Effect of Actin Filament on Deformation-Induced Ca$^{2+}$ Response in Osteoblast-Like Cells*

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Under the influence of mechanical environment, bone structure is formed and maintained by adaptive remodeling that involves osteoclastic resorption and osteoblastic formation. In the mechanotransduction system in osteoblasts, it is believed that intracellular calcium plays a fundamental role and cytoskeletal actin filament is a crucial component for the signal transduction process. To clarify the role of actin filament in deformation-induced Ca$^{2+}$ signaling, osteoblast-like cells (MC3T3-E1) with different actin filament densities controlled by cytochalasin D were subjected to tensile strain in vitro. The change in intracellular Ca$^{2+}$ concentration labeled by fluo-3 was observed using a confocal laser-scanning microscope. As a result, the disruption of the actin filament was found to significantly suppress the deformation-induced Ca$^{2+}$ response that was regulated according to the degree of actin filament organization. This result indicates that the actin filament is indispensable for the quantitative regulation of Ca$^{2+}$ signaling in response to a mechanical stimulus in osteoblasts.

Key Words: Biomechanics, Cellular Mechanics, Osteoblast, Mechanical Stimulus, Calcium Signaling, Mechanotransduction, Actin Filament, Bone Adaptation

1. Introduction

In recent years, tissue engineering, an interdisciplinary field that applies the principles of engineering and biology to develop biological substitutes that restore, maintain and improve the structure and function of damaged tissues, has been rapidly growing. Bone tissue regeneration has been targeted for investigation as one of the successful areas in tissue engineering, in which mechanical stimulation is a key factor in the regulation of new bone formation, in addition to biological factors such as growth factors and bone morphogenetic proteins. Therefore, it is essential to understand the response of bone-forming

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cells, osteoblasts, to a mechanical stimulus.

To understand the response of osteoblasts to a mechanical stimulus, in vivo study is necessary to reflect the actual environment. However, due to the complexity of the in vivo system that involves many different cells and unknown biological factors that interact with mechanical factors, it is difficult to examine the effect of a well-defined mechanical stimulus on individual osteoblasts. Thus, utilizing a controlled in vitro system, one can study the effect of a mechanical stimulus on osteoblastic activities. For example, in vitro studies have clarified that osteoblastic cells in culture responded to a mechanical stimulus by increasing PGE$_2$ concentration, altering the pattern of synthesized proteins, changing the cellular alignment, stimulating cell division, and increasing the gap junctional communication between osteoblastic cells.

The above-mentioned osteoblastic responses are understood to be a result of the signal transduction process from external mechanical stimulation to intracellular signaling cascades leading to many different biochemical pathways. In this mechanotransduction process, intracellular calcium ion, Ca$^{2+}$, plays
an important role as a second messenger^{9a,9b} and cytoskeletal actin filament may be involved in the regulation of the process as a crucial component^{10}--^{12}. Therefore, to gain an understanding of the mechanotransduction mechanism in osteoblastic cells, an investigation of the quantitative relationship between intracellular Ca^{2+} response and cytoskeletal actin filament is indispensable.

In this study, first, an in vitro experimental apparatus for applying tensile strain to osteoblastic cells in a culture dish was developed. Second, we examined a method for controlling the density of cytoskeletal actin filament using cytochalasin D by inhibiting actin polymerization. Third, to clarify the role of actin filament in the deformation-induced Ca^{2+} response, osteoblastic cells with different actin filament densities controlled by cytochalasin D were subjected to tensile strain in vitro.

2. Materials and Methods

2.1 Cell culture and application of mechanical stimulus

Osteoblast-like cells (MC3T3-E1) were obtained from RIKEN Cell Bank, and cultured in α-minimum essential medium (α-MEM : ICN Biomedicals) containing 10% fetal bovine serum (FBS: ICN Biomedicals), which was changed every three days, in a humidified atmosphere of 95% air and 5% CO_{2} at 37°C^{13}.

