Continuous application of compressive force facilitates the formation of osteoclast-like cells in RAW264.7 cells via upregulation of RANK and downregulation of LGR4

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Contents

The following artice and a new unpublished data (Figure 3a) are part of this doctoral thesis:

Continuous application of compressive force induces fusion of osteoclast-like RAW264.7 cells via upregulation of RANK and downregulation of LGR4.

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Abstract

During orthodontic treatment, osteoclastic bone resorption in the alveolar bone exposed to the compressive force (CF) is an important factor for tooth movement. The present study investigated the effect of CF stimulation on the differentiation of RAW264.7 cells from precursors to mature osteoclasts.

The cells were continuously stimulated with 0.3, 0.6, or 1.1 g/cm^2 CF, which was generated by increasing the volume of culture medium in the wells of a 96-well plate, in the presence or absence of receptor activator of nuclear factor κB (RANK) ligand (RANKL) for 4 days. The formation of osteoclast-like cells was estimated using tartrate-resistant acid phosphatase staining. The mRNA levels of RANK, leucine-rich repeat-containing G-protein-coupled receptor (LGR)4, dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast-stimulatory transmembrane protein (OC-STAMP) were determined using real-time PCR. The nuclear translocation of nuclear factor of activated T cells cytoplasmic 1 (NFATc1) was evaluated by immunofluorescent staining.

In the presence of RANKL, the number of TRAP-positive multinucleated cells and the mRNA levels of RANK, DC-STAMP and OC-STAMP were increased by application of 0.6 and 1.1 g/cm^2 CF as compared to 0.3 g/cm^2 CF. The mRNA level of RANK was upregulated whereas

that of LGR4, another RANKL receptor, was downregulated by 0.6 and 1.1 g/cm^2 CF as compared to 0.3 g/cm^2 CF in the absence of RANKL. The nuclear translocation of NFATc1 was increased by 0.6 and 1.1 g/cm^2 CF in the presence of RANKL.

Continuous application of CF induced the differentiation of RAW264.7 cells into TRAP-positive multinuclear cells by enhancing the expression of DC- and OC-STAMP and the nuclear translocation of NFATc1. This may result from the CF-induced increase in RANK and decrease in LGR4 expression.

Introduction

Bone becomes fragile in immobile patients, whereas physical activity, exercise, and adaptive stress increases bone strength. Thus, mechanical stress is an important factor for bone tissue homeostasis [1,2]. In medicine, mechanical force has been used to stimulate bone fracture healing or orthodontic tooth movement [3,4]. In the latter instance, continuous compressive force (CF) and tension force are usually applied to alveolar bone via the periodontal ligament [5]. Accordingly, facilitating osteoclastic bone resorption in alveolar bone exposed to CF is essential for tooth movement [6].

Osteoclasts play an important role in bone resorption by decomposing bone matrix components such as hydroxyapatite and extracellular matrix proteins. Osteoclastogenesis requires the presence of receptor activator of nuclear factor κB (RANK) ligand (RANKL), which is produced by osteoblasts, activated T cells, and synovial fibroblasts [7]. Osteoclast precursor cells express high levels of RANK on their membrane [8, 9] that binds to its ligand RANKL, thereby the activation of downstream signal pathways of RANK is related to osteoclastogenesis [7-10].

The biological effects of RANK/RANKL signaling are regulated by osteoprotegerin, a decoy RANKL receptor [11]. Osteoblasts express both RANKL and osteoprotegerin [12].

RANK/RANKL signaling induces the nuclear translocation of nuclear factor of activated T cells cytoplasmic 1 (NFATc1) [13], a transcription factor involved in osteoclast differentiation. NFATc1 translocates to the nucleus and binds to the promoter of dendritic cell-specific transmembrane protein (DC-STAMP), which is essential for osteoclast fusion and multinucleation [14]. Osteoclast-stimulatory transmembrane protein (OC-STAMP) also promotes the formation of multinucleated osteoclasts in the presence of RANKL [15]. Previous studies have reported the effects of mechanical loading on the expression of osteoclast differentiation-related factors including RANKL and osteoprotegerin in osteoblasts [16-19]. However, few studies have investigated the effects of mechanical loading of osteoclast precursor cells on osteoclast differentiation.

To address this issue, the effect of continuous CF stimulation in the presence of RANKL on the differentiation of RAW264.7 cells into osteoclasts was investigated in the present study. CF was generated by increasing the volume of culture medium in the well of culture plates. The proportion of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells, RANK, DC- and OC-STAMP expression, and NFATc1 nuclear translocation were evaluated in RAW264.7 cells stimulated with CF in the presence of RANKL. The expression of RANK and leucine-rich repeat-containing G-protein-coupled

receptor (LGR)4, another RANKL receptor that negatively regulates osteoclast differentiation [12, 20, 21], was also examined in RAW264.7 cells stimulated with CF in the absence of RANKL.

