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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

were imaged using a CCD camera, and the images were transferred to a computer-assisted image analysis system (MCID, Image Research, Brock University, Ontario, Canada).

2.3. Immunocytochemistry

Cultured rat osteoblastic cells were fixed in 90% ethanol for 10 min, and then washed in PBS. The fixed cells were pre-incubated in 0.1 M PBS with 1% normal goat serum for 30 min at room temperature. Then, the cells were incubated with rabbit polyclonal antibodies against NK_1-R (1:1000; Molecular Probes, Eugene, OR) for 2 h at 37 °C, washed in PBS and incubated with goat anti-rabbit IgG conjugated to fluorescein (1:100; Molecular Probes) for 1.5 h at 37 °C. Finally, the sections were washed in 0.1 M PBS and placed under a coverslip.

The samples were examined under a fluorescence microscope (Olympus Optical, Tokyo, Japan) equipped with a CoolSNAP CCD camera (RS Photometrics, Tucson, AZ, USA) or a confocal laser scan microscope (Radiance 2100, Bio-Rad, Herts, UK).

2.4. RT-PCR analysis

To confirm the expression of NK_1-R, rat calvarial osteoblastic cells and ROS osteoblastic cells were cultured for 7 and 14 days without SP in medium containing 10% FCS, dexamethasone, ascorbic acid, and β-glycerophosphate. The RNA in the rat striatum was extracted as a positive control.

To examine the time-dependent effects of SP on the differentiation of osteobalstic clls, semi-quantitative RT-PCR for runX2, Col I, and OCN expression was performed on 7-, 14- and 21-day-cultured calvarial osteoblastic cells with 10^{-7} M SP, 10^{-7} M SP + 10^{-6} M SP antagonist (spantide), 10^{-7} M SP + 10^{-6} M FK888, and 10^{-8} M calcitonin-gene-related peptide (CGRP; Peptide Institute), which is another neuropeptide in sensory neurons.

Cellular mRNA was extracted using a Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA), according to the manufacturer's protocol. To reduce DNA contamination, the RNA samples were treated with RNase-free DNaseI (Takara Bio, Shiga, Japan) for 3 h at 37 °C; cDNA was synthesized from 2 μg total RNA in 30 μL of a reaction buffer composed of 500 μM dNTPs, 20 U ribonuclease inhibitor (Promega, Madison, WI), and 200 U Superscript II reverse transcriptase (Invitrogen Life Technology, Carlsbad, CA). The reaction was carried out at 70 °C for 7 min, then at 45 °C for 60 min, followed by 10 min at 70 °C, with final cooling to 4 °C. The primers used to amplify NK_1-R were 5′-AGG ACA GTG ACC AAT TAT TTC CTG G-3′ and 5′-CTG CTG GAT GAA CTT CTT-3′ (GeneBank J05097; 745–769 and 1386–1410). The primers used to amplify runX2 were 5′-GCT TCA TTC GCC TCA CAA ACA-3′ and 5′-TGC TGT CCT CCT GGA GAA AGT T-3′ (GeneBank AF325502; 626–646 and 672–693). The primers used to amplify Col I were 5′-GGC AAC AGT CGA TTC ACC-3′ and 5′-AGG GCC AAT GTC CAT TCC-3′ (GeneBank Z78279; 4171–4188 and 4330–4347). The primers used to amplify OCN were 5′- GTC CCA CAC AGC AAC TCG-3′ and 5′-CCA AAG CTG AAG CTG CCG-3′ (GeneBank M25490; 611–628 and 1464–1481). The primers for the GAPDH internal control were 3′-TGA AGG TCG GTG TCA ACG GAT TTG GC-3′ and 5′-CAT GTA GGC CAT GAG GCT CAC CAC-3′ (GeneBank M17701; 35–60 and 994–1017). Each cycle consisted of denaturation (NK_1-R, 94 °C for 30 s; runX2, 94 °C for 1 min; Col I, 94 °C for 1 min; OCN, 94 °C for 1 min; GAPDH, 94 °C for 1 min), annealing (NK_1-R, 55 °C for 30 s; runX2, 52 °C for 1 min; Col I, 60 °C for 1 min; OCN, 61 °C for 40 s; GAPDH, 65 °C for 1 min), and 72 °C for 25 cycles of 1 min steps for NK_1-R, 40 cycles for runX2, 20 cycles for Col I, 24 cycles for OCN, and 38 cycles for GAPDH, with a final extension step (72 °C for 9 min). To confirm its reproducibility, we replicated the RT-PCR experiment three or more times. Each PCR mixture consisted of cDNA (reverse-transcribed RNA), Taq polymerase buffer and 1 μL each of the sense and anti-sense primers, for a total volume of 20 μL. The PCR products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The data were analyzed using NIH Image.

