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Substance P stimulates late-stage rat osteoblastic bone formation through neurokinin-1 receptors

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Abstract

Substance P (SP) is a widely distributed neuropeptide that works as a neurotransmitter and neuromodulator. Recently, SP receptors, particularly neurokinin-1 receptors (NK_1-Rs) that have a high affinity for SP, have been observed not only in neuron and immune cells, but also in other peripheral cells, including bone cells. To identify the role of SP in bone formation, we investigated the expression of NK_1-Rs in osteoblastic cells and the effects of SP on bone formation by rat calvarial osteoblastic cells. Rat calvarial osteoblastic cells were isolated and cultured for 3 weeks in alpha-MEM containing 10% serum, ascorbic acid, dexamethasone, and beta-glycerophosphate. We then investigated NK_1-R expression, SP effects on osteoblastic bone formation, and osteocalcin mRNA expression in osteoblastic cells. RT-PCR and immunocytochemistry showed that NK_1-R mRNA was expressed and NK_1-R was present in 14-day, but not 7-day, cultured calvarial osteoblasts. Bone formation by cultured osteoblastic cells significantly increased after the addition of 10^{-8}–10^{-6} M SP. During 3 weeks of culture, the addition of SP in the first week did not significantly increase bone formation, whereas adding SP during the first and second week or all 3 weeks significantly increased calvarial osteoblastic bone formation. Furthermore, semi-quantitative RT-PCR indicated that SP stimulated osteocalcin mRNA expression in the osteoblasts at day 14 or day 21, whereas SP did not stimulated the runX2 or type I collagen mRNA expression at day 7 but stimulated them at day 14. These results indicate that SP stimulates bone formation by osteoblastic cells via NK_1-Rs at late-stage bone formation. These effects were dependent on the expression of NK_1-R in osteoblastic cells. Our findings suggest that SP secreted from sensory neurons may modulate bone formation after the expression of SP receptors.

Keywords: Bone formation; Neurokinin-1 receptor; Osteoblastic cell; Substance P

1. Introduction

The neuropeptide substance P (SP) is widely distributed, particularly in the central and peripheral nervous systems, where it functions as a neurotransmitter of nociceptive messages (Nicoll et al., 1980; Otsuka and Yoshioka, 1993). SP is basically localized in thin, unmyelinated afferent nerve fibers, transported to peripheral endings, and released by axon reflexes (Olgart et al., 1977). At the periphery, SP is co-localized with calcitonin gene-related peptide (CGRP). It is synthesized in the dorsal root ganglion as a result of nerve-ending stimulations. Although SP has been implicated in inflammatory responses, such as vasodilatation and plasma extravasation (Lembeck and Holzer, 1979), recent studies have revealed that SP also modulates bone metabolism, such as osteoblastic bone formation or osteoclastic bone resorption (Mori et al., 1999; Lerner, 2002; Azuma et al., 2004).

Bone exhibits abundant sensory neuron innervation, particularly in the bone marrow of the patella and epiphyses and in the periosteum (Bjurholm et al.,

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1988). Patients with familial dysautonomia suffer from a loss of unmyelinated axons with reduced bone mineral density and increased bone fragility (Maayan et al., 2002). Neuropeptides such as SP and CGRP are synthesized in unmyelinated sensory neurons and released from their periphery. Therefore, under this syndrome decreased local neuropeptide levels in bone adversely may affect bone integrity. Although some previous studies have also revealed that SP is associated with bone metabolism, especially bone resorption (Mori et al., 1999; Goto et al., 2001), the effects of SP on bone formation are still unclear.

For SP to affect the osteoblast directly, the SP receptor must be present on the osteoblast. However, the presence of SP receptors on the osteoblast is controversial. Our previous immunocytochemical study (Goto et al., 1998) indicated that neurokinin-1 receptors (NK_1-R: SP receptors) were distributed *in vivo* on osteoblasts. However, using reverse-transcription polymerase chain reaction (RT-PCR), Togari et al. (1997) were unable to detect SP receptors in human periosteum-derived osteoblastic cells and human osteosarcoma-derived cells (SaOS-2, HOS, MG-63). This discrepancy calls for an explanation.