Materials and Methods

Osteoclast cultures and CF application

The RAW264.7 murine monocyte/macrophage cell line was purchased from Dainippon Pharmaceutical (Osaka, Japan). Cells were seeded in a 96-well culture plate at a density of $1 \times 10^5/cm^2$. After overnight incubation, 100 μl of α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 50 ng/ml soluble RANKL (Wako Pure Chemical, Osaka, Japan) was added. CF was increased by adding 100 or 250 μl of the medium without RANKL and FBS (Fig. 1). Cells at the bottom of the well were subjected to approximately 0.3, 0.6, or 1.1 g/cm^2 CF for up to 4 days. The medium with or without RANKL was replaced every other day. Cell morphology was observed by light microscopy daily. There were minimal floating dead cells in each CF loading condition.

Tartrate-resistant acid phosphatase (TRAP) staining

Cells were fixed and stained using a TRAP staining kit (Cosmo Bio, Sapporo, Japan) according to the manufacturer's protocol on days 1, 2, 3, and 4 of culture.

Real-time reverse transcription (RT)-PCR

Total RNA was isolated from cells using NucleoSpin RNA (Takara Bio, Otsu, Japan). After measuring the concentration using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), the cDNA was synthesized from 1 μg RNA using an RNA PCR kit (PrimeScript; Takara Bio). One microliter of mixture containing cDNA was subjected to real-time RT-PCR with SYBR Green I dye. Reactions were performed in 25 μl of SYBR Premix Ex Taq solution (Takara Bio) containing 20 μM sense and antisense primers (Table 1) on a Smart Cycler (Cepheid, Sunnyvale, CA, USA), and data were analyzed using Smart Cycler software (version 1.2d, Cepheid, Sunnyvale). The reaction for DC- and OC-STAMP, RANK, LGR4, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) repeated 30 cycles of 95°C for 3 s and 60°C for 20 s. Reactions were performed in triplicate, and the specificity of the PCR products was verified by Smart Cycler software on the basis of melting curve analysis. Target gene expression levels were normalized to that of GAPDH.

Immunofluorescence analysis of NFATc1 localization

Cells were seeded on glass coverslips at the bottom of each well of a 96-well plate and

stimulated with CF in the presence of 5 ng of RANKL and 10% FBS (Fig. 1) for 6 hours. The cells were then fixed with methanol for 10 min at -20°C, blocked with 1% bovine serum albumin, and incubated with mouse monoclonal antibodies against NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI, Vector laboratories, Burlingame, CA, USA). Immunoreactivity was detected with an epifluorescence microscope and images were acquired with a Pro600ES digital camera system (Pixera, Osaka, Japan). The proportion of cells exhibiting nuclear localization was calculated.

Statistical analysis

Each value is reported as the mean ± standard deviation. Significant differences were determined by one-way analysis of variance followed by Tukey's multiple comparisons test. Differences with $P < 0.05$ were considered statistically significant.

Results

CF induces RAW264.7 cell fusion and osteoclast differentiation

RAW264.7 cells were stimulated with 0.3, 0.6, or 1.1 g/cm^2 CF in the presence of 5 ng of RANKL (Fig. 1) for 4 days before staining and observation by light microscopy. Fewer TRAP-positive cells were observed starting from day 2 of culture (data not shown). TRAP-positive multinucleated cells more than three nuclei were clearly observed on days 3 and 4 of culture and the area of each osteoclast increased in response to CF (Fig. 2A). On day 4 of culture, the numbers of large (> 10 nuclei) and medium (6–9 nuclei) cells were higher in the 0.6 and 1.1 g/cm^2 CF groups than in the 0.3 g/cm^2 CF group, whereas the number of large cells was higher with 1.1 than with 0.6 g/cm^2 CF application (Fig. 2B). Real-time RT-PCR analysis revealed that RANK, DC- and OC-STAMP mRNA levels were higher in cells stimulated with 0.6 and/or 1.1 g/cm^2 CF than in those stimulated with 0.3 g/cm^2 CF, and their levels were higher with 1.1 g/cm^2 CF as compared to 0.6 g/cm^2 CF application on days 1, 3 and/or 4 (Fig. 3A, B, C).

CF induces RANK and LGR4 mRNA expression in RAW264.7 cells

The total amount of RANKL and FBS in the culture medium was similar across the three CF

loading conditions (Fig. 1), but these concentrations were reduced when CF was increased by adding α-MEM without RANKL and FBS. To eliminate the effects associated with reduced RANKL and FBS concentrations, RAW264.7 cells were stimulated with 0.3, 0.6, or 1.1 g/cm^2 CF in the absence of RANKL and FBS (Fig. 4) for 6 hours, and RANK and LGR4 expression was determined 3 and 6 hours later. The mRNA level of RANK was increased, whereas that of LGR4 was decreased in cells stimulated with 0.6 or 1.1 g/cm^2 CF as compared to 0.3 g/cm^2 CF at 3 and 6 hours (Fig. 5A, B); moreover, compared to application of 0.6 g/cm^2 CF, RANK expression was higher whereas LGR4 expression was lower with 1.1 g/cm^2 CF application at 3 or 6 hours. Thus, changes in RANK and LGR4 expression in RAW264.7 cells are dependent on CF magnitude in the absence of RANKL.