The oligonucleotide RT-PCR primer sequences were designed and their specificity was confirmed using a BLAST-assisted internet search of non-redundant nucleotide sequence databases (National Library of Medicine, Bethesda, MD).

2.5. Statistical analysis

The data were analyzed using Stat View (Abacus Concepts, Berkeley, CA, USA). An analysis of variance (ANOVA), followed by Fisher's protected least-squares difference (PLSD) and the Bonferroni correction, were used to analyze the difference in bone formation between the control and each group. The values were expressed as mean ± standard deviation (SD).

3. Results

3.1. Expression of NK_1-R in osteoblastic cells

To examine the expression of NK_1-R in osteoblastic cells, RT-PCR was performed using NK_1-R specific

primers. NK_1-R expression was not detected in rat calvarial osteoblastic cells or ROS osteoblastic cells cultured for 7 days in medium containing ascorbic acid and dexamethasone β-glycerophosphate (Fig. 1a). In 14-day cultures, NK_1-R was expressed in calvarial osteoblastic cells, but not in ROS osteoblastic cells (Fig. 1b). To confirm our findings regarding NK_1-R expression in calvarial osteoblast cultures, we performed immunocytochemistry using the antibody against NK_1-R, which showed that the 7-day cultures of calvarial and ROS cells lacked immunopositive reactions for NK_1-R (Fig. 1c and d). However, the 14-day cultures showed many punctate immunopositive reactions for NK_1-R localized in calvarial cells (Fig. 1e).

3.2. Time- and dose-dependent effect of SP on osteoblastic bone formation

Rat calvarial osteoblastic cells isolated in medium containing 10% FCS, dexamethasone, ascorbic acid, and β-glycerophosphate formed numerous mineralized bone nodules by day 21 (Fig. 2a). These were recognized as dark brown spots using von Kossa staining under normal light. To examine the dose-dependent effect of SP on osteoblastic bone formation, 10^{-12}–10^{-6} M SP was added to the culture medium from week 1–3 and maintained for 21 days. The addition of $\geqslant 10^{-8}$ M SP significantly increased the average bone formation by calvarial osteoblastic cells (Fig. 2b).

To establish the time-dependent effects of SP on osteoblastic bone formation, we added 10^{-7} M SP to 3-week osteoblast cultures during the first of the three weeks (week 1), the first two weeks of the three weeks (week 1–2), or all three weeks (week 1–3) when the medium was changed. In week 1, we observed no significant

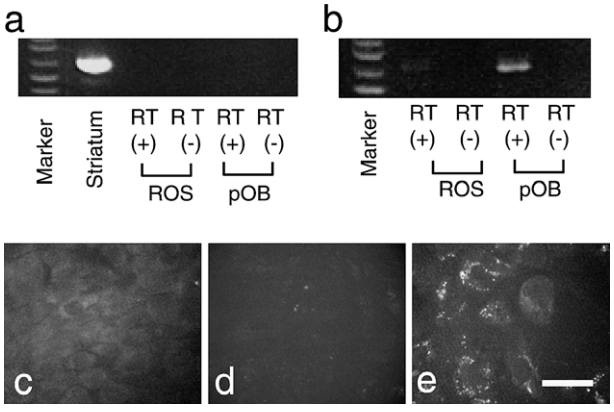

Fig. 1. Expression of NK_1-R in osteoblastic cells. (a) Expression of NK_1-R mRNA by ROS osteoblastic and primary osteoblastic (pOB) cells cultured for 7 days; and (b) ROS and pOB cells cultured for 14 days. The striatum is a positive control. RT(+) and RT(−): with and without reverse transcription, respectively. (c) Immunostaining for NK_1-R in pOB cells cultured for 7 days; and (d) ROS cells, (e) pOB cells cultured for 14 days. Bar = 10 μm.

