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博 士 論 文

Lactoferrin directly suppresses osteoclastogenesis

(ラクトフェリンは破骨細胞の分化誘導を
直接抑制する)

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Abstract

Recent research has shown that lactoferrin indirectly suppresses osteoclastogenesis by affecting osteoblasts and periodontal ligament fibroblasts. However, the mechanism by which lactoferrin directly affects osteoclastogenesis has not been reported. Therefore, the aim of this study was to examine the direct effects of lactoferrin on RANKL-induced osteoclast differentiation of murine osteoclastic RAW 264.7 cells. The number of osteoclasts was determined by counting the number of cells positive for TRAP staining. The effect of lactoferrin on the number of osteoclasts was measured, and the effect on the mRNA expression of osteoclast differentiation markers was assayed using real-time PCR. Lactoferrin decreased the number of osteoclasts (≥ 2 nuclei) and large osteoclasts (≥ 8 nuclei) in a dose-dependent manner without affecting the viability of RAW 264.7 cells. In addition, lactoferrin only mediated these effects within 48 h of the beginning of culturing the RAW 264.7 cells with RANKL. Lactoferrin also significantly inhibited RANKL-induced mRNA expression of osteoclastic

differentiation genes such as NFATc1, RANK, DC-STAMP, and OC-STAMP.

These findings suggest that lactoferrin directly suppresses osteoclastogenesis within 48 h of the beginning of culturing the RAW 264.7 cells with RANKL. The use of lactoferrin may be a novel and innovative therapy for treating bone diseases.

Introduction

Lactoferrin, which was discovered by a North European scholar in 1939 as the "red protein of the milk," is an iron-binding glycoprotein with a molecular weight of 80,000 that can be separated from milk. Lactoferrin belongs to the transferrin family of proteins ¹⁾. It is produced by many exocrine glands and consequently, is widely distributed in body fluids including tears, saliva, bile, pancreatic fluid, vaginal secretions, semen, and milk ²⁾. Lactoferrin is also a major constituent of the secondary granules of neutrophilic leukocytes, which release lactoferrin during acute inflammation ³⁾. Serum levels of lactoferrin in healthy subjects range from 2 to 7 µg/ml and are

predominantly neutrophil derived, but during inflammation and sepsis, the local concentrations may be much higher ⁴⁾. Lactoferrin acts as an iron chelator, which may contribute to its antimicrobial activity ⁵⁾, but it also affects cell growth and differentiation ⁶⁾, embryonic development ⁷⁾, myelopoiesis ⁸⁾, endothelial cell adhesion ⁹⁾, cytokine ^{10, 11)} and chemokine ¹²⁾ production, regulation of the immune system ¹³⁾, and modulation of the inflammatory response ¹⁴⁾. However, the effects of lactoferrin on osteogenesis have received little attention. Recent studies reported that lactoferrin functions as a growth factor, acting at physiological concentrations to induce osteoblast growth and activity. Lactoferrin also indirectly inhibits osteoclast development *in vitro* and promotes bone growth *in vivo* ^{15, 16)}. Recent studies have also shown a possible use for lactoferrin as a dietary supplement for improving bone health.

Bone is continuously remodeled by osteoblasts and osteoclasts ^{17, 18, 19)}. The proper balance between bone formation and resorption is regulated by various hormones and cytokines that modulate bone metabolism. If this

balance is disrupted, metabolic bone diseases such as osteoporosis and osteopetrosis result ^{20, 21}). Osteoclasts are members of the monocyte/macrophage lineage and are formed by fusion of their mononuclear cellular precursors. The receptor activator of NF- κ B (RANK) ligand (RANKL) is essential and the most important cytokine for the process of osteoclast differentiation and activation ²²). RANKL is a member of the tumor necrosis factor family and is expressed as a membrane-bound protein in osteoblasts and stromal cells ¹⁹). The receptor for RANKL, termed RANK, is induced by macrophage colony-stimulating factor (M-CSF) in osteoclast precursor cells. M-CSF is also an essential factor for the survival of osteoclast precursor cells and for the formation of osteoclasts ²³). In this study, we used osteoclastic RAW 264.7 cells, which express endogenous RANK and can differentiate into osteoclasts following RANKL stimulation without M-CSF. Binding of RANKL to RANK induces activation of tumor necrosis factor receptor-associated factor 6 and c-Fos pathways, which lead to auto-amplification of nuclear factor of activated T cells (NFAT) c1, the

master transcription factor for osteoclast differentiation. Auto-amplification of NFATc1 is also dependent on calcium signaling and regulates many osteoclast marker genes such as cathepsin K, tartrate-resistant acid phosphatase (TRAP), and the calcitonin receptor ²⁴).

Although recent research has shown that lactoferrin indirectly suppresses osteoclastogenesis by affecting osteoblasts and periodontal ligament fibroblasts ^{15, 16}), whether lactoferrin has a direct effect on osteoclast differentiation is unknown. Therefore, in this study, we investigated the direct effect of lactoferrin on osteoclastogenesis.

Materials and methods

Cell culture

The murine monocyte/macrophage cell line (RAW cells: TIB-71; American Type Culture Collection, Manassas, VA, USA) was used as osteoclast precursors. RAW cells differentiate into osteoclast-like cells in the presence

of RANKL ²⁵). Cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) and 66.7 µg/ml kanamycin sulfate (Meiji Seika, Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were seeded onto 100-mm standard dishes (BD Biosciences, Franklin Lakes, NJ, USA) and cultured overnight. For osteoclast differentiation, RAW cells were transferred into 24-well culture plates at 1.0×10^4 cells/well and cultured in α -minimum essential medium (Wako) supplemented with 10% heat-inactivated fetal bovine serum, 2 µM L-alanyl L-glutamine (Wako Pure Chemical), 284 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 66.7 µg/ml kanamycin sulfate, and 50 ng/ml RANKL (Oriental Yeast Co. Ltd., Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Medium containing these reagents was replaced every other day.

