Low Molecular-Weight Curdlan, (1→3)-β-Glucan Suppresses TLR2-Induced RANKL-Dependent Bone Resorption

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Fungal β-glucan is a potent immunological stimulator, and that it activates both the innate immune system and adaptive immunity. Curdlan is (1→3)-β-glucan, a linear form of β-glucan with a high molecular weight; it modulates the immune response. However, its role in bone tissue is controversial, and the effects of curdlan on bone tissues are unknown. Toll-like receptors (TLRs) play critical roles in innate immunity, and various ligands for TLRs are thought to regulate the host defense mechanisms against pathogens. TLR2 is known to form heterodimers with TLR6, and the TLR2-TLR6 heterodimer (TLR2/6) recognizes diacylated lipopolysaccharides from Gram-positive bacteria. In the present study, we prepared low molecular-weight curdlan, (1→3)-β-D-glucan, and examined its effects on bone resorption induced by TLR2/6 signaling. In co-cultures of bone marrow cells and osteoblasts, low molecular-weight curdlan suppressed the osteoclast formation induced by TLR2/6 ligand, and attenuated bone resorption in mouse calvarial organ cultures. Curdlan acted on mouse osteoblasts and suppressed the expression of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL), a key molecule for osteoclastogenesis. Curdlan also acted on mouse bone marrow macrophages and suppressed RANKL-dependent osteoclast differentiation from osteoclast precursor cells. The present study indicates that low molecular-weight curdlan attenuated TLR2-induced inflammatory bone resorption. Curdlan, (1→3)-β-glucan may be a natural agent with beneficial effects on bone health in humans.

Key words (1→3)-β-D-glucan; curdlan; osteoclast; Toll like receptor 2 (TLR2); bone resorption

Bone remodeling is regulated by bone resorption and bone formation, and osteoclasts, primary bone-resorbing cells are differentiated from monocyte-macrophages by a mechanism involving the receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) expressed on the cell surface of osteoblasts. Osteoclast precursors possess RANK, a receptor for RANKL, and can differentiate into mature osteoclasts via a RANK/RANKL-mediated mechanism.13

Toll-like receptors (TLRs; TLR1-TLR13) play critical roles in innate immunity, and various ligands for TLRs are thought to regulate the host defense mechanisms against pathogens.20 TLR4 was identified as the receptor for lipopolysaccharide (LPS), which is an outer membrane component of Gram-negative bacteria. We have reported that LPS induces inflammatory bone resorption by TLR4 signaling.22 TLR2 is known to form heterodimers with TLR1 or TLR6, and the TLR2-TLR6 heterodimer (TLR2/6) recognizes diacylated lipopolysaccharides from Gram-positive bacteria.23 We previously reported that TLR2 signaling induces osteoclast formation and inflammatory bone resorption.3

It is known that fungal β-glucan is a potent immunological stimulator, and that it activates both the innate immune system and adaptive immunity.6 Curdlan is (1→3)-β-glucan, a linear form of β-glucan with a high molecular weight; it modulates the immune response by a receptor-dependent mechanism. Several receptors of β-glucan have been identified, including dectin-1, complement receptor 3 (CR3), and lactosylceramide (LacCer).7 Dectin-1 is a major receptor for the recognition of β-glucan, and is found on various immune cells including macrophages and T cells. Dectin-1 dependent β-glucan signaling is thought to be coordinated by TLR2 in the innate immune system.8 In addition to immune system regulation, β-glucan exhibits anti-cancer activity, anti-viral activity and reduces the blood glucose level.9 However, the role of β-glucan in bone tissues is unclear. The biological activity of β-glucan is closely related to its molecular size and the degree of polymerization of glucose. In the present study, we prepared low molecular-weight curdlan, (1→3)-β-glucan, and examined its effects on the inflammatory bone resorption induced by TLR2/6 signaling.

MATERIALS AND METHODS

Animals and Reagents Newborn and six-week-old ddy mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). High molecular-weight curdlan, (1→3)-β-glucan (MW 80000), was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and heated to 180°C for 2h in distilled water (1 mg/mL) using high pressure reactor (Highpreactor BR, Berghof Group, Germany) to prepare low molecular-weight curdlan (MW 3000) (Fig. 1A). The molecular size of the sample was determined by Gel Permeation Chromatography using HPLC (Prominence, Shimadzu Co., Ltd., Japan) with columns (TSKgel G4000PWXL, G4000PWXL and G2000PWXL;...


