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Substance P stimulates bone marrow stromal cell osteogenic activity, osteoclast differentiation, and resorption activity in vitro

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Abstract

Introduction—SP is a neuropeptide distributed in the sensory nerve fibers that innervate the medullar tissues of bone, as well as the periosteum. Previously we demonstrated that inhibition of neuropeptide signaling after capsaicin treatment resulted in a loss of bone mass and we hypothesized that SP contributes to bone integrity by stimulating osteogenesis.

Materials and Methods—Osteoblast precursors (bone marrow stromal cells, BMSCs) and osteoclast precursors (bone marrow macrophages, BMMs) derived from C57BL/6 mice were cultured. Expression of the SP receptor (NK1) was detected by using immunocytochemical staining and PCR. Effects of SP on proliferation and differentiation of BMSCs were studied by measuring BrdU incorporation, gene expression, alkaline phosphatase activity, and osteocalcin and Runx2 protein levels with EIA and western blot assays, respectively. Effects of SP on BMMs were determined using a BrdU assay, counting multinucleated cells staining positive for tartrate-resistant acid phosphatase (TRAP⁺), measuring pit erosion area, and evaluating RANKL protein production and NF-κB activity with ELISA and western blot.

Results—The NK1 receptor was expressed in both BMSCs and BMMs. SP stimulated the proliferation of BMSCs in a concentration-dependent manner. Low concentrations (10^{-12} M) of SP stimulated alkaline phosphatase and osteocalcin expression, increased alkaline phosphatase activity, and up-regulated Runx2 protein levels, and higher concentrations of SP (10^{-8} M) enhanced

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mineralization in differentiated BMSCs. SP also stimulated BMSCs to produce RANKL, but at concentrations too low to evoke osteoclastogenesis in co-culture with macrophages in the presence of SP. SP also activated NF- κ B in BMMs and directly facilitate RANKL induced macrophage osteoclastogenesis and bone resorption activity.

Conclusions—NK1 receptors are expressed by osteoblast and osteoclast precursors and SP stimulates osteoblast and osteoclast differentiation and function *in vitro*. SP neurotransmitter release from sensory neurons could potentially regulate local bone turnover *in vivo*.

Keywords

Neuropeptide; Substance P; Bone Marrow Stromal Cell; Mouse; Osteogenesis; Osteoblast; Osteoclasts

Introduction

Bone is abundantly innervated by small diameter sensory nerves in the periosteum, bone marrow, and vascular canals.[1–3] In addition to conducting pain and information about thermal, mechanical, and chemical stimuli that have the potential to cause tissue damage, these skeletal sensory neurons produce a variety of peripherally released neurotransmitters including substance P (SP), calcitonin gene-related peptide (CGRP), and somatostatin. We have observed that capsaicin induced depletion of neuropeptides such as SP and CGRP in the unmyelinated sensory neurons of adult rats is accompanied by bone loss and increased bone fragility.[4] Furthermore, it was found that bone loss in the capsaicin treated animals was associated with a reduction in the bone formation rate and increased osteoclast numbers and osteoclast surface. Similarly, patients with familial dysautonomia, an autosomal recessive disease occurring mainly in Ashkenazi Jews, suffer from a loss of unmyelinated axons with a reduction in neuropeptide signaling, a reduction in BMD, and a concomitant increase in bone fragility.[5–7] These data support the hypothesis that neuropeptide signaling stimulates bone formation and inhibits bone resorption, and to further test this hypothesis we have selectively investigated the role of each of the 3 neuropeptides (SP, CGRP, somatostatin) that are depleted by systemic capsaicin treatment in adult rats.[4,8,9] In the current study we examined the effects of SP on bone cell proliferation, differentiation, and function.

