EXPERIMENTAL STUDY ON BONE REMODELING RATE EQUATION: EFFECTS OF MECHANICAL STIMULUS ON OSTEOBLASTIC ACTIVITIES *IN VITRO*

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Abstract. *An experimental study in vitro at the cellular level is fundamental to clarify the mechanism for adaptive bone remodeling. Many efforts have been made to study the cellular responses to mechanical stimuli in vitro, however, the mechanism for the mechano-sensory and mechano-transduction system of bone cells remains largely unknown. An investigation of the effects of mechanical stimuli on the osteoblastic activities in vitro will provide basic understanding about the mechanism and contribute to the development of a rate equation for adaptive bone remodeling. In this study, the effect of cell-to-cell communication of a mechanical signal on the activities of cultured osteoblasts was investigated. The propagation of an intracellular Ca^{2+} wave in the osteoblastic network, which can be considered as a messenger of mechanical signals to neighboring cells, was observed by applying mechanical deformation to a single cell with a micropipette. The measured propagation distance enabled the sensing distance of the osteoblasts to be estimated as about 300 μm. New experimental apparatus was developed to study the quantitative response of osteoblasts to a non-uniform strain field, in which cell-to-cell communication of the mechanical signal played an important role. This apparatus was used to monitor the effect of the mechanical signal on osteoblastic proliferation when considering cell-to-cell communication.*

1. INTRODUCTION

Under the influence of mechanical environment, bone structure is formed and maintained by adaptive remodeling. To clarify the mechanism for bone remodeling by which bone functionally adapts to a mechanical environment due to osteoclastic resorption and osteoblastic formation, an *in vitro* study at the cellular level is fundamental, since the mechanical environment of cells can be manipulated and quantitatively controlled. Previous *in vitro* studies with cells have examined the effects of magnitude, rate, and duration of mechanical stimuli on such osteoblastic activities, as the proliferation rate, collagen synthesis, and alkaline phosphatase activity (Harell *et al*., 1977; Yeh and Rodan, 1984; Hasegawa *et al*., 1985; Buckley *et al*., 1988; Murray and Rushton, 1990; Jones *et al*., 1991; Neidlinger-Wilke *et al*., 1994; Ziambaras *et al*., 1998).

A transient increase in Ca^{2+} concentration in cells under the influence of mechanical deformation has also been observed, and wave propagation of intracellular Ca^{2+} induced by mechanical deformation applied to a single cell with micropipette has been investigated (Xia and Ferrier, 1992; Guilak *et al*., 1994). These results indicate that a mechanism exists by which the cell can sense mechanical deformation and forward this signal to its neighboring cells. Even though the existence of cell-to-cell communication has been reported, none of the previous studies have considered the effect of the neighboring mechanical condition on the activities of the bone cells.

This study was conducted to clarify the effect of a mechanical stimulus on the osteoblastic activities *in vitro* by considering cell-to-cell communication for a basic understanding of the cellular mechanism which can be hypothesized in the rate equation for bone remodeling. The sensing distance of the osteoblast was first evaluated by observing the Ca^{2+} wave propagation due to mechanical deformation applied to a single cell. Second, a new mechanical index that considers cell-to-cell communication was introduced, and the effect of a non-uniform strain field on osteoblastic proliferation was observed with the newly developed experimental apparatus.

2. PROPAGATION OF INTRACELLULAR Ca^{2+} IN OSTEOBLASTS

2.1 Materials and Methods

Osteoblast-like MC3T3-E1 cells were obtained from RIKEN Cell Bank and cultured in α-minimum essential medium (α-MEM; ICN Biomedicals) with 10% fetal bovine serum (FBS; ICN Biomedicals) (Kodama *et al*., 1981). The cells were incubated to confluency at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. After subculturing, the cells were ready to be used in the experiment.

Osteoblasts were plated on a glass-bottomed dish (Mat-Tech) and loaded by exposure to 10μM Fluo3/AM (Wako Chemicals), a fluorescence indicator of Ca^{2+}, at 37℃ for 2 hours. After being rinsed with phosphate-buffered saline (PBS), the dish was mounted on a microscope stage. A confocal scanning laser microscope (CSLM; MRC-1024, Bio-Rad) was used to examine the spatial and temporal distribution of the intracellular fluorescence of Ca^{2+} in response to cell deformation. The transmission and confocal images were digitized at the rate of 1 image per 1.5 seconds.

A micromanipulator (Narishige) was used to deform a single osteoblast with the tip of a glass micropipette. The tip of the glass pipette was fitted with a micropipette puller (Narishige), and the diameter was about 20 μm. All experiments were performed at room temperature (21℃).

