Development of Three-Dimensional Micro Vibration Stage and Its Application to Control Device for Cell Culture*

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Abstract

In this study, a new three-dimensional micro vibration stage is developed, in which a doubly steric L-shaped clamped-free beam type vibrator is utilized. The developed three-dimensional micro vibration stage, which has very simple structure and is quite easily sterilized, is extended to control system of cell culture. Focusing on osteoblast character which is sensitive to external mechanical stimuli, a normal human osteoblast is chosen as a test subject. Dynamic stimulation is applied to the normal human osteoblast utilizing the developed three-dimensional vibration stage. In order to estimate effect of dynamic stimulation upon cultured cell, we define parameters of projected area and slenderness ratio of the cultured normal human osteoblast. Statically cultured cells of 4th and 6th passage were provided for experiments. It was observed that the projected area of normal human osteoblast increased, while the slenderness ratio decreased according to the number of cell passage increasing. Shape of the 6th passage of dynamically stimulated cells was not similar to that of same passage of control, but to that of 4th passage of control. From the viewpoint of morphology, the present study has shown the significant effect of dynamic stimulation upon cultured cells.

Key words: Three Dimensional Vibration Stage, Piezoelectric Vibrator, Three Dimensional Stimulation, Normal Human Osteoblast, Culture Control, Actin Filament

1. Introduction

All cells are more or less facing to extracellular mechanical forces. Presumably, all cells have a mechanism to detect mechanical stimulation from outside⁽¹⁾⁻⁽³⁾. Mechanical stimulation from outside is transduced to biochemical signal by intracellular mechanosensor molecules. Then, homeostatic reactions are induced such as cytoskeletal restructuring. This is so-called mechanotransduction^{(4),(5)}, and the understanding of this is one of the great importance in bioscience field. Starting with discovery of a channel activated by cell membrane stretching by Guharay and Sachs⁽⁶⁾ in 1984, many studies have been reported on the channel molecules, which are working as mechanosensor^{(7),(8)} on the cell membrane. Based on the evidences reported above, in this study, we assume that the effect of applied dynamic stimulation

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onto cultured cell will appear in its cytoskeletal morphology.

On the other hand, in this field, there are numerous reports investigating from a viewpoint of mechanics. The observation of cell reaction, caused by exerted extraneous mechanical stimulation of various conditions such as distribution, magnitude and cycles, is very important to clarify the detection and reaction ability to environmental mechanical stimulation. Various methodologies of exerting mechanical stimulation have been investigated such as compression^{(9),(10)}, fluid flow shear stress⁽¹¹⁾, tensile strain⁽¹²⁾, and local deformation by a microneedle⁽¹³⁾. In case of utilizing vibration stimulation, there are two typical types. The first case is a local vibration stimulation to a cultured cell imposed by a micro probe. Konno et al.⁽¹⁴⁾⁻⁽¹⁶⁾ developed the three-dimensional actuator to enforce dynamic stimulation on a living cell and tissue, and established the evaluation system by incorporating the sensor system developed previously^{(17),(18)}. However, this system was assumed for large cells, such as few hundred μm in diameter or more large tissue such as a cartilage tissue, so the system was not available for cultured cell directly. The other case is a whole vibration stimulation to cultured cells in well plate. Various cultured cells were stimulated by well plates which were placed on excitation devices⁽¹⁹⁾⁻⁽²¹⁾. Normally, these methods were only available in uniaxial vertical vibration.

In this study, a new compact three-dimensional vibration stage is developed. This device provides the ability to simulate mechanical environment of cells *in vivo*. Three piezoelectric ceramics are bonded in three orthogonal directions, so that the doubly steric L-shaped vibration stage is able to perform three-dimensional vibration up to around 30-60 μm in each direction. Cultured cells in artificial environments are normally free from mechanical stimulation. The developed three-dimensional vibration stage is extended to control device for cell culture. This device is useful as an effective means of investigation not only in load response of cultured cell but also initiation of cellular functions. In order to demonstrate the validity of the device, horizontal vibration stimulation is applied to the cultured normal human osteoblast utilizing the three-dimensional vibration stage. The cell morphology is observed to evaluate effect of the exerted vibration stimulation.