Previous investigators have reported conflicting results regarding the presence of the SP NK1 receptors in bone and bone cells.[10–13] Some investigators have reported that SP treatment can stimulate bone marrow stromal cell (BMSC) proliferation, protein production, and mineralization,[14,15] Paradoxically, SP inhibitory effects have been observed on alkaline phosphatase activity, mineralization, and osteoblastic gene expression, and in addition, SP had no effect on cAMP production in osteoblastic cell lines and in mouse calvarial osteoblastic cells.[15–17] Recently Goto et al demonstrated that SP treatment of calvarial osteoblasts increased mineralization and the expression of the bone related proteins Runx2, type I collagen, and osteocalcin.[12]

Bone formation involves a progression through several progenitor and precursor stages to achieve development of mature osteoblasts.[18] Bone marrow is heavily innervated by unmyelinated neuropeptide rich sensory neurons and it is the most likely source of bone progenitor cells.[19] Osteogenic differentiation *in vitro* is marked by three distinct stages of cellular activities: proliferation, extracellular matrix maturation, and matrix mineralization. Being directed by essential transcriptional regulators such as Runx2 (also known as Cbfa1), BMSCs can differentiate towards the osteoblastic lineage, characterized by appearance of osteoblast cell morphology and accompanied by increased alkaline phosphatase activity and production of type I collagen and osteocalcin.[20] In the current study we examined mouse

BMSCs at all phases of cell differentiation to determine at which time points these cells express NK1 receptors and when SP regulated cell proliferation and osteoblast differentiation occurs.

Osteoclasts are the sole bone-resorbing cells and play a critical role in bone remodeling. NK1 immunoreactivity has been observed in osteoclasts in rat bone tissue,[10,11] and several studies have observed that SP treatment of bone marrow derived osteoclasts can increase the number of multinucleated tartrate-resistant acid phosphatase (TRAP⁺) stained osteoclasts and stimulate bone resorption activity in these cell cultures.[21,22] Nuclear factor kappa B (NF- κ B) is an essential transcription factor for osteoclastogenesis and osteoclast resorption activity and SP effects on osteoclast formation and function may be the result of NF- κ B activation. In the current study we examined the expression of NK1 receptors in murine bone marrow derived macrophages (BMMs), in osteoclasts derived from BMMs, and in murine macrophage-like RAW 264.7 cells. We also evaluated the effects of SP on NF- κ B activation, osteoclast formation, and bone resorption in BMM cell cultures.

Materials and Methods

BMSC osteoblastic cell culture

Bone marrow stromal cells from 6 week old male C57 BL/6 mice were harvested using established techniques.[23,24] Briefly, in each experiment six to eight mice were sacrificed by CO₂ inhalation and both femur and tibia were excised aseptically, cleaned of soft tissues and the ends of bones were removed. Bone marrow was flushed out with the growth medium containing Minimum Essential Medium Alpha (α MEM, Invitrogen, Carlsbad, CA) supplemented with 10% (V/V) fetal bovine serum (FBS, Tissue Culture Biologicals, Los Alamitos, CA), 0.25 μ g/mL Fungizone (Invitrogen), and 1% (V/V) penicillin and streptomycin (Invitrogen). The cell suspension was prepared by repeatedly aspirating the bone marrow cells through a 20 gauge needle. Cells were then seeded into 6 well plates, 5.6×10^6 cells/well (Day 0) and grown in the growth medium in a humidified atmosphere of 5% CO₂ at 37°C. After 48 hours the growth medium was replaced and on day 5 the growth medium was changed to an osteogenic medium consisting of α MEM supplemented with 10% fetal bovine serum, 50 μ g/mL ascorbic acid (Sigma, St. Louis, MO), 5mM β -glycerol phosphate (Sigma), 0.25 μ g/mL Fungizone, and 1% penicillin and streptomycin.

BMM and RAW 264.7 osteoclastic cell culture

Bone marrow macrophages (BMMs) were cultured as previously described.[25] Macrophage colony-stimulating factor (M-CSF) dependent macrophages were obtained by culturing the nonadherent bone marrow cells in the presence of 20ng/ml M-CSF (R&D Systems, Minneapolis, MN) in culture medium for overnight. Starting on day 1, cells were treated in an osteoclastogenic medium consisting of α MEM supplemented with 10% fetal bovine serum, 50 ng/mL RANKL (R&D Systems), 10 ng/mL M-CSF, and various concentrations of SP. Mouse BMMs were purified for the western blot assay by collecting the non-adherent cells from the fresh mouse bone marrow cell cultures which were then layered on a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden). Cells mainly consisting of macrophages and monocytes at the gradient interface were collected and cultured for 3–4 days at 6×10^6 cells per 60-mm plate in α -MEM supplemented with 10% FBS in the presence of 10ng/ml M-CSF.

