Effect of Ultrasonic Vibration on Culture of Myoblast

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ABSTRACT

The effect of mechanical stimulation of vibration on cultured cells has been studied *in vitro*. To apply the vibration on the cultured cells, a piezoelectric element was attached on the outside surface of the bottom of the culture dish. The piezoelectric element was vibrated by sinusoidal alternating voltage (Vp-p < 16 V) at 1.0 MHz generated by a function generator. C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the test. Cells were seeded on the polystyrene bottom of the dish at the density of 1000 cells/cm^2. After adhesion of cells in 24 hour, cells are exposed to the ultrasonic vibration in two manners: intermittently (for thirty minutes a day) or continuously. The cells were observed with a phase contrast microscope every day during the cell culture. The experimental results show that C2C12 proliferates and differentiates even under continuous vibration.

Keywords: Biomedical Engineering, C2C12, Cell Culture and Vibration.

1. INTRODUCTION

Ultrasonic vibration has been applied to human body in several cases: measurements of deep structure of body, and lithotrity [1, 2].

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. In most cases, the vibration with low frequency was applied to cell culture: shaking the scaffold, or vibrating the scaffold at audible frequency.

Several methodologies have been clinically applied to regenerative medicine. The acceleration technique for proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [3-11]. 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, and differentiation. Several methods have been designed to apply the mechanical stress to cells [4-12].

In the present study, the effect of ultrasonic vibration on culture of myoblast has been studied *in vitro*.

2. METHODS

Experimental System

Mechanical vibration was applied to cultured cells with the following experimental system. A piezoelectric element (1Z28D-SYX, Fuji ceramics Corporation, Tokyo), which has 28 mm diameter and 1 MHz resonance frequency, is used for vibrator (Fig. 1). A polystyrene dish of 60 mm of internal diameter (IWAKI 3010-060-MYP) was used for cell culture. A polydimethylsiloxane (PDMS) disk, which contains a piezoelectric element, is attached on the outside surface of the bottom of the dish (Fig. 2).

Fig. 1: PDMS disk contains piezoelectric element. Waves on the drop of water reveal vibration.

The contact between the disk and the dish is kept by affinity between them without adhesiveness. To keep acoustic contact between the outside surface of the bottom of the dish and the piezoelectric element, water is filled between them.

The piezoelectric element was vibrated by sinusoidal alternating current at 1.0 MHz generated by a function generator (PM8572A, Tabor Electronics Ltd.) (Fig. 3). Variation was made on the amplitude (peak to peak) of the sinusoidal voltage (Vp-p): between 3V and 16 V (Fig. 4).

The surface of the medium was observed macroscopically, while the ultrasonic vibration is applied during cell culture.

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the test. In the case of intermittent vibration test, sixth passage of C2C12 was used. In the case of continuous vibration test, fifth passage of C2C12 was used. Cells were seeded on the polystyrene bottom of the dish at the density of 1000 cells/cm^2. Cells are cultured at 310 K with 5 % of CO_2 in an incubator. After adhesion of cells in 24 hour, cells are exposed to the ultrasonic vibration in two manners: intermittently (for thirty minutes a day) or continuously.

C2C12 was cultured with the D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (fetal bovine serum, after decomplementation). The medium containing penicillin and streptomycin was refreshed every two days.

The culture dish with the piezoelectric element was kept in the incubator to maintain both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent (Fig. 5). Sinusoidal voltage is transmitted to the piezoelectric element from the function generator placed outside of the incubator (Fig. 6).

The cells were observed with a phase contrast microscope (IX71, Olympus, Tokyo) every day during the cell culture for 5 days. On the outer surface of the bottom of the dish, a line of 0.2 mm width or a circle of 1 mm diameter is marked to trace the same area for the cell culture. When the number of cells is counted with Burker-Turk hemocytometer, cells are exfoliated with trypsin, and stained with trypan blue (GIBCO) to eliminate dead cells.

The electric pulses were applied to the cells through the medium at the end of the culture to confirm differentiation of C2C12 to myotubes.

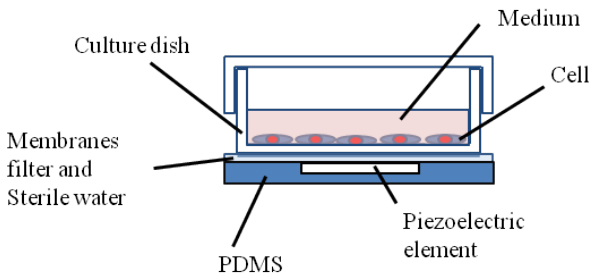

Fig. 2: Piezoelectric element attached on the bottom of the dish.

