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Effects of Strain Magnitude on Mechanical Responses of Three-Dimensional Gel-Embedded Osteocytes Studied with a Novel 10-Well Elastic Chamber*

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Abstract

Osteocytes inside bone matrix play an important role in detecting the local mechanical environment. In order to study their mechanical responses, we have developed a mechanical loading device that can apply physiological and supraphysiological strains to osteocytes embedded in a three dimensional (3-D) gel. The newly designed elastic chamber with ten separated culture wells can simultaneously apply five different strain magnitudes for the mechanical stretching of the cells. When the gel-embedded MLO-Y4 cells were prepared in the wells, they were subjected to mechanical stretching of physiological and supraphysiological strain levels for 24 h. The cell viability assay indicated that significant dead cells were observed for strain values greater than 8890 με. Beyond this threshold, the number of dead cells linearly increased with the strain magnitude. The supernatant of MLO-Y4 cells, which was exposed to strain levels beyond the threshold, showed a significant increase in tartrate-resistant acid phosphatase (TRACP) activity in the bone marrow culture. These experimental findings indicate that the local death of osteocytes provides an important mechanism to initiate bone resorption.

Key words: Osteocyte, Mechanical Stretching, Cell Death, Bone Remodeling, Cell Differentiation

1. Introduction

Mechanical loading is one of the factors that affect bone resorption and formation. Although adequate load can increase bone mineral density, repetitive mechanical loading sometimes induces microdamage in bone matrix, even if the magnitude is within the physiological range [1, 2]. The sites of microdamage must be sensed and promptly remodeled in order to avoid clinical bone fractures. Hence, mechanical loading and the consequent microdamage must be recognized by bone cells, which facilitates the regulation of bone mass and architecture. Although the mechanism for detecting microdamage and transducing specific signals has not been well elucidated, it is considered that osteocytes are the most promising candidates that can provide a cellular basis for mechanosensing and bone remodeling regulation [3-5].

In intact human bones, there are approximately ten times as many osteocytes as

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osteoblasts; [6] therefore, osteocytes are the most abundant bone cells. These cells are embedded deep within the mineralized bone matrix in lacunae spaces and connected to each other via gap junctional coupling through canalicular spaces. A three dimensional (3-D) network of osteocytes within the bone matrix provides a structure that is sufficient to detect matrix distortion induced by mechanical loading. However, their unique and almost inaccessible location has made it extremely difficult to study their functions. An osteocyte-like cell line, MLO-Y4, was recently developed from long bones of transgenic mice [7]. This cell line showed characteristics similar to those of primary osteocytes, such as less expression of alkaline phosphatase, high production of osteocalcin, and formation of long dendritic processes. MLO-Y4 cells also have a great potential to respond to mechanical stimulation [8-10].

Some investigators have recently focused on the crucial role of osteocytes in the initial resorptive phase of bone turnover. They reported that osteocyte death or apoptosis induced by fatigue microdamage coincided with osteoclastic bone resorption, which means that the initiation of bone remodeling can be attributed to microdamage [11]. We have recently indicated that damaged osteocytes could activate osteoclast precursors by soluble factors in which gel-embedded osteocytes were subjected to repetitive loading of 4000 με or 10,000 με; further, the M-CSF and RANKL could be secreted by supraphysiological loading [12,13]. However, the responses of osteocytes to mechanical stimuli ranging from physiological to supraphysiological strain magnitudes were not yet clear.

Therefore, the aim of this study is to examine the effects of strain magnitude on the mechanical responses of gel-embedded MLO-Y4 cells. We have developed a mechanical device that can apply physiological and supraphysiological strains to MLO-Y4 cells. The supernatants of the MLO-Y4 culture were evaluated by adding them to bone marrow cell culture and examining cell differentiation.