Application of lactoferrin

Lactoferrin was purchased from Wako Pure Chemical and dissolved in PBS. Stock solutions of lactoferrin were stored at 4°C until use. In the primary experiment, 10 µl of 1, 10, 25, or 100 µg/ml lactoferrin was added to RAW cells. Fresh medium with lactoferrin was added every other day. Control cells were treated with 10 µl PBS instead of lactoferrin. Second, the 6-day cultivation period was divided into three stages (2 days each); the stage when lactoferrin (25 µg/ml) was applied to the culture is indicated as (+), and the stage when lactoferrin was not applied to the culture is indicated as (-). The early, middle, and late stages are indicated in a row from left to right.

Pit assay

The pit formation assay was analyzed using the Corning Osteo Assay Surface Plate (Corning, Inc., Corning, NY, USA). RAW cells were seeded onto 24-well plates at a density of 1.0×10^4 cells/well with RANKL. Lactoferrin was added at various concentrations and schedules. After 6 days of culture,

plates were observed under a light microscope, and the resorption area was quantified using Image J software (v. 1.48a; National Institutes of Health, Bethesda, MD, USA). The pit areas were compared ^{26, 27}).

TRAP staining

After cell culture with lactoferrin, they were fixed in 10% neutral formalin, washed with distilled water, and stained with Fast Red Violet LB Salt (Sigma-Aldrich) in TRAP staining solution containing acetate buffer (pH 5.0) (Sigma-Aldrich), naphthol AS-MX phosphate (Sigma-Aldrich) as a substrate, and red violet LB (Sigma-Aldrich) as a stain in the presence of 50 mM sodium tartrate (Wako). TRAP-positive cells with 2-7 nuclei were considered to be small osteoclasts, and those with ≥ 8 nuclei were considered to be large osteoclasts ^{28, 29}). Osteoclasts were counted under a light microscope (magnification, $\times 100$; Olympus IMT-2; Olympus, Tokyo, Japan) in a 20 mm² rectangle within the circular field.

Analysis of cytotoxicity

The cytotoxicity of lactoferrin was analyzed using the Cell Counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). RAW cells were seeded on 24-well plates (Falcon) at a density of 1.0×10^4 cells/well with or without RANKL. Lactoferrin was added according to the various schedules. After 6 days of culture, medium was replaced with new medium (400 μ l), and 40 μ l Cell Counting kit-8 solution was added. After incubation for 1 h at 37°C, absorbance was measured at 450 nm using a micro plate reader (Model 550, Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR

RAW cells were seeded onto 100-mm standard dishes (Falcon) and treated with or without lactoferrin. After 3, 6, 12, 24, and 48 h, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. cDNA was synthesized from total RNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo

(dt) primers.

For analysis of mRNAs for osteoclastogenic proteins, such as NFATc1, RANK, dendritic cell-specific transmembrane protein (DC-STAMP), and osteoclast stimulatory transmembrane protein (OC-STAMP), the following primers were used: NFATc1 (Mm00479445_m1), RANK (Mm00437135_m1), DC-STAMP (Mm01168058_m1), OC-STAMP (Mm00512445_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1). Primers were purchased from Applied Biosystems (Carlsbad, CA, USA). Quantitative real-time PCR was performed using the ABI 7300 (Applied Biosystems). We used the comparative Ct method to calculate mRNA expression. We verified the Ct values for both the calibrator and samples of interest by normalization to GAPDH. The comparative Ct method is also known as the $2^{-\Delta\Delta Ct}$ method, in which, $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{reference}}$. $\Delta Ct_{\text{sample}}$ is the Ct value for the samples normalized to the GAPDH, and $\Delta Ct_{\text{reference}}$ is the Ct value for the calibrator, which was also normalized to the GAPDH ^{30, 31}.

Statistical analysis

All data are expressed as the means $\pm$ SD. Comparisons between samples of interest and respective controls were analyzed with the two-tailed unpaired Student's t-test. P-values of <0.05 were considered to be significant.

Results

Effects of the lactoferrin concentration on osteoclastogenesis

We analyzed the effects of lactoferrin on osteoclastogenesis by incubating RAW cells with different concentrations of lactoferrin (1, 10, 25, and 100 $\mu\text{g/ml}$) and RANKL, and counting the number of osteoclasts (2-7 nuclei) and the number of large osteoclasts (≥ 8 nuclei) after 6 days of culture. Lactoferrin decreased the number of osteoclasts (Fig. 1 A) and osteoclasts with multiple nuclei (Fig.1 B) in a concentration-dependent manner .

Osteo Assay Surface Plate

The pit area was confirmed to be 100% in the controls. Pit areas were 77.6, 25.7, 9.3, and 2.8 % at lactoferrin concentration 1, 10, 25, and 100 $\mu\text{g/ml}$, respectively. Lactoferrin treatment decreased the ratio of the pit area in a concentration-dependent manner (Fig. 2).