The RAW 264.7 monocyte/macrophage mouse cell line was obtained from ATCC and was cultured in the growth medium containing α MEM, 10% FBS, and 1% (V/V) penicillin and streptomycin. For differentiation, RAW 264.7 cells were cultured in a growth medium supplied with 50 ng/ml mouse RANKL. The medium was changed every other day. At the end of the culture period, TRAP⁺ cells with 3 or more nuclei per cell in BMMs or RAW 264.7 cells were counted.

Substance P (Sigma) was dissolved in distilled water to a final concentration of 10^{-3} M, and then was aliquoted and stored at -20°C . SP was diluted to the appropriate concentration in the culture medium. Starting on day 1 the mouse cells were continuously stimulated with SP at concentrations of 10^{-8} , 10^{-10} , and 10^{-12} M, and the medium was changed every two days. The concentrations used in this study were based on previous reports demonstrating in vitro efficacy for these concentrations.[15,26–28]

Cell proliferation

The cell proliferation assay in BMSCs was performed as previously described.[29] Briefly, BrdU (3.1 $\mu\text{g/mL}$; Sigma) was added to BMSC cultures at 72h post seeding incubated for 4h. After washing with PBS, cultures were fixed in ice-cold 70% ethanol for 10 minutes and denatured in 4 M HCl for 20 min. After washing in PBS and blocking in 5% FBS plus 1% bovine serum albumin, cultures were incubated in mouse anti-BrdU (Santa Cruz Biotech, CA) for 1 h at 37°C and then incubated with FITC conjugated anti-mouse IgG antibody (Santa Cruz Biotech) for 1h at 37°C . Cell nuclei were counterstained with propidium iodide (Invitrogen). Under a magnification of 200x, proliferation was quantified as the percentage of cells positive for BrdU incorporation relative to the total number of nuclei.

Effect of SP on the proliferation of BMMs was also determined by measuring BrdU incorporation using a Cell Proliferation ELISA kit (Roche Applied Science, Indianapolis, IN). Briefly, the BMMs cells were plated onto 96 well plates at a seeding density of 1×10^5 cells/well and cultured as previously described. On day 4, BrdU was added to the culture medium 4 hour prior to the assay. The incorporated BrdU in each culture was quantified according to the manufacturer's instruction.

Immunofluorescence confocal microscopy

Mouse BMSCs and BMMs were cultured on cover slips. The BMSCs were separately stained on days 7, 14, and 21 post-seeding and the BMMs were stained on day 7 or 8 post-seeding. The cells were fixed for 20 minutes with 3.7% (V/V) formaldehyde at room temperature and then permeabilized with ice-cold ethanol for 5 min.[30] BMSCs were incubated with 5% FBS plus 1% bovine serum albumin at room temperature for 1h to reduce background staining, then treated with primary antibodies against mouse NK1 receptor (Santa Cruz Biotechnology) and mouse alkaline phosphatase (R&D Systems) for overnight at 4°C . Cells were then treated with the Texas Red- and FITC-conjugated secondary antibodies (Santa Cruz Biotechnology) in 1:200 dilution for 1h at room temperature. BMMs were stained with primary antibodies against mouse NK1 receptor and mouse CD14 (Santa Cruz Biotechnology). Control slides were stained with just the secondary antibody. Immunostained cells were visualized with a Zeiss LSM 510 META laser scanning confocal microscope and the confocal software was used for acquisition of the data and merging of the digital images.

Alkaline phosphatase activity

Cultured BMSCs were fixed with fresh 10% neutral buffered formalin on days 7, 14, and 21 before each well was assayed for alkaline phosphatase activity. Activity was determined using a commercially available kit (Sigma). The absorbance was measured with a spectrophotometer at 410 nm and the cell number was assayed using crystal violet staining as previously described. [23]

Mineralization

After the alkaline phosphatase activity assay was completed, day 21 BMSC cultures were stained with 2% Alizarin red (Sigma) for 10 minutes as previously described.[23] The Alizarin red bound to the calcium salts in the cell matrix was eluted with 10% cetylpyridinium chloride

(Sigma) for 1 to 2 hours before proceeding to assay cell number. The total absorbance of Alizarin red was measured with a spectrophotometer at 540 nm and normalized by the absorbance of crystal violet staining at 590 nm.[23]