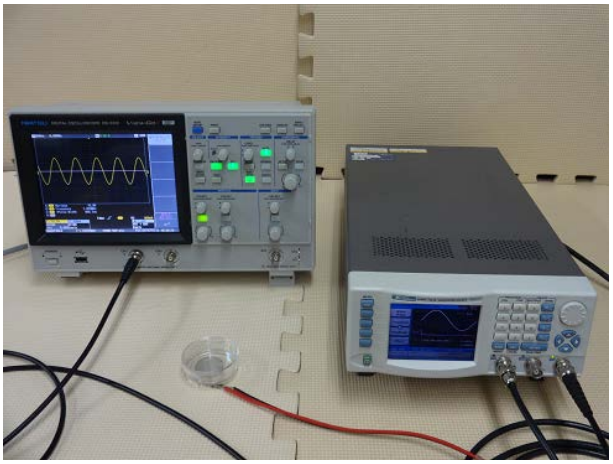

Fig. 3: Electric circuit: oscilloscope (left), piezoelectric element (middle), function generator (right).

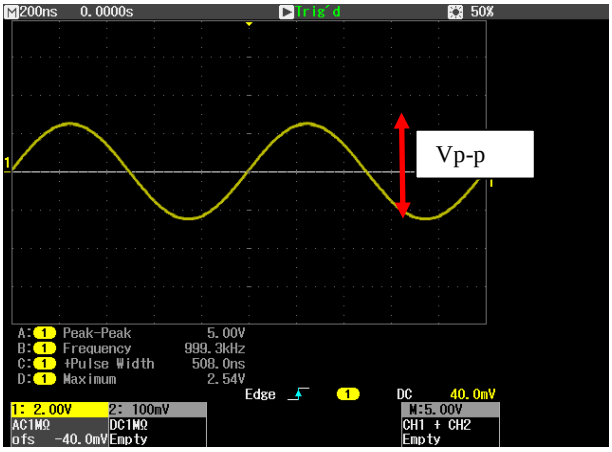

Fig. 4: Voltage tracings of 1 MHz with function generator.

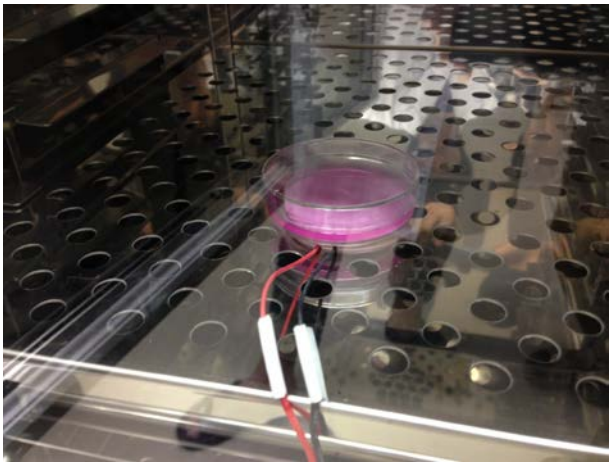

Fig. 5: Cell culture dish with piezoelectric element in incubator.

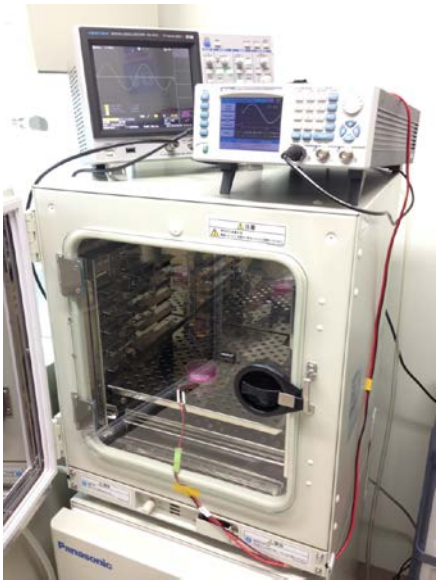

Fig. 6: Sinusoidal voltage is transmitted to the piezoelectric element from function generator placed outside of the incubator.