Effects of the stage of osteoclastogenesis by lactoferrin

We also analyzed the effects of lactoferrin when it was added at 0, 24, and 48 hours after the beginning of RANKL-treated RAW cells culture the number of osteoclasts (2-7 nuclei) and the number of large osteoclasts (≥ 8 nuclei) after 6 days of culture. Lactoferrin decreased the number of osteoclasts (2-7 nuclei) when added within 48 h of the beginning of culturing the RAW cells with RANKL. Similarly, the number of large osteoclasts (≥ 8 nuclei) was decreased by lactoferrin when added within 48 h of the beginning of culturing the RAW cells with RANKL (Fig. 3).

Cytotoxicity of lactoferrin on RAW cells and osteoclasts

We analyzed the cytotoxicity of lactoferrin on RAW cells in the absence of RANKL. Lactoferrin did not decrease the number of RAW cells, was not cytotoxic to RAW cells, and did not induce cell death (Fig. 4).

Effects of lactoferrin on mRNA expression of osteoclastic differentiation genes and osteoclast-specific markers in RAW cells

We investigated the effects of lactoferrin on the mRNA expression of genes associated with osteoclastogenesis and osteoclast-specific markers. RAW cells stimulated with RANKL were treated with or without lactoferrin for 3, 6, 12, 24, and 48 h. We analyzed the mRNA expression of NFATc1, RANK, DC-STAMP, and OC-STAMP using quantitative real-time PCR analysis. Lactoferrin reduced the mRNA expression of NFATc1 and RANK between 6 and 24 h. Lactoferrin also reduced the mRNA expression of DC-STAMP and OC-STAMP between 12 and 48 h (Fig. 5).

Discussion

Lactoferrin inhibits osteoclastogenesis via osteoblasts ^{15, 16)}. The number of newly developed osteoclasts, assessed as multinucleated cells staining positively for TRAP, was significantly decreased by lactoferrin at concentrations of 10 $\mu\text{g/ml}$, and at 100 $\mu\text{g/ml}$, osteoclastogenesis via osteoblasts was completely arrested. In a study about bone resorption, lactoferrin was found to reduce bone-resorbing activity in a rabbit mixed bone cell culture ³²⁾. Therefore, in the present study, we investigated the direct effect of lactoferrin on osteoclast differentiation without using a coculture system with osteoblasts and osteoclasts.

To examine the direct effect of lactoferrin on RANKL-stimulated osteoclast differentiation, RAW cells were cultured with lactoferrin (1, 10, 25, and 100 $\mu\text{g/ml}$). Lactoferrin reduced the number of osteoclasts (2-7 nuclei) and large osteoclasts (≥ 8 nuclei) in a dose-dependent manner (Fig. 1). Generally, osteoclasts are resorptive and are thought to mainly resorb bone; thus, the preliminary and

post-resorption stages must be relatively short when compared to the actual resorption stage. We investigated the resorption activity of osteoclasts treated with lactoferrin (1, 10, 25, and 100 $\mu\text{g/ml}$). In the pit assay, the pit area was significantly reduced in a dose-dependent manner after treatment with lactoferrin (Fig. 2). These results suggest that lactoferrin impairs the resorption activity of osteoclasts.

We further investigated the effects of lactoferrin at different stages of osteoclast differentiation. The numbers of osteoclasts (2-7 nuclei) and large osteoclasts (≥ 8 nuclei) were reduced when RAW cells were cultured with lactoferrin at an early stage of differentiation within 48 h of RANKL addition (Fig. 3A, B). We did not observe large multinucleated osteoclasts in the plate when cells were cultured with lactoferrin at an early stage (Fig. 3C). Similarly, in the pit assay, we did not observe significant changes in the pit area after treatment with lactoferrin at an early stage of differentiation (Fig. 3D). These findings suggested that lactoferrin inhibits osteoclast differentiation and fusion at an early stage.

Lactoferrin was suspected to be cytotoxic to RAW cells and to contribute to the suppression of osteoclastogenesis. However, lactoferrin was not cytotoxic to RAW cells in the absence of RANKL, suggesting that the effect of lactoferrin on osteoclastogenesis was not due to cytotoxicity of RAW cells in the absence of RANKL (Fig. 4).

We further investigated the effect of lactoferrin on mRNA expression of osteoclast-associated genes in RAW cells at an early stage of differentiation (3, 6, 12, 24, and 48 h). The expression of NFATc1 and RANK mRNA was decreased when lactoferrin was added within 48 h of the beginning of culturing the RAW cells with RANKL. NFATc1 is a master regulator of RANKL-induced osteoclast differentiation and plays a pivotal role in osteoclast fusion and activation via up-regulation of various osteoclast-related genes ^{33, 34}). Therefore, suppression of NFATc1 mRNA by lactoferrin indicates inhibition of osteoclast differentiation ³⁴). RANK regulates the expression of osteoclast marker genes ³⁵). NFATc1 induces osteoclast fusion via up-regulation of DC-STAMP, which is essential for

cell-cell fusion in osteoclasts. DC-STAMP-positive cells provide the first mechanistic insight into the process of fusion ^{36, 37}). A new protein called OC-STAMP has recently been characterized and shows functional similarities with DC-STAMP ³⁸). In this study, DC-STAMP and OC-STAMP mRNAs were decreased when lactoferrin was added from 12 to 48 h, suggesting that lactoferrin inhibits osteoclast differentiation and fusion (Fig. 5).

In summary, this is the first study of the direct effects of lactoferrin on RANKL-induced osteoclast differentiation. We found that lactoferrin inhibited RANKL-stimulated osteoclasts at an early stage of differentiation. Although further studies are needed, our data indicate that the application of lactoferrin may be a beneficial strategy as an alternative therapy for bone diseases such as osteoporosis.

