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Neurokinin B activates the formation and bone resorption activity of rat osteoclasts

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ABSTRACT

Neurokinin B (NKB) is a neuropeptide in the tachykinin family that acts as a neurotransmitter and neuromodulator, primarily in the central nervous system. The distribution and role of NKB and its receptor, the neurokinin-3 receptor (NK-3R), in peripheral tissues are poorly understood. In this study, we investigated the distribution of NKB and NK-3R in peripheral tissues as well as the role of NKB in bone metabolism, especially in osteoclast formation and bone resorption activity through NK-3R. The distributions of NKB in intact rat neurons of the trigeminal ganglion (TG) and in axons of periodontal tissue were investigated by immunohistochemistry. Osteoclasts from cultured rat bone marrow cells were used to examine the distribution of NK-3R by immunocytochemistry and RT-PCR and to investigate the effects of NKB on the resorption activity of osteoclasts on ivory slices. We found that NKB immunopositive neurons were localized in the rat TG and that NKB immunopositive axons were distributed in periodontal tissues. Immunoreactivity for NK-3R was found in cultured osteoclasts, and NK-3R mRNA expression in the osteoclasts was confirmed by RT-PCR. The addition of NKB significantly increased the number of osteoclasts and the resorption area compared with the control. These findings suggest that NKB was localized in peripheral neurons and may involve the activation of osteoclast formation and bone resorption through NK-3R.

1. Introduction

Neurokinin B (NKB) is a neuropeptide in the tachykinin family that acts as a neurotransmitter and neuromodulator in the sensory nervous system (Maggi et al., 1993). The other major tachykinin family peptides are substance P (SP), neurokinin A (NKA), neuropeptide K, and neuropeptide gamma (Otsuka and Yoshioka, 1993; Pennefather et al., 2004). Tachykinins belong to a family of neuropeptides that share a common C-terminal region, characterized by the presence of the Phe-Xaa-Gly-Leu-Met-NH_2 motif (Salthun-Lassalle et al., 2005). The biological action of the tachykinins is mediated through three subtypes of receptors, neurokinin-1, -2, and -3 receptors (NK-1R, -2R, and -3R), belonging to the G-protein-coupled receptor family. SP and NKA preferentially bind to NK-1R and NK-2R, respectively, whereas NKB has a high affinity for NK-3R. It is now recognized that expression of NKB and NK-3R is mainly in the central nervous system, including the hypothalamus, solitary nuclei, and intermediolateral nucleus of the spinal cord (Otsuka and Yoshioka, 1993; Nagano et al., 2006). The distribution of NK-3R in the periphery has been examined in only a few tissues, such as the airways and uterus (Pennefather et al., 2004).

Osteoclasts are multinucleated cells derived from hematopoietic progenitors in the bone marrow that also give rise to monocytes in peripheral blood and to the various types of tissue macrophages (Teitelbum, 2000). The presence of "receptor activator of nuclear factor-kappa B ligand" (RANKL) and macrophage-colony-stimulating factor (M-CSF) are essential for the formation and fusion of multinucleated cells, expressing osteoclast-specific markers such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and the calcitonin receptor (see reviews in [Bar-Shavit, 2007; Rauner et al., 2007]).

We have been investigating the association between bone metabolism and tachykinins, especially the distribution of NK1-Rs and the function of SP (Goto et al., 1998, 2001, 2007). To date, there is no reported information about the effects of NKA or NKB on osteoclastogenesis or the function of osteoclasts. Our preliminary experiments suggested that bone formation increased after the addition of NKB, but not NKA. Thus, in this study, we focused on the effects of NKB on osteoclast formation and bone resorptive activity.

The purpose of this study was to investigate (1) the localization of NK-3R in osteoclasts and preosteoclast-like cells, (2) the distribution of NKB in peripheral bone tissue and in the neurons of the trigeminal ganglion (TG), and (3) the effects of NKB on the formation and bone resorptive activity of osteoclasts through NK-3R.

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2. Materials and methods

2.1. Osteoclast culture

The experimental protocols involving rats were reviewed and approved by the Animal Care Committee of Kyushu Dental College, Kitakyushu, Japan.

The bone marrow cells of 7-week-old male Sprague–Dawley rats were isolated by a modified version of a previously published method (Morikawa et al., 2008). Briefly, the ends of the bones were removed, and the marrow cavity was flushed with α-minimum essential medium (α-MEM; Gibco, Grand Island, NY) by injecting the medium into one end of the bone using a sterile 22-gauge needle. The marrow cells were collected in tubes, centrifuged (1500*g*, 5 min) aspirated, and mixed with 0.83% NH_4Cl. They were centrifuged again (1500*g*, 5 min) and then aspirated and covered with fresh medium. Finally, the cells were plated in 35-mm culture dishes on cover glasses (5×10^5 cells/dish) and in 96-well plates on dentin slices (7×10^5 cells/well) and cultured for up to 10 days at 37 °C under 5% CO_2 in α-MEM containing 10% fetal bovine serum (Gibco), 1% gentamicin/amphotericin B/penicillin G (Wako, Osaka, Japan), 10^{-7} M 1α,25-dihydroxyvitamin D_3 (BIOMOL, PA, USA), 25 ng/mL human M-CSF (Wako), and 20 ng/mL human RANKL (Wako). Fresh medium was added every 3 days.

