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Cyclic mechanical stretch contributes to network development of osteocyte-like cells with morphological change and autophagy promotion but without preferential cell alignment in rat

Nao Inaba, Shinichiro Kuroshima*, Yusuke Uto, Muneteru Sasaki, Takashi Sawase

Department of Applied Prosthodontics, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1, Sakamoto, Nagasaki-city, Nagasaki 852-8588, Japan

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ABSTRACT

Osteocytes play important roles in controlling bone quality as well as preferential alignment of biological apatite *c*-axis/collagen fibers. However, the relationship between osteocytes and mechanical stress remains unclear due to the difficulty of three-dimensional (3D) culture of osteocytes *in vitro*. The aim of this study was to investigate the effect of cyclic mechanical stretch on 3D-cultured osteocyte-like cells. Osteocyte-like cells were established using rat calvarial osteoblasts cultured in a 3D culture system. Cyclic mechanical stretch (8% amplitude at a rate of 2 cycles min^{-1}) was applied for 24, 48 and 96 consecutive hours. Morphology, cell number and preferential cell alignment were evaluated. Apoptosis- and autophagy-related gene expression levels were measured using quantitative PCR. 3D-cultured osteoblasts became osteocyte-like cells that expressed osteocyte-specific genes such as *Dmp1*, *Cx43*, *Sost*, *Fgf23* and *RANKL*, with morphological changes similar to osteocytes. Cell number was significantly decreased in a time-dependent manner under non-loaded conditions, whereas cyclic mechanical stretch significantly prevented decreased cell numbers with increased expression of anti-apoptosis-related genes. Moreover, cyclic mechanical stretch significantly decreased cell size and ellipticity with increased expression of autophagy-related genes, *LC3b* and *atg7*. Interestingly, preferential cell alignment did not occur, irrespective of mechanical stretch. These findings suggest that an anti-apoptotic effect contributes to network development of osteocyte-like cells under loaded condition. Spherical change of osteocyte-like cells induced by mechanical stretch may be associated with autophagy upregulation. Preferential alignment of osteocytes induced by mechanical load *in vivo* may be partially predetermined before osteoblasts differentiate into osteocytes and embed into bone matrix.

1. Introduction

Bone quality, which has been defined as “the sum of all characteristics of bone that influence the resistance to bone fracture [1],” is completely independent of bone mineral density (BMD). Bone quality plays a crucial role in determining bone strength with BMD. Particularly, both preferential alignment of biological apatite (BAp) *c*-axis/collagen fibers and osteocytes are key factors affecting bone quality [2]. Osteocytes, which reside within lacunae of bone matrix, play a central role in controlling bone quantity (bone volume). Mechanical load, in particular, is considered to be one of the essential factors regulating bone quantity, as mechanical unloading results in load-associated osteoporosis [3]. Mechanical load on bone tissue is converted to various mechanical stimuli such as fluid shear stress, hydrostatic pressure and direct deformation of osteocytes [4]. Recently, we reported that cyclic mechanical load on bone tissue via dental implants led to the development of osteocyte networks with preferential alignment of BAp *c*-axis/collagen fibers and increased bone volume around dental implants [5]. Moreover, it has been reported that preferential alignment of osteocytes occurred in response to mechanical stimulation [6]. However, when preferential alignment of osteocytes occurs, how osteocytes control bone quality and why osteocyte networks are improved in response to mechanical load remain unclear.

Autophagy and apoptosis, which are two distinct self-destructive processes, play an important role in degradation of cytoplasmic organelles and cell death, respectively. Autophagy is an intracellular degradation system that delivers cytoplasmic degraded components to lysosomes fused to autophagosomes [7]. Apoptosis is one of several types of programmed cell death that occurs by altering the balance between apoptosis-related proteins and anti-apoptosis-related proteins.

