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ORIGINAL

Suppression of osteoclastogenesis by lactoferrin

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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 is yet to be reported. Therefore, this study examined 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 tartrate-resistant acid phosphatase 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. Additionally, it only mediated these effects within 48 h of culturing the RAW 264.7 cells with RANKL. Lactoferrin also significantly inhibited RANKL-induced mRNA expressions of osteoclastic differentiation genes, such as NFATc1, RANK, DC-STAMP, and OC-STAMP. Thus, these findings suggest that lactoferrin directly suppresses osteoclastogenesis within 48 h of culturing the RAW 264.7 cells with RANKL. Therefore, lactoferrin may be a novel and innovative therapy for treating bone diseases.

Key Words : lactoferrin, osteoclast, osteoclastogenesis

Introduction

Lactoferrin, discovered by a North European scholar in 1939 as the "red protein of 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 is widely distributed in body fluids, including tears, saliva, bile, pancreatic fluid, vaginal secretions, semen, and milk[2)]. It 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, though 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)]. However, 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 have 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 of lactoferrin as a dietary supplement for improving bone health.

The bone is continuously remodeled by osteoblasts and osteoclasts[17, 18, 19)]. Various hormones and cytokines regulate the proper balance between bone formation and resorption that modulates bone metabolism. If this balance is disrupted, metabolic bone diseases, such as osteoporosis and osteopetrosis, occurs[20, 21)]. Osteoclasts

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are members of the monocyte/macrophage lineage and are formed through the fusion of their mononuclear cellular precursors. The receptor activator of the NF-κB (RANK) ligand (RANKL) is essential and the most important cytokine for 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 a 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 the formation of osteoclasts[23]. This study used osteoclastic RAW 264.7 cells, which express endogenous RANK and can differentiate into osteoclasts following RANKL stimulation without M-CSF. The binding of RANKL to RANK induces the activation of tumor necrosis factor receptor-associated factor 6 and c-Fos pathways, which lead to autoamplification of nuclear factor of activated T cells (NFAT) c1, the master transcription factor for osteoclast differentiation. Autoamplification of NFATc1 is also dependent on calcium signaling and regulates many osteoclast marker genes, such as cathepsin K, tartrate-resistant acid phosphatase (TRAP), and calcitonin receptor[24].

Although recent research has shown that lactoferrin indirectly suppresses osteoclastogenesis by affecting osteoblasts and periodontal ligament fibroblasts[15, 16], whether lactoferrin directly affects 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℃ in a humidified atmosphere of 95% air and 5% CO_2. 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℃ in a humidified atmosphere of 95% air and 5% CO_2. 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℃ 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. First, control cells were treated with 10-μl phosphate-buffered saline instead of lactoferrin. Second, the six-day cultivation period was divided into three stages (two 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 into 24-well plates at a density of 1.0×10^4 cells/well with RANKL. Lactoferrin was added at various concentrations and schedules. After six days of culture, plates were observed under a light microscope, and the resorption area was quantified using ImageJ (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 tartrate-resistant acid phosphatase (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 as the small osteoclasts, and those with ≥8 nuclei were considered as the large osteoclasts[28, 29]. Osteoclasts were

counted under a light microscope (magnification, ×100; Olympus IMT-2; Olympus, Tokyo, Japan) in a 20 mm^2 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 six days of culture, the medium was replaced with a new medium (400 µl), and a 40-µl Cell Counting kit-8 solution was added. After incubation for 1 h at 37℃, absorbance was measured at 450 nm using a microplate 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) following the manufacturer's instructions. cDNA was synthesized from total RNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo (dt) primers.

For mRNAs analysis 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 conducted 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 $2^{-\triangle\triangle Ct}$ method, in which $\Delta Ct = \Delta Ct$ sample $- \Delta Ct$ reference. ΔCt sample is the Ct value for the samples normalized to the GAPDH, and ΔCt 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 ± SD. Comparisons between samples of interest and respective controls were analyzed using the two-tailed unpaired Student's t-test. *P*-values of <0.05 were considered significant.