Quantitative real time PCR

Total RNA from BMSCs, MC3T3-E1, BMMs, and RAW 264.7 cells grown in 6-well plates or 100 mm culture dishes was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and the purity and concentration were determined spectrophotometrically. The cDNA (20 μ L final volume) was subsequently synthesized from 1 μ g RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Real time PCR reactions were conducted using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The primer sequences used in these experiments are listed in Table 1. To validate the primer sets used in this study, the dissociation curves were performed to document single product formation and the agarose gel analysis was carried out to confirm the size. The data from real time PCR experiments were analyzed by the comparative CT method as described in the manual for the ABI prism 7900 real time system. All results were confirmed by repeating the experiment 3 times.

Osteocalcin and RANKL levels

Osteocalcin secreted by mouse BMSCs was measured from the conditioned cell culture medium by using a mouse osteocalcin ELISA kit (Biomedical Technologies, Stoughton, MA) according to the manufacturer's instruction. RANKL secreted by mouse BMSCs was measured from the conditioned culture medium and cell layer by using a R&D Systems EIA kit.

Western blot

Western blot analysis of Runx2 was performed as previously described.[31] Cultures were harvested and homogenized in 56.8 mol/L Tris buffer pH 6.8 (1.8% (V/V) β -mercaptoethanol, 9.1% glycerol) and the homogenate was centrifuged at 13,000 g for 15 min at 4°C. The supernatant was decanted and the pellet was used for western blot analysis. The results of this assay were confirmed by repeating the experiment 3 times.

For western blot analysis of NF- κ B p65, nuclear extract was prepared as previously described. [22] Briefly, BMMs were washed with ice-cold PBS, scraped and briefly centrifuged. The cell pellet was then resuspended in a hypotonic lysis buffer containing 10mM 4-(2-hydroxyethyl)-1-piperazinethansulfonic acid (HEPES), 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 μ g/ml leupeptin, and 6.4% Nonidet P-40 and incubated for 15 minutes on ice. After brief centrifugation, the nuclear pellet was collected and suspended in nuclear extraction buffer containing 20 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 5 μ g/ml pepstatin A and 5 μ g/ml leupeptin. After incubation on ice for 30 minutes, the nuclear extract was collected, boiled with 3 x sodium dodecyl sulfate (SDS) sample buffer, and then subjected to SDS electrophoresis. The results of this assay were confirmed by repeating the experiment 3 times.

The concentration of protein in the samples containing Runx2 or NF- κ B was measured by using a DC Protein Assay kit (Bio-Rad Laboratories). Equal amounts of protein were size fractionated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluorided membrane. The blot was blocked for overnight in 5% non-fat dry milk in Tris-buffered saline with 0.5% Tween-20 (TBST), and incubated with primary antibody to mouse Runx2 (1:1000, Santa Cruz Biotechnology) or mouse NF- κ B p65 (1:200, Santa Cruz Biotechnology) on a rocking platform at 4°C for 24h. After washing in TBST, the blots were incubated in horseradish peroxidase conjugated anti-rabbit or anti-mouse antibody (Santa Cruz Biotechnology) (diluted in 1:5000) for 1h at room temperature. The membrane was then washed again and exposed to film following chemiluminescence reagent treatment

with the ECL plus western blotting reagents (Amersham, Piscataway, NJ). Bands were quantified using densitometry of digitalized images. Each blot was then stripped and re-probed with anti- β actin antibodies, thus allowing normalization of expression between samples.

Tartrate-resistant acid phosphatase (TRAP) staining and pit resorption assay

To evaluate the effects of SP treatment on osteoclastogenesis, BMMs were plated in 48-well plates at a seeding density of 5×10^5 cells/well and cultured for 7 days in media containing 10 ng/mL M-CSF and 50 ng/mL RANKL. RAW 264.7 cells were seeded in 48 well plates at a density of 2×10^4 cells/well and cultured for 5 days. At the end of the culture, TRAP staining was performed using a Leukocyte Acid Phosphatase kit (Sigma) to identify osteoclasts as TRAP⁺ cells containing 3 or more nuclei. TRAP⁺ osteoclast numbers were counted using a Bioquant Image Analysis system (Nashville, TN).

