R848, a toll-like receptor 7 agonist, inhibits osteoclast differentiation but not survival or bone-resorbing function of mature osteoclasts

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Abstract R848, also known as resiquimod, acts as a ligand for toll-like receptor 7 (TLR7) and activates immune cells. In this study, we examined the effects of R848 on differentiation, survival, and bone-resorbing function of osteoclasts. R848 inhibited osteoclast differentiation of mouse bone marrow-derived macrophages (BMMs) and human peripheral blood-derived monocytes induced by receptor activator of NF- κ B ligand in a dose-dependent manner. In addition, it inhibited mouse osteoclast differentiation

induced in cocultures of bone marrow cells and osteoblasts in the presence of dihydroxyvitamin D₃ [1,25(OH)₂D₃]. However, R848 did not affect the survival or bone-resorbing activity of mouse mature osteoclasts. R848 also upregulated the mRNA expression levels of interleukin (IL)-6, IL-12, interferon (IFN)- γ , and inducible nitric oxide synthase in mouse BMMs expressing TLR7. IFN- β was consistently expressed in the BMMs and addition of neutralizing antibodies against IFN- β to the cultures partially recovered osteoclast differentiation inhibited by R848. These results suggest that R848 targets osteoclast precursors and inhibits their differentiation into osteoclasts via TLR7.

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Introduction

Osteoclasts are multinucleated giant cells responsible for bone resorption (Edwards and Mundy 2011). It is generally accepted that osteoblasts control osteoclast differentiation and bone-resorbing activity through cell–cell physical contact (Takahashi et al. 1988; Takami et al. 1999). Osteoblasts produce a membrane-bound cytokine known as receptor activator of NF- κ B ligand (RANKL) that triggers osteoclast differentiation from osteoclast precursors in the presence of macrophage colony-stimulating factor (M-CSF) (Edwards and

Mundy 2011). A receptor for RANKL, termed RANK, is expressed on osteoclast precursors and activates intracellular signaling pathways, which regulate the expressions of genes related to osteoclastogenesis (Edwards and Mundy 2011).

Many investigators have explored the mechanism of osteoclast differentiation using mouse bone marrow-derived macrophages (BMMs) and human peripheral blood monocytes (CD14⁺ cells), which possess the potential of differentiation into osteoclasts by stimulation with recombinant RANKL and M-CSF in vitro (Azuma et al. 2000; Kobayashi et al. 2000; Matsuzaki et al. 1999). On the other hand, osteoclast differentiation is also induced in cocultures of mouse bone marrow cells and osteoblasts in the presence of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], which induces production of RANKL by osteoblasts (Takahashi et al. 1988, 1999). Those osteoclasts appear as tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (TRAP⁺-MNCs), which are typical characteristics of osteoclasts in vivo and in vitro.

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns and play important roles in the innate immune system (Iwasaki and Medzhitov 2010). Peptidoglycan (PGN) and lipoteichoic acid (LTA) act as ligands of TLR2. Double-stranded RNA [poly(I:C) RNA], LPS, flagellin, and the CpG motif of unmethylated DNA (CpG DNA) act as ligands of TLR3, TLR4, TLR5, and TLR9, respectively (Akira et al. 2006). In response to TLR ligands, immune cells produce several inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, interferon (IFN)- β , IFN- γ , and IL-12 to activate immune systems (Akira et al. 2006). We previously reported that stimulation of TLR2, 3, 4, and 9 with their specific ligands inhibited osteoclast differentiation from BMMs (Takami et al. 2002). Those results suggest that TLRs may be critical to prevent the pathogenic effects of microbial invasion on bone. On the other hand, the functions of other TLRs in bone metabolisms have not been fully elucidated.

Among the family of TLRs, TLR7 recognizes viral single-stranded RNAs (ssRNAs) and imidazoquinolin family compounds, including R848 (C₁₇H₂₂N₄O₂, molecular weight: 314), also termed resiquimod (Hemmi et al. 2002; Kawai and Akira 2007). R848 possesses anti-viral activity that is mediated by activation of immune cells via TLR7 (mouse) and TLR7/TLR8 (human) (Jurk et al. 2002). In addition,

R848 has been shown to protect mice from experimental asthma and have antitumor activities (Hemmi et al. 2002; Kawai and Akira 2010). R848 is often administered for treatment of skin lesions as a topical cream in current clinical practice, including those caused by herpes simplex virus, or as an adjuvant agent for increasing the effectiveness of vaccines. However, the effects of R848 on bone metabolism have not been elucidated (Wu et al. 2004).

