Journal of Biomechanics 47 (2014) 3750–3757

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Effects of low frequency cyclic mechanical stretching on osteoclastogenesis

Yousef Shafieyan [a,c], Kerstin Tiedemann [b,c], Svetlana V. Komarova [b,c], Thomas M. Quinn [a,*]

[a] Department of Chemical Engineering, McGill University, 3610 University Street, Montreal, Canada H3A 2B2
[b] Faculty of Dentistry, McGill University, Montreal, Canada
[c] Shriners Hospital for Children, 1529 Cedar Avenue, Quebec, Montreal, Canada H3G IA6

ARTICLE INFO

Article history:
Accepted 20 June 2014

Keywords:
Osteoclast
Mechanical stimulation
Low frequency
Gene expression
Signaling pathway

ABSTRACT

Bone cells are continuously exposed to mechanical deformations originating from movement. Mechanical stimulation at fundamental frequencies associated with most frequent normal locomotion (0.167–10 Hz) has been reported to suppress differentiation of osteoclasts. However, the effects of very low frequency (0.01 Hz) stimulation (which could be a frequency component of normal movement and also relevant to locomotion of movement-impaired individuals) on osteoclasts are poorly understood. We examined differentiation of osteoclasts from mouse bone marrow precursors and RAW 264.7 monocytes cultured on an extendable silicone surface that was dynamically stretched at 0.01 Hz. Three stimulation regimes were applied: (i) continuously during 4 days of differentiation, (ii) non-continuously, 8 h/day for 4 days, and (iii) post-differentiation, when stimulation was applied for 24 h after osteoclasts were noted. Low frequency mechanical stimulation did not inhibit osteoclastogenesis. Moreover, the expression of osteoclast marker genes was upregulated in mechanically stimulated cells compared to static control. Conditioned medium collected from osteoclast cultures stimulated non-continuously or post-differentiation induced differentiation of osteoclast precursors plated in standard tissue culture plates. Extracellular signal-regulated kinase (ERK) phosphorylation was increased in mechanically-stimulated cultures compared to static control. Thus, low frequency mechanical stimulation has qualitatively different effects on osteoclast formation compared to stimulation associated with the fundamental frequencies of normal movement.

1. Introduction

Control of bone resorption is of interest for a range of physiological and pathophysiological processes including bone remodeling, osteoporosis and fracture healing (Boyle et al., 2003; Robling et al., 2006; Roodman, 1999). Failure to regulate activity of osteoclasts, cells responsible for bone resorption, can result in inappropriate bone loss and long-term disease, which may require medical intervention including replacement of lost tissue with bone substitutes (Shafieyan et al., 2010). Osteoclast differentiation may be affected by a range of environmental conditions. Growth factors, proteins, hormones, mechanical stimulation and cell interactions with their mechanically dynamic surroundings are among the parameters capable of affecting osteoclastogenesis (Horowitz et al., 2001; Rubin et al., 2000; Shafieyan et al., 2012). Dynamic mechanical strain has been reported to influence bone remodeling mostly by enhancement of bone formation and inhibition of bone resorption (Robling et al., 2006; Rubin et al., 1999; Rubin et al., 2000; Suzuki et al., 2008). In response to imposed mechanical stimulation, cells often transduce the detected mechanical signal by producing biochemical signaling factors. These factors mediate cell responses to the stimulus and in case of osteoclasts can modulate osteoclast bone resorptive activity. Thus, the response of osteoclasts to mechanical stimuli which is mainly transduced and regulated by osteoblasts and osteocytes is especially important as bone cells are exposed to a mechanically dynamic environment *in vivo*.

Mechanical stimulation varies widely in both amplitude and frequency depending on the individual's habitual activities. Most normal locomotion activities associated with walking and exercise are performed at frequencies near 1 Hz (Fritton et al., 2000; Thompson et al., 2012). While in a movement-impaired individual high frequency activities are mostly absent, the low frequency stimulations due to limited movement and change in body position persist. Furthermore, the loading waveforms associated with normal movement are complex, such that a very wide range of frequency components are present. The studies of mechanically induced bone formation demonstrate that effective stimulation of

* Corresponding author. Tel.: +1 514 398 4276; fax: +1 514 398 6678.
E-mail address: thomas.quinn@mcgill.ca (T.M. Quinn).