2. Materials and methods

2.1 Mechanical loading apparatus

An apparatus for stimulating the cells was designed to stretch the elastic chamber. The elastic chamber comprised ten separated culture wells. The inner size of the well was 8 x 17 x 10 mm (Fig. 1). The wall thickness of each well was increased in a stepwise manner from the peripheral to the center region so that the wall cross-sectional area of well No.1 was 1.2 times that of well No.2. The wall cross-sectional area was decreased from well No.1 to No.5 in a similar manner. This design theoretically realized the requirement that the strain magnitude at well No.2 was 1.2 times that at well No.1. Additionally, the neutral axis of the geometrical moment of inertia in the well cross-section was fitted to the center of the rods, which prevented the silicone chamber from bending or twisting.

The elastic chamber was molded with a silicone elastomer (SILGARD 184 Silicone Elastomer Kit, World Precision Instruments, Inc., Sarasota, FL). The elastomer was poured into a cast, cured overnight under vacuum, and sonicated in pure water. The molded chamber was sterilized in 70% ethanol before use. The stretching was accomplished with reciprocating stainless steel rods that were inserted into both the sides of the chamber. One of the rods was firmly fixed to a basement frame, while the other rod was held on a movable frame. The movable frame was reciprocated with a motor-driven shaft. The frequency and displacement of stretching were regulated by a ball screw, stepping motor, and motor controller (Fig. 2). All the devices, except the motor controller, were fixed inside a CO_2 incubator, and thus the mechanical stretching could be applied to the cells during the culture.

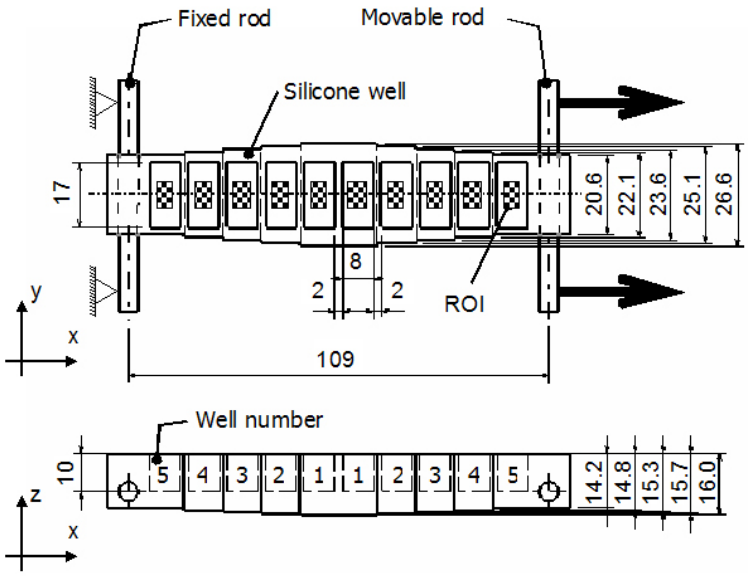

Fig.1 Elastic chamber with ten separated culture wells.

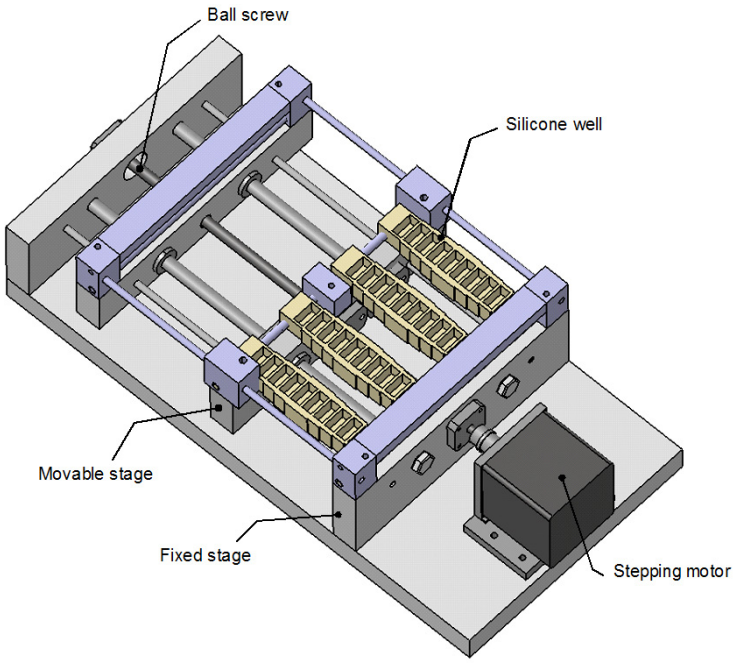

Fig. 2 Mechanical loading apparatus.