To evaluate the effects of SP treatment on osteoclast resorption activity, BMMs were cultured on dentine discs (Immunodiagnostic System, Boldon, UK) at a seeding density of 1×10^5 cells/well for 10 days in media containing 10 ng/mL M-CSF and 50 ng/mL RANKL. All cells were then removed and erosion pits were visualized after 0.1% toluidine blue staining and the main pit area was measured as previously described.[32] The results of this assay were confirmed by repeating the experiment 3 times.

Statistical analysis

Statistical analysis was done by using the Prism 4.02 (GraphPad Software, San Diego, CA). All data was evaluated using an analysis of variance (ANOVA) followed by Bonferroni post hoc testing. Data are presented as the mean $\pm$ standard error of the mean (SEM) and a $p \leq 0.05$ was considered statistically significant.

Results

Expression of NK1 receptors in osteoblasts

Confocal microscopy in adherent BMSCs demonstrated an overlapping presence of NK1 receptors with alkaline phosphatase expression in the subcellular compartments (Fig. 1A–C). Immunofluorescence for both proteins was detected in the cytoplasm and on the plasma membrane. To further characterize NK1 receptor mRNA expression during osteogenic differentiation, RT-PCR was performed to quantify mRNA levels of NK1 receptors in adherent BMSCs (Fig. 1D). Mouse brain was used as a positive control for the primer and the MC3T3-E1 cell line was used to confirm the presence of NK1 receptors in osteoblastic cells (Fig. 1E). After normalization to 18S, no temporal differences were observed in NK1 receptor expression over time in the BMSC cultures (Fig. 1D). As measured by RT-PCR, NK1 receptor mRNA was expressed in BMSCs and MC3T3-E1 cells and in mouse brain, which was used as a positive control (Fig. 1E).

Effect of SP on osteoblast precursor proliferation

SP effects on cellular proliferation were assessed using the BrdU incorporation assay. The relative change of the percentage of cells positive for BrdU incorporation relative to total nuclei was calculated. SP significantly increased BrdU incorporation in BMSCs in a dose-dependent manner (Fig. 2A), with a 50% ($P < 0.05$) increase after treatment with the 10^{-8} M concentration.

Effect of SP on osteoblast differentiation

Real time PCR was used to characterize the well-defined phenotypic transitions that occur over the life cycle of an osteoblast from stromal cell to cell death (Fig. 3). Genes of interest included alkaline phosphatase, osteocalcin, collagen type I, and Runx2. The relative mRNA levels at

days 7, 14, and 21 were compared to day 3 mRNA expression. Alkaline phosphatase, collagen type I, and Runx2 mRNA levels started to increase on day 7 and peaked by day 14 or 21. Expression of alkaline phosphate increased 12.2 fold ($p < 0.05$), collagen type I increased 10.8 fold and Runx2 increased 3.2 fold, compared to day 3 mRNA levels. Osteocalcin mRNA levels began increasing on day 14 and reached a 2 fold increase by day 21 ($p < 0.05$, Fig. 3B).

Next, we evaluated the effects of SP on gene expression for markers of osteoblast differentiation (Fig. 4). After 7, 14, and 21 days of SP treatment, mRNA was isolated from BMSCs and expression levels were compared to mRNA levels in untreated BMSCs collected at the same time points of cell culture. The 10^{-12} M concentration of SP increased alkaline phosphatase and osteocalcin expression after 14 and 21 days, respectively (Fig. 4A and 4B). No significant SP treatment effects were observed on Runx2 or type I collagen gene expression in BMSCs (Fig. 4C and 4D).

SP treatment also stimulated BMSCs to express bone related proteins. Alkaline phosphatase activity was increased 142% after 14 days of SP treatment (10^{-12} M, vs day 14 untreated BMSCs, Fig. 4E). SP treatment had no effect on osteocalcin secretion in BMSC cultures (Fig. 4F), but did increase protein levels of Runx2 in BMSCs at day 21 (10^{-12} M, vs day 21 untreated BMSCs, Fig. 4G).

Finally, we examined the effect of SP on BMSC mineralization (Fig. 4H). Continuous SP treatment (10^{-8} M) over 21 days significantly stimulated the mineralization in BMSC cultures at day 21 post-seeding. There was no SP effect on the protein content of day 21 BMSC cultures as measured by crystal violet staining or on total protein (Fig. 2B, C). When SP treatment was limited to the first 7 days of cell culture there was no effect on mineralization at day 21 post-seeding (data not shown).