2. Methods of experiments

2.1 Development of the three-dimensional vibration stage

Figure 1 shows a construction of the proposed three-dimensional vibration stage, which is designed to exert vibration stimulation non-invasively and three-dimensionally to cultured cells on a dish. This vibration stage is assumed to be used in a CO_2 incubator, so it has a compact and simple structure with clamped-free boundary conditions. In order to excite vibrations in three orthogonal directions, it was manufactured from one L-shaped stainless steel plate (thickness: 0.2 mm, width: 5 mm) into a doubly steric L-shaped vibrator by bending 90 degrees at two mid points. Furthermore, a square-shaped stage section was formed by bending 90 degrees three times at the tip of the vibrator. Consequently, a whole structure of the three-dimensional vibration stage was made from one sheet of stainless steel plate. An acrylic square plate was bonded in the bottom of the stage section to mount a small-sized culture dish.

Figure 2 shows a close-up of the real three-dimensional vibration stage and its maximum amplitudes. Three piezoelectric ceramics (PZT, 10 x 5 x 0.3 mm) were bonded in X, Y and Z direction, respectively, so that the vibration stage is able to perform first mode of vibration up to around 30-60 μm in each direction. The electric wires to input signals to each PZT were bonded by a few amount of electrically

conductive adhesive. Very fine (diameter: 0.1 mm) polyurethane wires were selected in order to remove the kinetic ill effect to vibrating condition caused by the stiffness of electric wires connected to the PZTs. One end of the vibrator (13 mm margin for fixation) was clamped by two brass blocks (13 x 16 x 5 mm) to realize clamped boundary condition. By inputting signals to each PZT, the vibration stage is able to generate any vibration in needed axial direction. Furthermore, the vibration stage is capable to perform complicated two or three-dimensional vibration simultaneously.

At the tip of the vibrator, a square-shaped stage section (10 x 10 mm) was

Fig. 1 Construction of the 3D vibration stage. Three piezoelectric ceramics (green colored portion) are bonded in X, Y and Z direction, respectively, so that the vibration stage is able to perform first mode of vibration in the three orthogonal directions.

Fig. 2 Close-up of the real 3D vibration stage and its maximum amplitudes.

designed to mount a small-sized culture dish (made of polystyrene, surface-treated for cell culture, inside diameter: 7.0 mm, outside diameter: 9.0 mm, height: 12.5 mm). The developed three-dimensional vibration stage has a quite simple structure, since the vibrator and the stage section are made from one stainless steel plate. Therefore, the sterilization treatment, which should be done before setting the vibration stage in a CO₂ incubator to avoid contamination, can be carried out without difficulty. On the other hand, there is a risk of occurring short-circuit due to forming dew condensation because of the condition of 100 % humidity inside the CO₂ incubator. Therefore, to prevent the risk, the water-resistant silicone was coated to cover all PZT surfaces of the three-dimensional vibration stage. As a result of this treatment, the long-term operation of the three-dimensional vibration stage has been realized.

2.2 Experimental setup

The schematic diagram of the experimental setup is shown in Fig. 3. By using signal output function of a FFT analyzer (ONO-SOKKI, CF-360Z) and an amplifier (NF Electric Instruments, HSA4012), a continuous sine wave is generated and applied to the PZT of the vibration stage via a signal selector/distributor. Then the cultured cells on a dish mounted on the vibration stage are stimulated dynamically with a given frequency, amplitude and direction. On the other hand, vibrating condition of the stage inside the CO₂ incubator can be monitored through unexcited PZT and the other FFT analyzer (ONO-SOKKI, CF-5220Z).

The overall view of the real experimental setup is shown in Fig. 4. In order to avoid contamination, a clean booth (AIRTECH JAPAN, LTD., MAC-II-31) was installed at the laboratory to keep a clean air space for cell culture experiment. A HEPA filter unit was set at the top of the booth and cleaned air (cleanliness class: 10000) was blown down into the booth constantly. A bio-clean bench (SANYO, MCV-B131F) was connected next to the clean booth and a CO₂ incubator (SANYO, MCO-18AIC(UV)) was placed inside the clean booth. All the cell culture-related manipulations were conducted inside the bio-clean bench. Eight developed three-dimensional vibration stages were set inside the CO₂ incubator after appropriate sterilization treatment (Fig. 5). Practically, these eight vibration stages were electrically connected in parallel via a signal selector/distributor. FFT analyzers and an amplifier were set outside of the clean booth and were connected to all three-dimensional vibration stages inside the CO₂ incubator.