2.2 Three-dimensional gel-embedded culture for osteocytes

The mouse primary osteocyte-like cell line MLO-Y4 was kindly provided by Dr. Lynda Bonewald (University of Missouri at Kansas City, MO) and Dr. Yoichi Kato (Asahi Kasei Corporation, Tokyo, Japan) [7]. The cells were routinely maintained on type I collagen-coated plates (0.15 mg/ml, rat tail collagen type I, BD Biosciences, Bedford, MA) in an α–minimum essential medium (α-MEM, Gibco BRL, Paisley, Scotland) supplemented with 2.5% fetal bovine serum (FBS, Gibco BRL), 2.5% iron-supplemented calf serum (iCS, HyClone Laboratories, Inc., Logan, UT), and antibiotics (100IU/ml penicillin and 100μg/ml streptomycin, Gibco BRL).

According to a previous study, MLO-Y4 cells were embedded in collagen gel and cultured three-dimensionally *in vitro* [12, 13]. In brief, neutralized collagen solution

(Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan) was mixed with Matrigel Basement Membrane Matrix (BD Biosciences) at a ratio of 1:1 because preliminary experiments revealed that Matrigel was effective for MLO-Y4 cells in extending cell processes and forming cell-to-cell contacts inside the gel. As a base layer, 100 μl of plain collagen gel was poured into an elastic culture well. After allowing the base layer to solidify by incubation for15 minutes at 37°C, 200 μl of the cell-suspended collagen-Matrigel mixture was overlaid. The initial cell density was 2×10^6 cells/ml. The gel in the culture well was solidified by additional incubation for 15 min, and then 400 μl of the culture medium was added. The culture medium for gel-embedded culture consisted of α -MEM, 5% FBS, 5% iCS, and antibiotics.

2.3 Evaluation of cell-to-cell strain magnitude in the gel-embedded culture

The relationship between the displacement of the movable rod and the strain generated inside the gel was preliminary evaluated by measuring the deformation of the gel-embedded cells. The MLO-Y4 cells were cultured inside the gel for four days, as described above. After the four-day culture, the nuclei of the MLO-Y4 cells were stained under dark conditions for 10 min with Hoechst33258 (Hoechst33258 solution, Dojindo, Tokyo, Japan) with a dilution of 1:600 (stock solution: 1mg/ml). By mounting the elastic chamber on the stage of a microscope (IX 71, Olympus, Japan), the location of the Hoechst-stained cells was observed with a 10x objective lens (UPlanFl, 22.4 μm depth of field, Olympus, Japan). When the chamber was stretched from a displacement of 0 to 3 mm in steps of 0.25 mm, fluorescent microscopic images of the stretched cells were obtained in each region of interest (ROI, Fig.1) with a CCD camera (FX380, Olympus, Japan) connected to a personal computer. The ROI (4 x 8 mm) was defined at the center of each well. Ten microscopic images were randomly recorded in the central plane between the well bottom and gel surface in the ROI.

The cells which emitted bright hoechst fluorescence and visually in focus were manually selected from the obtained images. The centroid of the cell nuclei were marked, and then identical cells were selected from each microscopic image. When several pairs were formed, the change in the cell-to-cell distance was measured along the stretching (axial) and the orthogonal directions. The generated strain was calculated by dividing the increased distance between the two cells by their initial distances. The spatial resolution of the measurement was approximately 1.4 μm. The measurement was repeated thrice by using three independent gel cultures.