2.2 Calcium Wave Propagation

A series of fluorescent confocal images of deformed and adjacent osteoblasts is shown in Fig. 1. The application of a mechanical deformation with the micropipette to a single osteoblast (cell D) resulted in an increased fluorescence intensity in the cell within 6 seconds after the cell was deformed (t=0 sec); i.e., a significant increase in intracellular calcium concentration ($[Ca^{2+}]_i$) resulted. After 12 seconds, $[Ca^{2+}]_i$ remained elevated and was propagated from the deformed cell to neighboring cells (cells 1, 2, and 3). The time-course data for $[Ca^{2+}]_i$ in these cells showed a transient increase in $[Ca^{2+}]_i$, and peaked with the time delay shown in Fig. 2.

The increase in fluorescence intensity in the neighboring cells (cells 1, 2, and 3) was smaller than that in the deformed cell (cell D).

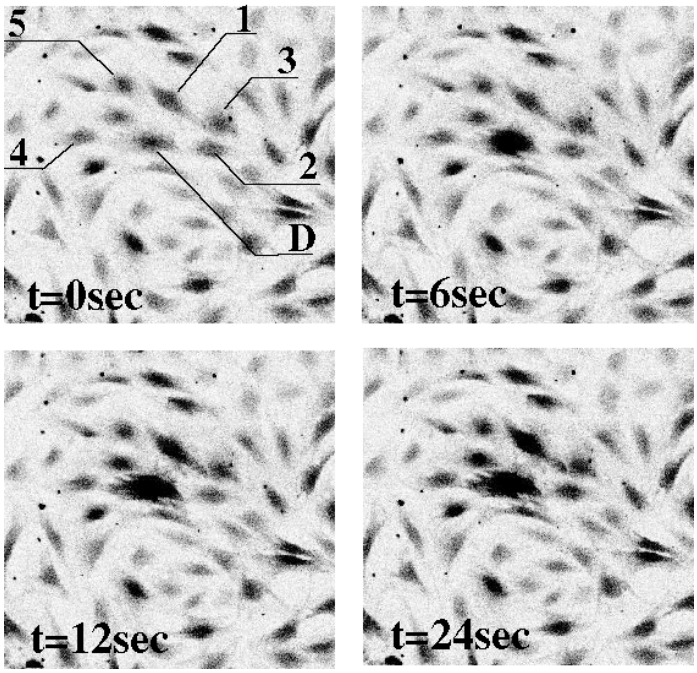

Fig. 1 Propagation to the neighboring cells (1-5) of the Ca^{2+} signal induced by deformation applied to a single cell (D) with a micropipette

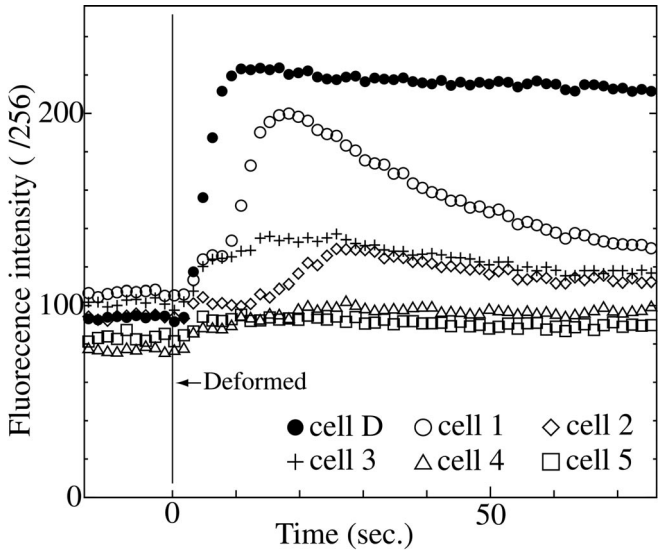

Fig. 2 Change in the fluorescence intensity, $[Ca^{2+}]_i$, with time in osteoblasts

Figure 3 presents the relationship between the increase in $[Ca^{2+}]_i$, defined as $\Delta I = (I - i)/i$ where i and I are fluorescence intensity before and after the mechanical deformation, in the adjacent cells and the distance L from the deformed osteoblast. The results

in Fig. 3 indicate that the elevation of $[Ca^{2+}]_i$ decreased with increasing distance from the deformed cell. Assuming a linear relationship, the maximum propagation distance of the Ca^{2+} wave was estimated to be about 300 μm.