In the present study, to explore the roles of TLR7 in regulation of osteoclasts in bone metabolism, we examined the effects of R848 on osteoclast differentiation, survival, and bone-resorbing function. R848 inhibited mouse and human osteoclast differentiation, whereas it did not have effects on survival or bone-resorbing function of mature osteoclasts. Our results suggest that TLR7 plays an inhibitory role in osteoclast differentiation.

Materials and methods

Cytokines, chemicals, antibodies, animals, and cell culture medium

Human M-CSF (Leucoprol) was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). R848 and RANKL were purchased from Enzo Life Sciences (Farmingdale/NY, USA) and R&D Systems Inc. (Minneapolis/MN, USA), respectively. Neutralizing antibodies against IFN- β , IFN- γ , and IL-12 were purchased from R&D Systems. Mice (C57BL/6 strain, males, newborn, 6-week old) were purchased from Sankyo Laboratories Animal Center (Tokyo, Japan). In all experiments, α -modified MEM (α MEM) (Sigma-Aldrich, Tokyo, Japan) containing 10% fetal bovine serum (GIBCO, Carlsbad/CA, USA) was used for the cell cultures.

Mouse BMM cultures

Mouse bone marrow cells were collected from the femora and tibiae of 6-week-old mice, and cultured in 10 ml of α MEM and M-CSF (50 ng/ml) in 10-cm cell culture dishes (BD Biosciences, Sparks/MD, USA). After 3 days, BMMs formed in the dishes were collected by trypsin treatment and used as mouse osteoclast precursors. BMMs were then cultured in 96-well cell culture plates (2 $\times$ 10⁴ cells/well) (BD Biosciences) to which RANKL (50 ng/ml), M-CSF (50 ng/ml), and

various concentrations of R848 were added. After 3 days, cells were fixed with formalin and stained for TRAP using a conventional method described previously (Mochizuki et al. 2006; Zhao et al. 2009).

Mouse cocultures

Primary mouse osteoblasts were obtained from calvaria of newborn mice using a conventional method with collagenase (Wako Pure Chemical Industries, Tokyo, Japan). Primary mouse osteoblasts (5×10^3 cells/well) and bone marrow cells (1×10^5 cells/well) were cocultured for 6 days in α MEM containing 10% FBS, 10 nM $1,25(\text{OH})_2\text{D}_3$, and various concentrations of R848 in 96-well cell culture plates (BD Biosciences). Medium was replaced with fresh medium every 3 days. The cells were fixed with formalin and stained for TRAP.

Human PBMC cultures

CD14⁺ cells (monocytes) were obtained from peripheral blood using MACS® separation columns

with human CD14 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) and used as human osteoclast precursors. The cells were cultured in α MEM in 96-well cell culture plates (2×10^4 cells/well) (BD Biosciences) to which RANKL (50 ng/ml), M-CSF (50 ng/ml), and various concentrations of R848 were added. After 4 days, the cells were fixed with formalin and stained for TRAP using a conventional method described previously (Mochizuki et al. 2006; Zhao et al. 2009).

Pit formation assay

The mouse BMM and human PBMC cultures described above were also performed with dentin slices (4 mm in diameter, 0.3 mm thick) placed in 96-well cell culture plates. After culturing for 24 h, cells were removed from the dentin slices by wiping with cotton and resorption pits formed on the surface of dentin slices were visualized by staining with 1% toluidine blue solution.