2.4 Determination of dead cells induced by mechanical stretching

In order to determine the influence of mechanical stretching with wide strain ranges, the number of dead cells was assessed in the gel-embedded MLO-Y4 culture after mechanical loading for 24 h. After the cells were incubated in the elastic chambers for four days, the chambers were mounted on the loading apparatus and were subjected to triangular-wave stretching for 24 h at a frequency of 2 Hz. The cells were subjected to the following strain magnitudes: 933 (Strain 1), 1780 (Strain 2), 2240 (Strain 3), 2640 (Strain 4), and 3000 με (Strain 5) in strain pattern I; and 4670 (Strain 6), 8890 (Strain 7), 11200 (Strain 8), 13200 (Strain 9), and 15000 με (Strain 10) in strain pattern II (Table 1). The control cells were cultured without applying mechanical stretching.

After the application of mechanical stretching, the cells were stained with 2 mM ethidium homodimer-1 (EthD-1, Molecular Probes, Inc., Eugene, OR) for 20 min. EthD-1, which binds to nucleic acids, shows red florescence in dead cells. The images were obtained as described above. The stained cultures were rinsed twice with phosphate buffered saline

(PBS) and immediately observed under a fluorescence microscope. The experimental was repeated thrice by using three independent gel cultures obtained from various strain magnitudes.

In order to examine the number of live cells, the gel-embedded cultures stretched at strain pattern II and non-stretched control were treated with 0.02% collagenase (Collagenase, Nitta Gelatin, Osaka, Japan). The cells were further trypsinized, mixed with 0.5% trypan blue, and then counted by using a hemacytometer.

Table 1 Pattern of strain magnitude in the experiment.

Pattern	Well number	Group	ε_x (με)	ε_y (με)
Non-stretched	-	Control	0	0
Strain I	1	Strain 1	933	-265
	2	Strain 2	1780	-715
	3	Strain 3	2240	-918
	4	Strain 4	2640	-975
	5	Strain 5	3000	-1020
Strain II	1	Strain 6	4670	-1320
	2	Strain 7	8890	-3570
	3	Strain 8	11200	-4590
	4	Strain 9	13200	-4880
	5	Strain 10	15000	-5120

2.5 Effect of supernatant of the stretched MLO-Y4 culture on bone marrow cell differentiation

The gel-embedded MLO-Y4 culture, which formed a 3-D cellular network, was prepared as described above. All the culture media were changed to fresh ones, and then the cells were subjected to the stretching regimes of patterns I and II. The non-stretched culture was also prepared under control. After application of mechanical stretching for 24 h at 2Hz, the supernatants of the culture media were collected and mixed with fresh medium at a concentration of 20%. These conditioned media (CM) were immediately used for the following bone marrow cell culture. Bone marrow cells were obtained from 8-week-old ICR mice (Kyudo, Saga, Japan) according to the method previously reported [12,13]. In brief, the tibiae and femora were removed and dissected free from soft connective tissues. The bone marrow was flashed out with antibiotics containing α-MEM after both the metaphyses were cut off with a scalpel. Un-fractioned bone marrow cells were collected by centrifugation at 50 x g for 10 min and seeded onto a culture plate with α-MEM containing 10% FBS and antibiotics. After cultivation (37°C, 5% CO_2) for 2 h, non-adherent cells were collected and then applied into a 24-multi well plate at a density of 10^6 cells/well. The cells were cultured for a week with 1 ml of the CM. Half milliliter of the medium in each well was replaced with a new batch of CM collected from another stretched MLO-Y4 culture on the third day. After a week of incubation, the cellular tartrate-resistant acid phosphatase (TRACP) activity was evaluated. The cells were carefully washed with PBS and lysed into 200 μl of lysing buffer (10 mM Tris, 2 mM EDTA, 10 mM NaCl, 0.5% Triton X-100, 0.3% SDS, 1x protease inhibitor) [14]. The enzyme activity in 25 μl lysates was detected by using 8 mM p-nitrophenylphosphate (pNPP, Sigma Chemical, St. Louis, MO) as a substrate in 0.1 M sodium acetate buffer (pH 5.7) containing 40 mM L(+)-tartrate. After a 30-min incubation at 37°C, the enzyme reactions were terminated by the addition of 20 μl of 1.625 M NaOH, and then the optical density at 405 nm was measured with FLUOstar OPTIMA (BMG LABTECH GmbH, Germany). The TRACP activity was determined as the enzyme activity that hydrolyzed 1 μmol of pNPP/min at 37°C.