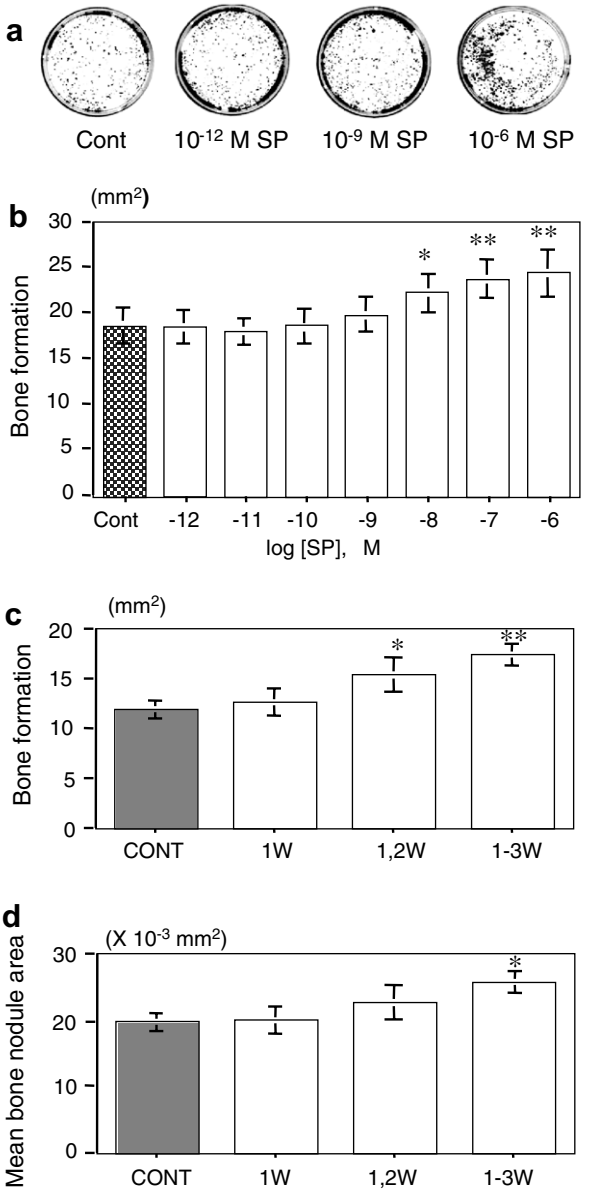

Fig. 2. Dose- and time-dependent effects of SP on bone formation by primary osteoblastic (pOB) cells. (a) Bone nodule formation by rat calvarial osteoblastic cells cultured for 21 days without SP (cont) or with 10^{-12} M, 10^{-9} M, and 10^{-6} M SP. (b) Calvarial osteoblastic cells cultured for 3 weeks with 10^{-12}–10^{-6} M SP. Bone formation increased significantly compared to the control ($^{*}p < 0.05$, $^{**}p < 0.01$). (c) and (d) Calvarial osteoblastic cells cultured for three weeks with or without SP (cont). 10^{-7} M SP was added during the first of the three weeks (1w), the first two weeks of the three weeks (1, 2w), or all three weeks (1–3w) when the medium was changed. After three weeks culture, the formation of bone nodule were assessed. (c) Bone formation increased significantly compared to the control ($^{*}p < 0.05$, $^{**}p < 0.01$). Data represent the mean total bone nodule area ± SD ($n = 10$). (d) Mean bone nodule area (total bone nodule area/number of bone nodules). The differences were statistically significant ($^{*}p < 0.05$). Data represent the mean bone nodule are ± SD ($n = 10$).

increase in bone formation by calvarial osteoblasts in the presence of 10^{-7} M SP compared to bone formation without SP. However, during weeks 1–2 and 1–3, the

addition of 10^{-7} M SP to the culture medium significantly increased bone formation (Fig. 2c). Furthermore, the mean area of bone nodule was significantly increased only when the cells were cultured with SP for 1–3 week (Fig. 2d).

3.3. Effects of SP and SP-receptor antagonists

To examine whether the effect of SP on bone formation was dependent on the interaction of SP with its receptor, we investigated the effects of an SP antagonist and SP-receptor (SP-R) antagonist on the SP-induced increase in bone formation of calvarial osteoblasts. Enhanced bone formation in the presence of 10^{-7} M SP was inhibited by the addition of 10^{-6} M spantide or 10^{-6} M FK888 (Fig. 3). The increased bone formation with 10^{-8} M SP was inhibited by the adding 10^{-6} M spantide.