Fig. 1 Effects of R848 on mouse osteoclast differentiation by BMMs. **a**, **b** Mouse BMMs were cocultured in the presence of RANKL (50 ng/ml) and M-CSF (50 ng/ml) with the indicated concentrations of R848 for 3 days. After culturing, cells were fixed and stained for TRAP (**b**), and the numbers of TRAP⁺-MNCs in the wells counted (**a**). **c**, **d** Dentin slices were placed in 96-well cell culture plates and exposed to the same experimental conditions as shown in **a**. After culturing, cells were removed from the dentin slices. The slices were stained with toluidine blue to visualize resorption pits formed on the surfaces (**d**) and the numbers of pits counted (**c**). Data are expressed as the mean $\pm$ SD of 4 cultures. ** $P < 0.01$

Ethical review of experiments using mouse and human cells

The Animal Care Committee of Showa University approved all experimental procedures using animals (approval number: 11039). The Medical Ethics Board of Showa University approved the use of human cells in the experiments (approval number: 2006-12).

PCR amplification of reversed-transcribed mRNA (RT-PCR)

Total RNA from BMMs in culture dishes was prepared using TRIzol solution (Invitrogen Life Technologies, CA, USA) and synthesized from total RNA using Superscript III (Invitrogen Life Technologies), then subjected to amplification with GoTaq DNA polymerase (Promega Corporation, CA, USA) using the following specific PCR primers: GAPDH, 5'-GAAG GTCGGTGTGAACGGATTTGGC-3' (forward) and 5'-CATGTAGGCCATGAGGTCCAACAC-3' (reverse); IFN- β , 5'-CCCTATGGAGATGACGGAGA-3' (forward) and 5'-GCAACCACCACTCATTCTGA-3' (reverse); IFN- γ , 5'-GCGTCATTGAATCACACCT G-3' (forward) and 5'-CGCAATCACAGTCTTGGCT A (reverse); IL-12, 5'-ACGGCAGGAAAACTGA A-3' (forward) and 5'-CAGATAGCCCATCACCCCTG T-3' (reverse); TLR7, 5'-GCGTGTC AAGGGTATGT CT-3' (forward) and 5'-AGTTTGGCCAATGGTGT TCT-3' (reverse); iNOS, 5'-ACGCTTGGGTCTTGTT CACT-3' (forward) and 5'-GTCTCTGGGTCCTCTG GTCA-3' (reverse); and IL-6, 5'-CACAGAGGATAC CACTCCCAACA-3' (forward) and 5'-TCCACGATT TCCCAGAGAACA-3' (reverse).

Results

R848 inhibits mouse osteoclast differentiation induced by RANKL

First, we examined the effects of R848 on mouse osteoclast differentiation from BMMs induced by RANKL. R848 decreased the number of TRAP⁺-MNCs, which are identical to osteoclasts, in a dose-dependent manner (Fig. 1a, b). In the presence of 1.0 nM of R848, the number of TRAP⁺-MNCs was decreased to half of the control and 100 nM of R848 was sufficient to decrease the number to less than 10% of the control

(Fig. 1a, b). No cytotoxic effects were observed, even in the presence of 100 nM of R848 (data not shown). When these experiments were performed on dentin slices placed in the cultures, the number of resorption pits formed by osteoclasts was significantly decreased in the presence of 100 nM of R848 (Fig. 1c, d).

R848 inhibits human osteoclast differentiation induced by RANKL

To examine the effects of R848 on human osteoclast differentiation, various concentrations of R848 were added to human PBMC cultures in the presence of RANKL and M-CSF (Fig. 2a, b). R848 decreased the

Fig. 2 Effects of R848 on human osteoclast differentiation from PBMCs. **a, b** Human PBMCs were cultured in the presence of RANKL (50 ng/ml) and M-CSF (50 ng/ml) with the indicated concentrations of R848 for 3 days. After culturing, cells were fixed and stained for TRAP (**b**), and the numbers of TRAP⁺-MNCs in the wells counted (**a**). Data are expressed as the mean $\pm$ SD of 4 cultures. ** $P < 0.01$

number of human TRAP⁺-MNCs in a dose-dependent manner (Fig. 2a). Addition of 1.0 μ M of R848 to the cultures decreased the number of TRAP⁺-MNCs to half of the control without cytotoxicity.