To examine the differentiation of osteoclasts from precursor cells, mouse bone marrow cells were collected and cultured with macrophage colony-stimulating factor (M-CSF) and soluble RANKL (sRANKL) to generate bone marrow macrophages. The macrophages were further cultured for 3 days in the presence of M-CSF and sRANKL. After the cultures, TRAP-positive multinucleated cells were counted as osteoclasts.

RT-PCR Analysis Total RNA was extracted from cultured osteoblasts, and an RT-PCR was performed to examine the mRNA expression of RANKL and osteoprotegerin (OPG) in osteoblasts. The primer sequences were as follows: mouse RANKL: 5'-AGGCTTGGCAGATCTCTA-3' (forward) and 5'-GTCCTGATTAGCTCTTCCC-3' (reverse), mouse OPG: 5'-AGGAGGTGCAACCACCC-3' (forward) and 5'-TTCCAGCTTGCACCAAGCC-3' (reverse).

Bone-Resorbing Activity in Mouse Calvarial Organ Cultures To measure the bone-resorbing activity in the mouse calvarial organ cultures, the calvariae were collected from newborn mice, dissociated into halves, and cultured for 24 h in BG/Jb medium containing bovine serum albumin (BSA) (1 mg/mL). After 24 h, the calvariae were transferred into new medium with or without test compounds, and cultured for 5 days. The bone-resorbing activity was determined by measuring the calcium in the medium.

Statistical Analyses The data were analyzed by one-way ANOVA, followed by Tukey’s test for a post hoc analysis, and expressed as the mean ± standard error of the mean (S.E.M.).

RESULTS Effects of Curdlan, (1→3)-β-Glucan, on Osteoclast Formation We examined the effects of low molecular-weight curdlan, (1→3)-β-glucan, on osteoclast formation in co-cultures of bone marrow cells and osteoblasts. As reported previously, the TLR2/6 ligand markedly induced osteoclast formation, and TRAP-positive multinucleated osteoclasts were formed. The addition of curdlan (0.001–10 μg/mL) suppressed the osteoclast formation induced by TLR2/6 ligand in a dose-dependent manner (Fig. 1B). Curdlan (10 μg/mL) did not induce osteoclast formation in the absence of TLR2/6 ligand (Fig. 1B).

Curdlan Acts on Both Osteoblasts and Osteoclast Precursor Cells We next examined the action mechanism of curdlan in osteoclast formation. An RT-PCR revealed that curdlan suppressed the differentiation of osteoclasts from the precursor cells in a dose-dependent manner (Fig. 2A). Curdlan did not inhibit the expression of RANKL in osteoblasts in the absence of TLR2/6 ligand (Fig. 2A). The expression of OPG mRNA was not influenced by TLR2/6 ligand or curdlan (Fig. 2A). In bone marrow macrophage cultures, sRANKL induced the differentiation of macrophages into mature osteoclasts. The addition of curdlan suppressed the differentiation of osteoclasts from the precursor cells in a dose-dependent manner (Fig. 2B). Curdlan (10 μg/mL) did not inhibit osteoclast differentiation in the absence of sRANKL (Fig. 2B). Thus, curdlan acts on both osteoblasts and osteoclast precursors to suppress osteoclast formation.

Effects of Curdlan on Bone Resorption in Mouse Calvarial Organ Cultures Mouse calvarial organ culturing is a typical ex vivo assay system used to define the effects of test compounds on bone resorption. In this assay, the TLR2/6 ligand markedly induced the bone-resorbing activity detected by the increase levels of medium calcium. Adding low molecular-weight curdlan significantly suppressed the bone-
Resorbing Activity in Mouse Calvarial Organ Cultures

Fig. 2. The Effects of Curdlan on the TLR2/6 Ligand-Induced Expression of RANKL mRNA in Primary Osteoblasts, and on the Differentiation of Osteoclasts from Bone Marrow Macrophages