3. RESULTS

The observation of the surface of the medium shows no macroscopic vibration during cell culture in the present experiment (Fig. 7B). Two lines of lights are reflected on the surface in Fig. 7. The surface of the medium shows several slightly convex parts, while the ultrasonic vibration is applied during cell culture with the condition in the present study. At the intensity of the vibration, the electric current is estimated to 0.1 A. The value is calculated from the voltage between the ends of the resistance (Vp-p = 12 V), when the resistance of 51 S^{-1} is connected in series of the piezoelectric element.

To select the density of seeding, C2C12 was cultured without vibration (Figs. 8 & 9). Fig. 8 shows that C2C12 proliferates to confluent state in three days, when C2C12 seeded in the density of 5000 cells/cm^2. Fig. 9 shows, on the other hand, that C2C12 proliferates to sub-confluent state in three days, when C2C12 seeded in the density of 1000 cells/cm^2. For the density of seeding, 1000 cells/cm^2 is selected in the following cell culture.

The experimental results show no significant effect of continuous vibration at 10 kHz for four days on proliferation of C2C12 (Figs. 10-12). The frequency of 10 kHz makes audible sound, which confirms continuous vibration at the piezoelectric element. Some cells of C2C12 show morphological change after vibration: extended pseudopodia, flake of pseudopodia, decrease of cytoplasm, and exfoliation of cells (Fig. 13).

Figs. 14-17 exemplify C2C12 cultured under intermittent vibration at 1 MHz for 30 minutes a day compared with control study. Fig. 18 shows the number of cells in relation to amplitude of the sinusoidal voltage of vibration at 1 MHz. In every cases, the number of cells increases more than 10 times in 5 days. The number of cells is standardized with that of control in Fig. 18. The proliferation of C2C12 tends to accelerate with the ultrasonic vibration of Vp-p lower than 13 V.

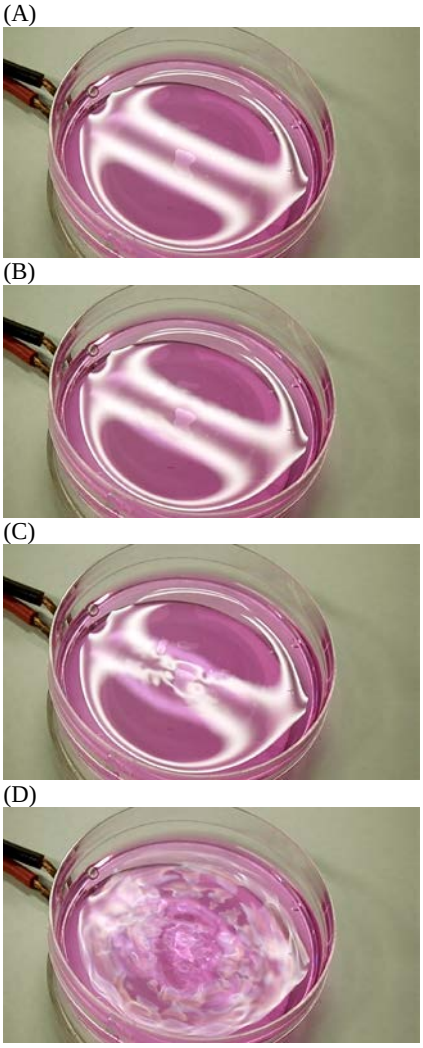

Fig. 7: Surface of medium before vibration (A) and during vibration at 6 V (B), 10 V (C), 16 V (D).

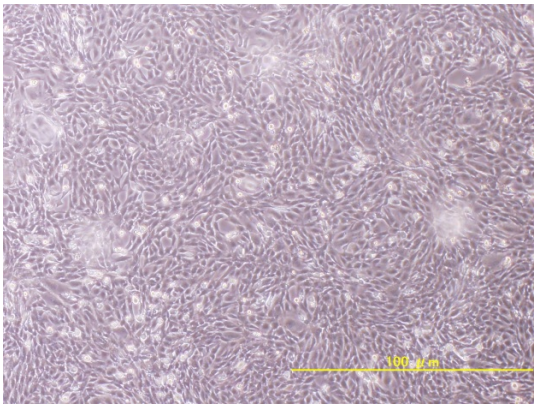

Fig. 8: C2C12 after 3 days after seeding of 5000 cells/cm^2 without vibration. Dimension from left to right is 2 mm.

Figs. 19-21 show C2C12 culture under continuous vibration compared with control study. Even under continuous vibration, C2C12 differentiate to myotubes, which shows repetitive contraction with stimulation of electric pulses.