CF induces NFATc1 translocation to the nucleus

The mRNA expression level of RANK was upregulated, but that of LGR4 was downregulated with increasing CF within 6 hours of stimulation. To test the hypothesis that CF enhanced RANK/RANKL signaling in the presence of RANKL during the same period in response to CF, RAW264.7 cells were stimulated with 0.3, 0.6, or 1.1 g/cm^2 CF in the presence of 5 ng of RANKL and FBS (Fig. 1) for 6 hours, and NFATc1 localization was determined by

immunofluorescence analysis at 3 and 6 hours. Nuclear localization of NFATc1 (light green nuclei in the merged image) was markedly observed in the 0.6 and 1.1 g/cm^2 CF groups than in the 0.3 g/cm^2 CF group (Fig. 6A). The proportion of NFATc1 expression was increased 3 or 6 hours after application of 0.6 and 1.1 g/cm^2 CF (Fig. 6B). Compared to the 0.6 g/cm^2 CF group, its level was higher in the 1.1 g/cm^2 CF group at 3 hours (Fig. 6B).

Discussion

The results of this study demonstrate that continuous application of CF, which was generated by increasing the volume of culture medium, induced the fusion of osteoclast precursor cells and the differentiation of TRAP-positive osteoclast-like cells. These positive effects of CF on osteoclastogenesis were in partial agreement with those of a previous study in which stimulation of RAW264.7 cells with CF (~0.3 g/cm^2) for 24 hours after pre-incubation with RANKL for 3 days increases DC- and OC-STAMP expression [22].

Orthodontic mechanical stress is applied to alveolar bone via the periodontal ligament, which contains fibroblasts, osteoblasts, osteoclast precursor cells, and osteoclasts [5]. In a previous study in which continuous or intermittent CF (~5 g/cm^2), which was generated by varying the volume of culture medium, was applied to human periodontal ligament cells for 4 days, RANKL and interleukin-1β were upregulated whereas osteoprotegerin was downregulated by a function of CF magnitude on days 2 and 4 of culture [23]. In the present study, RAW264.7 cells were continuously stimulated with CF (0.3–1.1 g/cm^2) in the presence of RANKL for 4 days by a similar method, as osteoclast precursors as well as osteoclasts on the alveolar bone surface are exposed to mechanical stress. Under these conditions, increasing CF induced the expression of RANK, DC- and OC-STAMP from day 1 of culture, and promoted

the number of TRAP-positive multinucleated cells on day 4 of culture. Moreover, the size of multinucleated cells increased with applied CF. These results suggest that applying CF to osteoclast precursors induces the expression of DC- and OC-STAMP, which are involved in cell fusion [18, 19], at an early stage of RANKL-induced osteoclastogenesis and facilitates their subsequent differentiation into multinucleated osteoclasts.

In the present study, RAW264.7 cells were stimulated with CF generated by increasing the volume of culture medium; 100 or 250 μl of α-MEM without RANKL and FBS was added to 100 μl of medium containing 5 ng of RANKL and 10% FBS. As a result, the magnitude of CF applied to cells at the bottom of the well was increased by 2.0 or 3.7 fold whereas the RANKL and FBS concentrations were reduced. Nevertheless, the number of TRAP-positive multinucleated cells as well as RANK expression was increased. Therefore, it was considered that CF might induce the alteration of RANKL receptor expression, independent of RANKL concentration in the culture medium. LGR4 is also a RANKL receptor having seven transmembrane regions [12, 21, 22]. In *Lgr4* knockout mice, the number and size of osteoclasts were found to be increased, in addition, RANKL treatment increased their number and size in *Lgr4*-depleted RAW264.7 cells [20]. Based on these findings, RAW264.7 cells were stimulated with CF for 6 hours in the absence of RANKL in order to confirm that CF would

alter the expression of RANK and LGR4, which have opposite effects on osteoclast differentiation, independent of RANKL. RANK was upregulated, whereas LGR4 was downregulated by 1.1 g/cm^2 CF as compared to 0.6 or 0.3 g/cm^2 CF application (Fig. 5). Moreover, nuclear translocation of NFATc1 was induced under these conditions. These results are consistent with the results of TRAP staining and DC- and OC-STAMP expression analyses; the number of large TRAP-positive cells (> 10 nuclei) and DC- and OC-STAMP levels were higher with application of 1.1 g/cm^2 CF as compared to 0.3 or 0.6 g/cm^2 CF. Thus, CF induced RANK and suppressed LGR4 expression within several hours, and cell fusion and osteoclast differentiation were enhanced as a function of CF magnitude via activation of RANK/RANKL signaling.