These results show that the osteoblast can sense mechanical deformation and forward this signal to its neighboring cells. Therefore, to investigate the osteoblastic activities under mechanical stimuli, the mechanical conditions at neighboring points should be considered.

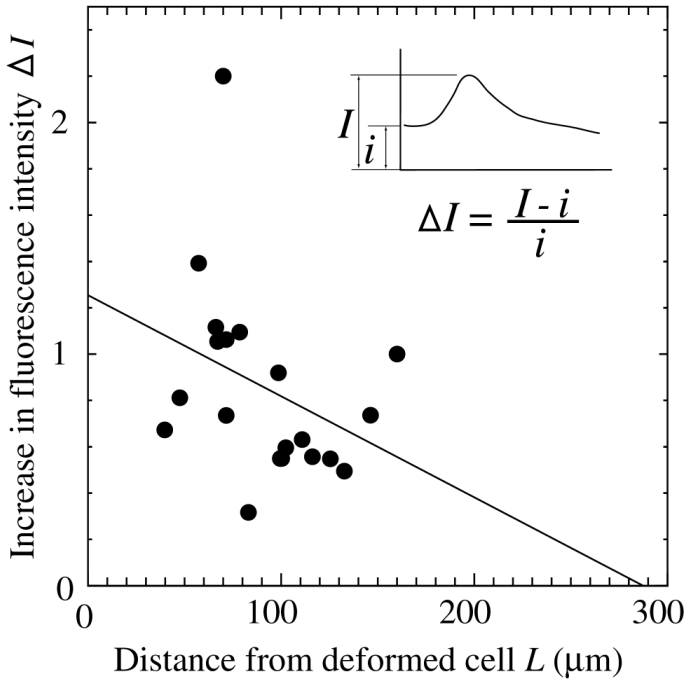

Fig. 3 Relationship between the increase in fluorescence intensity of Ca^{2+} and the distance from the deformed cell

3. OSTEOBLASTIC ACTIVITITY ON A NON-UNIFORM STRAIN FIELD

3.1 Index of Non-uniformity for the Mechanical Stimulus

Considering that bone cells communicate with each other, a mechanical stimulus in the neighbors will affect the cellular response as well as that at the point. Thus, the local strain non-uniformity on the substrate surface should be evaluated (Adachi *et al*., 1997). Let ε_c denote the strain at point x_c on the surface, and ε_d the representative strain in the area around point x_c. We introduce a new index, Γ, representing the local strain non-uniformity at point x_c, which can be evaluated by the relative value of ε_c to ε_d :

$$\Gamma = \ln(\varepsilon_c / \varepsilon_d) . \tag{1}$$

By using the integral form, representative strain ε_d is defined by

$$\varepsilon_d = \int_S w(l)\varepsilon_r dS \Big/ \int_S w(l) dS , \tag{2}$$

where S denotes the surface, ε_r the strain at point x_r on the surface, l the distance between points x_c and x_r, and $w(l)$ [$w(l) > 0$ $\left(0 \le l < l_L\right)$] is a weighting function depending on

distance l. Based on the experimental results for the mechanical deformation by a micropipette in the previous section, we assumed that sensing distance l_L, representing the area where cells can sense a mechanical stimulus, to be 300 μm. For simplification, weighting function $w(l)$ is assumed to be a linear decreasing function.

3.2 Experimental Apparatus

To investigate the effect of Γ on the osteoblastic activity, new experimental apparatus was developed. Osteoblasts were subjected to a cyclic strain *in vitro* by controlled stretching of the polymer substrate on which cells were being cultured. The substrate was constructed from a transparent polystyrene base that could be stretched elastically. The stretching device consisted of a piezoelectric actuator driven by amplified voltage signals from a function generator and clamps provided to hold the polystyrene base. The strain was applied cyclically in the form of a sinusoidal wave with a frequency of 1 Hz for the duration of the test period in the incubator.