2.6 Statistical analysis

All of the experiments were repeated at least three times and values were presented as mean value ± standard deviation (SD). Statistical analysis was performed with use of a one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference among groups, the difference was evaluated using Fisher's protected least significant difference (PLSD). A confidence level of 95% ($p < 0.05$) was chosen for statistical significance.

3. Results

3.1 Cell-to-cell strain magnitude under mechanical loading

At each culture well, the strain magnitudes along the axial (Fig. 3A) and orthogonal (Fig. 3B) directions were calculated by determining the change in the nuclear-to-nuclear distances of cells embedded in the gel. The relationship between displacement and the generated cell-to-cell strain was linear. According to this strain measurement, a tensile strain up to 48,000 με or 4.8% could be applied to the cells when the rod was displaced from 0 to 3 mm. The strain magnitude in well No.5 was 3.21 times that in well No.1. In the orthogonal direction, compressive strain was generated; the absolute magnitude of this strain was approximately 70% less than that along the axial direction.

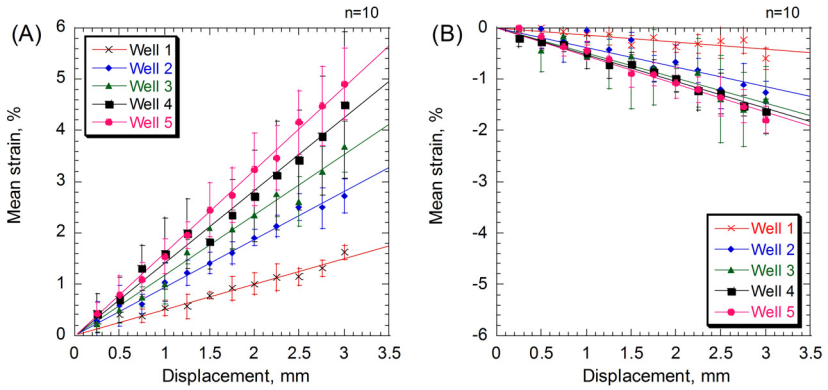

Fig. 3 Strain magnitude along the axial (A) and orthogonal (B) directions.

3.2 Dead cell induction by the application of mechanical stretching

In order to confirm the cell viability after the application of mechanical stretching, the gel-embedded MLO-Y4 cell was stained with EthD-1 (Fig.4). EthD-1 dye, which was bound to nucleic acids, were used to show the distribution of dead cells. The cells emitting red fluoresce gradually increased according to the strain magnitude. Apparently, the distribution of dead cells became more dense in the culture stretched over 5000 με (Fig. 4C and D).

The number of EthD-1 positive cells was counted in each macroscopic image, and this number is indicated in Fig.5. The numbers of dead cells in each stretched group was normalized with that in the groups under non-stretched control. The application of mechanical stretching increased the number of dead cells in a magnitude-dependent manner, and a significant amount of the cells were injured due to the stretching that exceeded the Strain 7 group. The number of dead cells in Strain 1~6 groups showed no significant differences as compared to the control groups.

On the other hand, the number of live cells in Strain 6~10 and control groups were $25.7 \pm 1.9 \times 10^4$, $24.8 \pm 3.1 \times 10^4$, $27.4 \pm 5.7 \times 10^4$, $26.7 \pm 4.0 \times 10^4$, $24.4 \pm 1.6 \times 1\,0^4$, and $26.8 \pm 2.4 \times 10^4$ cells, respectively. This implies that the application of mechanical stretching had no significant effect on cell proliferation.