Expression of NK1 receptors in osteoclasts and their precursors

Expression of NK1 receptor protein was studied in the mouse bone marrow derived osteoclast cultures by confocal microscopy. Immunofluorescence for NK1 receptor was mainly detected in the cytoplasm and on the plasma membrane of TRAP⁺ BMMs and osteoclasts (Fig. 5A–C). Expression of NK1 mRNA was also studied in the mouse BMM cultures on days 4 and 7 post-seeding in the presence of M-CSF and RANKL (Figs. 5G, 5H). As measured by RT-PCR, NK1 mRNA expression was observed in BMMs, RAW 264.7 cells, and in mouse brain, which was used as a positive control. NK1 mRNA levels in BMMs were 30% lower at day 7 post-seeding compared to day 4 levels.

Effect of SP on osteoclast precursor proliferation

SP (10^{-8} M) treatment had no effect on BrdU incorporation in osteoclast progenitor cells with or without the addition of M-CSF and RANKL to the cell culture media (Fig. 6A).

Effect of SP on osteoclast differentiation and function

SP concentration-dependently increased the number of TRAP⁺ multinucleated cells in the BMM culture, but only in the presence of M-CSF and RANKL. When SP was added to BMMs lacking M-CSF and RANKL in the media no TRAP⁺ multinucleated cells were observed. The peak osteoclastogenic effect in BMM cultures was observed with 10^{-8} M SP, resulting in a 23-fold increase in TRAP⁺ multinucleated cells (Fig. 6C). This concentration of SP also increased bone resorption activity in BMM cultures by 15-fold (Fig. 6B). In addition, we found SP increased osteoclast formation in the RAW 264.7 cell culture (Fig. 6D). The peak osteoclastogenic effect in the RAW 264.7 cell culture was observed with 10^{-8} to 10^{-10} M SP, resulting in a 2.9 and 3-fold increase in TRAP⁺ multinucleated cells, respectively. When SP

was added to RAW 264.7 cells not exposed to RANKL we failed to observe any TRAP⁺ multinucleated cells in the culture.

SP activation of NF- κ B in BMMs

As expected, the receptor activator of NF- κ B (RANKL) induced the nuclear translocation of NF- κ B p65 in BMMs in a time-dependent manner. Similarly, we also observed that SP (10^{-8} M) induced NF- κ B p65 nuclear translocation in BMMs, and this activation was not enhanced by the addition of RANKL (Fig. 7B).

SP increases RANKL protein expression in osteoblasts

We further examined whether SP stimulated osteoclastogenesis through regulating RANKL production in osteoblasts. BMSCs were cultured for 7 days and then treated with 10^{-8} M SP for 12 hours, then RANKL protein was assayed by EIA. SP significantly increased RANKL protein levels in the cell layers of BMSCs, but not in the conditioned medium (Fig. 7C). When BMSCs were co-cultured with RAW 264.7 cells no TRAP⁺ multinucleated cells were observed with or without the addition of SP (10^{-8} M). These results provide further evidence that SP has direct osteoclastogenic effects on RAW 264.7 cells not dependent on the induction of RANKL in BMSCs or fibroblasts.

Discussion

This study is unique in that it comprehensively investigated the concentration-dependent effects of SP signaling on primary osteoblast and osteoclast progenitor cells throughout the period of cell differentiation, looking specifically at cellular proliferation, differentiation, function, and intracellular and transmembrane signaling. SP receptor (NK1) expression was also observed at various time points during cell differentiation in both primary osteoblast and osteoclast cell cultures and in cell lines.