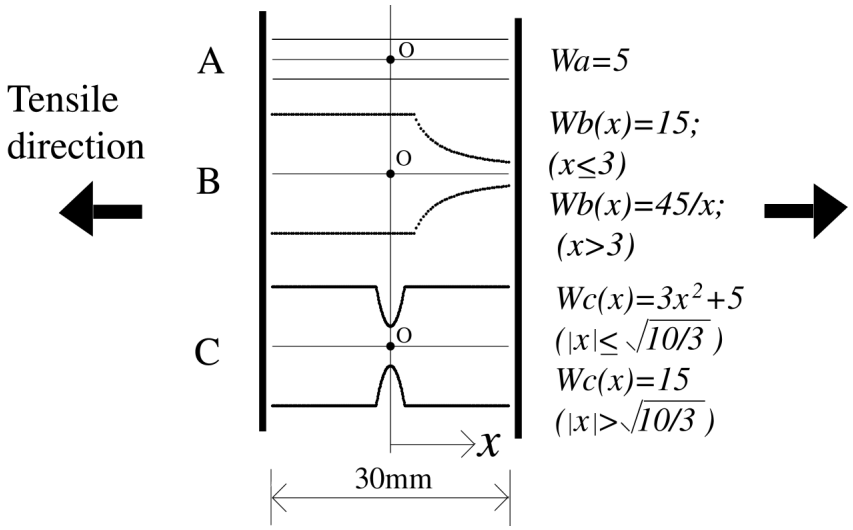

Fig. 4 Shapes of the specimens used for the non-uniform strain field

Three types of specimen, A, B and C as shown in Fig. 4, were clamped and stretched. The coordinate axes, x and y, are defined as being along the longitudinal and lateral directions of a specimen, respectively, and the origin O is set at the center of the specimen. Specimen A was a rectangle of W_a=5mm in width and L=30mm in length. Specimen B was $W_b|_{x<3}$=15mm, $W_b|_{x>3}$=45/x mm in width, where $W_b|_{x>3}$ is inversely proportional to x. Specimen C had notch $W_c|_{|x|\le\sqrt{10/3}}=3x^2+5$mm which is proportional to x^2, and $W_c|_{|x|>\sqrt{10/3}}$=15mm. By observing the osteoblastic activity on these specimens, the effect of a neighboring mechanical condition, i.e., the non-uniformity of strain, was examined.

Equivalent strain ε along the x axis (y=0) of a specimen was calculated as a plane-stress problem by a finite element analysis, and is plotted in Fig. 5. The strains ε on specimens A and B were homogeneous and monotonically increased with respect to the x axis. On specimens C, strain ε repetitively increased and decreased. In respect of these strain distributions, the non-uniformity of local strain Γ along the x axis was calculated by equation (1), and is plotted in Fig. 6. Index Γ for the specimen A was zero, and that for B had a small

value. On specimen C, index Γ repetitively increased and decreased, the value being larger than the other two. This device was used to examine the effect of a mechanical stimulus at the point or in the neighbors on the osteoblastic proliferation. For example, we could observe the points at x=0 and x=5 mm on specimen C with almost the same magnitude of ε of about 650 μ with a different sign for Γ. Thus, the osteoblastic activity was examined by observing the cellular proliferation at points with various combinations of strain ε and strain non-uniformity Γ.