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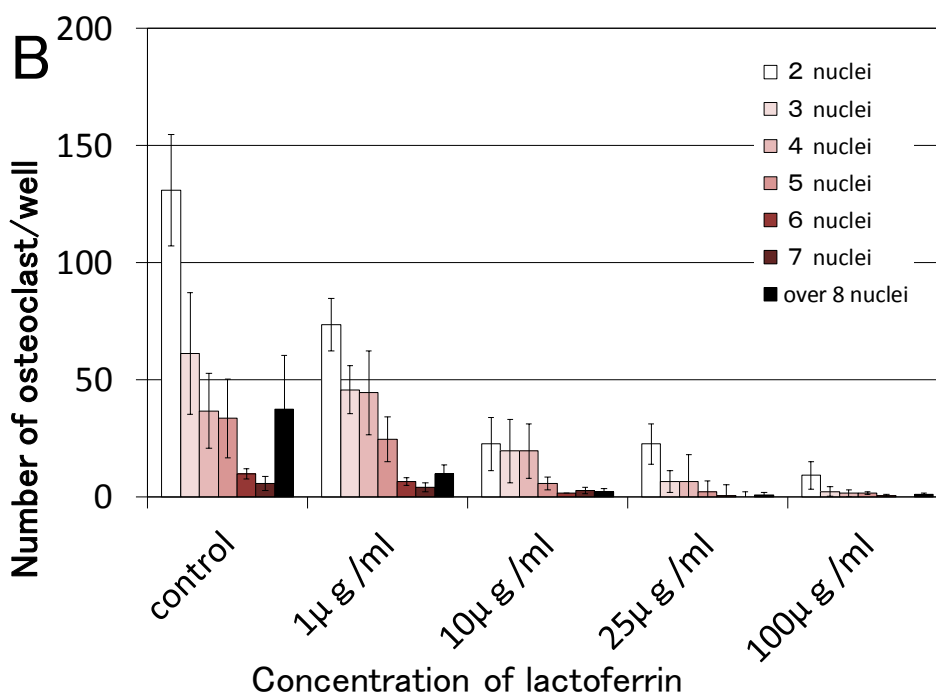
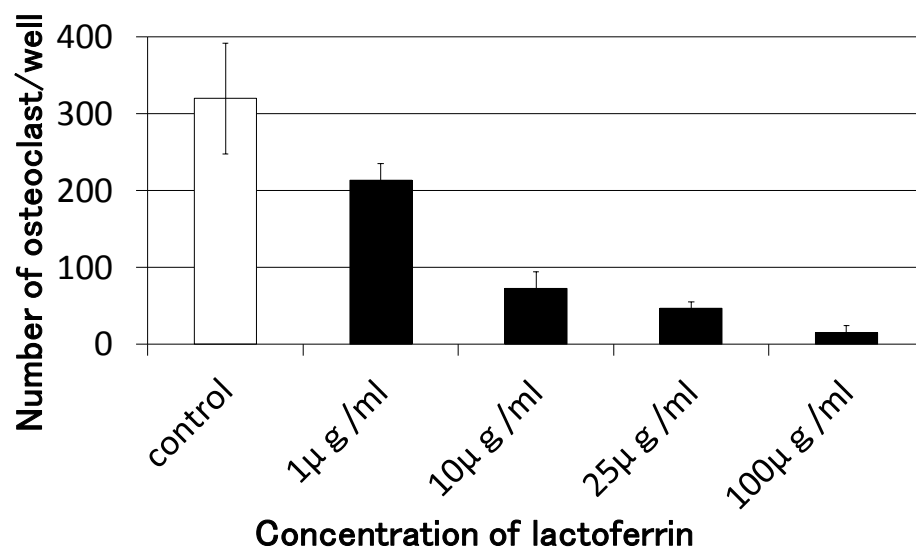
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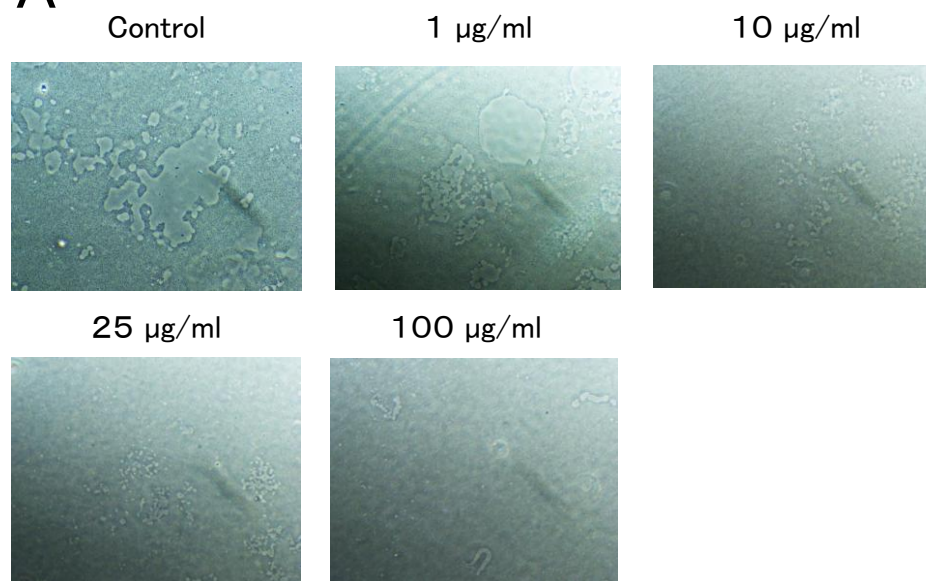