With immunofluorescence techniques we detected robust colocalized immunoreactivity for the NK1 receptor and alkaline phosphatase on the plasma membrane and in the cytoplasm of BMSCs (Fig. 1). These results support previous immunohistochemical observations demonstrating weak NK1 receptor immunoreactivity on the plasma membrane and in the cytoplasm of osteoblasts in bone sections and in calvarial osteoblastic cells.[10,12,33] In addition, using real time PCR we observed stable levels of NK1 receptor mRNA in BMSCs over the cell life cycle and in murine osteoblast-like MC3T3-E1 cells (Fig. 1). Previous studies had failed to observe NK1 mRNA expression in rat ROS osteoblastic cells and human periosteum-derived and osteosarcoma-derived (SaOS-2, HOS, MG-63) osteoblastic cells.[12,13] One study did observe low NK1 mRNA levels in rat calvarial osteoblastic cells after 14 days cell culture, but not at 7 days, leading the authors to postulate that SP could only affect osteogenesis at the later stages of cell differentiation.[12]

The life cycle of an osteoblast from stromal cell to cell death is approximately 28 days, during which time the cell undergoes a well-defined series of phenotypic transitions.[34] Stromal cells in this study exhibited early expression of collagen I and alkaline phosphatase and late expression of osteocalcin (Fig. 3), data indicating that mouse BMSCs develop into mature osteoblasts under the conditions used in this study. The presence of NK1 receptors in mouse BMSCs throughout the cell life cycle indicates that SP could directly modulate bone metabolism via receptor activation at any time point in osteoprogenitor differentiation and maturation. Several groups have studied SP actions on BMSC or primary osteoblastic cell proliferation, differentiation, or function, but the results of these studies have been contradictory and none have examined the effects of various concentrations of SP throughout the osteoprogenitor life cycle. Adamus et al observed that SP (10^{-10} M) stimulated proliferation

of rat first passage BMSCs, while higher concentrations of SP (10^{-9} and 10^{-8} M) stimulated production of cell protein.[15] Paradoxically, alkaline phosphatase activity decreased with SP treatment in Adamus's study. Another recent study found that higher concentrations of SP (10^{-7} to 10^{-5} M) dose-dependently inhibited alkaline phosphatase activity and bone nodule formation, as well as gene expression for osteocalcin in fetal rat calvarial osteoblasts.[16] Shih et al used similar concentrations of SP (ranging from 10^{-8} M to 10^{-6} M) to stimulate rat BMSCs and found that SP increased, rather than decreased, the number and size of bone colonies in a concentration-dependent manner.[14] Similarly, Goto et al found that higher concentrations of SP (ranging from 10^{-8} M to 10^{-6} M) increased the size of bone colonies in rat calvarial osteoblastic cells.[12]

Similar to the SP proliferative effects observed by Adamus et al in rat BMSC culture,[15] we observed in the current study that higher concentration (10^{-8} M) SP stimulated mouse BMSC proliferation (as measured by BrdU incorporation) during the first 72 h post-seeding, but SP treatment for 21 days had no effect in the later stages of BMSC cellular proliferation as measured by crystal violet staining or protein assay at 21 days post-seeding (Fig. 2). Among the osteoblastic genes screened for in this study, alkaline phosphatase and osteocalcin were most sensitive to SP stimulation and alkaline phosphatase activity and Runx2 protein were also up-regulated by SP treatment (Fig. 4). SP (10^{-8} M) treatment for 21 days also increased BMSC mineralization, as measured by the Alizarin red assay (Fig. 4H). SP treatment limited to the first 7 days of cell culture did not affect mineralization at day 21 post-seeding, consistent with Goto et al's findings in rat calvarial osteoblastic cells.[12] These investigators also looked at the effects of SP stimulation on Runx2, collagen 1, and osteocalcin gene expression in calvarial osteoblastic cells and observed that SP had no effect on osteoblastic gene expression at day 7, but up-regulated Runx2 and collagen 1 expression at day 14, similar to our results showing no SP effects on osteoblastic gene or protein expression at day 7 (Fig 4). Collectively, these results suggest that SP stimulates osteogenesis in the later stages of osteoprogenitor differentiation and maturation. Previously we observed that neuropeptide depletion in rats caused trabecular bone loss and inhibited bone formation,[4] and hereditary small fiber sensory neuropathy in man is associated with neuropeptide loss, reduced BMD and increased bone fragility.[5–7] These diverse data support the hypothesis that SP signaling contributes to the maintenance of bone mass by regulating osteogenic differentiation in BMSCs.

