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Inhibitory effect of CGRP on osteoclast formation by mouse bone marrow cells treated with isoproterenol

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Abstract

The present study was designed to elucidate the mode of action of isoproterenol (Isp; adrenergic β-agonist) and to characterize the effect of the calcitonin gene-related peptide (CGRP; sensory neuropeptide) on osteoclast formation induced by Isp in a mouse bone marrow culture system. Treatment of mouse bone marrow cells with Isp generated tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (MNCs) capable of excavating resorptive pits on dentine slices, and caused an increase in receptor activator of NF-κB ligand (RANKL) and a decrease in osteoprotegerin (OPG) production by the marrow cells. The osteoclast formation was significantly inhibited by OPG, suggesting the involvement of the RANKL–RANK system. CGRP inhibited the osteoclast formation caused by Isp or soluble RANKL (s-RANKL) but had no influence on RANKL or OPG production by the bone marrow cells treated with Isp, suggesting that CGRP inhibited the osteoclast formation by interfering with the action of RANKL produced by the Isp-treated bone marrow cells without affecting RANKL or OPG production. This in vitro data suggest the physiological interaction of sympathetic and sensory nerves in osteoclastogenesis in vivo.

Keywords: Osteoclastogenesis; Isoproterenol (Isp); Calcitonin gene-related peptide (CGRP); Receptor activator of NF-κB ligand (RANKL); RANK; Osteoprotegerin (OPG)

Bone envelopes have been demonstrated to be innervated by both myelinated and unmyelinated fibers belonging to the sensory and sympathetic nervous systems. These sympathetic and sensory innervations are required for regulating bone metabolism under physiological conditions [2,5,7,9,24]. Although bone-resorbing activity has been reported to be modulated by sensory neuropeptides and norepinephrine, little attention has been given to the physiological interaction of sensory neuropeptides with norepinephrine with respect to osteoclastic activity and osteoclast formation. The recent discovery of RANKL–RANK interaction confirms the well-known hypothesis that osteoblasts play an essential role in osteoclast differentiation [10]. Osteoblasts/stromal cells express RANKL as a membrane-associated factor and OPG as a decoy receptor for RANKL. Osteoclast precursors that express RANK, a receptor for RANKL, recognize RANKL through cell–cell interaction and differentiate into osteoclasts. This RANKL–RANK system has been reported to underlie the effect of osteotropic factors on osteoclast differentiation. Epinephrine has been reported to increase the expression of osteotrophic factors such as interleukin (IL)-6, IL-11, prostaglandin (PG)-E_2, and RANKL in osteoblastic cells [11,22], as well as the formation of osteoclast-like cells from mouse bone marrow cells by activating adrenergic β-receptors [22]. These findings suggest that the β-adrenergic stimulation induces osteoclastogenesis via the RANKL–RANK system in culture systems. On the other hand, CGRP has been demonstrated to inhibit bone-resorbing activity of isolated osteoclasts [1,13], calcium release in bone tissue culture [18,23], and osteoclast formation from bone marrow cells treated with $1,25(OH)_2D_3$ [4,17]. In this study, we elucidated the involvement of RANKL and OPG in the osteoclast formation from mouse bone marrow cells treated with Isp, an adrenergic β-agonist, for 7 days, and characterized the effect of the sensory neuropeptide CGRP on Isp-

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induced osteoclastogenesis to gain a better understanding of the physiological interaction between the sympathetic and sensory nerves in bone resorption.

Mouse bone marrow cells were obtained from tibiae of 6- to 8-week-old ddY male mice (Japan SLC Inc., Hamamatsu, Japan) as previously described [20] in accordance with the Guidelines for Animal Experiments at the School of Dentistry, Aichi-Gakuin University. Marrow cells were cultured in alpha-modified minimum essential medium (α-MEM; Invitrogen Co., Carlsbad, CA, USA) containing 10% fetal calf serum (Sigma Co., St. Louis, MO, USA) at 1.5×10^6 cells in 24-well plates in a humidified air with 5% CO_2 at 37 °C. One-half of the culture medium was exchanged for fresh medium every two days, and Isp (Sigma Co.), recombinant mouse RANKL (s-RANKL), which is extracellular domain of RANKL and has an ability to induce osteoclast differentiation, OPG (R&D Systems, Minneapolis, MN, USA) or CGRP (Peptide institute Inc., Osaka, Japan) was added at the beginning of cultures and at each change of medium. To estimate the osteoclast formation induced by Isp or s-RANKL, we counted the number of TRAP-positive MNCs by the following method: adherent cells were fixed in a mixture of 10% formalin in phosphate-buffered saline and ethanol–acetone (50:50, v/v), and stained for TRAP by incubating the cells in 0.1 M sodium acetate buffer (pH 5) containing naphthol AS-MX phosphate, and red violet LB salt in the presence of 10 mM sodium tartrate, as described previously [15]. Cells containing three or more nuclei were designated as MNCs. Moreover, to confirm the bone-resorbing capability of the TRAP-positive MNCs, we used six or eight cultures grown on dentine slices in each group.