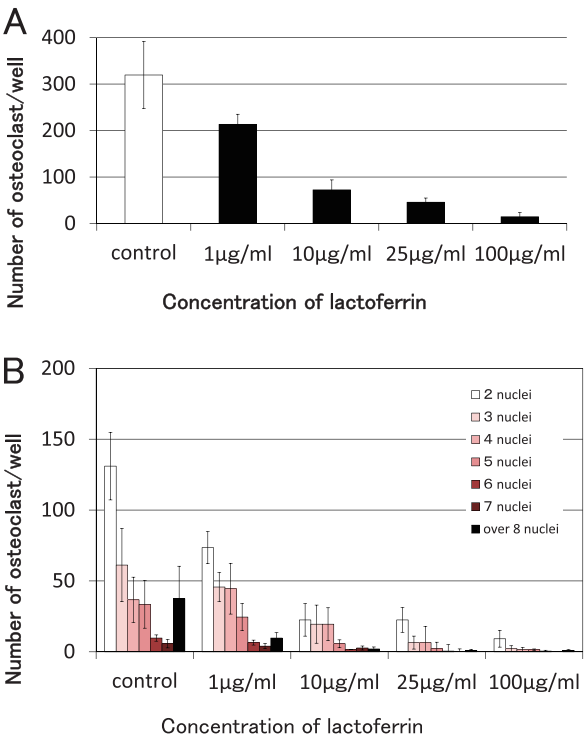

Figure 1. Effect of lactoferrin on osteoclastogenesis.
(A) Number of osteoclasts at each concentration of lactoferrin up to day 6. (B) The number of osteoclasts with multiple nuclei up to day 6. The control group was cultured with PBS instead of lactoferrin. The number of cells positive for tartrate-resistant acid phosphatase staining with 2-7 nuclei and those with ≥8 nuclei was counted. Values are expressed as the mean ± standard deviation (n = 4).

Results

Effects of 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 µg/ml) and RANKL, and counting the number of osteoclasts (2–7 nuclei) and the number of large osteoclasts (≥8 nuclei) after six days of culture. Lactoferrin decreased the number of osteoclasts (Fig. 1A) and osteoclasts with multiple nuclei (Fig. 1B) 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 µg/ml, respectively. Lactoferrin treatment decreased the ratio of the pit area

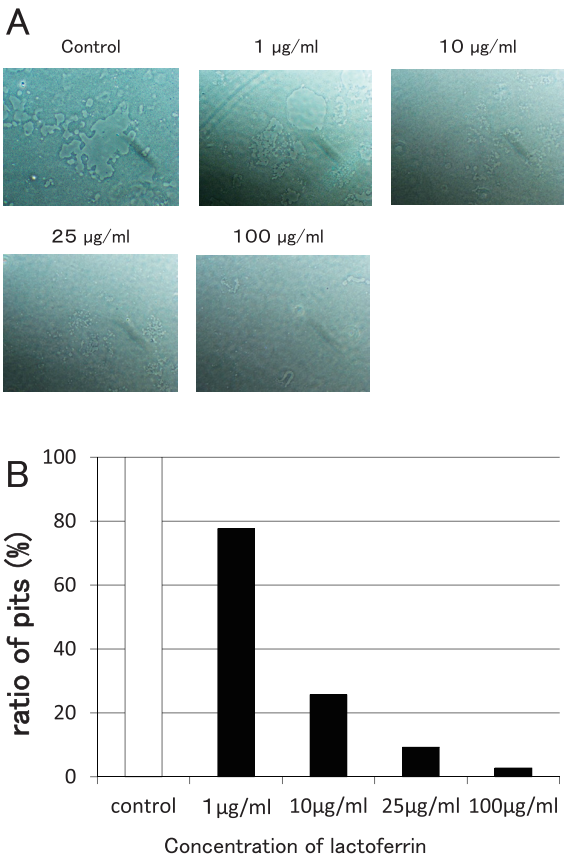

Figure 2. Pit assay.

We investigated the resorption activity of osteoclasts treated with lactoferrin (1, 10, 25, and 100 μg/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 ×100. (B) The control group was set as 100%. The resorption area was quantified using ImageJ software.

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 h, after the beginning of RANKL-treated RAW cells culture the number of osteoclasts and large osteoclasts after six days of culture were 2–7 and ≥ 8 nuclei, respectively. Lactoferrin decreased the number of osteoclasts (2–7 nuclei) when added within 48 h 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 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. Lactoferrin reduced the mRNA expression of NFATc1 and RANK between 6 and 24 h. Lactoferrin also reduced DC-STAMP and OC-STAMP mRNA expression 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 μg/ml, and at 100 μg/ml, osteoclastogenesis via osteoblasts was completely arrested. Furthermore, in a study on bone resorption, lactoferrin was found to reduce the bone-resorbing activity in a rabbit-mixed bone cell culture[32]. Therefore, in this 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 μg/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 thought to resorb bone mainly; thus, the preliminary and postresorption stages must be short compared to the actual resorption stage. Therefore, we investigated the resorption activity of osteoclasts treated with lactoferrin (1, 10, 25, and 100 μg/ml). In the pit assay, the pit area was significantly reduced in a dose-dependent manner after treatment with lactoferrin (Fig. 2). Thus 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