Numerous studies have reported that mechanical stress influences the differentiation and function of osteoblasts and osteoclasts [13-16, 22-28], but the effect of mechanical stress on osteoclastogenesis remains controversial. Several in vitro studies have demonstrated that tension force generated by the Flexercell tension system suppressed osteoclastogenesis; stimulation with 10% elongation at 30 cycles per min, which was controlled by Flexercell tension system, inhibited osteoclast differentiation as well as the expression of DC- and OC-STAMP [26-28], whereas the release from elongation enhanced osteoclastogenesis [27].

One study found that a CF of ~0.3 g/cm^2 induced osteoclastogenesis, with CF > 0.3 g/cm^2 having no effect [22], when RAW264.7 cells were stimulated with CF generated by increasing the number of coverslips placing on the cells for 24 h after pre-incubation with RANKL. It is possible that the decrease in the partial pressure of oxygen in the closed environment under the coverslip or pre-incubation with RANKL before CF stimulation in the previous study affected the optimal magnitude of CF for inducing osteoclastogenesis.

It has been considered that cells respond to mechanical loading via mechanotransducer systems including focal adhesions and F-actin stress fiber or cadherins [29,30]. Studies using osteoblasts reported that the P2X7 receptor, which is a receptor for ATP, or angiotensin II type 1 receptor functions as a mechanoreceptor [31-33]. Less information is available about mechanosensors in monocytes/macrophages, but Kim et al. [34] reported that increased expression of tumor necrosis factor-α, dependent on the magnitude of mechanical stress generated by centrifugation, was associated with intracellular calcium ion release and Toll-like receptor-2 in human monocyte-like cell line. These mechanotransducer systems might be involved in CF-induced osteoclast differentiation.

Several in vitro studies reported that optimal mechanical stress induced osteogenesis; in particular, 1.0 g/cm^2 CF using a lead weight, or a tension force of 5% elongation with a

increase of bone nodule formation as well as expression of Runx2 and Osterix, which are osteoblast differentiation-related transcription factors for the induction of type I collagen, bone sialoprotein, osteopontin, and bone morphogenetic proteins [25,35,36]. A previous study also reported that 2.0 g/cm^2 CF did not induce in vitro mineral nodule formation in osteoblasts, and 3.0 g/cm^2 CF inhibited nodule formation [35]. Thus, the effect of CF on osteoclastogenesis may be depending on the magnitude. In the present study, CF applied to RAW264.7 cells was in the range of magnitude, which induced osteoblast differentiation and bone formation. Further studies are needed to clarify the effect of continuous CF more than 2.0 g/cm^2, which inhibits osteogenesis in osteoblasts, or intermittent CF on the differentiation of RAW264.7 cells from precursors to mature osteoclasts.

Conclusion

This study demonstrated that continuous application of CF induced the differentiation of RAW264.7 cells into TRAP-positive multinucleated cells by stimulating the expression of DC- and OC-STAMP as well as the nuclear translocation of NFATc1, which is a downstream signal of RANK/RANKL. This effect may involve the upregulation of RANK and downregulation of LGR4 induced by CF. These results provide novel insight into the molecular mechanism of osteoclastogenesis that can be applied to orthodontics and bone healing.

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Table

Table 1. PCR primers used in the experiments

Target	Forward primer	Reverse primer	Genbank acc no.
DC-STAMP	5'-CTAGCTGGCTGGACTTCATCC-3'	5'-TCATGCTGTCTAGGAGACCTC-3'	NM_029422.4
OC-STAMP	5'-AGCTGTAGCCTGGGCTCAGAAG-3'	5'-AGCCTGTGGTAGATGACAGTCGTG-3'	NM_029021.1
RANK	5'-TGCAGCTCAACAAGGATACG-3'	5'-GAGCTGCAGACCACATCTGA-3'	NM_009399.3
LGR4	5'-CTGATTGCCACGTGGGTTTAAGTAG-3	5'-AGGACATTGCCAGTCCAGATGAG-3'	NM_172671.2
GAPDH	5'-AAATGGTGAAGGTCGGTGTG-3'	5'-TGAAGGGGTCGTTGATGG-3'	XM_001473623