The inverted phase contrast microscope (OLYMPUS, IX71) with the fluorescent equipment (OLYMPUS, IX-FLA) was used in occasion to observe the cultured cell. Microscopic images are displayed on a monitor (EIZO NANA0 Corp., FlexScan S1721) through the microscope digital camera (OLYMPUS, DP71), and are saved to a computer (OLYMPUS, DP71-WPCXP).

2.3 Materials

In this study, focusing on osteoblast character which is sensitive to external mechanical stimuli, normal human osteoblast (NH0st, Cambrex inc., obtained under informed consent, Lot No.5F0582) was chosen as a test subject. Normal cell has a limit of total population doublings and a senescence according to the number of cell passage. Generally, senescent normal cell shows enlarged and flattened morphology^{(22),(23)}. As the effect of senescence, the change of morphology can be recognized in the case of NH0st. Therefore, the effects of the senescence and the applied dynamic stimulation were assumed to be evaluated by observing cell morphologies.

NH0st provided for the experiment, was seeded on culture dishes and statically

(without any mechanical stimulation) cultured in α -MEM (alpha-minimum essential medium, COSMO BIO Co., LTD.) + 10 % FBS (fetal bovine serum, Invitrogen, Lot No: 915045) + 1 % antibiotic substance (penicillin - streptomycin solution stabilized, SIGMA-Aldrich) in the CO₂ incubator (temperature: 37 °C, humidity: 100 % and 5 %

Fig. 3 Schematic drawing of experimental setup.

Fig. 4 Overall view of the real experimental setup. 3D vibration stages are set inside the CO₂ incubator (middle of picture, facing left) and almost all measuring devices are locating outside of the clean booth (far right side).

CO₂- 95 % Air). The passage number of the cell for the experiment was chosen at four or six. These numbers include the cell passage before obtaining.

2.4 Experimental methods

Vibration stimulation was applied to NHOst for several hours continuously utilizing the developed three-dimensional vibration stage and above mentioned experimental system. To evaluate the cell reaction against the applied vibration stimulation, the changes of cell morphologies were observed.

The experimental procedures are as follows. The cells, whose passage reached to four or six by static culture, were reseeded at 6.0×10^2 cell/dish density in the small-sized culture dishes for the developed vibration stages. The amount of the culture medium in each dish is 150 μ l. Cells, immediate after seeding, have generally spherical shape because of trypsin activity. The cell-seeded eight dishes were mounted on the vibration stages quickly. These dishes did not slip on the stage because of friction due to dishes' own weight. Starting from this state, unidirectional sinusoidal vibration was applied to the culture cells by exciting the vibration stages. In case of control, cells were statically cultured in the dish and environments completely same as those of the stimulated case.

On pilot experiment where vibration stimulation with 5, 10 and 20 Hz were applied to cultured NHOst, the biggest changes in cell morphologies were observed with 20 Hz. Based on the result, the frequency of vibration stimulation of this experiment was decided at 20 Hz. Also, this frequency of 20 Hz has been suggested as the upper limit of the normal vibration environment of bone cells *in vivo*^{(20),(24)}. Proliferation of first passage adult human osteoblast-like cells has been optimally stimulated at a frequency of 20 Hz⁽²⁰⁾. The vibration direction was decided along with X axis (horizontal) direction of Fig. 2. The vibration stages were excited around 27 μ m total amplitude. In order to apply smooth sine vibration, total amplitude of the vibration stages was controlled within 90 % of its maximum amplitude. On this condition, the maximum acceleration applied to the cultured cells was approximately estimated as 0.02 G (G: acceleration of gravity). Stimulation period was decided at 12, 24, 36, 48, 60 and 72 hours. Additionally, temperature increase due to heat generation arising from the vibrating stage has not been observed in culture medium.

Fig.5 Parallel-connected eight vibration stages set in the CO₂ incubator.