http://dx.doi.org/10.1016/j.jbiomech.2014.06.028
0021-9290/

osteogenesis requires loading frequencies of greater than 0.5 Hz (Hsieh and Turner, 2001; Robling et al., 2006; Turner et al., 1994). Previous studies of mechanical loading on osteoclasts, however, were only performed at frequencies between 0.167 and 10 Hz and have shown that these frequencies are inhibitory for osteoclastogenesis (Kulkarni et al., 2013; Rubin et al., 1999; Rubin et al., 2000; Suzuki et al., 2008). It was recently demonstrated that relatively low frequencies of mechanical stimulation near 0.01 Hz can influence stem cell differentiation to adipocyte and osteocyte (Khayat et al., 2012). Hence, determination of osteoclast responses in this physiological range of low frequencies can provide valuable insight into mechanisms involved in osteoclastogenesis under low frequency mechanical stimulation.

In general, osteoclasts are considered to be weak mechanosensors since they lack rigid attachment and cytoskeletal organization. However, since osteoclasts are known to be regulated by multiple local and systemic factors, including cytokines and growth factors produced or activated by osteoclasts themselves, it is conceivable that mechanical stimulation of osteoclasts includes generation of biochemical autocrine factors. Intracellular signaling by mitogen-activated protein (MAP) kinases (MAPKs), a family of serine/threonine protein kinases, is particularly implicated in mechanosensing and is known to be pivotal in osteoclastogenesis (Hotokezaka et al., 2002; Kumar et al., 2001; Matsumoto et al., 2000; Miyazaki et al., 2000; Tiedemann et al., 2009). Extracellular signal-regulated kinase (ERK) and p38, two major subfamilies of MAPK, are primarily responsible for cell survival and differentiation, respectively (Matsumoto et al., 2000; Miyazaki et al., 2000). Despite their known function in mediating mechanical signals which can induce physiological feedback in response to the stimuli in other cell types (Khayat et al., 2012; Tanabe et al., 2004), the exact role of these MAP kinases in osteoclast differentiation under mechanostimulation remains to be clarified.

In this study, we used a motorized mechanical device that is automated and programmable to permit expansion of stretchable membranes in a nearly isotropic manner by arranging the points of attachment in a circular fashion (Khayat et al., 2012; Majd et al., 2009). An extendable culture surface was used in conjunction with the device which results in highly uniform, controllable strain applied to cells at a range of frequencies. This device was employed to investigate the influences of low frequency mechanical stimulation applied during and after osteoclast differentiation on cell function. RAW 264.7 monocytes and mouse bone marrow cells were used as precursors for osteoclast formation. Culture time for the RAW 264.7 and bone marrow cells were chosen such that not to exceed the length of standard protocol for culturing osteoclasts (Guo et al., 2008; Shafieyan et al., 2012), as it has been previously shown that prolonged culture time would result in significant decrease in number of osteoclasts due to their death, primarily by apoptosis (Akchurin et al., 2008).

2. Methods

2.1. Mechanical stimulation

High-extension silicone rubber (HESR) culture dishes (Cytomec GmbH, Spiez, Switzerland) were modified by covalent attachment of collagen type I to the surface as described previously (Shafieyan et al., 2012; Tan and Desai, 2003), and mounted within a motorized iris-like device which was used for mechanical stimulation (Fig. 1A and B) (Khayat et al., 2012; Majd et al., 2009). We have previously confirmed that osteoclast differentiation is not affected by modified collagen-coated membrane (Shafieyan et al., 2012). Dynamic mechanical stimulation was applied to the surface area of 20 cm^2 with amplitude of 10% (± 2 cm^2) and frequency of 0.01 Hz (36 cycles/h) with three different loading protocols: (a) continuous stimulation applied for 4 days (Fig. 1C), (b) non-continuous stimulation applied for 8 h/day during 4 days (Fig. 1D), and (c) post-differentiation stimulation applied from the beginning of day 4 (for bone marrow cells) or 5 (for RAW 264.7 cells) for 24 h (Fig. 1E). Strain has been shown to be uniform over the culture surface if nominal area of the HESR kept between 15 and 25 cm^2 (Khayat et al., 2012). The static (unstimulated) control devices were kept at fixed surface area of 20 cm^2 throughout experiments.

