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Original Article

The W9 peptide directly stimulates osteoblast differentiation via RANKL signaling

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ABSTRACT

Objective: A RANKL-binding peptide, WP9QY (W9), is known to inhibit mouse osteoclastogenesis by stimulating the production of autocrine factors such as bone morphogenetic proteins (BMPs) to induce osteoblast differentiation. In the present study, we investigated whether osteoblastic differentiation is mediated by RANKL signaling.

Methods: The effect of W9 on the differentiation of osteoclasts and osteoblasts was examined in mouse bone-marrow cultures, and in a mouse co-culture system consisting of primary osteoblasts derived from RANKL-deficient or wild-type (WT) newborn mouse calvariae, with WT-derived bone marrow mononuclear cells.

Results: The addition of the W9 peptide to the WT mouse bone-marrow culture simultaneously inhibited RANKL-induced tartrate-resistant acid phosphatase (TRAP)-positive osteoclast differentiation, and stimulated alkaline phosphatase (ALP)-positive osteoblastic calcified nodule formation. RANKL-deficient osteoblasts exhibited weak ALP activity compared to WT osteoblasts. W9 treatment strongly inhibited TRAP-positive osteoclast formation, and stimulated ALP-positive osteoblast differentiation in co-cultures of WT-derived osteoblasts and bone-marrow cells, in the presence of bone-resorbing factors. In contrast, W9 exerted only a weak effect on ALP-positive osteoblast differentiation in co-cultures with RANKL-deficient osteoblasts, even in the presence of the W9 peptide, parathyroid hormone, and/or BMP-2.

Conclusions: The W9 peptide inhibited RANKL-mediated osteoclast formation in osteoblasts. It also directly stimulated osteoblast differentiation, both via RANKL signaling-mediated autocrine factors, and alternative mechanisms.

1. Introduction

Bone is continuously destroyed by osteoclasts, and reformed by osteoblasts, to maintain bone volume and calcium homeostasis throughout the life span of vertebrates [1–4]. Osteoclasts are multinucleated cells that resorb bone, and derive from monocyte/macrophage-lineage cells [5]. In contrast, osteoblasts mediate osteoclastogenesis [1,6] by producing macrophage colony-stimulating factor (M-CSF), which is essential for osteoclast differentiation [7]. The receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) is another cytokine that is essential for osteoclastogenesis, and is expressed by osteoblasts as a membrane-associated cytokine [8]. Osteoclast precursors express RANK (a RANKL receptor), recognize RANKL expressed by osteoblasts via cell-cell interaction, and differentiate into osteoclasts in the presence of M-CSF [9]. Osteoprotegerin (OPG) is a soluble RANKL decoy receptor that is predominantly produced by osteoblasts [10,11], and that prevents osteoclast formation, and osteoclastic bone resorption, by inhibiting the RANKL-RANK interaction. In contrast, bone resorption-stimulating hormones and cytokines enhance RANKL expression in osteoblasts. Mature osteoclasts also express RANK, and RANKL both supports osteoclast survival, and stimulates osteoclast bone-resorbing activity.

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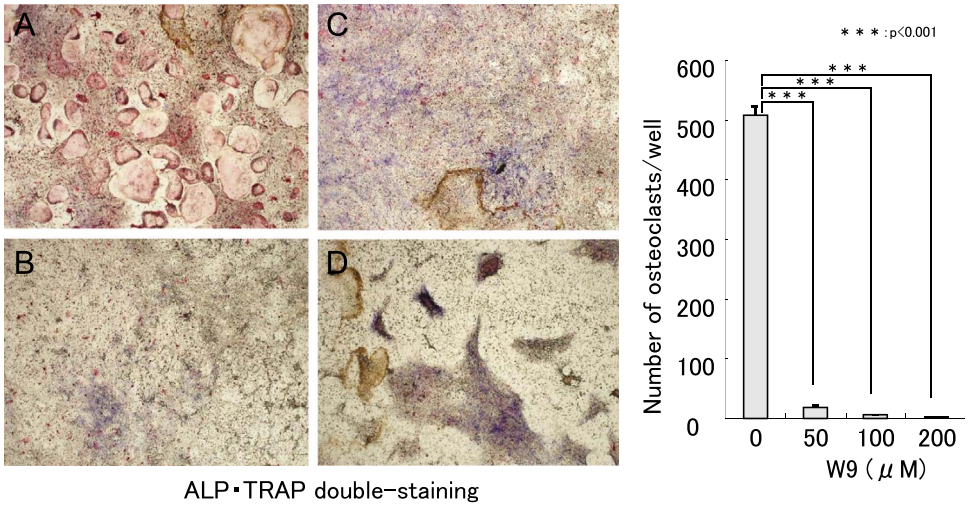