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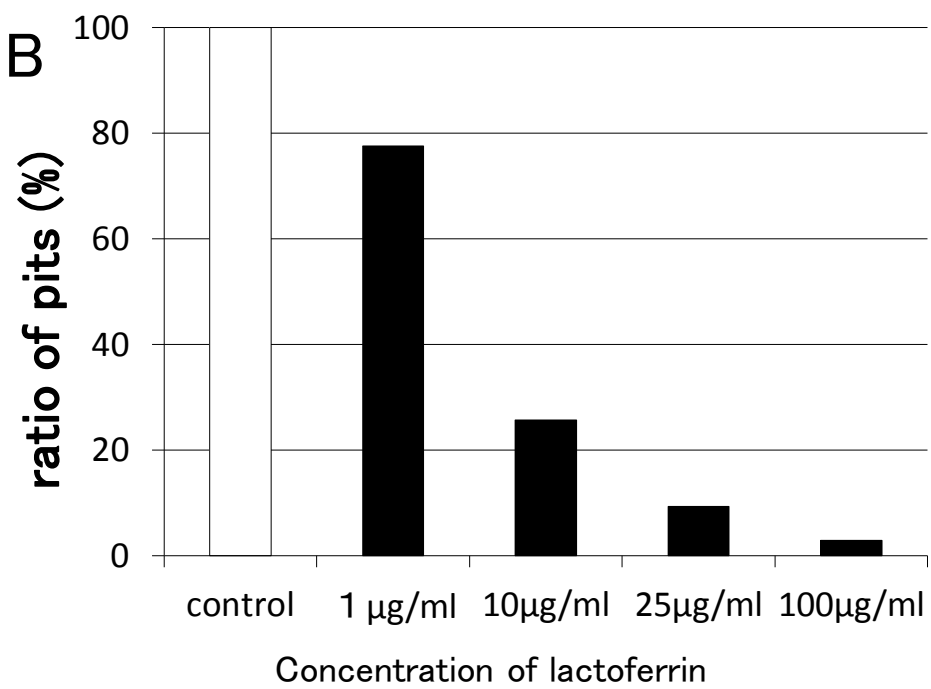
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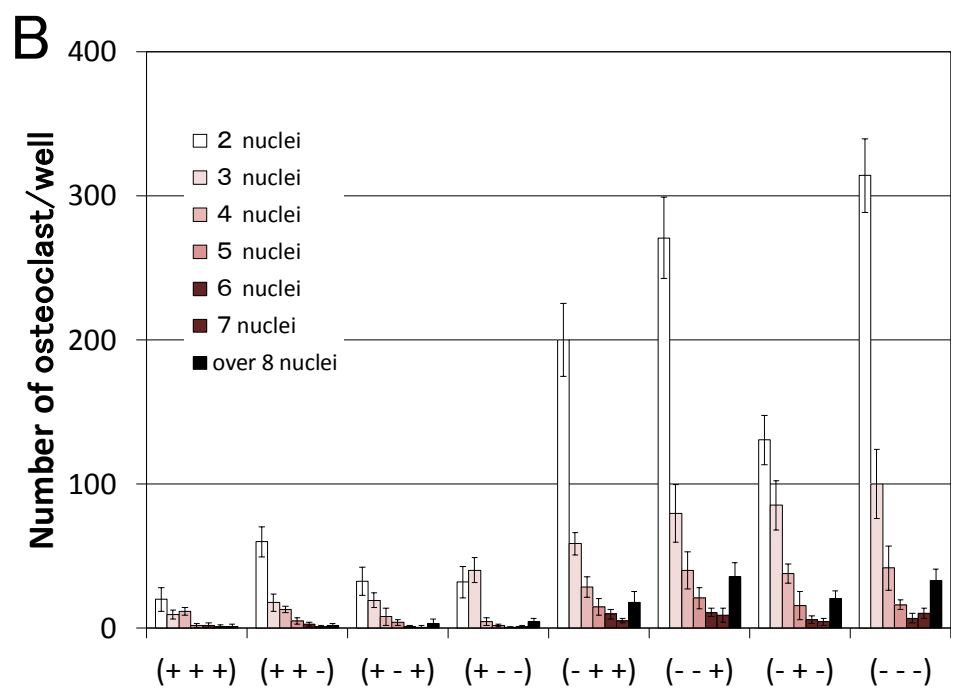
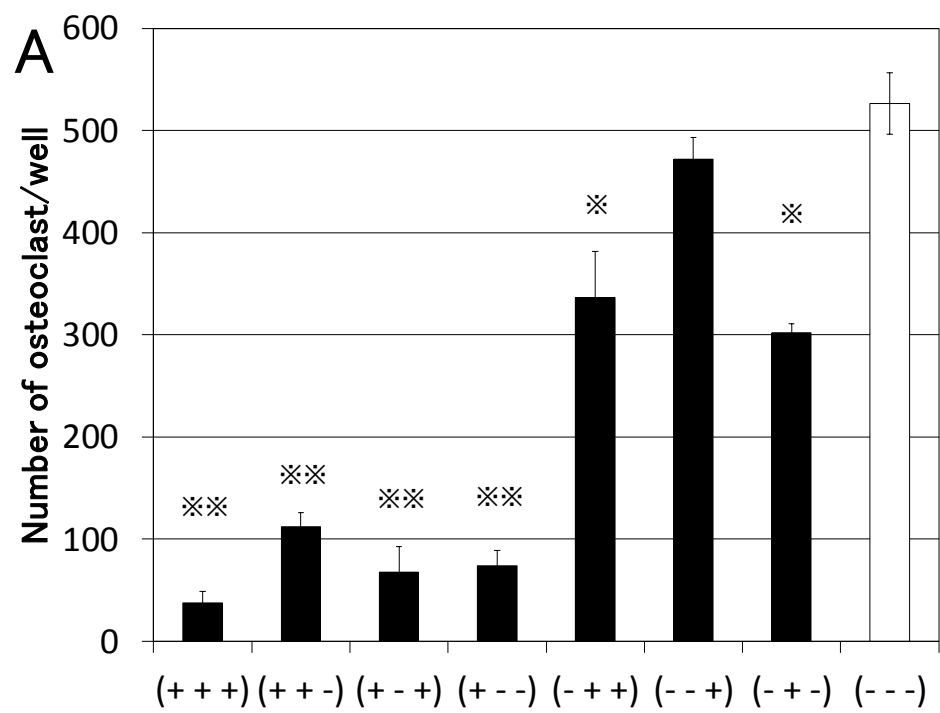