Figures

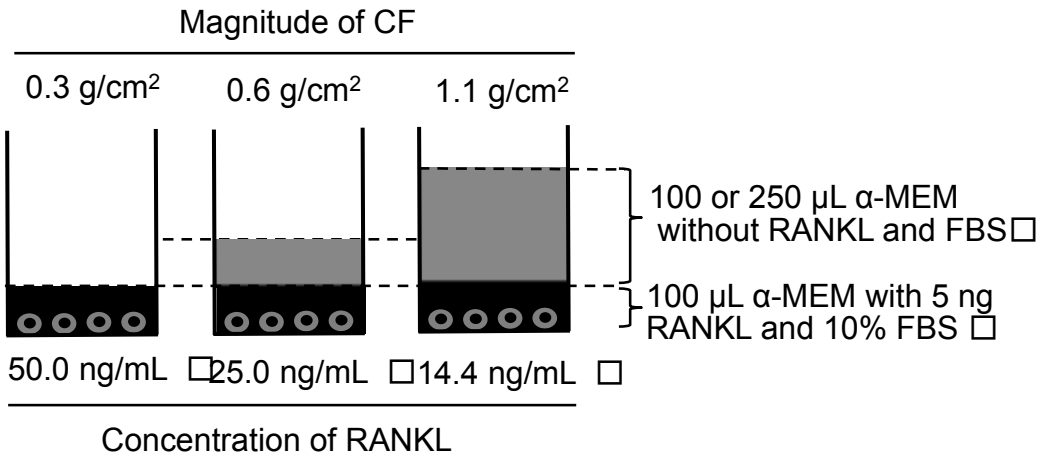

Figure 1. Schematic representation of CF loading in the presence of RANKL.

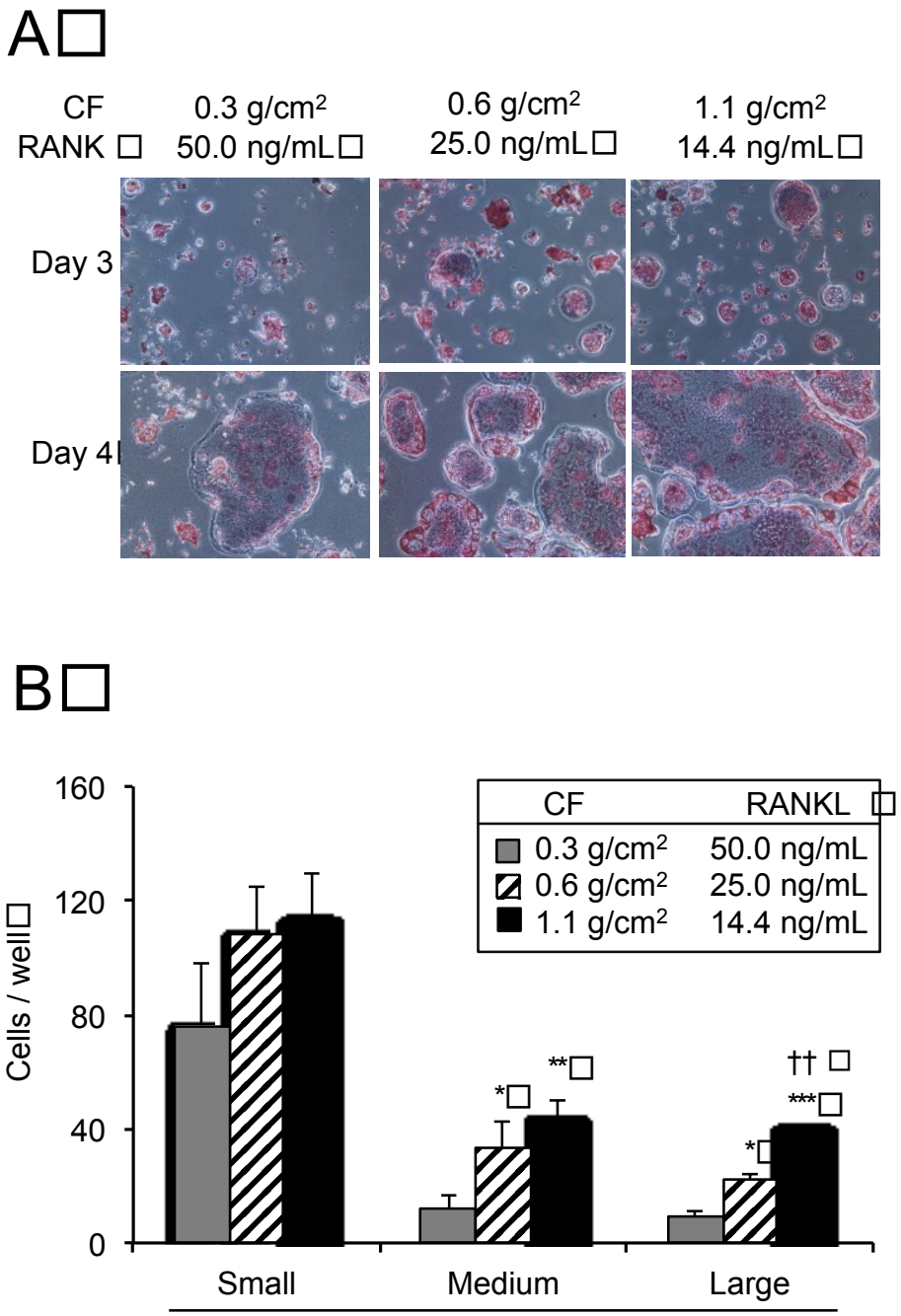

Figure 2. Effect of CF on TRAP staining. The cells were stimulated with 0.3, 0.6, or 1.1 g/cm^2 CF in the presence of 5 ng of RANKL for 4 days, and then stained and observed by light microscopy (original magnification: 100×) (A); TRAP-positive multinucleated cells were counted and classified as large (> 10 nuclei), medium (6–9 nuclei), or small (3–5 nuclei) on day 4 of culture (B). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (vs. 0.3 g/cm^2), ††$P < 0.01$ (0.6 vs. 1.1 g/cm^2).