The experimental apparatus shown in Fig. 1 was developed to apply a mechanical stimulus to the osteoblastic cells in culture. The cells were subjected to tensile strain by bending a cantilever glass plate on which the cells were cultured with a magnetic force. Cover glass A (50×24 mm, 0.1 mm thick) was cantilevered in a stainless steel (SUS304) block. The origin of the x-coordinate was taken at the fixed point of cover glass A, and a magnetic body was mounted at x=36 mm. Osteoblastic cells were cultured on the underside of cover glass A (Fig. 1), and the region including the observation point (x=3 mm) was covered with cover glass B (18×18 mm, 0.1 mm thick). By putting the culture medium between cover glasses A and B, it was possible to prevent changes in osmotic pressure and pH, and to keep the cellular condition stable during the experiment. The entire apparatus was put in a common culture dish (Ø=85 mm, 15 mm height) on which an electromagnet was fixed to pull up the magnetic body by magnetic force so as to bend cover glass A.

Using this apparatus, tensile strain of a single rectangular wave with a magnitude of 1500 μ strain, as shown in Fig. 2, was applied to the osteoblastic cells for 3.5 sec, and the change in intracellular calcium ion concentration, [Ca^{2+}], was measured. The strain magnitude was determined by referring to the literature^{14} that reported that the mean peak strain in the dorsal aspect of the second metatarsal was approximately 1900 μ strain under normal walking conditions.

The change in [Ca^{2+}], was observed with the fluorescent indicator dye fluo-3 (Dojindo Molecular Technologies) supplied in a cell-permeant form (fluoro-3-AM). Fluo-3 is a fluorescent probe that shows spectral response upon binding with Ca^{2+}; thus changes in [Ca^{2+}], can be relatively evaluated by measuring the intensity of the fluorescent luminescence. Cells on the cover glass were loaded with 11 μM fluo-3-AM, incubated for 3 hours at 37°C, rinsed with phosphate-buffered saline (PBS), and used for the experiment in α-MEM. Fluorescence images were obtained using a confocal laser-scanning microscope (MRC 1024/MP: Bio-Rad). Each fluorescence image was scanned at a rate of 3.5 sec, and the fluorescent intensity of each pixel was digitized into 8 bits.

2.2 Control of actin filament density

To clarify the role of the cytoskeletal actin filament in the deformation-induced Ca^{2+} response in osteoblastic cells, a method for controlling actin filament density was examined using cytochalasin D (Calbiochem) that inhibits actin polymerization. By incubating the cells in the medium with 50 μM cytochalasin D for 1 hour, the actin filament structure was disrupted. Then, the cells were rinsed with PBS, and were put back into the normal α-MEM. By altering the incubation period after the PBS rinse for 0, 30, 60,
and 90 min, the degree of reorganization of the actin filament by polymerization was adjusted to control actin filament density. After each reorganization period, the cells were fixed in 5% paraformaldehyde solution, and the actin filament was labeled with rhodamine phalloidin (Molecular Probes) and observed using a confocal laser-scanning microscope.

Fluorescence images of osteoblastic cells treated by cytochalasin D in Fig. 3 show the reorganization of the actin filament structure with time, namely, 0, 30, 60 and 90 min after the PBS rinse. At 0 min immediately after the treatment by cytochalasin D, the actin filament structure is hardly observed, as shown in Fig. 3(a). This result confirms that the inhibition of actin polymerization using cytochalasin D disrupts the actin filament structure. In addition, it was clearly shown that the disruption of the actin filament caused the contraction of the cells. After 30 min of PBS rinse, though the cell stretched to some extent as shown in Fig. 3(b), the internal actin filament structure was still unclear. Then, the actin polymerization progressed with increasing period of reorganization, and the structure spread out into the whole cell after 90 min, as shown in Fig. 3(d). Thus, it was shown that the actin filament disrupted by cytochalasin D could gradually reorganize its structure by incubating it in the normal medium.

