Strain magnitude dependent intracellular calcium signaling response to uniaxial stretch in osteoblastic cells

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Abstract

In osteoblast cells, cells change their intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) as the response to mechanical stimuli. Although it has been reported that osteoblast cells responded to many kinds of mechanical stimuli including stretch of substrate, shear stress in fluid flow, direct indentation of glass microneedle and hydrostatic pressure etc., the detail of the characteristics of intracellular calcium signaling response to substrate stretch still remains unclear because motion artifact during stretch application causes out of focus plane and observation area, and complicates the in situ time lapse observation of change in [Ca\(^{2+}\)], in a single cell level. In this study, we combined our originally developed cell stretching MEMS device with the ratiometric microscopy method with two kinds of visible wavelength calcium indicator dyes. The cell stretching micro device and the ratiometric method reduce the influence of motion artifact during stretch application, and enable us to quantitatively evaluate the characteristics of cellular calcium signaling response to stretch. MC3T3-E1 osteoblastic cells were plated onto the cell stretching micro device and fluorescently labeled by Ca\(^{2+}\) indicator Fluo 3 and Fura Red. A uniaxial stretch with three magnitudes of strain 5%, 10% and 15% with constant strain rate were applied to the cells, and in situ time lapse observation of cellular calcium signaling response to stretch was conducted with high temporal and spatial resolution. We succeeded in obtaining time lapse fluorescent image sequences during stretch application without excessive out of focus and blank time. The results revealed that MC3T3-E1 cells change the intensity of calcium signaling response to stretch according to the stretch strain magnitude. As stretch strain magnitude was increased, the amount of change in fluorescent ratio value of Ca\(^{2+}\) indicators in stretched cells also increased. This result suggests the possibility that osteoblastic cells can sense the magnitude of mechanical stimuli at upstream of mechanotransduction pathway such as influx of extracellular Ca\(^{2+}\).

Key words: Cell biomechanics, Mechanotransduction, Osteoblast, Calcium signaling, Stretch stimuli

1. Introduction

Bone structures are generally considered to be remodeled adaptively to the mechanical environment (Duncan and Turner, 1995). This remodeling is based on the sensitivity of bone cells such as osteoblasts, osteoclasts and osteocytes to mechanical stimulus. Osteocytes are considered as one of the mechanotransducers, and many studies have reported that they can sense and respond to fluid shear stress and direct mechanical perturbation (Genetos et al., 2007, Adachi et al., 2009a, Adachi et al., 2009b). And also osteoblasts, which form new bone on the bone surface, could sense the bone surface strain caused by external mechanical loading. It has been reported that osteoblasts can respond to variety of mechanical stimulus such as fluid shear stress (Jacobs et al., 1998, Genetos et al., 2005, Jing et al., 2013), hydrostatic compression (Roelofsen et al., 1995, Rath et al., 2008), direct indentation of microneedle (Adachi et al., 2003, Sato et al., 2007) and substrate stretch strain (Kaspar et al., 2002, Matsugaki et al., 2013).

It is generally believed that strain magnitude influences bone remodeling in tissue level. There is a threshold strain
magnitude at which bone remodeling occurs to adapt mechanical environment (Rubin and Lanyon, 1985, Frost, 1992). Therefore, bone cells could possibly sense the magnitude of mechanical stimuli and change their bone forming and resorption activities. Actually, osteoblasts change their response to varied magnitude of fluid shear stress (Jacobs, et al., 1998, McAllister and Frangos, 1999) and stretch strain (Tang, et al., 2006, Li, et al., 2010).

Intracellular calcium ion plays an important role as second messenger in cellular signaling cascade. Although many studies have reported that osteoblasts change intracellular calcium concentration ([Ca$^{2+}$]) as the response to applied mechanical stimuli (Jacobs, et al., 1998, Chen, et al., 2000, You, et al., 2001, Adachi, et al., 2003, Tanaka, 2012), the characteristics of osteoblastic [Ca$^{2+}$] response to varied magnitude of stretch strain remains unclear. Stretching stimulus causes non-negligible motion artifacts during observation such as out of focus or moving out of the field of view. This motion artifacts during stretch application disturb continuous in situ time lapse observation of [Ca$^{2+}$] dynamics under stretch application with high spatial and temporal resolution.