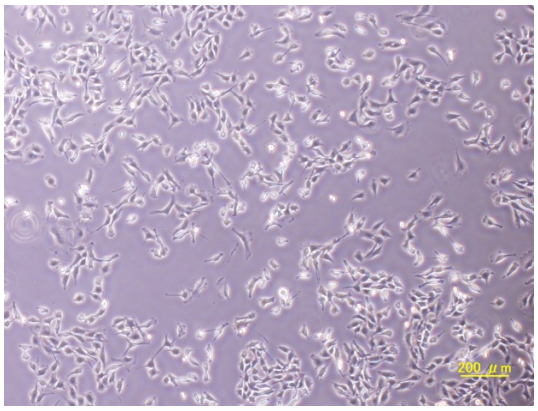

Fig. 9: C2C12 after 3 days after seeding of 1000 cells/cm^2 without vibration. Dimension from left to right is 2 mm.

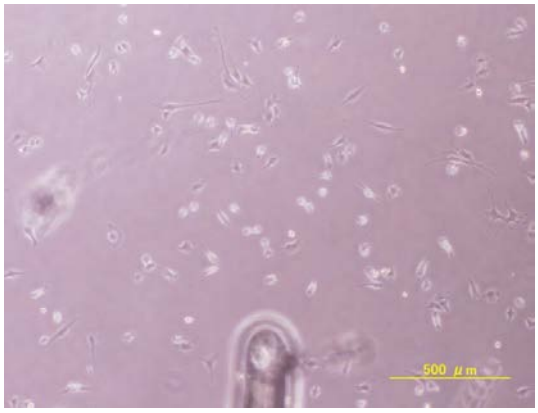

Fig. 10: C2C12 after 1 days after seeding of 1000 cells/cm^2 without vibration. Dimension from left to right is 2 mm.

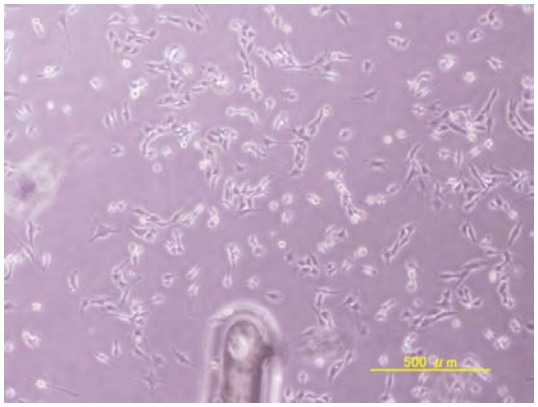

Fig. 11: C2C12 after 3 days after seeding of 1000 cells/cm^2 without vibration. Dimension from left to right is 2 mm.

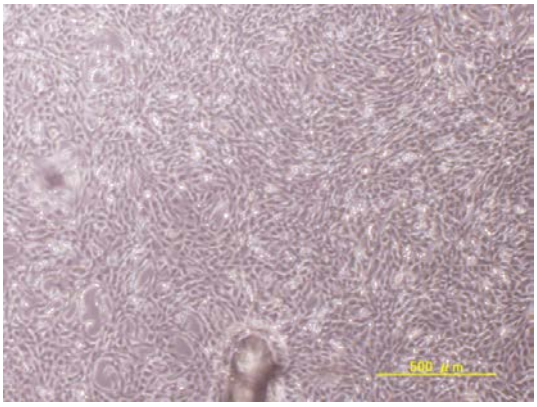

Fig. 12: C2C12 after 5 days after seeding of 1000 cells/cm^2 without vibration. Dimension from left to right is 2 mm.

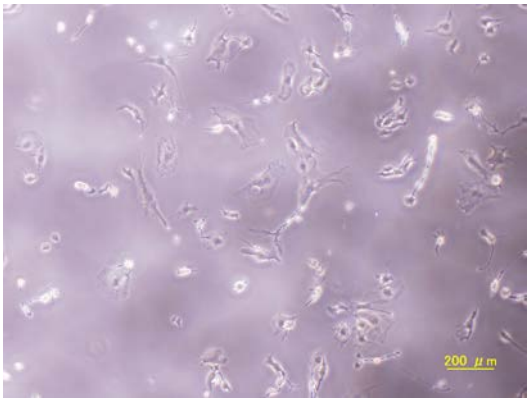

Fig. 13: C2C12 after vibration of 16 V for 30 min.