2.2. Immunocytochemistry and NKB binding

Bone marrow cells, which were cultured for 1 week as described above, were fixed in 4% paraformaldehyde for 10 min and then treated with 0.5% Triton X-100 for 10 min. After washing with phosphate-buffered saline (PBS, pH 7.4) three times, the cells were blocked with 1% whole goat serum (Nichirei, Tokyo, Japan) for 30 min, washed three times in PBS, and incubated with anti-NK-3R rabbit IgG antibodies (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 2 h at 37 °C. After another three washes with PBS, the samples were incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:100; Molecular Probes, Eugene, OR, USA) for 2 h at 37 °C. After washing, the cells were incubated with TRITC-labeled phalloidin (1:40) (Molecular Probes) for 1 h at 37 °C. Finally, the sections were washed in PBS and placed under coverslips.

To confirm the binding of NKB on the cultured osteoclasts, NKB (Peptide Institute Inc., Osaka, Japan) was biotin-labeled using a Biotin Labeling Kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Bone marrow cells were cultured in 35-mm culture dishes on cover glasses for 1 week, as described above, and transferred into 1.5 mL of α-MEM with 10% FBS containing 10 μL of 250 μg/mL biotin-labeled NKB and cultured overnight. The cells were fixed in 4% paraformaldehyde for 10 min, washed three times with PBS, and incubated with Alexa Fluor 488 conjugated avidin (1:100; Molecular Probes) for 1 h at 37 °C. The samples were again washed in PBS, placed under coverslips, and observed under a fluorescence microscope (Olympus Optical, Tokyo, Japan).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Bone marrow cells were cultured for 1 week and then incubated with 0.01% Pronase/0.02% EDTA for 10 min at 37 °C to obtain an osteoclast-rich population (Chikazu et al., 2000). mRNA was then extracted using a Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA) according to the manufacturer's protocol. To reduce DNA contamination, samples were treated with RNase-free DNase I (Takara Bio, Shiga, Japan) for 3 h at 37 °C. cDNA was synthesized from 2 mg of total RNA in 30 μL of reaction buffer containing 500 mM dNTPs, 20 U of ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U of Superscript reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). The reaction was carried out for 7 min at 70 °C, 60 min at 45 °C, and 10 min at 70 °C, and then cooled to 4 °C.

The primers used to amplify NK-3R were 5′-GAGAGATCCCAGGAGACA-3′ and 5′-TGGGGTCAAACAGCACGG-3′ (GenBank J05189), giving a PCR product of 417 bp (Cintado et al., 2001). The primers used to amplify cathepsin K were 5′-CCCAGACTCCATCGACTATCG-3′ and 5′-CTGTAGCCTCTGCACTTAGCTGCC-3′ (GenBank BC078793), giving a PCR product of 297 bp (Corisdeo et al., 2001). Each cycle consisted of denaturation (94 °C for 1 min), annealing (NK-3R, 49 °C for 1 min; cathepsin K, 60 °C for 1 min), 72 °C for 43 cycles of 1-min steps for NK-3R, and 30 cycles of 1-min steps for cathepsin K, with a final extension step (72 °C for 2 min). To confirm the reproducibility of our results, each experiment was repeated at least three times. Each reaction mixture consisted of cDNA, Taq polymerase buffer, and 1 μL of each of the sense and antisense primers, in a total volume of 20 μL. The amplified products were electrophoresed in 2% agarose gels and visualized with ethidium bromide.

2.4. Immunohistochemistry of trigeminal ganglion (TG)

Rats were anesthetized with intramuscular injection of xylazine (13 mg/kg, Bayer, Tokyo, Japan). Rats were perfused transcardially with 4% paraformaldehyde in 0.1 M PBS containing 0.2% picric acid. TG were rapidly frozen then cut into sections 6 μm in thickness using a cryostat (Leica Instruments GmbH, Wetzlar, Germany). Double labeling for NKB and protein gene product 9.5 (PGP9.5) was carried out as follows. First, the sections were preincubated in 0.1 M PBS with 1% normal goat serum (ICN Pharmaceuticals, Aurora, OH, USA) for 30 min at room temperature. Sections were then incubated with rabbit monoclonal antibodies against NKB (1:200; Phoenix Pharmaceuticals Inc., Belmond, CA, USA) for 2 h at 37 °C. After rinsing with PBS, the sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:100; Molecular Probes) for 2 h at 37 °C. After rinsing with PBS, the sections were incubated with guinea pig polyclonal antibodies against PGP-9.5 (1:500; Neuromics, Edina, MN, USA) for 2 h at 37 °C, washed in PBS, and incubated with Alexa Fluor 568 goat anti-guinea pig IgG antibody (1:100; Molecular Probes) for 2 h at 37 °C. Finally, the sections were washed in PBS and covered with coverslips.

2.5. Immunohistochemistry of maxilla

The fixed maxillae were then decalcified through incubation for 2 weeks in 10% EDTA in 0.1 M PBS. After decalcification, the maxillae were frozen and cut into 10-μm sections using a cryostat (Leica). For immunostaining for NKB, the sections were pre-incubated for 5 min at room temperature in 0.1 M PBS with 0.3% H_2O_2, rinsed with PBS, and pre-incubated for 30 min at room temperature in 0.1 M PBS with 1% normal goat serum. Sections were incubated with rabbit polyclonal antibodies against NKB (1:200; Phoenix Pharmaceuticals) for 2 h at 37 °C. After rinsing with PBS, the sections were incubated with goat anti-rabbit IgG (1:1000; Molecular Probes) and then with avidin–biotin–peroxidase complex using a VECTASTAIN Elite ABC KIT (Vector Laboratories, Burlingame, CA, USA). The peroxidase activity was developed using 0.02% 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan) and 0.02% hydrogen peroxidase (Wako) solution. The sections were then observed under an optical microscope (Olympus Optical, Tokyo, Japan).