Although the distribution of SP receptors on osteoblasts is uncertain, previous studies have demonstrated that SP affects osteoblastic bone formation. Shih and Bernard (1997) found that SP has a dose-related osteogenic-stimulating effect, and suggested that the increase in the number and size of bone colonies by SP was most likely caused by the stimulation of stem cell mitosis, osteoprogenitor cell differentiation, or osteoblastic activity. The stimulatory effect of SP on bone formation was supported by experiments using rat bone-marrow-derived cells (Adamus and Dabrowski, 2001). However, a more recent study demonstrated that SP inhibits osteoblastic cell differentiation in rat calvarial osteoblastic cells (Azuma et al., 2004). Thus, the effect of SP on osteoblastic bone formation is not fully understood.

The aim of this study was to investigate the effect of SP on bone formation. Therefore, we investigated: the expression of NK_1-R mRNA and the distribution of NK_1-R on primary cultures of rat osteoblastic cells or ROS osteoblastic cells; the dose- and time-dependent effects of SP on bone formation by primary osteoblastic cells; and the effects of SP on bone related proteins; runX2, type I collage (Col I), and osteocalcin (OCN), mRNA in primary osteoblastic cells.

2. Materials and methods

2.1. Cell isolation and primary culture of osteogenic cells

Rat osteogenic cells were isolated essentially as previously described (Bellows et al., 1986). Briefly, calvarias were dissected aseptically from 3-day-old Wistar rats. They were minced and digested in a collagenase-containing enzyme mixture at 37 °C for 10, 20, 30, and 50 min, yielding populations I through IV. Cells retrieved from each step of the digestion sequence were plated in 60 mm culture dishes in α-minimal essential medium (α-MEM) containing 10% heat-inactivated fetal bovine serum (FBS; Wako Pure Chemical Industries, Osaka, Japan), 0.3 μg/mL fungizone (Gibco, Grand Island, NY), plus antibiotics: 100 μg/mL penicillin G (Wako) and 50 μg/mL gentamicin (Gibco). After 2 days, the cultures were washed with phosphate-buffered saline (PBS) to remove nonviable cells and other debris, then incubated with 0.25% trypsin (Gibco), and counted using a hemocytometer. Cells from populations II through IV were pooled and resuspended in α-MEM containing 10% FBS and antibiotics and plated in 35 mm culture dishes or on coverslips (22 mm diameter; Matsunami Glass Ind., Osaka, Japan) in 35 mm culture dishes at 3×10^4 cells/dish. After 24 h, the culture medium was changed to the above medium, supplemented with 50 μg/mL ascorbic acid (Wako), 10 nM dexamethasone (Wako), and 10 mM β-glycerophosphate (Nacalai Tesque, Kyoto, Japan). These conditions are optimal for the formation of mineralized osteoid nodules. The culture medium was changed three times each week, and cells were cultured up to 21 days. Fresh SP or compounds were added when the culture medium was changed.

To examine the dose-dependent effects of SP, 10^{-12}–10^{-6} M SP was added and cultured for 21 days. To examine the effects of SP antagonists or SP receptor antagonists, cells were cultured with 10^{-7} M or 10^{-8} M SP, 10^{-7} M or 10^{-8} M SP + 10^{-6} M spantide (SP antagonist; Peptide Institute, Osaka, Japan), and 10^{-7} M or 10^{-8} M SP + 10^{-6} M FK888 (NK_1-R antagonist; Fujisawa Pharmaceutical, Osaka, Japan), as described by Fujii et al. (1992). To examine the time-dependent effects of SP on osteoblastic bone formation, 10^{-7} M SP was added during the first of the three weeks, the first two weeks of the three weeks, or all three weeks when the medium was changed. All experiments for bone formation assay were assessed after 3 weeks culture.

2.2. Bone formation analysis

To detect minerals in bone-like nodules, the samples were stained with von Kossa reagent. In summary, the specimens were washed three times in PBS and fixed with 3.7% formaldehyde in PBS for 10 min, washed in distilled water three times, and then incubated for 1 h in 0.5% silver nitrate (Wako). They were then washed three times in distilled water, incubated for 3 min in 0.3% sodium thiosulfate pentahydrate (Nacalai Tesque), washed in distilled water, and dried. The stained mineralized bone-like nodules