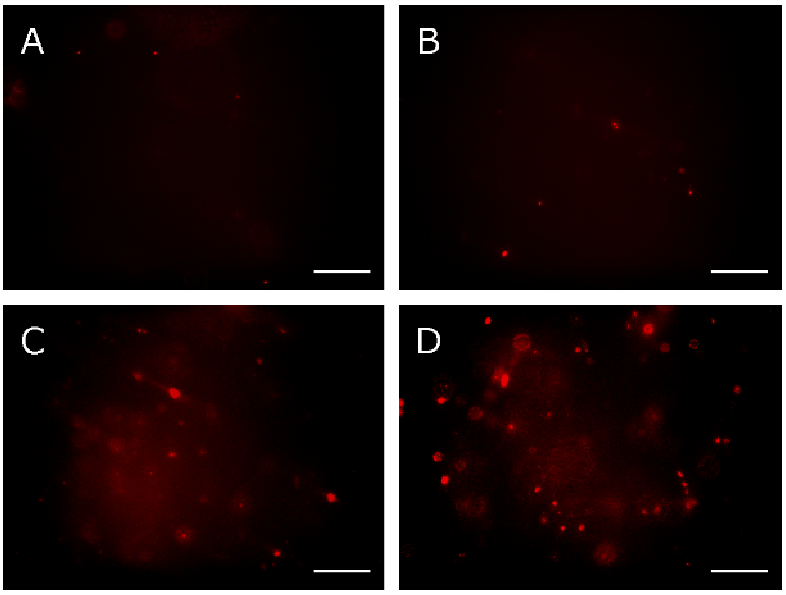

Fig. 4 Dead cell labeling with mechanical stretching in non-stretched groups (A), 3000 με (B), 5000 με (C), and 15,000 με (D). Bar; 100 μm.

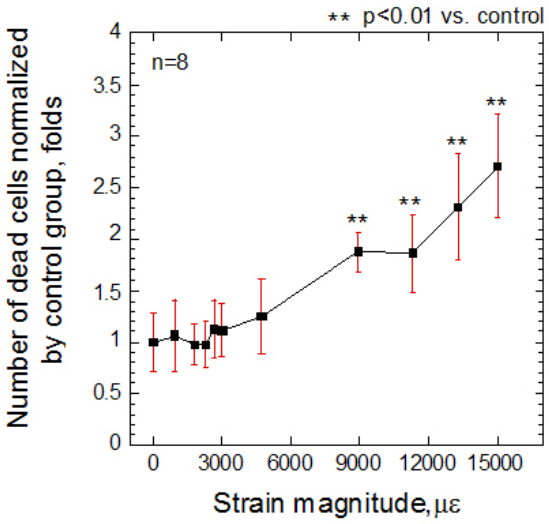

Fig. 5 Number of dead cells normalized by non-stretched control culture (Mean ± SD).

3.3 TRACP-positive cell differentiation induced by conditioned media

In order to evaluate the effects of mechanically stretched osteocytes on cell differentiation, the bone marrow cells were incubated in the presence of the CM collected from MLO-Y4 culture. Figure 6 shows the TRACP activity in each stretching group in which each enzymatic activity was normalized by that of non-stretched control. The TRACP activities were normalized without mechanical stretching in a non-stretched control group. The media from Strain 1~6 groups showed no significant potential to induce TRACP-positive cells as compared with that from non-stretched control. The CM from the stretched MLO-Y4 at Strain 7~10 significantly increased the TRACP activity in the bone marrow cells.

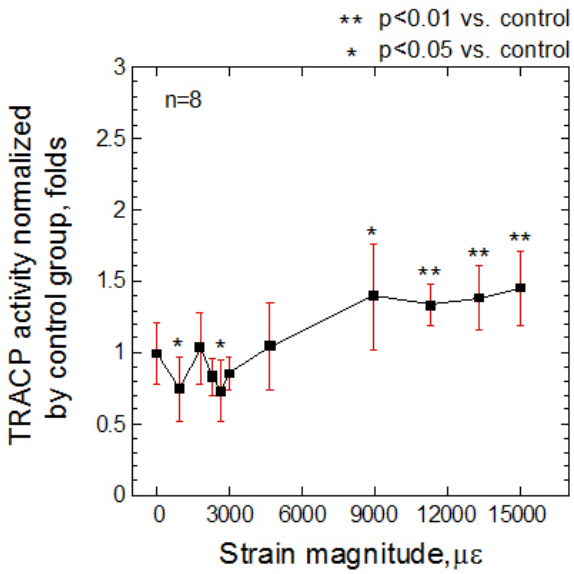

Fig. 6 TRACP activity normalized by non-stretched groups (Mean ± SD).