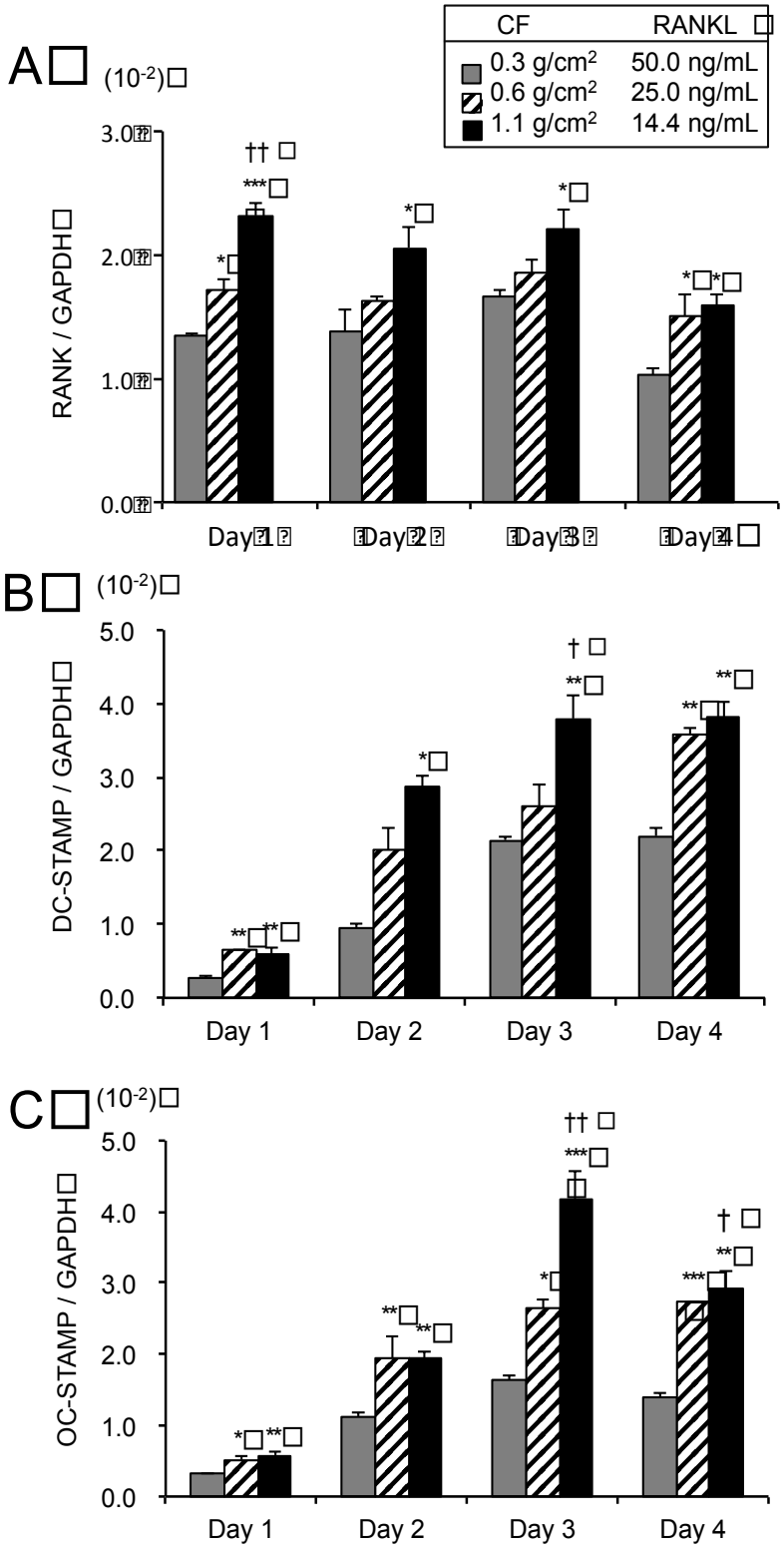

Figure 3. Effect of CF on RANK, DC- and OC-STAMP expression in RAW264.7 cells. The mRNA expression of RANK (A), DC-STAMP (B) and OC-STAMP (C) was determined by real-time RT-PCR on days 1, 2, 3, and 4 of culture. Bars indicate the mean ± standard deviation of three independent experiments. *$P < 0.05$, **$P < 0.01$ (vs. 0.3 g/cm^2), ***$P < 0.001$, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (0.6 vs. 1.1 g/cm^2).