Fig. 1. Effects of W9 on the differentiation of osteoclasts and osteoblasts in mouse bone-marrow cultures. Bone marrow cells obtained from the tibiae of 6–9-week-old male ddY mice were cultured in 48-well plates in α-MEM supplemented with 10% FBS, in the presence of sRANKL (100 ng/ml) and M-CSF (50 ng/ml), **(A)** without, or with **(B)** 50 μM, **(C)** 100 μM, or **(D)** 200 μM W9 peptide. After seven days, the cells were fixed, and then stained for TRAP and ALP as described. Violet-stained, multinucleated cells with more than three nuclei were categorized as osteoclasts. Each experiment was repeated at least five times, and obtained results expressed as the mean ± SEM of three cultures. *P* values calculated using a Dunnett's test are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Inhibition of RANKL-RANK signaling in bone can increase bone mass by preventing osteoclastic bone resorption. RANKL- and RANK-deficient mice have been shown to exhibit severe osteopetrosis, and an accompanying lack of osteoclast differentiation [12,13]. In contrast, OPG-deficient mice exhibit severe osteoporosis arising from enhanced adult-stage osteoclastogenesis [14,15]. Accordingly, OPG and soluble RANK have been investigated as potential therapeutic targets, and an anti-human RANKL-antibody called denosumab has been employed in the clinical setting, for the treatment of osteoporosis and cancer-related bone disorders [16,17].

WP9QY (W9) is a peptide that was designed to be structurally similar to one of the cysteine-rich TNF-receptor-type-I domains, and was demonstrated to bind to TNFα and block its activity [18]. W9 also binds RANKL, and inhibits RANKL-induced osteoclast differentiation and function both in vitro and in vivo [19]. Furthermore, we previously demonstrated that W9 stimulates RANKL-mediated osteoblast differentiation in vitro via the production of autocrine factors including bone morphogenetic proteins (BMPs), and showed an anabolic effect in cortical bone in mice [17].

In the present study, we used RANKL-deficient mouse-derived osteoblasts to evaluate whether osteoblastic differentiation is mediated by RANKL signaling in vitro. We found that RANKL-deficient osteoblasts exhibited weak alkaline phosphatase (ALP) activity compared to wild-type (WT) osteoblasts, even in the presence of W9, parathyroid hormone (PTH), and/or BMP-2. In addition, RANKL-deficient osteoblasts displayed no supporting tartrate-resistant acid phosphatase (TRAP)-positive osteoclast formation activity when co-cultured with WT bone-marrow hematopoietic cells in the presence of bone-resorbing factors. Together, these results suggest that the RANKL-RANK signaling in osteoblasts may be essential for the dynamic regulation of bone formation and resorption.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6 and ddY male mice were obtained from Japan SLC (Shizuoka, Japan). RANKL-deficient mice (C57BL/6) were generated in the laboratory of Josef M. Penninger.

2.2. Reagents

Recombinant soluble human RANKL (sRANKL) was prepared by Oriental Yeast Co., Ltd. Recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN). Recombinant human M-CSF (Leukoprol®) was obtained from Kyowa Hakko (Tokyo, Japan). 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and collagenase were purchased from Wako Pure Chemical Industries (Osaka, Japan). Human PTH (1–34) was purchased from the Peptide Institute (Osaka, Japan). Type I collagen gel (cell matrix type I-A) was obtained from Nitta Gelatin Inc. (Osaka, Japan). α-MEM was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical grade.

2.3. Osteoclast formation in bone marrow cultures

Bone marrow cells obtained from the tibiae of 6–9-week-old male ddY mice were cultured (seven days) in 48-well plates in α-MEM supplemented with 10% FBS, and in the presence of sRANKL (100 ng/ml), and M-CSF (50 ng/ml), with or without W9 peptide (50–200 μM). The cells were fixed, stained initially for TRAP, and then some culture wells were subsequently also stained for ALP, as described [17]. Violet-stained TRAP-positive multinucleated cells with more than three nuclei were categorized as osteoclasts. Each experiment was repeated at least five times. The results presented for each typical experiment are expressed as the mean ± SEM of three cultures.

2.4. Preparation of osteoblasts and spot co-cultures for osteoclast formation

To isolate primary osteoblasts from either RANKL-deficient or WT mice, calvaria from 2-day-old mice (male and female) were cut into small pieces, and cultured for five days in a type-I collagen gel that was prepared in α-MEM supplemented with 10% FBS, as described [20]. Osteoblasts derived from the calvarium were collected by treating the collagen-gel cultures with collagenase, and storing them at −80 °C until use. Bone marrow cells obtained from the tibiae of 6–9-week-old male mice were suspended (16 h) in α-MEM supplemented with 10% FBS, in the presence of 50 ng/ml M-CSF. Next, non-adherent cells were harvested as hemopoietic cells.

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