3.4. Effects of SP on the expression of runX2, Col I, and OCN

The effects of SP on the early differentiation of calvarial osteoblastic cells were examined by measuring the expressions of runX2 and Col I in the cells cultured for 7 days (Fig. 4). The relative density of runX2 and Col I expression revealed that SP had no effects on the early stage of osteoblastic cell differentiation. However, the relative density of runX2 and Col I expression were increased at day 14. Using semi-quantitative RT-PCR analysis, OCN expression was not enhanced by the addition of 10^{-7} M SP at day 7. However, OCN expression was apparently increased in the presence of 10^{-7} M SP compared with control value at day 14 and 21. Adding 10^{-6} M spantide or 10^{-6} M FK888 inhibited the stimulated SP expression. We also found that OCN mRNA expression was not enhanced in cells cultured with 10^{-7} M CGRP.

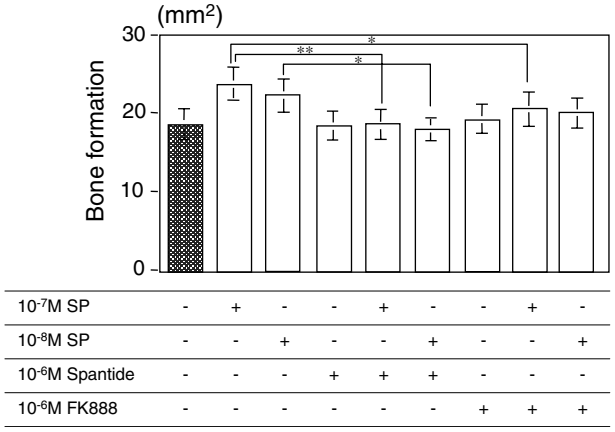

Fig. 3. Effects of 10^{-6} M SP antagonist (spantide) and 10^{-6} N SP-receptor antagonist (FK888; NK_1-R antagonist) on bone formation by calvarial osteoblastic cells stimulated with 10^{-7} M or 10^{-8} M SP. Data are mean ± SD ($n = 10$). The differences were statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$).

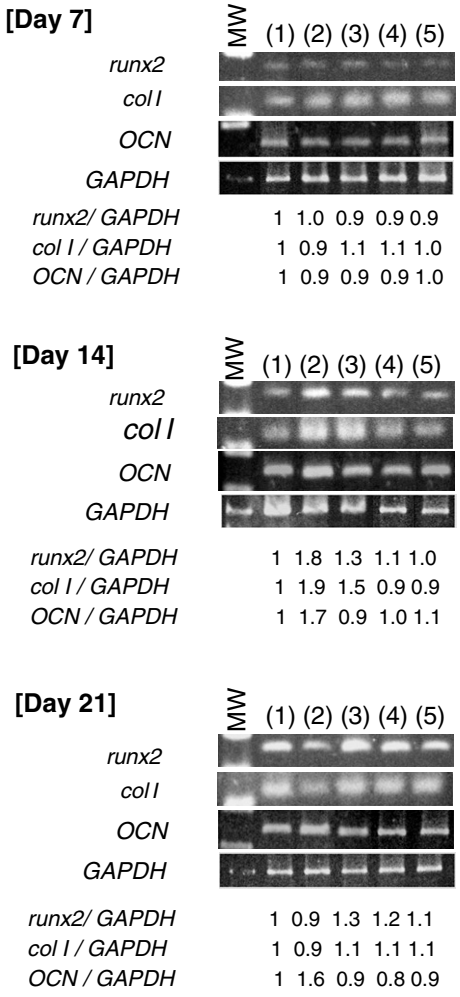

Fig. 4. Semi-quantitative RT-PCR for runX2, Col I, and OCN expression on 7-, 14- and 21-day-cultured calvarial osteoblastic cells (control; (1)) with 10^{-7} M SP (2), 10^{-7} M SP + 10^{-6} M SP antagonist (spantide) (3), 10^{-7} M SP + 10^{-6} M FK888 (4), and 10^{-8} M calcitonin-gene-related peptide (CGRP; Peptide Institute) (5). The total RNA was analyzed using RT-PCR with runX2-, Col I-, OCN- and GAPDH-specific primers. MW = molecular weight markers. The bottom lows indicate the relative density of runX2-, Col I-, or OCN expression versus control. NM = molecular marker.

4. Discussion

SP has been associated with both osteoblastic bone formation and osteoclastic bone resorption. Previous studies agree on the stimulatory effects of SP on osteoclastic bone resorption (Sherman and Chole, 1995; Mori et al., 1999; Goto et al., 2001). On the other hand, the effects of SP on osteoblastic bone formation are controversial, especially the effects of SP receptor expression on osteoblastic bone formation. We demon-

strated the stimulatory effects of SP-receptor expression on late-stage bone formation by calvarial osteoblastic cells.