For quantitative evaluation of the change in the actin filament structure, the average value of the fluorescent intensity in the region surrounding the contour of each cell was measured, and the results are shown in Fig. 4 where all data are expressed as mean and standard deviation. This figure indicates that the average value of the fluorescent intensity of actin filament increased with increasing reorganization period. The statistical significance of the increase in the fluorescent intensity depending on the reorganization period was determined using the analysis of variance. As a result, it was found that the fluorescent intensity significantly increased ($p < 10^{-5}$) depending on the reorganization period. Significant differences between 0 and 30 min ($p < 0.05$), 0 and 60 min ($p < 0.01$), 0 and 90 min ($p < 0.01$), and 30 and 90 min ($p < 0.05$) were found by Tukey's post hoc test for group comparison. Thus, the actin filament structure disrupted by cytochalasin D can reorganize again in the normal medium and its density increases with time. From these results, it was found that quantitative control of actin filament density is possible by adjusting the reorganization period after treatment with cytochalasin D.

3. Results and Discussion

3.1 Changes in deformation-induced Ca$^{2+}$ response

The effect of actin filament density on the deformation-induced Ca$^{2+}$ response in osteoblastic cells was examined. Using a newly developed experimental apparatus (Fig. 1), tensile strain, as shown in Fig. 2, was applied to the osteoblastic cells with different actin filament densities controlled by cytochalasin D treatment, and the deformation-induced change in intracellular Ca$^{2+}$ concentration, [Ca$^{2+}$], labeled by fluo-3 was observed using a confocal laser-scanning microscope.

For normal osteoblastic cells that were not treated with cytochalasin D, that is, the control cells, the increase in [Ca$^{2+}$], induced by tensile strain is shown in Fig. 5, where Figs. 5(a) and (b) are the fluorescence images of Ca$^{2+}$ before and after stimulation,
respectively. This figure shows that the area with high intensity spread widely inside the whole cell and that the average intensity increased, where the fluorescent intensity indicates the relative concentration of $\text{Ca}^{2+}$. This change implies that the increase in $[\text{Ca}^{2+}]$, in osteoblastic cells was induced by the stimulation of tensile strain.

For quantitative evaluation of the fluorescent images, the change in intensity with time was measured for individual cells, where the average value of the fluorescent intensity in the rectangular region at the center of each cell was measured. The size of each rectangle was adjusted to obtain the average value of 100 measured by 256 resolutions, immediately before the stimulation, so that the relative change in intensity induced by tensile strain can be compared. Intracellular $\text{Ca}^{2+}$ responses to the stimulation by tensile strain are plotted in Fig. 6 for the control cells ($\bigcirc$ : $n = 15$) that were not treated with cytochalasin D, and the treated cells with the corresponding reorganization periods after PBS rinse for 30 min ($\blacklozenge$ : $n = 15$), 60 min ($\bullet$ : $n = 12$), and 90 min ($\bullet$ : $n = 10$). Data during strain application for 3.5 sec from 20 sec in Fig. 6 was not available because the cells moved out of the focal plane of the microscope due to the deflection of the cantilevered cover glass A.

Control cells that were not treated with cytochalasin D showed an increase in $\text{Ca}^{2+}$ fluorescent intensity from 100 to $132 \pm 26.7$ (Mean $\pm$ S.D.) at the peak, followed by a gradual decrease toward the initial value before stimulation. In contrast, the treated cells with a reorganization period of 30 min did not show any change in the fluorescent intensity, indicating no deformation-induced $\text{Ca}^{2+}$ response. In the case of the treated cells with 60-min reorganization period, the fluorescent intensity increased to $118.8 \pm 19.8$ at the peak. The treated cells with 90-min reorganization period showed a considerable increase in intensity to $130.9 \pm 26.4$, which is almost the same as that in the control cells, followed by a gradual decrease to the initial value.