In order to have clear-cut morphology of the taken microscopic digital images, actin, the major component of cytoskeleton, was fluorescently labeled by the following steps. At first, cells were fixed by 4 % paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, LTD.). After treatment with Triton X (PBS with 0.1 % Triton X-100, Amersham Biosciences), the actin was stained by 0.13 μM Alexa Fluor 568-Phalloidin (Invitrogen).

As the indicator of cell morphology changes, projected area and slenderness ratio (defined as shown in Fig. 6) were measured. The slenderness ratio is defined as l/b . Here, a cell length in longitudinal direction is l and a distance between two straight lines which are parallel to l direction and circumscribing to both sides of the cell profile line is b . Three microscopic fluorescent images were taken from one cultured dish. Then, three bigger cells without overlapping were chosen randomly from each image. Projected area and slenderness ratio of the chosen cells were calculated by using generic software, i.e. image processing and spreadsheet. The profile line of cell was visually determined. Very thin pseudopodium-like regions were omitted in measurement of the projected area and slenderness ratio.

3. Results and discussion

Figure 7 shows the comparison of projected area of static cultured control cells of 4th passage (black bar), 6th passage (white bar), and dynamically stimulated 6th passage cells (green bar). Differences are statistically analyzed by ANOVA and Tukey-Kramer post hoc test and are considered to be significant when the probability, p , was less than 5 percent. In this figure, projected area of 6th passage of control is always larger than that of 4th passage. It is noted that the results are not depending on culture time. This difference in cell morphology is considered as the effect of senescence^{(22),(23)}. On the other hand, in 6th passage, the dynamically stimulated is smaller than the control of same passage. Projected area of the dynamically stimulated decreased by around 25 % and came slightly close to 4th passage at all the

Fig. 6 Parameters are defined on the microscopic fluorescent images of actin filaments to estimate effect of dynamic stimulation upon cell morphology.

Fig. 7 Comparison of projected area of static cultured control of 4th passage, 6th passage, and dynamically stimulated 6th passage cells. The projected area of the dynamically stimulated decreased by around 25 % and came slightly close to that of 4th passage at all the culture time. It is observed that the effect of applied dynamic stimulation to the cultured cell was significant.

Fig. 8 Comparison of slenderness ratio of static cultured control cells of 4th passage, 6th passage, and dynamically stimulated 6th passage cells. The slenderness ratio of the dynamically stimulated increased by 50 % and came close to that of 4th passage until 48 hours of culture time. But at 60 hours or later, the differences between the 6th passage of control and the stimulated are clearly decreasing.

culture time. It is observed that the effect of applied dynamic stimulation to the cultured cell was significant.

Figure 8 shows the comparison of slenderness ratio of static cultured control cells of 4th passage (black bar), 6th passage (white bar), and dynamically stimulated 6th passage cells (green bar). In this figure, the slenderness ratio of 6th passage of control is always smaller than that of the 4th passage of control. It is also considered as the effect of cell senescence. The slenderness ratio of the dynamically stimulated increased by 50 % and came close to the 4th passage until 48 hours of culture time. But at 60 hours or later, the differences between the 6th passage of control and the stimulated were disappeared. One reason of this is assumed to be adaptation or sensitivity decreasing of intracellular sensing system to detect external forces. These are considered to occur in case of constant applied stimulation with long period of time. Another probable reason is the inhibition of cell adhesion and extension due to the vibration stimulation.

Figure 9 shows the microscopic images of fluorescently labeled actin filaments of the NHOst of static cultured control cells of 4th passage, 6th passage and dynamically stimulated 6th passage cells with 48 hours of culture time. The cell size of 6th passage of control was larger in projected area compared to that of the 4th passage. However, cell shapes of the 6th passage of stimulated are not similar to that of the same passage of control but to that of the 4th passage of control. Thus, morphologically, the effect of dynamic stimulation was clearly observed. On the

Fig. 9 Shapes of control and stimulated cells at 48 hours. In these images, the actins are fluorescently labeled. (a): Cells have elongated shapes. (b): The cell shapes are wide and large in projected area compared to the 4th passage. (c): Cell shape was similar to that of the control of 4th passage.

other hand, a relationship between vibration direction and orientation of actin filaments is not observed.