The authors have developed a novel cell stretching micro device to reduce motion artifacts during stretch application (Sato, et al., 2010). In this study, we combined our cell stretching micro device with the fluorescent ratiometric method (Lipp and Niggli, 1993) to conduct quantitative and continuous in situ time lapse observation of [Ca$^{2+}$], under stretch application in a single cell level. The objective of this study was to evaluate the characteristics of osteoblastic intracellular calcium signaling response to stretch with varied magnitude of stretch strain.

2. Materials and Methods

2.1 Cell culture and fluorescent labeling

The mouse osteoblast cell line MC3T3-E1 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were cultured in α-MEM containing 10% FBS and maintained in 5% CO$_2$ at 37°C. Prior to the experiment, cells were seeded onto fibronectin coated 35mm glass bottom dish with built-in cell stretching...
microdevice developed by the authors at cell density approximately $5.0 \times 10^4$ cell / dish. After 18 hours preincubation to wait sufficient cell adhesion, fluorescent Ca$^{2+}$ indicator Fluo 3 and Fura Red were loaded into the cells. Hank’s balanced salt solution (HBSS) containing 6µM Fluo 3-AM (Dojindo), 12µM Fura Red (Life Technologies) and 0.1% Pluronic F-127 (Life Technologies) was used for loading buffer. Fluo 3 increases their fluorescent intensity as increase in Ca$^{2+}$ concentration. On the other hand, Fura Red decreases their fluorescent intensity as increase in Ca$^{2+}$ concentration. To calculate their ratio value of fluorescent intensities (Fluo 3 / Fura Red), we can evaluate the change in C$^{2+}$ concentration quantitatively and reduce the motion artifact due to the rigid displacement during stretch application. Normal culture medium was replaced by loading buffer, and the cells were cultured for 30 minutes at room temperature to load the fluorescent indicators into the cells. After indicator loading, the cells were rinsed twice using PBS, and loading buffer was replaced by normal culture medium and used for fluorescent image acquisition.

2.2 Cell stretching microdevice and stretch condition

In the present study, the cell stretching microdevice developed by the authors was slightly modified from the previously reported (Sato, et al., 2010). Fig.1 shows the schematic of the microdevice. Six microdevices are fabricated onto the 22mm square cover glass, and the cover glass is attached to the 35mm dish with a φ18mm hole at the bottom. Each microdevice consists of one pair of arms fabricated from photoresist SU-8 and a cell stretching chamber fabricated from silicone elastomer. Figure 1 (c) shows a microscopic image of cell stretching microdevice. Two metal needles are set onto the arms. The needle on right side is held by the micro manipulator mounted on the microscope stage to fix the one end of the stretching chamber. The needle on the left side is held by the micro manipulator via piezo electric actuator (MC-140L, Mess-tek) to apply uniaxial stretch to the other end of the chamber with well controlled strain magnitude and strain rate. In the present study, uniaxial strain with three different magnitudes (5%, 10% and 15%) were applied to the cell with constant strain rate 5% / sec.

2.3 Image acquisition and analysis

Time lapse fluorescent images of the Ca$^{2+}$ indicators were obtained by using an inverted confocal laser scanning microscope (A1R, Nikon) with x60 oil immersion objective lens. Fluo 3 and Fura Red were excited by 488nm Ar laser, and their fluorescence were splitted by using 525nm band-pass filter with 50nm band width and 561nm long-pass filter. 3 images of fluo 3 fluorescence, Fura Red fluorescence and transmitted image were acquired simultaneously by using 3 independent photomultiplier tubes. Recorded image size was 512 × 256 pixels with 12bit resolution. Image acquisition rate was approximately 4 frames / sec. All experiment were conducted in normal atmosphere and at room temperature (approximately 24℃). To prevent influences on the cell condition, image acquisitions were finished within 1 hour. Obtained time lapse images were analyzed using ImageJ software (NIH). To analyze time course change in fluorescent intensity of the cell, the targeted cell has to be fixed in the image during stretch. The alignment of sequential time lapse images were manually adjusted to fix the position of the targeted cell using ImageJ plug-in (Align slice) according to the position of the cell nuclei and outline of fluorescent image of the cell. The average value of fluorescent intensity in a cell was used for analysis. Although shape of the cell deforms due to stretch application, the average value of fluorescent intensity was calculated using the ROI defined by initial (before stretch) outline shape the cell. To reduce the motion artifact due to out of focus during stretch, the change in concentration of intracellular calcium was evaluated by ratiometric microscopy method. The fluorescent intensity ratio was defined by (Fluo 3 / Fura Red). Actual stretch strain magnitude on each experiment was confirmed by measuring the deformation of the chamber. The transmitted image shows a contrast image of cells and the chamber together. Change in the length between two noticeable spot marks on the chamber was measured and used to calculate equivalent strain on the chamber. Correlation between the increase in intracellular calcium concentration and the magnitude of applied stretch strain was statistically analyzed by one-way ANOVA and multiple comparison using Welch’s $t$-test with bonferroni correction.
3. Result