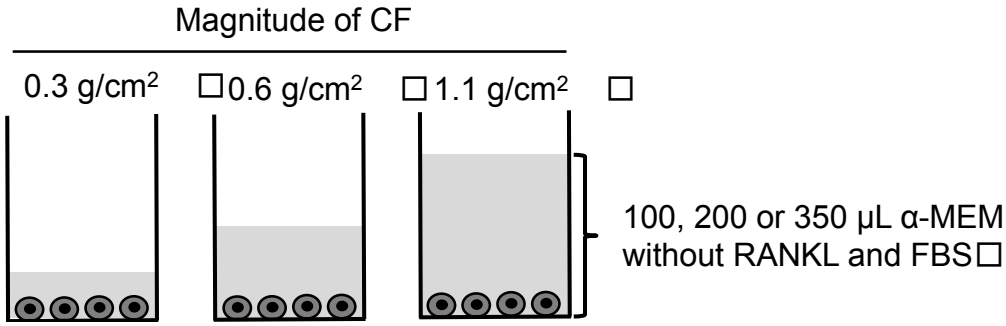

Figure 4. Schematic representation of CF loading in the absence of RANKL.

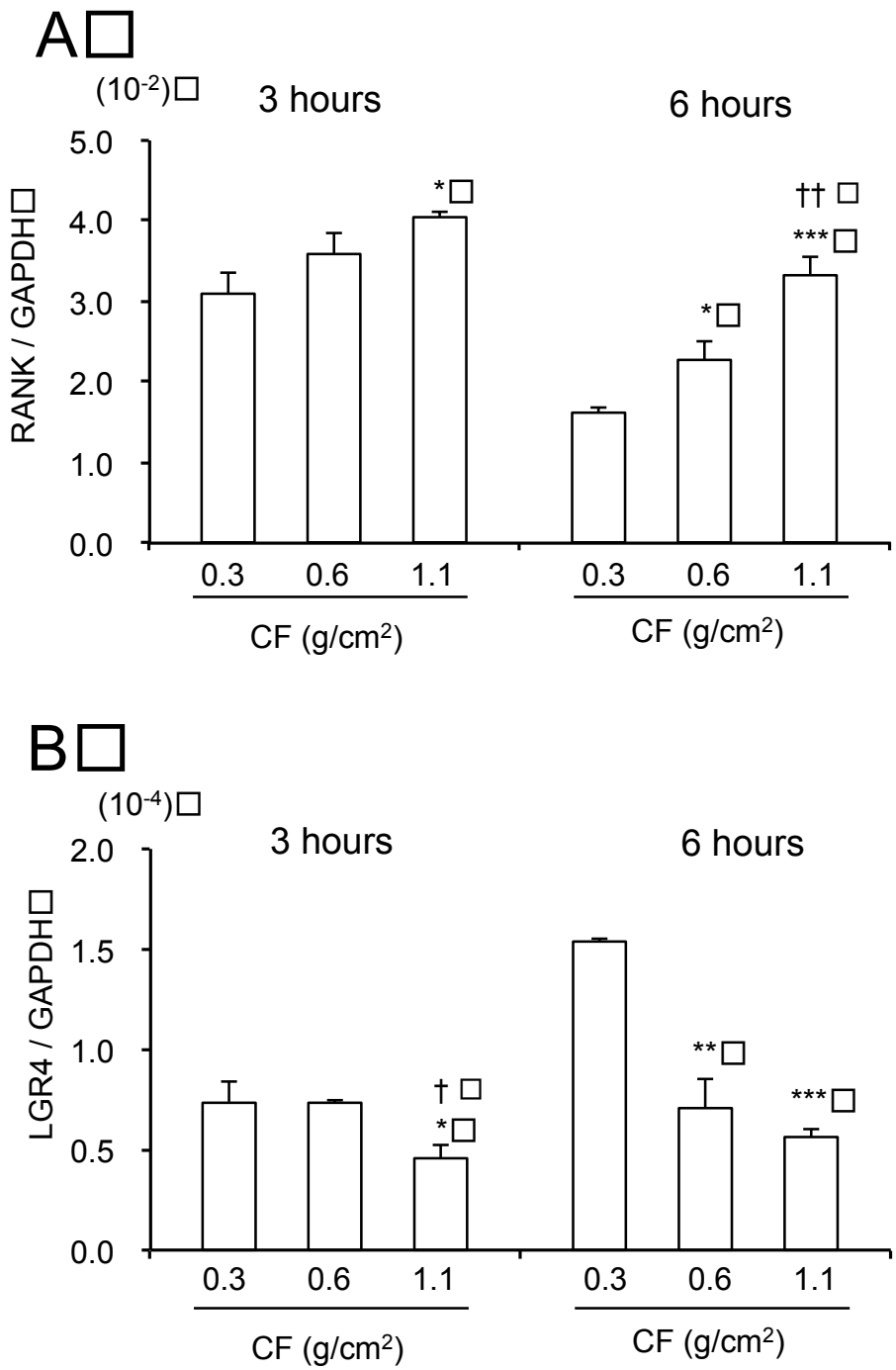

Figure 5. Effect of CF on RANK and LGR4 expression in RAW264.7 cells. The cells were stimulated with, 0.3, 0.6, or 1.1 g/cm^2 CF in the absence of RANKL for 6 hours, and the mRNA expression of RANK (A) and LGR4 (B) was determined by real-time RT-PCR 3 and 6 hours later. Bars indicate the mean ± standard deviation of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (vs. 0.3 g/cm^2), †$P < 0.05$, ††$P < 0.01$ (0.6 vs. 1.1 g/cm^2).