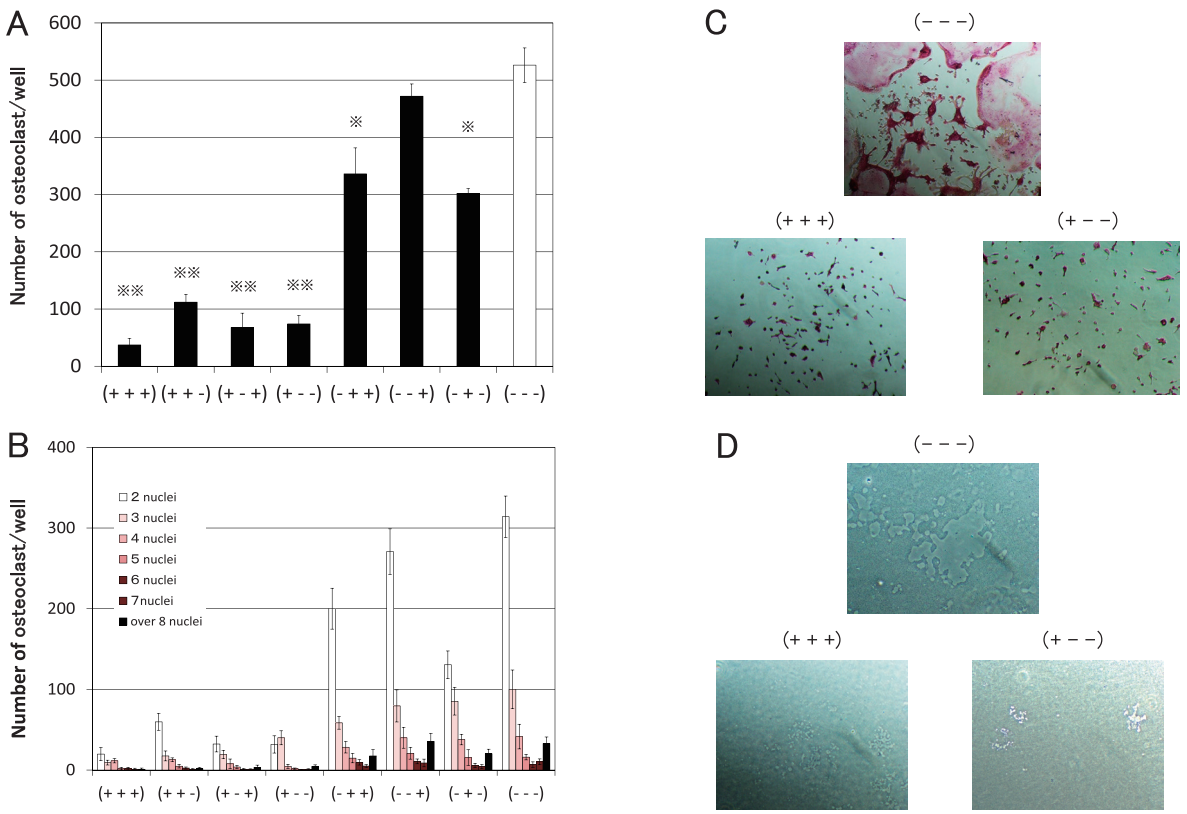

Figure 3. Effects of lactoferrin on osteoclastogenesis after treatment according to the various schedules.

The 6-day cultivation period was divided into three stages (2 days each): the stage when lactoferrin (25 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: (+ + +), (+ + −), (+ − +), (+ − −), (− + +), (− + −), (− − +), 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 ± standard deviation (n = 4). *P < 0.05, **P < 0.01 vs. control.

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 the cytotoxicity of RAW cells in the absence of RANKL (Fig. 4).

We further investigated the effect of lactoferrin on

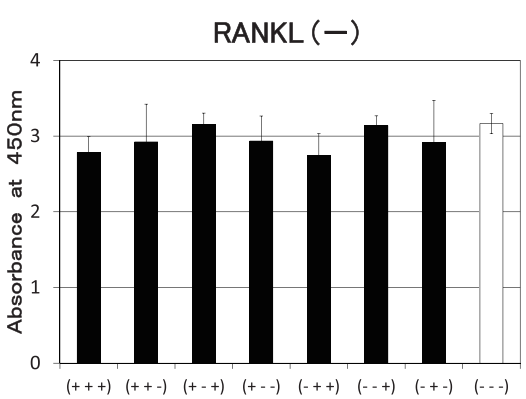

Figure 4. Effects of lactoferrin to cytotoxicity on osteoclastogenesis.