R848 inhibits osteoclast differentiation supported by osteoblasts

In cocultures of mouse bone marrow cells and osteoblasts, TRAP⁺-MNCs were induced in the presence of 1,25(OH)₂D₃ (Fig. 3a, b), while the addition of 100 nM of R848 significantly reduced their number (Fig. 3a, b). In addition, the number of resorption pits formed on the surfaces of the dentin slices was significantly decreased when these cells were cultured on dentin slices (Fig. 3c, d).

R848 does not affect survival or bone-resorbing activity of mature osteoclasts

To examine the effects of R848 on survival of mature osteoclasts, we added R848 to BMM cultures after mouse osteoclasts were formed. The number of TRAP⁺-MNCs was not changed in the presence of R848, even after 24 h (Fig. 4a, b). To examine the effects of R848 on bone-resorbing activity of mature osteoclasts, we obtained mature osteoclasts from the cocultures and cultured them on dentin slices in culture wells for 24 h. The number of resorption pits formed on the surface of dentin slices was not significantly different between the control and R848-treated osteoclasts (Fig. 4c, d).

Fig. 3 Effects of R848 on mouse osteoclast differentiation in cocultures. **a, b** Mouse bone marrow cells and osteoblasts were cocultured in the presence of 10 nM 1,25(OH)₂D₃ with the indicated concentrations of R848 for 6 days. After culturing, cells were fixed and stained for TRAP (**b**), and the numbers of TRAP⁺-MNCs in the wells counted (**a**). **c, d** Dentin slices were placed in 96-well cell culture plates and exposed to the same experimental conditions as shown in **a**. After culturing, cells were removed from the dentin slices. The slices were then stained with toluidine blue to visualize resorption pits formed on the surfaces (**d**) and the numbers of pits counted (**c**). Data are expressed as the mean $\pm$ SD of 4 cultures. ** $P < 0.01$

R848 regulates expressions of mRNAs related to inflammation in BMMs

Since stimulation of TLRs regulates gene expressions related to inflammation, we analyzed the mRNA expression levels of TLR7, IL-6, IL-12, IFN- β , IFN- γ , and iNOS in BMMs before and after R848 treatment (Fig. 5). TLR7 and IFN- β were expressed in the BMMs, and their expression levels were not affected by R848 treatment (Fig. 5). Furthermore, R848 upregulated the mRNA expression levels of IL-6, IL-12, IFN- γ , and iNOS in BMMs within 24 h (Fig. 5).

IFN- β partially involved in inhibition of osteoclast differentiation by R848

To examine whether the factors produced by BMMs mediate the inhibitory action of R848, we added

neutralizing antibodies against IFN- β , IFN- γ , and IL-12, which have been reported to inhibit osteoclast differentiation, to BMM cultures (Fig. 6). Among the examined antibodies, anti-IFN- β partially recovered the formation of TRAP⁺-MNCs in the presence of R848, whereas anti-IFN- γ and anti-IL-12 antibodies did not (Fig. 6).

Discussion

Although R848 has been reported to activate immune cells, including neutrophils, T cells, and dendritic cells, the effects of this compound on osteoclasts have not been studied. The present results clearly demonstrated that R848 has inhibitory effects on osteoclast differentiation of mature osteoclasts, but not their survival or bone-resorbing function. Our findings also

Fig. 4 Effects of R848 on survival and bone-resorbing function of mature osteoclasts. **a, b** Mouse BMMs were differentiated into mature osteoclasts by treatment with RANKL (50 ng/ml) and M-CSF (50 ng/ml) for 3 days in 96-well cell culture plates. Subsequently, mature osteoclasts were further cultured in the presence or absence of R848 (100 nM) for 24 h. After culturing, cells were fixed and stained for TRAP (**b**), and the numbers of TRAP⁺-MNCs in the wells counted (**a**). **c, d** Mature osteoclasts were obtained from mouse cocultures, placed on dentin slices in culture dishes, and cultured for 24 h with or without R848 (100 nM). After culturing, cells were removed from the dentin slices. The slices were then stained with toluidine blue to visualize resorption pits formed on the surfaces (**d**) and the numbers of pits counted (**c**). Data are expressed as the mean $\pm$ SD of 4 cultures. *NS* not significant