* Corresponding author.
E-mail addresses: bb55313205@ms.nagasaki-u.ac.jp (N. Inaba), kuroshima@nagasaki-u.ac.jp (S. Kuroshima), y-uto@nagasaki-u.ac.jp (Y. Uto), m-sasaki@nagasaki-u.ac.jp (M. Sasaki), sawase@nagasaki-u.ac.jp (T. Sawase).

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Upregulation and downregulation of autophagy or apoptosis are influenced by multiple factors such as physiological stress, hormonal stimulation, drug exposure, bacterial infections, innate immune signals, senescence and several types of diseases including cancers and immune diseases [8,9]. Recently, it has been reported that both autophagy and apoptosis are also influenced by mechanical stress in mammalian cells [10]. However, the effect of mechanical stimulation on autophagy and apoptosis in osteocytes has not been investigated due to the difficulty in monitoring osteocytes *in vitro*. Here, we developed a three-dimensional (3D) culture system in which rat calvarial osteoblasts were differentiated into osteocyte-like cells *in vitro*. The aim of this study was to investigate the effect of cyclic mechanical stretch on cultured osteocyte-like cells in rat.

2. Materials and methods

2.1. Establishment of osteocyte-like cells derived from rat calvarial osteoblasts in a 3D culture system

Three female Wistar rats (12-week-old) were purchased (Biotek Co. Ltd., Saga, Japan). Calvarial osteoblasts were isolated as previously described [11]. To establish osteocyte-like cells *in vitro*, a 3D culture system was developed with modification of previous studies [12,13]. A silicone chamber (STREX Inc., Osaka, Japan) was coated with collagen type I rat tail (Corning, Inc., NY, USA) and 0.02 N acetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Gel constructs were made using a 1:1 ratio of Matrigel (BD Biosciences, NJ, USA) and a collagen solution consisting of 58% collagen type I tail, 26% 5× DMEM, 2.5% FBS, 2.5% fetal calf serum (Gibco by Life Technologies, CA, USA), 0.05 N sodium hydroxide (Wako Pure Chemical Industries, Ltd.) and a 5% cell suspension (1×10^6 cells/mL). The gel was transferred to the collagen-coated silicon chamber and incubated at 37 °C for 60 min. Osteoblasts were cultured in this 3D culture system to facilitate network formation with cell process-like structures. Osteoblasts cultured on collagen type I rat tail and 0.02 N acetic acid coated-silicon chambers were used as control (designated as 2D culture). All experiments were performed in accordance with the guidelines for the Animal Experimentation of Nagasaki University, and the current protocol was approved by the Ethics Committee for Animal Research of Nagasaki University.

2.2. Mechanical stimulation of osteocyte-like cells in vitro

A loading device (ST-140, STREX Inc.) was used for mechanical stimulation. Silicon chambers were directly connected to the loading device. Cyclic mechanical stimulation was then initiated 24 hours after the start of 3D culture. Cells were cultured under continuous cyclic stretch of 8% amplitude at a rate of 2 cycles min^{-1}. Cyclic stretch amplitude > 8% was not used because the gel could not maintain its structure without tearing. Cyclic mechanical stimulation was applied for 24, 48 and 96 hours with medium changes every 2 days (Fig. 1A–D).