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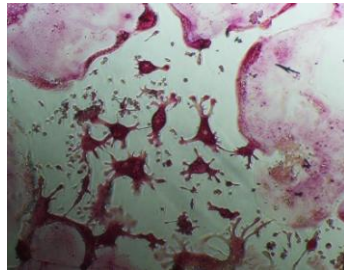
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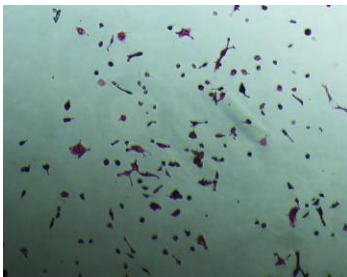


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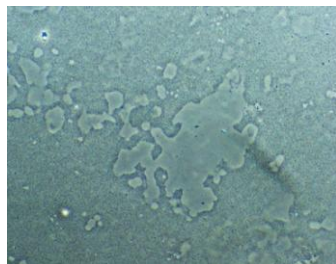


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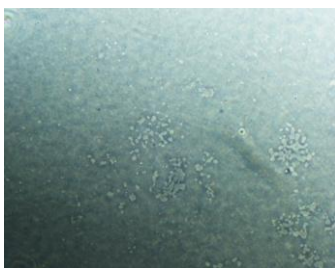


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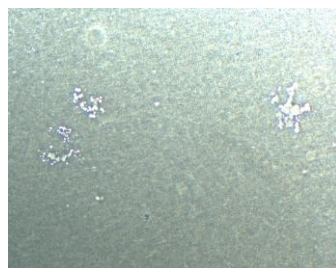
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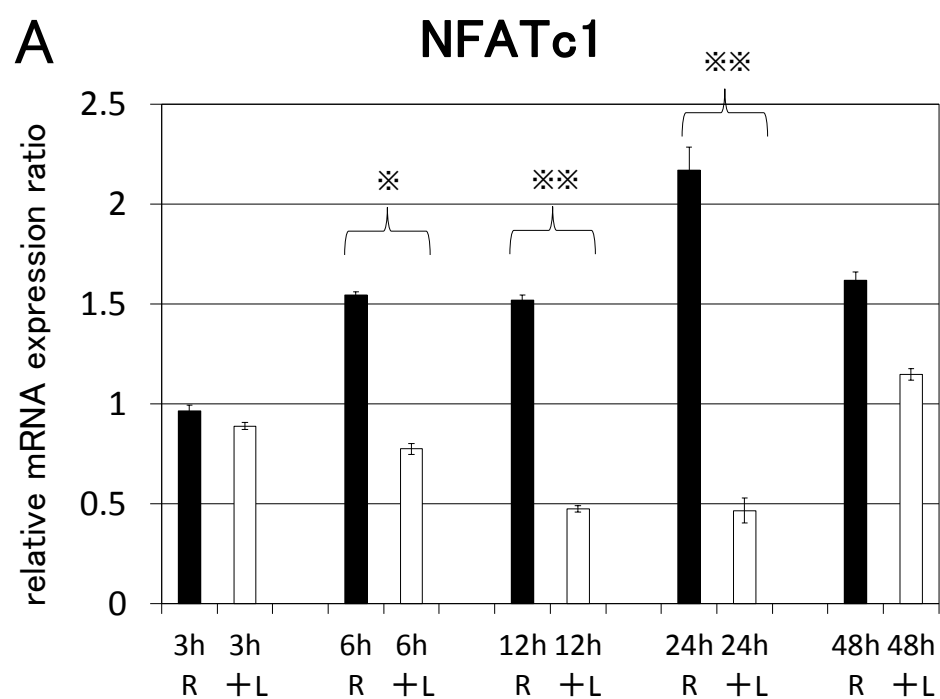
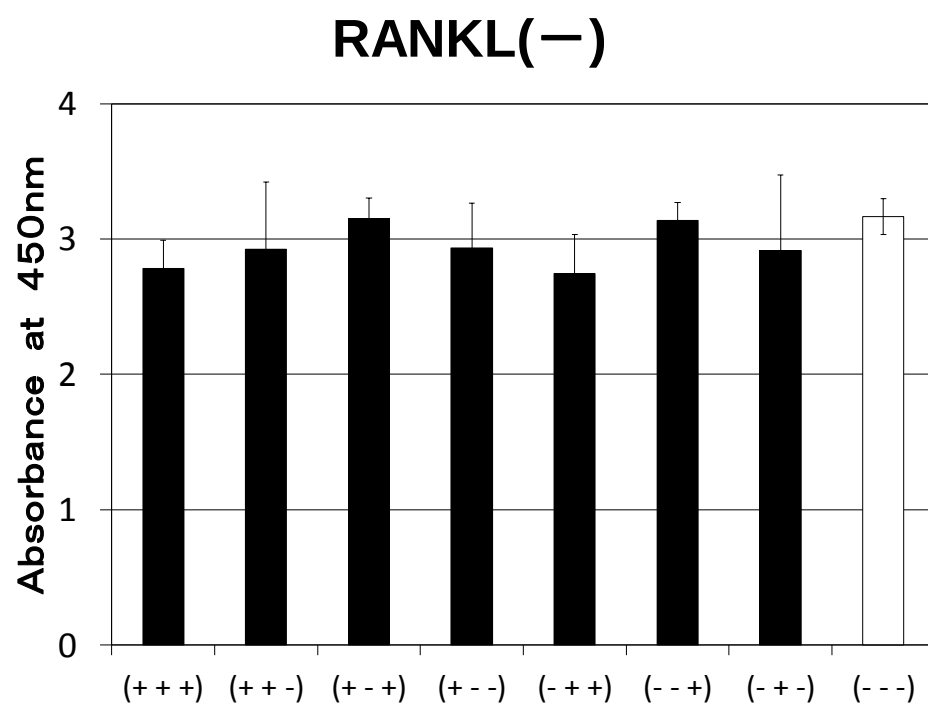