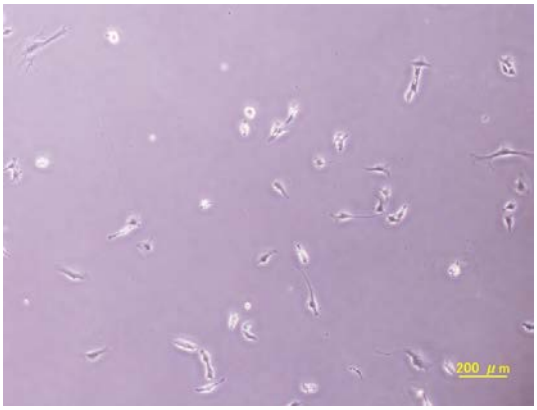

Fig. 14: C2C12 before vibration.

4. DISCUSSION

When C2C12 is seeded with the density of 5000 cells/cm^2, cells proliferate to confluent density in 3 days, which is too short to observe the effect of vibration to proliferation of cells. That is the reason why the density of 1000 cells/cm^2 is selected in the present experiment.

When the voltage, which is applied to the probe, increases, the surface of the medium becomes convex and vibrates (Vp-p > 16 V). The prominent vibration might generate macroscopic flow, which has stirring effect. In the present sturdy, variation has been made on Vp-p in the range smaller than 16 V, although micro vibration might have local stirring effect.

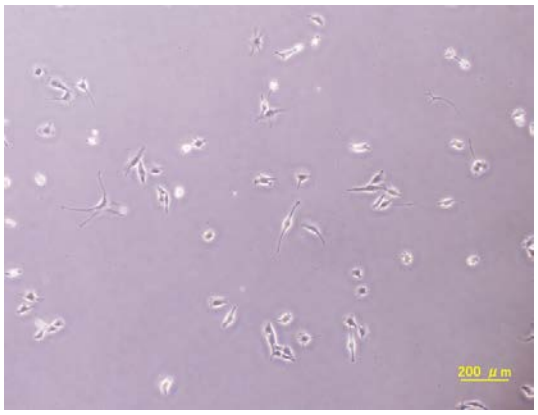

Fig. 15: C2C12 after vibration of 3 V for 30 min.

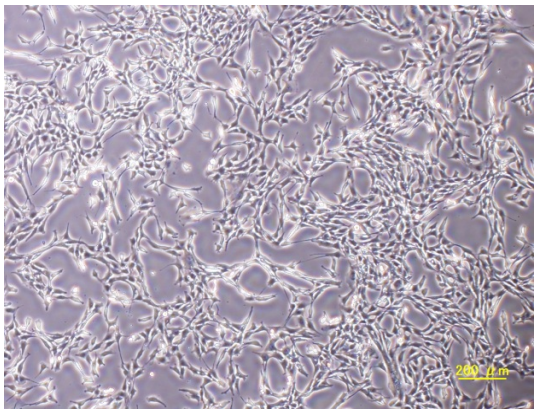

Fig. 16: C2C12 after 4 days without vibration.

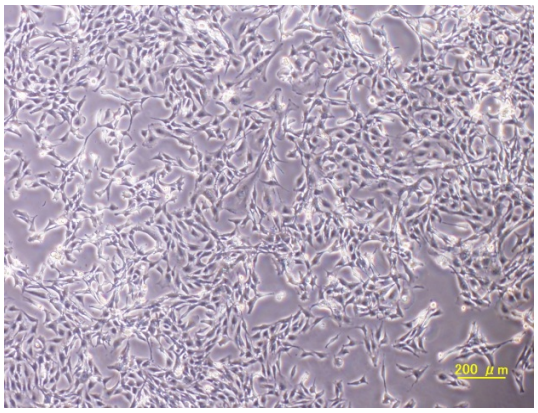

Fig. 17: C2C12 after 24 hours after three days' vibration of 3 V for 30 minutes a day.

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 does not vibrate macroscopically during cell culture in the present experiment. The surface of the medium shows slight convex, while the ultrasonic vibration is applied during cell culture with the condition in the present study (Fig. 7).