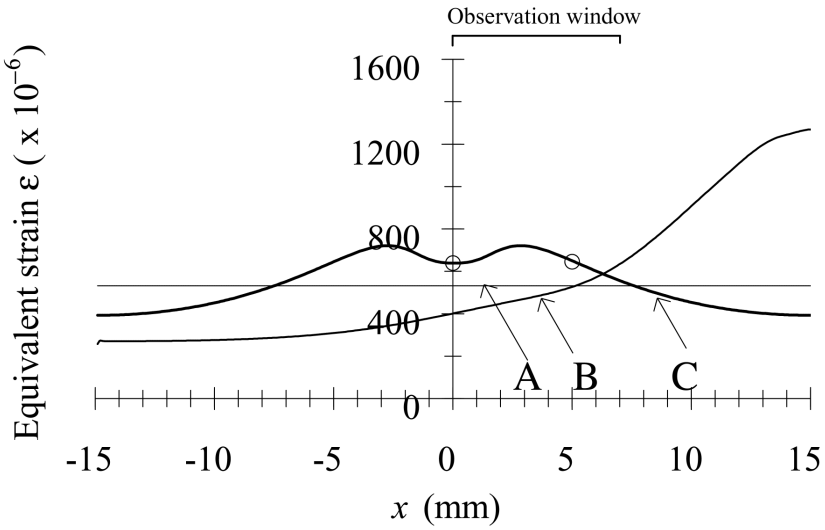

Fig. 5 Equivalent strain ε in the specimens

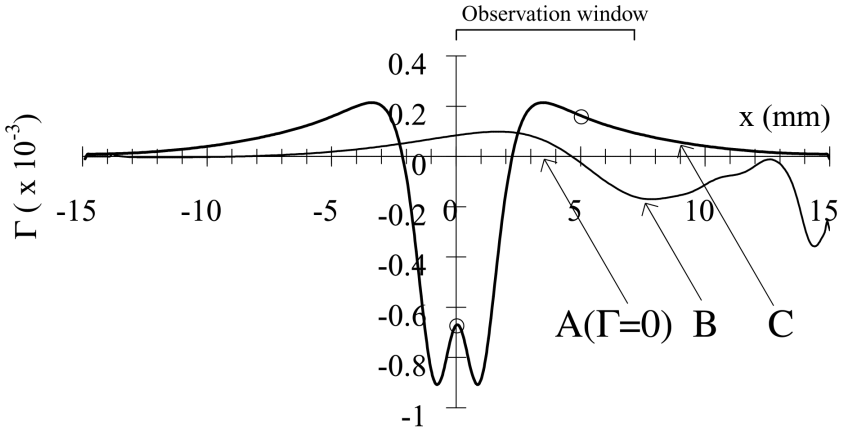

Fig. 6 Strain non-uniformity Γ in the specimens

3.3 Results and Discussion

Osteoblasts were seeded at a density of 100 cells / mm^2 on specimens A, B, and C. After being incubated for 2 and 24 hours, the number of cells was counted by using phase-contrast microscope. The mean value for the proliferation rate, which is defined as the number of cells after 24 hours divided by that after 2 hours, on the substrate (n=2) was calculated as shown in Fig. 7. The magnitude of the proliferation rate is indicated by the diameter of the circles, and

is plotted in the $\varepsilon - \Gamma$ field. As shown in Fig. 7, it was found that the proliferation rate increased with both increasing strain ε and non-uniformity Γ. These results imply that the proliferation rate of an osteoblast depends on the strain non-uniformity as well as absolute strain. Further investigation with a larger number of experiment is necessary to elucidate this characteristic in more detail.

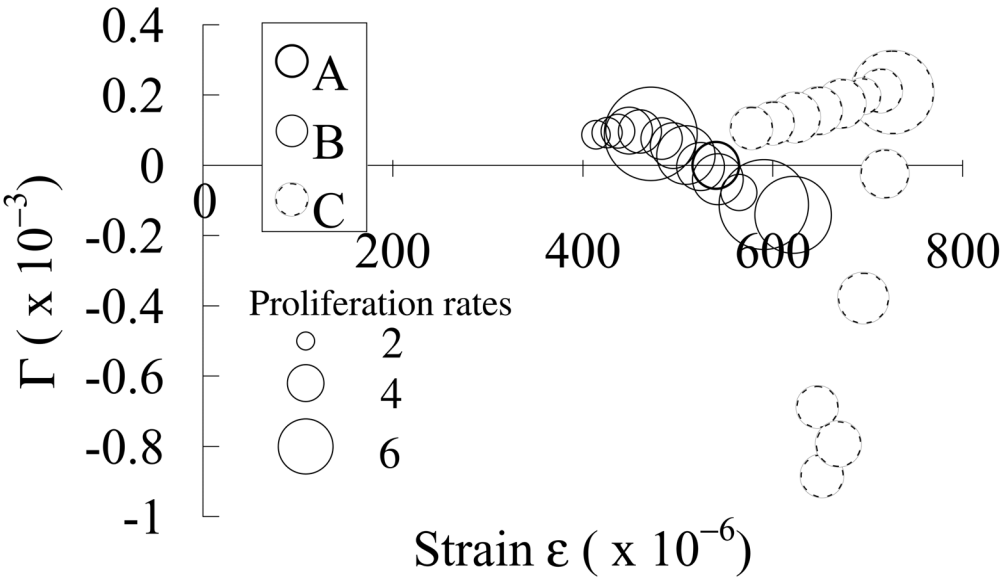

Fig. 7 Proliferation rate in the ε - Γ field

4. CONCLUSION

Previous studies have reported that a mechanism exists by which a cell can sense mechanical deformation and forward this signal to its neighboring cells. Therefore, to investigate the osteoblastic activity under a mechanical stimulus, the mechanical condition at neighboring points should be considered. To estimate the sensing distance, Ca^{2+} wave propagation, which is the intracellular messenger for mechanical signals in osteoblastic network, was observed under a mechanical deformation applied to a single cell with a micropipette. The sensing distance of an osteoblast was estimated by this method to be about 300 μm. In order to study the effect of the mechanical condition of neighboring cells, new apparatus to stimulate osteoblasts with a non-uniform strain field was developed. This device was used to examine the osteoblastic activity in a non-uniform strain field in more detail.

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