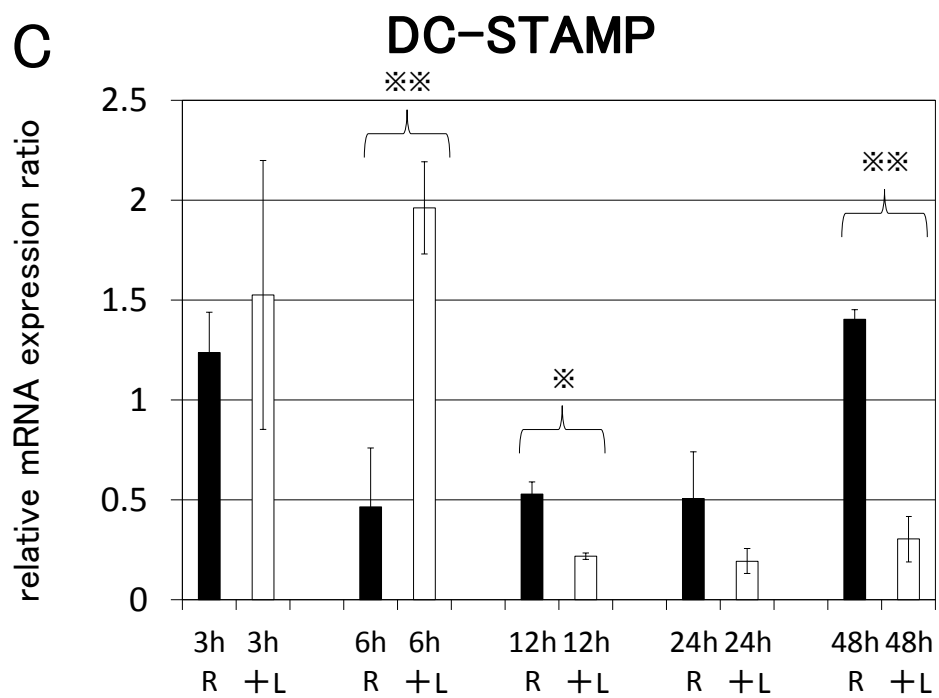
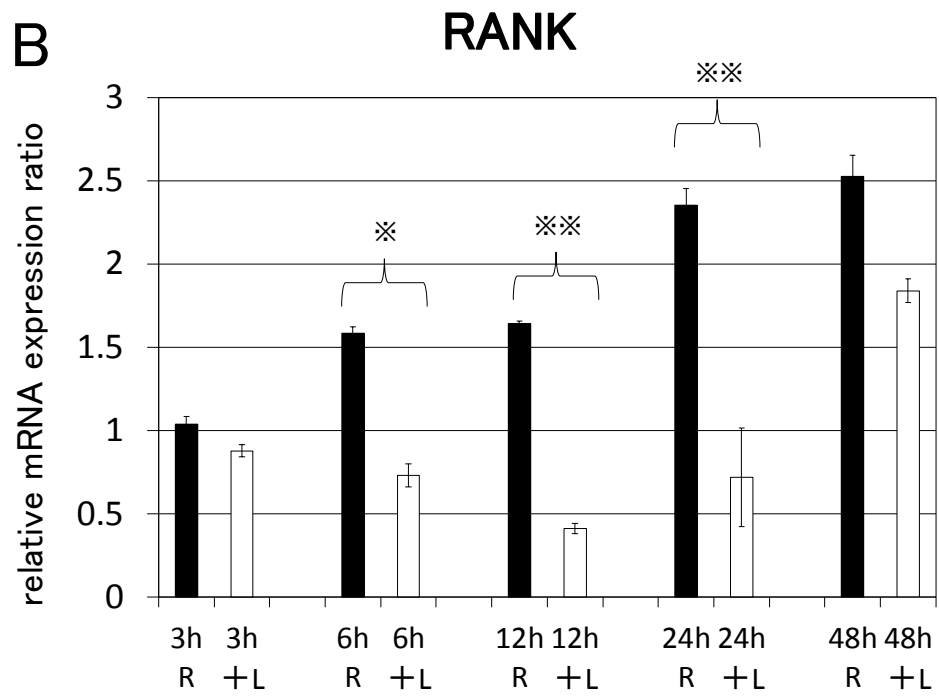
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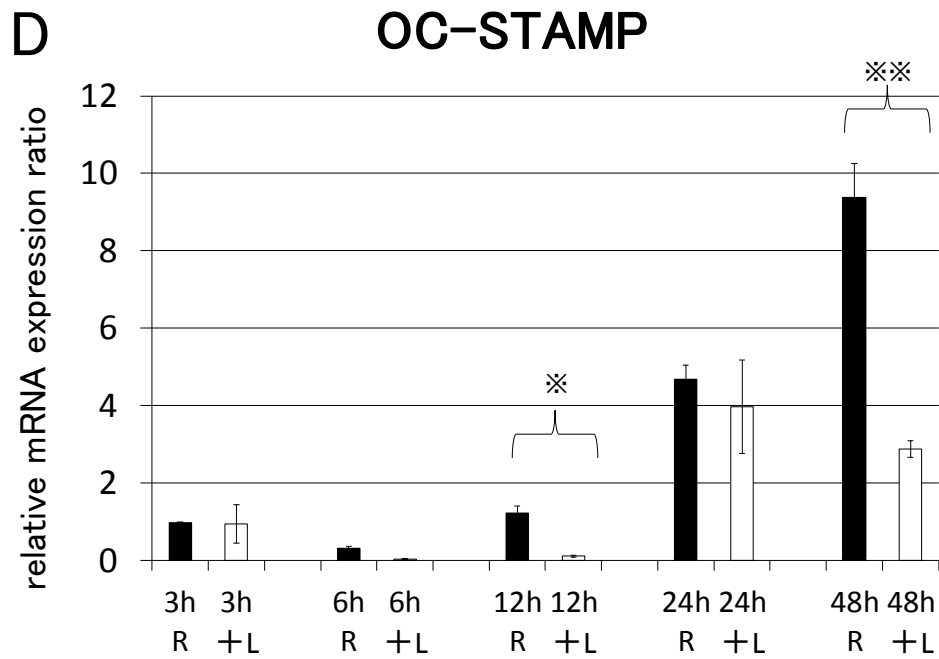