3.1 Calcium signaling response to stretch with varied strain magnitude

Figure 2 shows representative time course fluorescent images of stretched cell with 5% strain magnitude. In the figure, fluorescent images are shown with pseudo color using LUT to improve visibility. $t = 0$ was defined as the time point at which image acquisition started. Equivalent strain magnitude on the chamber ($\varepsilon$) was measured by using transmitted images simultaneously obtained with fluorescent images ($t = \text{sec. from starting time point of image acquisition}$).
to the chamber in left direction (shown as a white arrow in the figure). There were two cells in the field of view. The time course change in the average fluorescent intensity of each cells are shown in Fig.3. Immediately after stretch, both fluorescent intensities of Fluo 3 and Fura Red in each cells showed synchronized transient increase in a few seconds. In contrast, the time course change in fluorescent ratio value (Fluo 3 / Fura Red) showed different response in each cells. Cell 1 showed transient increase in fluorescent ratio value with several hundred milliseconds delay after stretch. Cell 2 did not show significant change in fluorescent ratio value despite stretch application. The magnitude of increase in fluorescent ratio value in cell 1 was approximately 0.1.

Figure 5 shows representative time course fluorescent images of stretched cells with 10% strain magnitude. There were four cells in the field of view. The time point \( t \) and equivalent strain \( \varepsilon \) are defined in the same way shown in Fig.2. Figure 6 shows the time course change in fluorescent intensities of cells shown in Fig.5. The cell numbers shown in the
Fluo 3 correspond to the ones in Fig.5. Fluorescent intensities changed in diverse ways in each cell. Cell 1 shows transient increase of both Fluo 3 and Fura Red immediately after stretch. In contrast, Cell 2, 3 and 4 showed transient decrease of both Fluo 3 and Fura Red. Fig.7 shows the time course change in fluorescent ratio value of each cell. In this observation case, all cells showed transient increase as the response to stretch in the same manner. The magnitude of increase in fluorescent ratio value was approximately 0.2.

Fig.8 Time course fluorescent images of stretched cells with 15% strain. Left column shows fluorescent images of Fluo 3 and right column shows Fura Red. Pairs of images in same row were recorded at same time point. The white arrow indicates direction of stretch. (t = sec. from starting time point of image acquisition)

Fig.9 Time course change in fluorescent intensities of stretched cells with 15% strain. The cell numbers correspond with the ones shown in Figure 8. An arrow indicates the time point of stretch application.

Fig.10 Time course change in fluorescent ratio value of stretched cells with 15% strain. The cell numbers correspond with the ones shown in Figure 8. An arrow indicates the time point of stretch application.

graph correspond to the ones in Fig.5. Fluorescent intensities changed in diverse ways in each cell. Cell 1 shows transient increase of both Fluo 3 and Fura Red immediately after stretch. In contrast, Cell 2, 3 and 4 showed transient decrease of both Fluo 3 and Fura Red. Fig.7 shows the time course change in fluorescent ratio value of each cell. In this observation case, all cells showed transient increase as the response to stretch in the same manner. The magnitude of increase in fluorescent ratio value was approximately 0.2.