4. Discussion

Our newly designed silicone chamber consists of ten symmetrical and separated culture wells. Five different strain magnitudes can be applied to the separated cell culture simultaneously, and thus the experimental data can be obtained for five different conditions with a single experiment. This can be very useful in increasing the accuracy of the experiment as well as in reducing the risk of contamination. From well No.1 to well No.5, the wall cross-sectional area of the inner well is designed to be 1.2 times that of the outer well. The strain magnitude generated at the well is theoretically proportional to the wall cross-sectional area, and therefore the strain at well No.2 should be 1.2 times that at well No.1. Our measurement of the cell-to-cell strain magnitude indicated that this design concept was mostly accomplished by our loading system, although the strain magnitude at well No.1 was slightly smaller that the theoretical expectation. In practice, the differences between theoretical and experimental strain magnitude do not matter because the mechanical loading conditions were defined by the microscopic observation of cell-to-cell strain in the gel culture.

The stretching apparatus can apply tensile strain up to 48,000 με (4.8%) to gel-embedded osteocytes. With regard to the strain magnitude, a number of previous studies have been reported in which both *in vivo* and *ex vivo* strain measurements using animal cortical bones were performed. Turner *et al*. examined the surface strain magnitude on rat tibia by using the four-point bending model and observed that a loading threshold above 1050 με activated bone formation on endocortical surfaces [15]. Rubin loaded a functionally isolated ulna of young and old turkeys at 3000 με for eight weeks. The geometrical properties of the young bones significantly increased after the loading period because of the increased periosteal mineralization [16]. These reports showed that bone tissue can sense a mechanical strain of approximately 3000 με and respond to it by activating bone formation.

Frost suggested that bone mass in a healthy animal adapts to mechanical usage by three tissue-level biological mechanisms: growth, modeling, and remodeling [17, 18]. Since these three biological activities were considered to be switched on or off by mechanical signals with the threshold, it was called as a mechanostat. In this mechanostat theory, it is mentioned that bone strains above 1500~3000 με cause bone modeling to increase the bone

mass while strains below 100~300 με induce bone loss. When the strain magnitude reaches 25,000 με, a normal lamellar bone gets fractured. Considering these measurements and theoretical findings, the cells in our apparatus might be exposed to much excessive strain level. However, osteocytes, which are considered to be mechanosensing cells, are embedded deep within the mineralized bone matrix in lacunae-canalicular spaces. From the viewpoint of material mechanics, these spaces can be regarded as material defects in the bone matrix, which induce stress concentration. There is a possibility that the matrix around osteocytes would have suffered from a strain significantly higher than the reported values. A recent report showed that bone matrix strain around osteocyte lacunae typically reached levels of approximately 12,000～16,000 με and the peak local strains exceeded 30,000 με when a bulk strain of 2500 με was applied to fresh bone specimen under a microscope [19]. Our newly developed apparatus can apply controllable strains widely ranging up to 48,000 με, which can be useful to study the mechanical responses of osteocytes by mimicking the *in vivo* situation.

Although a number of experimental devices have been established to apply mechanical stimulation to cells, the mechanical responses of osteocytes were examined only when they were grown on a 2-D plane substrate [20, 21]. Considering the environment around osteocytes *in vivo*, they are 3-D distributed in the bone matrix and form 3-D cellular networks. In order to examine the appropriate responses of osteocytes, the cells should be cultured in a 3-D substrate, which can sustain their original organization and function during *in vitro* culture. The 3-D gel-embedded cultures of various cells have already been reported to support the cell proliferation, differentiation, and functional expression of phenotypes [22-25]. With regard to bone-derived cells, the growth and matrix production were enhanced by collagen substratum in an osteoblast-like cell culture [26]. Furthermore, osteoblast-like cells grown in collagen gel expressed the osteocytic phenotypes, such as a spindle-like shape and cytoplasmic projections toward neighboring cells [27, 28]. Thus, our culture system that mimicks cell environment inside bone matrix is suitable for studying osteocytes, although it lacks the other major components of bone mineralized matrix.