The effects of SP-receptor expression on osteoblastic cells have been unclear. SP receptors in primary human osteoblasts were not detected by RT-PCR (Togari et al., 1997). Here, we demonstrated that NK_1-Rs (SP receptors) mRNA is expressed at the late stage of osteoblast differentiation, but not at the early stage. Interestingly, Lundberg et al. (2001) reported a similar differentiation-dependent expression of neuropeptide receptors. They reported that in mouse calvarial osteoblasts, the receptor of vasoactive intestinal peptide (VIP), a major neuropeptide of parasympathetic neurons, was expressed at 8–20 days, but not at 4 days, after cell seeding in the presence of β-glycerophosphate. They suggested that the expression of VIP-1 receptor is associated with osteoblast differentiation. Although we cannot exclude the species difference, we also demonstrated that the expression of rat calvarial osteoblastic SP receptors is most likely at late-stage cell differentiation.

The effects of SP on osteoblasts are also debated. Bjurholm (1991) reported that osteoblastic cell lines UMR106-1, SaOS-2, and MC3T3-E1 did not increase their intracellular cyclic AMP after the addition of SP. SP reduced the alkaline phosphatase activity of rat bone-marrow-derived cells (Adamus and Dabrowski, 2001) and inhibited bone nodule formation and alkaline phosphatase activities of rat calvarial cells (Azuma et al., 2004). On the other hand, SP was reported to stimulate osetogenesis in a dose-dependent manner (Shih and Bernard, 1997). Here, we demonstrated that SP stimulated bone nodule formation when added at late-stage cell differentiation in the presence of β-glycerophosphate. To explain the discrepancy, SP-receptor expression and SP dose should be taken into account. We could not detect the expression of SP receptors on the ROS osteoblastic cell line. Because SP-receptor expression has not been detected in osteoblastic human, rat, or mouse cell lines, it is reasonable to conclude that SP did not affect these cell lines. We found that SP stimulated bone nodule formation at $\geqslant 10^{-8}$ M concentrations and elevated bone formation only when added at late-stage cell differentiation. Such effects of SP on the late stage of osteoblastic cell differentiation were confirmed by the measurements of mean bone nodule area or the expression of runX2, Col I, or OCN. Regarding the physiological concentration of SP, the average concentration of SP in the synovial fluid of normal subjects and of rheumatoid arthritis patients was reported as 1.3×10^{-10} M and 3.0×10^{-10} M, respectively (Grimsholm et al., 2005). Thus, SP may stimulate bone formation when the local concentration of SP increases in a limited region and remains at a high concentration (possibly $\geqslant 10^{-8}$ M) for at least 1 week, or when the well-differentiated osteoblasts are present. By now it has not been found the disease that induce bone formation with high levels of SP. However, SP might involve in the unusual hyperplastic reaction of periosterium to chronic osteomyelitis such as Garre osteomyelitis. Though it is difficult to distinguish between the effects of inflammatory cytokines and the effects of sensory neuropeptides, sensory neuropeptide, such as SP, is one of the candidates that cause hyperplastic reaction of periosteum.

The neuropeptide CGRP is co-localized with SP in sensory neurons. A number of studies have demonstrated the effects of CGRP on osteoblasts. CGRP enhances cyclic AMP production by chicken, mouse, and rat osteoblastic cells (Michelangeli et al., 1989; Bjurholm et al., 1992). It is also a potent stimulator of interleukin-6 production in osteoblasts (Sakagami et al., 1993). Although these reports suggest that CGRP stimulates osteoblast activity, the stimulatory effect of CGRP on bone formation is still unclear. We found that 10^{-8} M SP stimulated OCN mRNA expression in calvarial osteoblastic cells cultured for 21 days, whereas 10^{-8} M CGRP did not. CGRP receptors were found in both primary osteoblastic cells and various cell lines, such as SaOS-2, MG-63, ROS, and MC3T3-E1 (Bjurholm, 1991; Togari et al., 1997; Kawase et al., 2005). Taken together, these findings may indicate that CGRP modulates the activity of immature osteoblasts, whereas SP stimulates osteoblastic activity only at late-stage bone formation.

In conclusion, we demonstrated that SP receptors are expressed in calvarial osteoblastic cells at the late stage of cell differentiation. SP stimulates calvarial osteoblastic-cell bone formation with dose- and time-dependent increases of OCN mRNA. These findings suggest that SP may be associated with the bone formation accompanied with chronic pain.

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