Figure 8 shows representative time course fluorescent images of stretched cells with 15% strain magnitude. There were three cells in the field of view. Figure 9 shows the time course change in fluorescent intensities of cells shown in Fig.8. The cell numbers shown in the graph correspond to the ones in Fig.8. Fluorescent intensities changed in diverse way in each cell. As shown in Fig.9, fluorescent intensity of Fluo 3 in cell 1 gradually increased with several seconds delay after stretching. While fluorescent intensity of Fura Red in cell 1 gradually decreased after stretching. In contrast,
fluorescent intensities in cell 2 and 3 did not show significant change despite stretch application. In the time course change in fluorescent ratio value shown in Fig.10, only cell 1 showed transient increase after stretching. The magnitude of increase in fluorescent ratio value in cell 1 was approximately 0.25.

3.2 Characteristics of calcium signaling response to stretching with varied strain magnitude

As shown in representative observation examples with stretch strain magnitude with 5% and 15%, although several cells were observed simultaneously with same stretch magnitude, not all of the cells responded to stretching. The percentage of responded cells to stretch application is shown in Fig.11. As shown in the graph, approximately 23% cells responded to the stretch with 5% strain magnitude, and 40% cells in 10% strain and 32% cells in 15% strain, respectively.

By considering the increase in fluorescent ratio value, an amount of increase in intracellular calcium ion concentration can be quantitatively evaluated. The relation between the increase in fluorescent ratio value and applied strain magnitude is shown in Fig.12. As applied strain magnitude is increased, an amount of increase in fluorescent ratio...
value also rises. There is statistically significant correlation ($p < 0.01$ by ANOVA) and ($p < 0.01$ by Welch’s $t$-test with Bonferroni correction between 5% stretch group and 10% stretch group, and $p < 0.05$ between 5% stretch group and 15% stretch group). A dispersion of increase in fluorescent ratio value also became larger in larger amount of stretch strain. There are not statistically significant difference between 10% stretch group and 15% group.

4. Discussion

In the present study, we combined the originally developed cell stretching micro device with fluorescent ratiometry method and successfully conducted continuous in situ observation of osteoblastic intracellular calcium signaling response to uniaxial stretch with high spatial and temporal resolution. Although some research groups have reported cell stretching device to observe the cellular response during stretching, their devices could not sufficiently reduce motion artifact during stretching, especially focus drift (Gerstmair, et al., 2009, Shao, et al., 2013). In our stretching device, we applied uniaxial stretch to the micro chamber by holding down the device arms to the cover glass by using metal needles. The amount of focus drift during stretch application was estimated to be approximately several microns by refocusing to the target cell after observation (data not shown). Even this slight focus drift significantly affects the intensities of each fluorescent indicators Fluo 3 and Fura Red. As increase in $[\text{Ca}^{2+}]$, Fluo 3 is supposed to increase their fluorescent intensity, in contrast Fura Red is supposed to decrease it. In some representative observation examples with no focus drift, such as the cell 1 shown in Fig.7, 8 and 9, fluorescent intensity of Fluo 3 gradually increased in response to stretch application, and that of Fura Red decreased. On the other hand, as the observation case shown in Fig.2, 3 and 4, fluorescent intensities of each cells showed synchronous up and down immediately after the stretch application. While these fluctuations are supposed to be caused by the motion artifact during stretch application, we can reduce the effect of motion artifact and evaluate the change in $[\text{Ca}^{2+}]$, by using the fluorescent ratio value calculated from Fluo3 / Fura Red. Actually, as shown in Fig.2, 3 and 4, there were two cells in the field of view and simultaneously observed during stretch application. One cell showed a steep increase in the fluorescent ratio value after stretch application, while the other cell showed no significant changes. These observation results support our opinion that we can successfully suppress the motion artifact during stretch application and evaluate the intracellular calcium signaling response quantitatively.

In this study, we calculated the average fluorescent intensities in the ROI which is defined by using the outline of the cell shape at the initial state (before stretch). Since the cell shape is changed due to the stretch application, the ROI does not represent the same region in the cell body in the strict sense. From our results, cells which did not respond to the stretch showed no significant change in fluorescent ratio value before and after the stretch application. These results suggest that discordance of regions between ROI and actual cell shape does not affect the evaluation results using change in fluorescent ratio value. However, we consider that the definition method of the ROI to represent the same position in stretched cell is very important issue to be solved. For further understanding about the intracellular calcium signaling response to stretch such as to clarify the initiation point of the intracellular calcium signaling response to stretch, more accurate analyzing procedure has to be invented.

