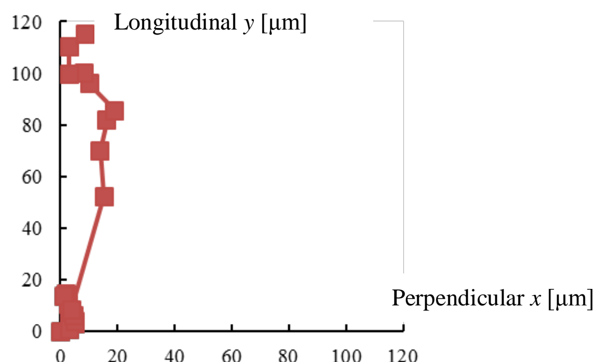

Fig. 12: Migration of C2C12 on 0.003 mm width for 3 hour with vibration.

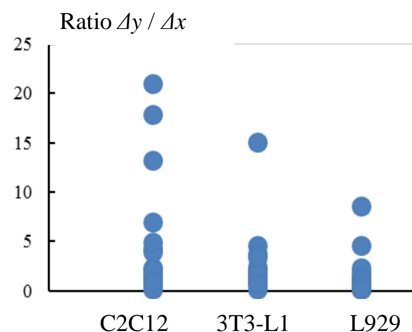

Fig. 13: Direction of migration of C2C12 on 0.003 mm width for 3 hour with vibration.

Table 3: Number of cells. vib: vibration. Orientation/ Adhesion.

C2C12	Flat	Width of ridge [mm]		
		0.001	0.003	0.005
3 h	0/3	1/7	2/9	0/8
+3 h vib	0/4	3/6	2/9	5/7

3T3-L1	Flat	Width of ridge [mm]		
		0.001	0.003	0.005
3 h	0/4	0/4	0/9	0/5
+3 h vib	0/5	0/3	1/5	0/5
+24 h	2/8	5/8	3/7	3/12

Hepa1-6	Flat	Width of ridge [mm]		
		0.001	0.003	0.005
1 h	0/35	0/33	0/13	0/14
+24 h vib	0/19	1/26	0/20	1/35
+24 h	0/14	5/14	0/23	0/18

L929	Flat	Width of ridge [mm]		
		0.001	0.003	0.005
3 h	0/5	0/5	0/9	0/14
+3 h vib	0/4	0/5	1/10	0/12

4. DISCUSSION

The effect of the height of micro ridges on the orientation of C2C12 was studied in the previous study. The experimental results show that myoblasts adhere on the top of the ridge and align to the longitudinal direction of the micro ridges with the height between 0.00015 mm and 0.0025 mm [1]. The height of the micro ridge is selected to be 0.001 mm in the present study. The time-lapse is convenient not only to catch the moment of the behavior of the cell, but also to observe the movement of the cell. The migration of the cell is able to be traced, because the micro ridges play a role as markers. In the present methodology of lithography technique, the dimension of lines of rectangular ridges smaller than 0.001 mm is difficult to control. The dimensions of the lines of rectangular ridges of the present study are selected to save the thickness of parylene coating.

When the voltage, which is applied to the probe, increases, the surface of the medium becomes convex and vibrates (Vp-p > 16 V) [11]. In the present sturdy, Vp-p is 16 V, although micro vibration might have local stirring effect. Vibration makes agitation in the liquid. Vibration at low frequency makes flow in the liquid. Vibration with high energy destroys structure. The surface of the medium is not vibrating macroscopically during cell culture in the present experiment.

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. The previous study shows that electrical stimulation enhances differentiation of muscle cells [15]. The previous studies show that a mechanical field,

on the other hand, governs behavior of cells. The shear flow governs the orientation of endothelial cells [17, 18]. Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [16]. The mechanical stimulation can decrease proliferation of cells [16]. The mechanical stress also exfoliates several cells, which makes vacancy around the adhered cell. The differentiation might be optimization of cells to the changing environment. The mechanical stress can accelerate differentiation of C2C12 into myotubes.

Low-intensity ultrasonic treatment might increase mass transport, and enhance C2C12 cell proliferation, metabolic activity, and differentiation of cells [10]. The wave length of ultrasonic vibration at frequency of 1 MHz through water is 1 mm, when the velocity of ultrasonic vibration through water is 1 km/s. The wave length is near the dimension of aggregation of cell. Vibration accelerates adhesion of C2C12 on the micro ridges, although vibration decelerates adhesion of 3T3-L1 on the micro ridges. C2C12 migrates and extends along the longitudinal axis of the ridges, although 3T3-L1 migrates regardless of the direction of the ridges.

5. CONCLUSION

The effect of micro ridges on cells cultured at vibrating scaffold has been studied *in vitro*. Four kinds of cells were used in the test: C2C12 (mouse myoblast), 3T3-L1 (mouse fat precursor cells), Hepa1-6 (mouse hepatoma), L929 (fibroblast connective tissue of mouse). The experimental results show that the cells adhere, deform and migrate on the scaffold with micro pattern regardless of the ultrasonic vibration. The effects of the vibration and the micro pattern depend on the kind of cells.

6. ACKNOWLEDGMENT

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