Many studies have used elastic culture substrates to apply mechanical stretching to bone-derived cells. For instance, Miyauchi *et al*. cultured chicken and rat primary osteocytes on a flexible membrane and cyclically stretched the cells by using the Flexcercell unit [29-31]. In their study, the cells were subjected to 2000 με- and 4000 με-stretching, which was defined by the strain magnitude acting on the elastic membrane. Danciu *et al*. cultured MC3T3-E1 cells on the bottom of a rectangular silicone chamber and stretched it at 200,000 με by using the Cell Stretcher System [32, 33]. They also defined the strain magnitude in terms of the elongating percentage of the culture substrate. It was not mentioned how much strain the cells suffered from. When the relationship between strain magnitude and cellular responses is discussed, cell deformation is much more important than substrate elongation. Although osteocytes are generally attached to culture substrates with tight focal adhesions, it is not clear whether the cells deform their bodies following substrate deformation. Thus, we calculated the strain magnitude from the axial strain acting on a representative cell in the middle plane of the gel.

Mechanical stretching of 8890 με induced significant cell death in the gel, which extends upto supraphysiological strain levels according to the mechanostat theory [17, 18]. Although the mechanism of the cell death is not certain, the cellular cytoplasmic membrane might rupture by excessive stretching and the cell would undergo necrosis. When mineralized bone matrix is subjected to supraphysiological strain, microcrack occurs in the matrix, which results in the rupture of the cells that exist across the microcrack. Nicollela *et al*. reported that microcracks can be expected at the lacunae for strain of the order of 10,000 με [19]. Prendergast and Huiskes showed that microcracks occurs at a critical local strain of the order of 8000 με [34]. It was not mentioned whether or not osteocytes were damaged by

microcracks because only the material behavior was studied; however, the osteocytes near microcracks are likely to rupture in *in vivo* situations. Although our experimental system lacks a mineralized component of the bone matrix, the gel-embedded cells can be damaged by supraphysiological stretching and can undergo necrotic cell death, which resembles the osteocytes behavior *in vivo*.

In order to evaluate the effects of mechanically stretched osteocytes on bone marrow cell differentiation, the gel-embedded MLO-Y4 cells were cultured in the apparatus under mechanical stretching of various strain magnitudes. The conditioned medium collected from mechanically damaged MLO-Y4 cells stretched over 8890 με could promote a significant TRACP-positive cell induction from the bone marrow cells. TRACP is one of the marker enzymes secreted by osteoclasts and has been widely used as a specific indicator for osteoclasts [35-37]. This result was consistent with that of our previous study [12, 13], in which mechanically damaged osteocytes were reported to have an influence on the initial phase of osteoclast formation. This implies that an osteocyte is a key instrument for bone remodeling regulation. A detailed mechanism of osteocytic mechanosensing, transduction of mechanical/chemical signals, and induction of bone remodeling coincident with microcracks must be further examined by focusing on osteocyte functions. For this purpose, our newly developed apparatus can be a useful experimental system.

5. Conclusions

A new mechanical loading apparatus that could apply repetitive stretching with wide strain ranges was developed to examine the mechanical responses of gel-embedded osteocytes. Excessive stretching greater than approximately 8980 με induced significant amount of cell death. The conditioned medium obtained from the mechanically damaged osteocyte culture could induce TRACP-positive cell differentiation, which indicates that soluble factors secreted by the damaged osteocytes have a potential to promote osteoclastic cell differentiation. These findings show that the newly developed culture system is useful to study the relationship between the wide-ranging strain magnitude and functional responses of osteocytes.

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