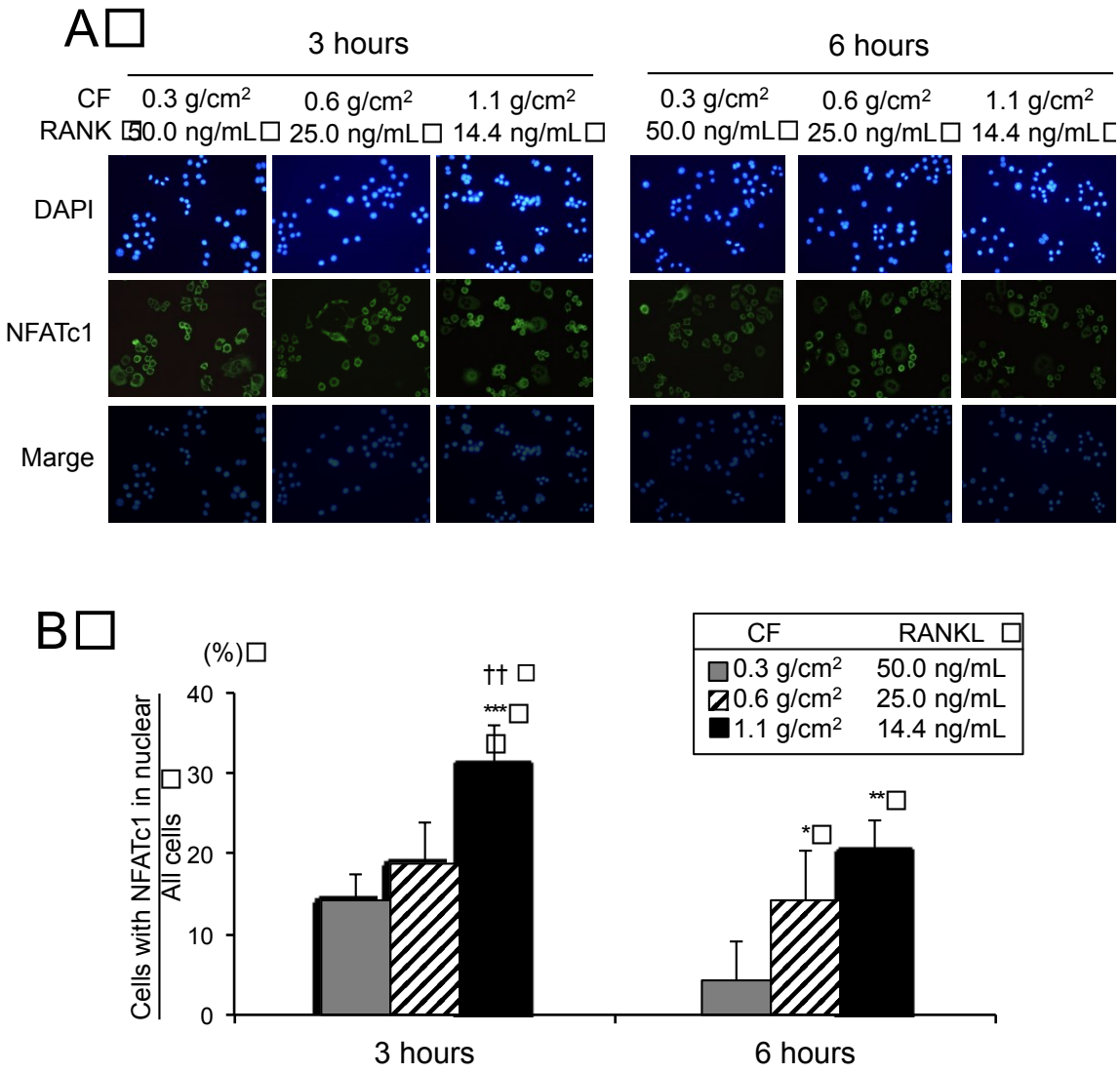

Figure 6. Effect of CF on NFATc1 nuclear translocation in RAW264.7 cells. The cells were stimulated with 0.3, 0.6, or 1.1 g/cm^2 CF in the presence of 5 ng of RANKL for 3 and 6 hours. Nuclear localization of NFATc1 was observed with a fluorescence microscope (A); the proportion of cells showing nuclear localization was calculated (B). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (vs. 0.3 g/cm^2), ††$P < 0.01$ (0.6 vs. 1.1 g/cm^2).