To estimate RANKL protein expression, we lysed bone marrow cells in 6-cm tissue culture dishes with lysis buffer containing 50 mM phosphate buffer, 0.1% Triton X-100, phenylmethylsulfonylfloride, and protease inhibitor cocktail (Sigma Co.). The RANKL proteins in the lysate were measured by using an ELISA kit (R&D Systems); and for quantitative analysis, RANKL was normalized to the total protein content, as determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). The amount of OPG in the conditioned medium was quantified by performing an ELISA using commercially available antibodies and recombinant mouse OPG (R&D Systems). Data are presented as means ± S.D. The statistical significance of differences between control and experimental group was determined using Student's *t*-test.

The effect of OPG on osteoclast formation was evaluated by examining the formation of TRAP-positive MNCs generated by the treatment of bone marrow cells with Isp in the presence or absence of OPG. As shown in Fig. 1A, treatment with Isp (1 μM) for 7 days significantly increased the formation of TRAP-positive MNCs. OPG (10 ng/ml) inhibited this osteoclast formation generated by Isp (1 μM). The bone-resorbing activity of osteoclasts was also evaluated based on the formation of pits on dentine slices. Osteoclasts newly generated by Isp (1 μM) were capable of excavating resorptive

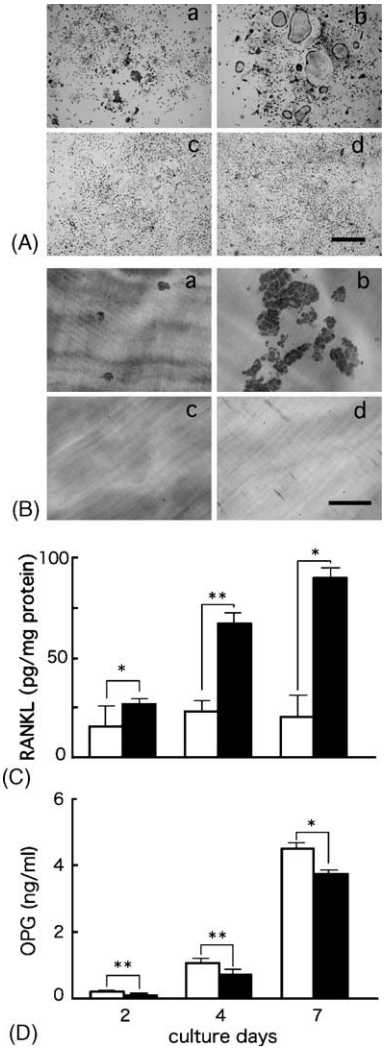

Fig. 1. Effect of isoproterenol on osteoclastogenesis and on the production of RANKL and OPG in mouse bone marrow cells. Mouse bone marrow cells were treated with vehicle (a), Isp (1 μM; b), OPG (10 ng/ml; c) or Isp and OPG (d) for 7 days. (A) TRAP-positive MNC formation from mouse bone marrow cells. Bar = 2.0 mm. (B) Bone-resorbing activity of TRAP-positive MNCs. Bar = 1.0 mm. (C) Time-course of the effect of Isp (1 μM) on RANKL production. (D) Time-course of the effect of Isp (1 μM) on OPG production. The accumulation of RANKL and of OPG was determined by ELISA assays; and for quantitative analysis, RANKL was normalized to the total protein content. Values are the mean ± S.D. ($n = 3$). (□) Control, (■) 1 μM Isp. Significantly different from the control values at $^{*}P < 0.01$, and $^{**}P < 0.001$, respectively.

pits on dentine slices, but no excavated pits were observed when OPG (10 ng/ml) was also present (Fig. 1B). The time-course of the effect of Isp (1 μM) on osteoclast formation showed a sharp increase between days 4 and 7 in the cultures of mouse bone marrow cells (data not shown).

Fig. 1C and D shows the time-course of alteration of both RANKL and OPG production in mouse bone marrow cells treated with Isp. Isp (1 μM) caused a significant increase in RANKL production at days 2, 4 and 7, with a 192% increase at day 4. It also caused a significant decrease in OPG production, with a 33% inhibition at day 4.