2.3. Fluorescent staining and quantitative morphological analyses

Cells were fixed with culture gel and stained for phalloidin A (Cytoskeleton Inc., Denver, CO, USA) and DAPI (TAKARA Bio Inc., Tokyo, Japan) for 30 minutes at 37 °C. Fluorescent microscopy (BZ9000, Keyence, Osaka, Japan) and CellProfiler software (Broad Institute, Cambridge, MA, USA) were used to visualize cells and for morphological analyses [14]. The images at half thickness from the bottom area were used for all analyses. Cell number, cell area, cell perimeter, major axis length, minor axis length, cell eccentricity, cell solidity and cell orientation were automatically measured using CellProfiler. Each parameter was determined as follows: (1) cell number and area (pixel) = the actual number of pixels in the region; (2) cell perimeter (pixel) = the total number of pixels around the boundary of each region in the image; (3) cell eccentricity = the eccentricity of the ellipse that has the same second-moments as the region. Eccentricity is the ratio of the distance between the foci of the ellipse and its major axis length; (4) major axis length (pixel) = the length of the major axis of the ellipse that has the same normalized second central moments as the region; (5) minor axis of length (pixel) = the length of the minor axis of the ellipse that has the same normalized second central moments as the region; (6) cell solidity (ratio) = the proportion of pixels in the convex hull that are also in the object; and (7) cell orientation (degree) = the angle between the x-axis and the major axis of the ellipse that has the same second-moments as the region. Absolute values of the cell alignment angle were used in this study.

2.4. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using TRIZOL (Invitrogen, CA, USA) and the phenol/chloroform method [15]. First strand cDNA was synthesized using the Promega system (Promega, WI, USA). Quantitative real-time PCR (qPCR) was conducted using the Thermal Cycler Dice system (TAKARA, Shiga, Japan) with SYBR green (Invitrogen). Samples were run in triplicate. All results were normalized to *β-actin* expression. Relative quantification of data generated using this system was performed using the standard curve method. The following primer sets were used: osteoblast-specific genes (*Runx2* and *Opn*), osteocyte-specific genes (*Cx43*, *E11*, *Mepe*, *RANKL*, *Dmp1*, *Fgf23* and *Sost*), apoptosis-related genes (*Bcl-XL*, *Bcl2*, *Bim*, *Bid*, *Bak1*, *Bax*, *PUMA*, *Casp3* and *Casp7*) and autophagy-related genes (*Ulk1*, *LC3b*, *Becn1*, *AMPKα1*, *AMPKα2*, *Mtor*, *p62/sqstm1*, *p62*, *atg7* and *p53*) (Table 1).

2.5. Statistics

Statistical analyses were conducted in a blinded manner. The Shapiro-Wilk test was performed to test for normality. The independent *t*-test and one-way analysis of variance were used for parametric data. The Kruskal-Wallis test was used for non-parametric data. All statistical analyses were conducted using SYSTAT 12 (Systat Software, Chicago, IL, USA). An α-level of 0.05 was used for statistical significance. All data are represented as the mean ± SEM.

3. Results

3.1. Effect of 3D culture for 120 hours on rat calvarial osteoblasts

Representative fluorescent images of 2D and 3D culture are shown in Fig. 2A. 2D- and 3D-cultured cells exhibited distinct morphology. 3D-cultured cells appeared to have cell process-like structures that connected to adjacent cells. *Runx2* expression was similar between 2D- and 3D- cultured cells (Fig. 2B). *OPN* expression in 3D-cultured cells was significantly higher than that in 2D-cultured cells (Fig. 2C). *Mepe* and *E11* expression levels were almost the same between 2D- and 3D-cultured cells (Fig. 2D, E), whereas *Dmp1*, *Cx43*, *Sost*, *Fgf23* and *RANKL* expression levels in 3D-cultured cells were significantly increased compared with those in 2D-cultured cells (Fig. 2F–J).

3.2. The effect of cyclic mechanical stretch on cell number and apoptosis

Cell numbers under loaded conditions at 96 hours after mechanical stretch appeared to be increased compared to that under non-loaded conditions (Fig. 3A, B). Cell number under non-loaded conditions was significantly decreased in a time-dependent manner, whereas it was not decreased at 48 and 96 hours after cyclic mechanical stretch (Fig. 3C). Cyclic mechanical stimulation significantly suppressed *Casp3* expression (Fig. 3D), whereas *Casp7* expression did not significantly change (Fig. 3E). Cyclic mechanical stimulation significantly increased *Bcl-XL* expression (Fig. 3F). *Bcl2* expression did not change, regardless of mechanical stimulation (Fig. 3G). Additionally, cyclic mechanical