The developed three-dimensional vibration stage has capability to vibrate its stage in three orthogonal directions with various amplitude and frequency. However, as a first step, the experiment in this study was carried out only in X direction with amplitude about 27 μm and frequency of 20 Hz. The results show that the vibration affects cell morphology. This suggests the evidence in which the cultured cell detects and reacts to the applied vibration stimulation. By conducting the experiment with various vibration conditions, it might be possible to find an optimal stimulation condition to initiate effective response of the cell.

Other than the horizontal acceleration of the vibration stage, it is speculated that the cultured cells are under the effect of shearing forces raised by fluid flow of the culture medium. By supposing a Couette flow formed in a dish, fluid flow shear stress τ is defined as $\tau = \mu U/h$. Here, μ is a viscosity of the culture medium, U : maximum velocity of the bottom of a dish, and h : height of the medium. If the viscosity of the medium is regarded as equal to 40°C water, μ is given as $66.6 \times 10^{-6} \text{ kgf}\cdot\text{s}/\text{m}^2$. The value of U is $1.70 \times 10^{-3} \text{ m/s}$ considering sinusoidal vibration with 27 μm amplitude, while h is 3.9 mm. Using these values, the maximum fluid flow shear stress is roughly estimated as $\tau_{\text{max}} = 2.8 \times 10^{-4} \text{ Pa}$. A simulated shear stress of interstitial fluid loaded onto *in vivo* osteoblasts has been given between 0.8 and 3.0 Pa⁽²⁵⁾. The estimated value of shear stress of this study is small enough than the result of simulation, and that the effect of fluid flow shear stress may be limited. It might be possible to rotate the culture medium in the dish by excitation actuation with appropriate phase difference in X-Y plane of the vibration stage. This could be used as another type of dynamic stimulation. On the other hand, some studies have been reported on the effect of vertical Z direction vibration upon cultured cells^{(19),(21)}, since preparation of apparatus and experimental procedure are comparatively not difficult. The most important feature property of the proposed three-dimensional vibration stage is to excite X, Y, Z vibration arbitrary, and it might be possible to provide completely different dynamic environment in cell culture.

The effect of vibration stimulation was evaluated in view of cell morphology. The adaptation or sensitivity decreasing-like phenomenon to the applied vibration stimulation was observed. Effect of vibration stimulation upon cell morphology is assumed to arise from either spontaneous morphological change as a result of cell reaction to the imposed vibration stimulation or inhibition in cell adhesion and extension due to the vibration stimulation. The channel molecules on the cell membrane⁽⁶⁾⁻⁽⁸⁾ are considered as a cell detection element for fluid flow shear stress. On the other hand, the cytoskeletal system⁽⁵⁾ including focal adhesions are considered as a cell detection element for mechanical strain induced on extracellular matrix. Although, it is difficult to point out a clear pathway of vibration stimulation in NHOst, the cytoskeletal system possibly detects the vibration stimulation through the focal adhesion points.

Cultured cells in artificial environments are normally free from mechanical stimulation. In this study, the three-dimensional vibration stage was introduced in cell culture to realize a dynamic environment. From the observed results, the applied vibration stimulation seems to have a significant effect on the cell culture. It is suggested that a certain cell function, which is initiated by senescence and causes expansion in cell area, may effectively be inhibited by the vibration stimulation. On the other hand, it is not completely deniable that the applied vibration stimulation inhibits the metabolism of the cultured cell. Therefore, a more comprehensive evaluation of cell reaction against vibration stimulation should be conducted in the

future, if it is possible to measure bone matrix generation as well as intracellular molecular reactions under the various conditions of dynamic stimulation.

4. Conclusion

In this study, a new compact vibration stage was developed and extended to control device for cell culture. A cell culture experiment using NHOst was carried out to demonstrate the validity of the device. The results can be summarized as follows.

1. The developed compact vibration stage has the capability to vibrate its stage in three orthogonal directions with various amplitude and frequency.
2. It is observed that the projected area of normal human osteoblast increases, while the slenderness ratio decreases according to the number of cell passage.
3. Shape of the 6th passage of dynamically stimulated cells are not similar to that of same passage of control, but to that of 4th passage of control. Thus, from the viewpoint of morphology, the present study reveals that the dynamic stimulation affects cultured cells.

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