2.2. RAW 264.7 cell cultures

Mouse monocytic cell line, RAW 264.7 (ATCC: TIB-71™; American Type Culture Collection, VA, USA), were grown in Dulbecco's Modified Eagle Medium (DMEM, 319-020-CL; Wisent) in the presence of 1% sodium pyruvate (Wisent), 1% antibiotics (10,000 IU penicillin, 10,000 μg/mL streptomycin, Wisent) and 10% fetal bovine serum (080-150, Wisent). Cells were plated on day 0 at 75×10^2 cells/cm^2 on the HESR culture dishes and supplemented with receptor activator of nuclear factor κB ligand (RANKL, 50 ng/mL) on day 1and 3. During experiments, culture dishes were completely filled with DMEM medium and covered to reduce the fluid perturbation and stimulatory and control devices were kept in the cell culture incubator at 37 °C and 5% CO_2. The stimulation started on day 2, osteoclasts were observed on day 5.

2.3. Primary cell cultures

All animal studies were conducted in accordance with the McGill University guidelines and regulations established by the Canadian Council on Animal Care. Mouse bone marrow cells were isolated from tibiae and femurs of BALB/c mice (male, 6-week old, Charles River), cultured in α-minimal essential medium (α-MEM, 12000-022, Gibco®) with 1% sodium pyruvate (Wisent), 1% antibiotics (10,000 IU penicillin, 10,000 μg/mL streptomycin, Wisent), 10% fetal bovine serum (080-150, Wisent) and 50 ng/mL human recombinant macrophage-colony stimulating factor (M-CSF: 300-25, PeproTech Inc.). On day 0, non-adherent bone marrow cells were collected, supplemented with M-CSF (50 ng/mL) and RANKL (50 ng/mL) and plated at density of 75×10^3 cells/cm^2 onto HESR culture dishes. Culture dishes were completely filled with α-MEM medium and then covered to minimize the fluid perturbation; stimulatory and control devices were kept in the cell culture incubator at 37 °C and 5% CO_2 during the experiments. Fresh media with M-CSF and RANKL was replaced on day 2. The stimulation started on day 1, osteoclasts were observed on day 4.

2.4. Osteoclast morphology and quantification

Cultures were fixed in 4% PBS buffered paraformaldehyde at room temperature for 10 min, washed with PBS and stained for tartrate-resistant acid phosphatise (TRAP, Sigma 387A-KT). For 3 independent experiments, 6 representative images per condition were taken from specific parts of the stimulation site using an inverted light microscope (TS 100, Nikon), and a digital camera (PL-A662 Color MegaPixel Firewire camera, PixeLINK) combined with PixeLINK Capture SE software. Osteoclasts were identified as TRAP-positive cells with 3 or more nuclei.

RNA Isolation and Real-time q-PCR-Total RNA was prepared using the RNeasy mini-kit (Qiagen, 74106) as previously described (Hussein et al., 2011; Shafieyan et al., 2012). Briefly, RNA was isolated from RAW 264.7 cells or mouse bone marrow cells and reverse transcribed to cDNA using a reverse transcriptase kit (Applied Biosystems, 4368814). Total RNA was quantified by the Quant-iT® RNA assay kit with a Qubit Fluorometer (Invitrogen). Real-time PCR was performed with Universal PCR Master Mix on an Applied Biosystems 7500 instrument. TaqMan probes from Applied Biosystemsfor TRAP (Mm00475698_m1), cathepsin K (CTSK, Mm00484039_m1), matrix metalloproteinase 9 (MMP9, Mm00600163_m1) and GAPDH (Mm99999915_g1) were used. Gene expressions were normalized to GAPDH and then to the static control.

2.5. Effect of conditioned media (CM) on osteoclast differentiation

Bone marrow precursors were plated in 24-well plates at 75×10^3 cells/cm^2, primed for 2 days with RANKL in the presence of M-CSF and washed. Fresh media supplemented with 0%, 5%, 10%, 20%, 30% or 40% of filtered CM collected from mechanically stimulated and static control cultures were added and the cultures were incubated for 1 h to assess signaling or for 2 days to examine osteoclast formation as described previously (Guo et al., 2008; Tiedemann et al., 2009).

2.6. Inhibition of MAPK activity

Bone marrow cells were plated at 75×10^3 cells/cm^2 in tissue culture plates and cultured with M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 2 days. On day 2 of the culture, cells were either; (1) incubated with 30% CM containing PD98059 (100 μM), SB203580 (1 μM), or DMSO as a vehicle, for additional 2 days to investigate osteoclastogenesis, or (2) were treated with α-MEM (data not shown), α-MEM with RANKL or CM with or without PD98059 (100 μM) or SB203580 (1 μM) for 1 h for protein extraction. Cells that were incubated with CM containing PD98059 or SB203580 were pretreated with these inhibitors for 45 min.