Fig. 5 Changes in expression levels of mRNAs in BMMs by treatment with R848. Mouse BMMs were cultured in the presence or absence of R848 (100 nM) for 24 h, then total RNA was extracted from the BMMs. The expression levels of mRNAs (TLR7, IL-12, IFN- β , IFN- γ , IL-6, iNOS, and GAPDH) were analyzed using an RT-PCR method with cDNA synthesized from total RNA

Fig. 6 Effects of neutralizing antibodies on osteoclast differentiation inhibited by R848. Mouse BMMs were cultured in the presence of RANKL (50 ng/ml) and M-CSF (50 ng/ml) with or without R848 (100 nM) alone or combinations of R848 (100 nM) with anti-IFN- β (1 μ g/ml), anti-IFN- γ (1 μ g/ml), or anti-IL-12 (1 μ g/ml) for 3 days. After culturing, cells were fixed and stained for TRAP, and the numbers of TRAP⁺-MNCs in the wells counted. ** $P < 0.01$. NS not significant

suggest that TLR7 in mice and TLR7/8 in humans regulate osteoclast differentiation in response to R848 or viral infections in bone.

R848 inhibited osteoclast differentiation by mouse BMMs and human PBMCs in the presence of RANKL. This suggests that R848 directly binds to TLR7 of mouse BMMs and TLR7/8 of human PBMCs, and activates intracellular signaling pathways. TLR7 and TLR8 are known to transduce

intracellular signals by recruiting MyD88 and regulating gene expressions related to inflammation (Iwasaki and Medzhitov 2010; Kawai and Akira 2007; Hemmi et al. 2002). Indeed, the expression levels of IL-6, IL-12, IFN- γ , and iNOS were upregulated in mouse BMMs by R848 treatment, while the expression of IFN- β mRNA was not upregulated, but consistently expressed in the BMMs. Among these genes, IL-6 has been shown to promote osteoclast differentiation (Herrera et al. 2011; Lee et al. 2004; O'Brien et al. 2005; Udagawa et al. 1995), while iNOS, IL-12, IFN- β , and IFN- γ inhibit it (Zheng et al. 2006, Horwood et al. 2001; Takayanagi et al. 2002; Takayanagi et al. 2000). Therefore, we added neutralizing antibodies against these molecules and found that neutralization of IFN- β by the antibody partially recovered osteoclast differentiation inhibited by R848. These results suggest that IFN- β partially contributes to inhibition of osteoclast differentiation by R848. In addition, IL-6 has been shown to indirectly promote osteoclast differentiation via upregulation of RANKL expression in osteoblasts (Palmqvist et al. 2002). Since IFN- β inhibits RANKL-induced osteoclast differentiation (Takayanagi et al. 2002), it can be considered that IFN- β inhibited IL-6 action. Unfortunately, we could not elucidate the contribution of iNOS to the inhibition of osteoclast differentiation by R848. Further analysis is necessary to elucidate the precise mechanism of the effects of R848 on osteoclast differentiation.

Inhibitory effects of R848 on osteoclast differentiation were observed not only in BMM cultures, but also cocultures of mouse bone marrow cells and osteoblasts in the presence of 1,25(OH) $_2$ D $_3$. These results indicate that R848 inhibits osteoclast differentiation supported by osteoblasts. Although we did not examine the effects of R848 on osteoblasts, studies of the precise effects of R848 on osteoblasts, such as differentiation, calcification, RANKL production, and proliferation, will be very important to understand the roles of TLR7 in bone metabolism.

Despite its inhibitory effects on osteoclast differentiation, R848 did not inhibit or promote survival and bone-resorbing activity of mature osteoclasts. We previously reported that TLR7 is expressed in BMMs, though its expression level is downregulated in mature osteoclasts (Takami et al. 2002). Therefore, R848 targets osteoclast precursors expressing TLR7, but not mature osteoclasts.

In conclusion, the inhibitory effects of R848 on osteoclast differentiation shown in this study are radically new findings, which will contribute to understanding of the relationships between innate immune systems and bone metabolism. Furthermore, our results suggest that R848 may be a candidate for a new drug to treat bone diseases such as osteoporosis.

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