(A) Mouse osteoblasts were cultured, and treated for 3h with TLR2/6 ligand (100 ng/mL) and/or curdlan (50 μg/mL), and the expression levels of RANKL and OPG mRNAs were examined by an RT-PCR. (B) Mouse bone marrow cells were cultured with M-CSF and for 3d, and further cultured for 5d with M-CSF and sRANKL with or without curdlan (0.1–10 μg/mL). The number of osteoclasts, indicated as TRAP-positive multinucleated cells, was counted. The data were expressed as the mean±S.E.M. of three independent wells. Significant differences between two groups were indicated as follows: *** p<0.001 vs. control, * p<0.05 vs. sRANKL.

Fig. 3. The Effects of Curdlan on the TLR2/6 Ligand-Induced Bone-Resorbing Activity in Mouse Calvarial Organ Cultures

Mouse calvariae were cultured for five days in BGJb containing 1 mg/mL of BSA with TLR2/6 ligand (100 ng/mL) and/or curdlan (10 μg/mL). The concentration of calcium in the medium was measured to calculate the bone-resorbing activity. The data were expressed as the mean±S.E.M. of three independent wells. Significant differences between two groups were indicated as follows: *** p<0.001 vs. control, ** p<0.01 vs. TLR2/6 ligand.

DISCUSSION

In the present study, we showed that low molecular-weight curdlan, (1→3)-β-D-glucan suppressed the osteoclast formation induced by TLR2/6 ligand, and attenuated TLR2-induced inflammatory bone resorption in mouse calvarial organ cultures. Curdlan acted on mouse osteoblasts and suppressed the expression of RANKL, and it also acted on bone marrow macrophages and suppressed RANKL-dependent osteoclast differentiation from osteoclast precursor cells.

Previous studies have shown that β-glucan is a potent immunological stimulator and that its biological activities are somehow similar to those of TLR2 signaling in immune cells. In the present study, however, curdlan did not induce osteoclast formation, but clearly suppressed TLR2-induced bone resorption. Thus, β-glucan may act as an inhibitor of inflammatory bone resorption. Since β-glucan signaling is reported to be coordinated by TLR2 signaling in the innate immune system, it is possible that β-glucan interacts with TLR2 molecule on the osteblast surface. Dectin-1 is a major receptor for β-glucan, but other receptors, such as CR3 and LacCer also recognize β-glucan. Yamasaki et al. reported that high molecule curdlan suppressed the differentiation of macrophage RAW 264.7 into osteoclasts through the overexpression of the dectin-1 gene. Further studies are needed to define the mechanisms of molecular interaction between curdlan and these receptors in osteoblasts and osteoclasts in bone.

The biological activity of β-glucan may be related to its molecular size, the degree of polymerization of glucose, and solubility. In the present study, we prepared curdlan (MW 3000) with low molecular solubility, and showed its effects on the regulation of bone resorption. We also tested the effects of other low molecular size β-glucan on osteoclast differentiation in the co-cultures of mouse bone marrow cells and osteoblasts. Three low molecular oligosaccharides, laminaritetraose (MW 666), laminarihexaose (MW 990), and laminarioligosaccharides (MW 1153) did not exhibit the suppressive effects on osteoclast differentiation (data not shown). High solubility may be important for it to exhibit biological activity, but the molecular size more than 1153 may be essential for the suppressive effects on osteoclast differentiation and bone resorption. Further studies are needed to define the role of the molecular size of curdlan in the biological activity and the mechanism of signaling pathway in osteoblasts and osteoclast precursors in bone tissues.

Periodontal diseases are infectious diseases that develop as a result of the accumulation of bacterial plaque in the periodontal pocket. In our original mouse model of periodontitis, we reported that the loss of alveolar bone was induced by not only the TLR4 ligand LPS but also TLR2 heterodimer signaling in vitro and in vivo. Thus, both Gram-positive and Gram-negative bacteria might be involved in alveolar bone loss. Silva et al. reported that β-glucan reduced glucose levels and attenuated alveolar bone loss in diabetic rats with periodontal disease. Further in vivo studies using β-glucan and TLRs are essential for defining the roles of β-glucan in the pathogenesis of periodontitis.
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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