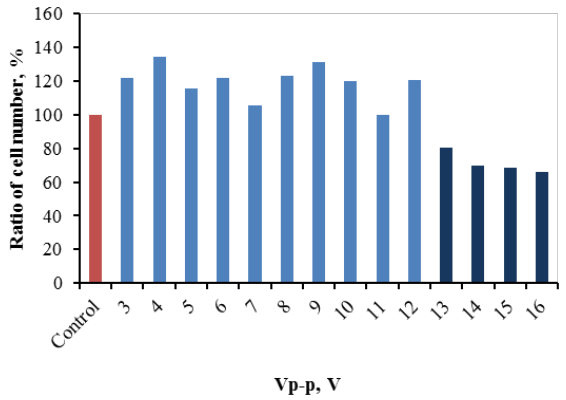

Fig. 18: Relation between number of cells and Vp-p after 5 days of culture. Datum at 0 V shows datum without vibration.

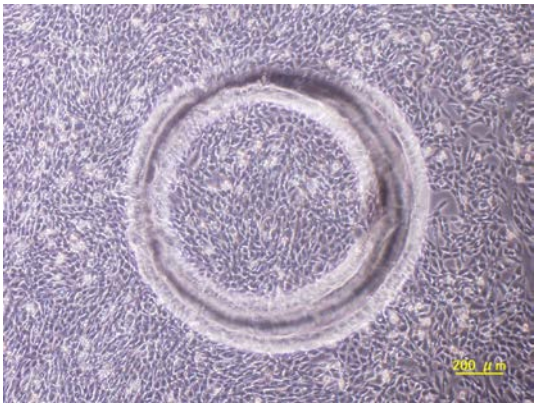

Fig. 19: C2C12 cultured for 48 hours under continuous vibration. Diameter of the circle is 1 mm. Circle marked on the outside of the bottom has 1 mm diameter to trace the same area of culture.

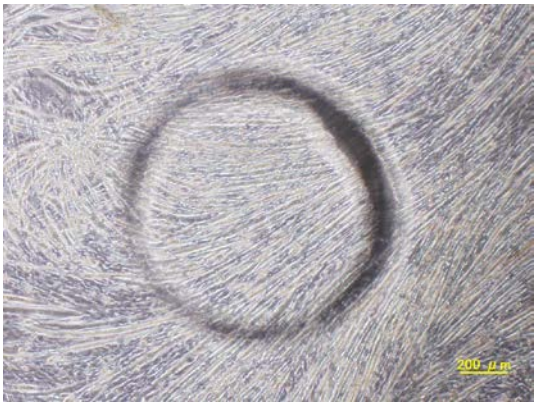

Fig. 20: C2C12 cultured for 11 days without vibration. Dimension from left to right is 2 mm.

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 [3]. Another study shows that mechanical stimulation improves a tissue-engineered human skeletal muscle [4].

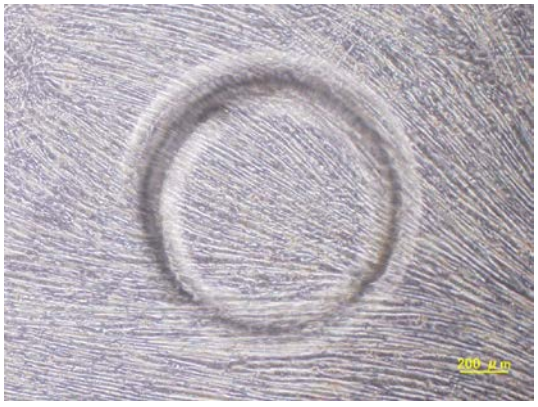

Fig. 21: C2C12 cultured for 11 days under continuous vibration. Dimension from left to right is 2 mm.

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 [9, 11]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [5]. The direction of the mechanical field affects fibroblasts [7].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [8]. The mechanical stimulation can decrease 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 the changing environment. The mechanical stress can accelerate differentiation of C2C12 to make myotubes (Fig. 17).

The effect of low-frequency ultrasound on neuronal activity was studied in the previous study [1]. Low-intensity ultrasound treatment might increase mass transport, and enhance C2C12 cell proliferation, metabolic activity, and differentiation of cells [2]. The wave length of ultrasound at frequency of 1 MHz through water is 1 mm, when the velocity of ultrasound through water is 1 km/s. The wave length is near the dimension of aggregation of cell of 0.01 mm.

5. CONCLUSION

Effect of ultrasonic vibration on culture of myoblast has been studied *in vitro*. To apply the vibration on the cultured cells, a piezoelectric element was attached on the outside surface of the bottom of the culture dish. The experimental results show that C2C12 proliferates and differentiates even under continuous vibration.

6. ACKNOWLEDGMENT

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