3.2 Discussion

It should be noted that the observed change in intracellular $\text{Ca}^{2+}$ concentration, $[\text{Ca}^{2+}]$, may be attributed to the magnetic force used in the experiment to bend cover glass A. To examine the effect of the magnetic force field on the change in $[\text{Ca}^{2+}]$, an experiment was performed by applying the same voltage to the electromagnet without a magnetic body, that is, the magnetic force field was applied to the cells without mechanical stimulation. The change in the average value of the $\text{Ca}^{2+}$ fluorescent intensity with time ($n = 7$) is plotted in Fig. 7 by a broken line, while that of the control cells ($n = 15$) is plotted by open circles. In contrast to the case of control cells with mechanical stimulation, the intensity did not change in the presence of only the magnetic force field. That is, the magnetic force field generated in the experimental apparatus used in this study had no significant influence on the change in $[\text{Ca}^{2+}]$. Thus, the increased $\text{Ca}^{2+}$ fluorescent intensity in Fig. 6 is due to the tensile strain applied to the osteoblastic cells.

The increase in $\text{Ca}^{2+}$ fluorescent intensity induced by tensile strain for different reorganization periods of the actin filament is shown in Fig. 8, in which mean and standard deviation are plotted as the peak values normalized by the intensity before stimulation. It was found that the increase in $\text{Ca}^{2+}$ fluorescent intensity induced by tensile strain was significant ($p < 10^{-5}$) with increasing reorganization period, which was determined by the analysis of variance. Tukey's post hoc test for group comparison yielded a significant difference ($p < 0.01$) in $[\text{Ca}^{2+}]$ increase between 30 and 90 min and between 60 and 90 min. In addition, it was found that $\text{Ca}^{2+}$ increases for 30 and 60 min were significantly different from those in the control cells ($p < 0.01$) by the two-tailed Student's $t$-test, but that for 90 min had no significant difference.

By rearranging Fig. 4, showing the reorganization...
of the actin filament structure, and Fig. 8, showing the increase in [Ca^{2+}], due to tensile strain, the relationship between actin filament density and deformation-induced Ca^{2+} response is summarized in Fig. 9. In this figure, the symbols ⋄, ■, and ▲ represent the fluorescent intensity of the cells with reorganization periods of 30, 60, and 90 min, respectively, normalized by that of the 0 min cells. A positive correlation ($r = 0.67$) was found between actin filament density and deformation-induced Ca^{2+} response.

Immediately after the treatment with cytochalasin D, in the 0 min cells with a disrupted actin filament structure, deformation-induced Ca^{2+} response was not observed. This result indicates that the cytoskeletal actin filament is indispensable in the Ca^{2+} signaling response to the mechanical stimulus. In addition, the positive correlation between actin filament density and deformation-induced Ca^{2+} response, as shown in Fig. 9, implies that the actin filament structure quantitatively affects the deformation-induced Ca^{2+} response in the osteoblastic cells.

4. Conclusion

In this study, we investigated the effect of the cytoskeletal actin filament on the deformation-induced Ca^{2+} response in vitro using osteoblast-like MC3T3-E1 cells. As a result, we found that the actin filament is indispensable for the regulation of the Ca^{2+} signaling response to a mechanical stimulus in osteoblastic cells. In addition, the deformation-induced Ca^{2+} response may be qualitatively related to the actin filament density.

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Appendix: Effect of Mechanical Stimulus on Actin Filament Organization

Bone cells are always exposed to a changing mechanical environment in vivo. Thus, the effect of dynamic mechanical stimulation on the actin filament
structure in osteoblastic cells was examined by applying repetitive tensile strain to the cells.

Cyclical tensile strain of 1500 μ strain with a rectangular waveform at 1 Hz, as shown in Fig. 10, was applied to the osteoblastic cells in culture for 10 min. After incubation for 30 min, the cells were fixed in 5% paraformaldehyde solution, and the actin filament was labeled by rhodamine phalloidin and observed using a confocal laser-scanning microscope.

Compared to the control cells, actin filament structure spread widely inside the whole cell and organized densely in the stimulated osteoblastic cells. For quantitative evaluation of the amount of actin filament in the cells, the average value of the fluorescent intensity of the rhodamine phalloidin in the region surrounding the contour of each cell was measured. The results are shown in Fig. 11, where data are expressed as mean ± standard deviation. The average intensity is 121.3 ± 23.3 in the control cells and 168.5 ± 22.6 in the stimulated cells, indicating the significant increase ($p < 0.01$) in actin filament density due to the mechanical stimulation, as determined by the paired Student’s $t$-test.

References