The cytotoxicity of lactoferrin (25 μg/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 ± standard deviation (n = 4).

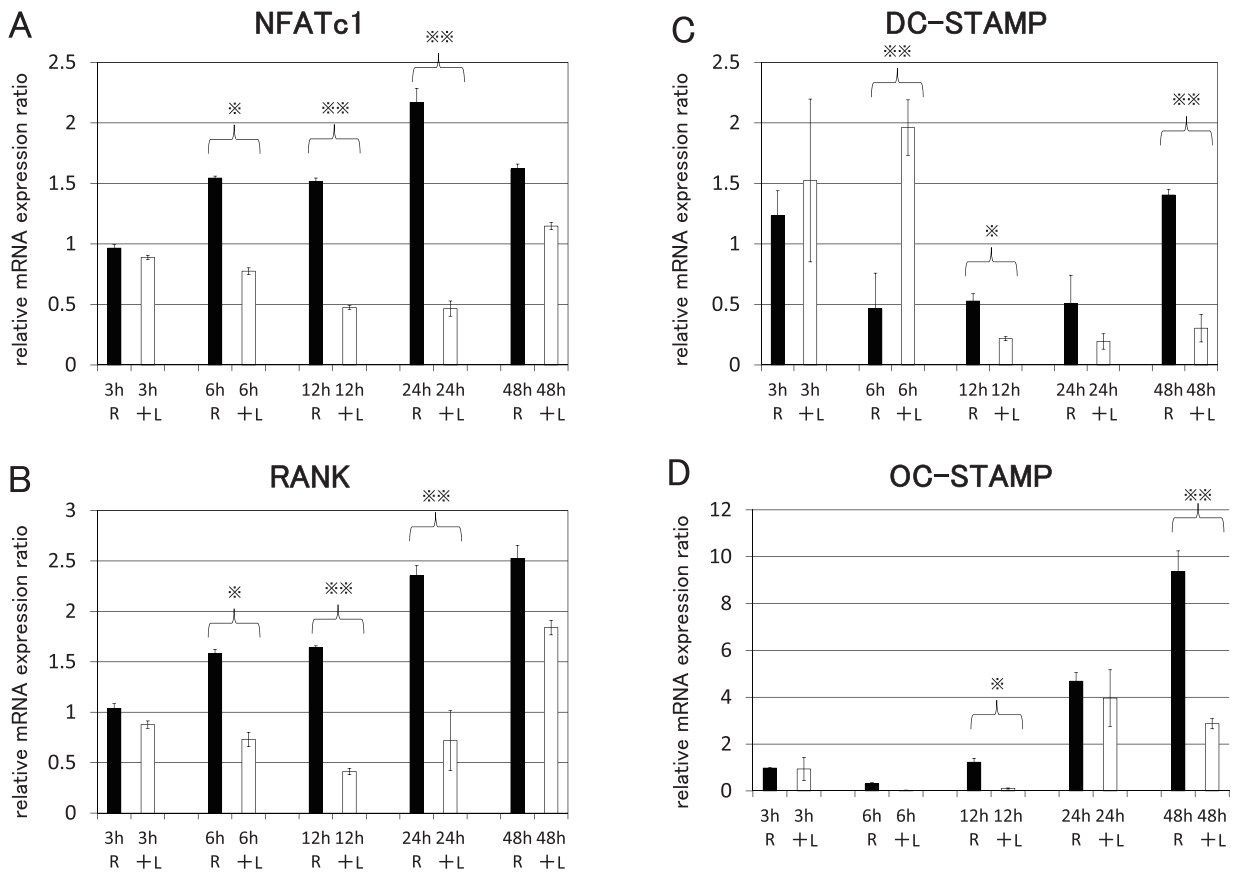

Figure 5. Effects of lactoferrin on mRNA expression.

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

mRNA expression of osteoclast-associated genes in RAW cells at an early stage of differentiation (3, 6, 12, 24, and 48 h). NFATc1 and RANK mRNA expression was decreased when lactoferrin was added within 48 h 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 upregulation of various osteoclast-related genes[33, 34]. Therefore, suppression of NFATc1 mRNA by lactoferrin indicates the inhibition of osteoclast differentiation[34]. RANK regulates the expression of osteoclast marker genes[35]. NFATc1 induces osteoclast fusion via upregulation 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 to examine 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 is beneficial as an alternative therapy for bone diseases, such as osteoporosis.

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