Figure legends

Figure 1. Effect of lactoferrin on osteoclastogenesis.

(A) Number of osteoclasts at each concentration of lactoferrin up to day 6. (B) The number of osteoclasts with multiple nuclei up to day 6. The control group was cultured with PBS instead of lactoferrin. The number of cells positive for tartrate-resistant acid phosphatase staining with 2-7 nuclei and those with ≥ 8 nuclei was counted. Values are expressed as the mean $\pm$ standard deviation (n = 4).

Figure 2. Pit assay.

We investigated the resorption activity of osteoclasts treated with lactoferrin (1, 10, 25, and 100 $\mu\text{g/ml}$) or PBS (control group) using the Corning Osteo Assay Surface Plate.

(A) After 6 days of culture, plates were observed under a light microscope magnification $\times 100$. (B) The control group was set as 100%. The resorption area was quantified using ImageJ software.

Figure 3. Effects of lactoferrin on osteoclastogenesis after treatment according to the various schedules.

The 6-day cultivation period was divided into three stages (2 days each): the stage when lactoferrin (25 $\mu\text{g/ml}$) was applied to the culture is indicated as (+), and the stage when lactoferrin was not applied to the culture is indicated as (-). The early, middle, and late stages are indicated in a row from left to right: (+ + +), (+ + -), (+ - +), (+ - -), (- + +), (- + -), (- - +), and (control). The control culture was treated with PBS. (A) Number of TRAP-positive osteoclasts (≥ 2 nuclei). (B) The number of osteoclasts according to the number of nuclei. (C) TRAP-positive osteoclasts were visualized with

light microphotography. Data are representative of results with lactoferrin (+ + +), (+ - -), and control. (D) The pit assay was visualized with light microphotography. Data are representative of results with lactoferrin (+ + +), (+ - -), and control. Values are expressed as the mean $\pm$ standard deviation (n = 4). *P < 0.05, **P < 0.01 vs. control.

Figure 4. Effects of lactoferrin to cytotoxicity on osteoclastogenesis.

The cytotoxicity of lactoferrin (25 μ g/ml) was analyzed. Cells were treated with lactoferrin as described in Fig. 3, after 6 days of culture, absorbance was measured at 450 nm using a microplate reader. The control culture was treated with PBS. RAW 264.7 cells were cultured with lactoferrin without RANKL. Values are expressed as the mean $\pm$ standard deviation (n = 4).

Figure 5. Effects of lactoferrin on mRNA expression.

We analyzed the mRNA expression of osteoclast-associated genes by real-time PCR 3, 6, 12, 24, and 48 h after the beginning of culturing the RAW cells with RANKL and lactoferrin or with RANKL and PBS (control group). (A) NFATc1, (B) RANK, (C)

DC-STAMP, and (D) OC-STAMP. Values are expressed as the mean $\pm$ standard

deviation (n = 4). *P < 0.05, **P < 0.01.