We applied a much larger amount of stretch strain to the cell than that expected in physiological condition in bone tissue, nevertheless large numbers of cells did not show calcium signaling response to stretch in all stretch condition. And the percentage of the responded cells to stretch was not correlated with the stretch strain magnitude. The one possibility for this reason is low strain rate employed in this study. Tanaka and other research groups reported that osteoblasts activities have frequency dependence in cyclic mechanical loading, and there is appropriated frequency range to enhance osteogenesis (Rubin, et al., 2001, Zhang, et al., 2007, Lam and Qin, 2008, Qin, et al., 2010, Tanaka, 2012, Tanaka, 2014). The proposed frequency range is around 5 to 50 Hz. The strain rate employed in the present study was much lower than that of these appropriate frequency ranges. To simplify the experimental condition, we tested only one strain rate. Further investigation has been conducted to clarify the strain rate dependency of the osteoblasts response to mechanical loading, especially in higher range. Another possibility to explain about this problem is the difference in stretching cycle. In this study, we applied the one-way stretch and hold it at the maximally stretched state. Cells might be more sensitive to compression or stretch-release or cyclic stretch. Although it is the different phenomenon from intracellular calcium signaling response to stretch, we found out that compressive strain, but not stretch strain, induces the dissociation of actin stress fibers as the initial state of the rearrangement of actin cytoskeleton in osteoblastic cells (Sato, el al., 2005). The further investigation is needed to discuss about this issue.

It has been reported that osteoblasts change their response to varied magnitude of mechanical stimulus. Osteogenic
related protein and gene expression increase in direct relation to the stretch strain magnitude (Tang, et al., 2006, Li, et al., 2010). These experimental results indicate that osteoblasts can sense the magnitude of stretch strain and change their activities. In these studies, cyclic stretch was applied to the cells for 24 hours. And after the application of mechanical loading, the amount of protein and gene expression was evaluated. Therefore it still remains unclear what is the mechanism to sense the difference of stretch strain magnitude in osteoblast cells. Intracellular calcium ion plays an important role in various signaling pathways, and functions as a second messenger which locates in the early stage of a mechanotransduction pathway. Our results suggest that osteoblastic cells can sense the difference of stretch strain magnitude at an early stage of mechanotransduction. Moreover, different amounts of increase in [Ca^{2+}], as the response to varied magnitude of stretch strain could induce the difference of various cellular responses which locate in the downstream of mechanotransduction pathway.

In osteoblastic mechanotransduction mechanisms, stretch-activated Ca^{2+} ion channels on the cell membrane are activated by the mechanical stimulus and modulate [Ca^{2+}], (El Haj, et al., 1999). Although the mechanism to generate the different increase in [Ca^{2+}], as the response to different magnitude of stretch strain is totally unclear, one possibility is that the stretch-activated channels have the open-close probability correlated with the magnitude of mechanical stimulus. Larger magnitude of stretch strain might activate larger number of stretch-activated channels. However, the strain magnitude on the cell membrane which is transmitted through the mechanical components of the cell such as focal adhesions, cytoskeletons and nucleus under the extracellular substrate stretching. To clarify the strain distribution on the cell membrane and intracellular mechanical components including cytoskeletons and nucleus during stretching stimulus is one of the research objectives in our future work.

In this study, we succeeded to conduct the continuous in situ observation of osteoblastic intracellular calcium signaling response to stretch with high spatial and temporal resolution. The seamless time lapse observation without defocus and moving out of the field of view revealed the detailed characteristics of osteoblastic cellular response to stretch stimulus. Our results suggest that osteoblastic cells can sense the difference of stretch strain magnitude at the early stage of the mechanotransduction pathway and change their activities which locate in the downstream of the pathway. Our device and experimental system enable us to assess the cellular responses which arise immediately after the stretch application in milliseconds order and in subcellular level, and are useful to further investigation of the mechanotransduction mechanism of the cells.

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