Using real time PCR we detected NK1 receptor mRNA in BMMs and in murine macrophage-like RAW 264.7 cells and observed NK1 receptor immunoreactivity in BMMs, as well as in the TRAP⁺ osteoclasts formed by RANKL induced differentiation (Fig. 5). Although SP had no effect on cellular proliferation of osteoclast progenitors, it dramatically stimulated primary osteoclast differentiation and resorption activity in BMMs (Fig. 6A–C). SP also increased the number of TRAP⁺ multinucleated cells in RAW 264.7 cell cultures in the presence of RANKL (Fig. 6D), evidence of a direct osteoclastogenic effect on macrophages. When SP was added to BMM cultures without the addition of M-CSF and RANKL, or to RAW 264.7 cells without the addition of RANKL, no TRAP⁺ osteoclasts formed. Thus, while SP alone does not induce osteoclastogenesis, SP does directly enhance RANKL-induced osteoclastogenesis.

Several investigators have observed that SP can induce fibroblasts to express RANKL.[35, 36] RANKL, a member of the TNF family of cytokines, is expressed on the plasma membrane of osteoblasts. Membrane-bound RANKL is essential in maintaining skeletal homeostasis through regulating differentiation and activation of osteoclasts by activating downstream signaling pathways such as NF- κ B. SP stimulated fibroblasts, when co-cultured with blood monocytes, can induce osteoclastogenesis, and when media from SP stimulated fibroblasts is added to osteoclast cultures it promotes bone resorption. We observed that SP stimulated RANKL production in BMSCs (Fig 7C), but the SP stimulated RANKL protein levels were extremely low, approximately 18 pg/ml of media, which is several orders of magnitude below

the minimal concentration of soluble RANKL (4 ng/ml) required to induce osteoclastic differentiation.[37,38] Furthermore, we failed to observe any TRAP⁺ multinucleated cells in a co-culture of SP stimulated BMSCs and RAW 264.7 cells (data not shown), indicating that SP stimulated BMSCs did not express enough RANKL to induce osteoclastogenesis in monocyte/macrophage cultures, even in the presence of SP. These data provide further support for the hypothesis that SP stimulatory effects on osteoclastogenesis and resorption activity are not due to an indirect effect (eg, by promoting RANKL expression in osteoblasts or fibroblasts), but rather a direct effect on the osteoclast precursors that express the SP receptor (Fig. 5).

Substance P added to the culture media rapidly induced NF- κ B nuclear translocation in BMMs, similar to the effects of RANKL, and this SP stimulatory effect was not enhanced by the addition of 50 ng/ml of RANKL (Fig. 7). These results corroborate those of Sun et al, who observed NF- κ B nuclear translocation in RAW 264.7 cells 15 min after adding SP (10^{-8} M) to the cell culture, indicating a direct effect on macrophage NF- κ B activation.[49] We also observed that although SP up-regulated osteoclastogenesis in BMMs and RAW 264.7 cells, and increased bone resorption in BMMs, SP alone was not sufficient to initiate osteoclastic differentiation or function without the presence of RANKL. Substance P appears to potentiate RANKL-induced osteoclastogenesis and bone resorption in the same way as TNF- α . [39] The addition of RANKL to osteoclast cell cultures evokes the release of Ca^{2+} from intracellular stores, resulting in a transient increase in cytosolic free Ca^{2+} that accelerates nuclear translocation of NF- κ B.[40,41] Similarly, SP activation of NF- κ B requires transient increases of intracellular Ca^{2+} in endothelial and astrocytoma cells,[42,43] and SP treatment increases cytosolic Ca^{2+} levels in rabbit osteoclasts due to an influx of extracellular Ca^{2+} . [21] Intracellular Ca^{2+} mobilization may be a common signaling pathway for RANKL and SP activation of NF- κ B in macrophages and osteoclasts. The results of our study confirm and extend other reports of SP activation of NF- κ B in murine peritoneal and bone marrow macrophages and human alveolar macrophages.[22,44]

In conclusion, the SP receptor NK1 exists in bone cell precursors, including BMSCs and BMMs, and on osteoblasts and osteoclasts. SP stimulates BMSC proliferation and mineralization at higher concentrations, while lower SP concentrations increase BMSC osteogenesis at later stages of osteoblastic differentiation, data suggesting that SP osteogenic effects are not the consequence of enhanced cellular proliferation, but rather due to facilitated differentiation of post mitotic precursors. Higher concentrations of SP also had stimulatory effects on BMM NF- κ B activation, osteoclastogenesis and bone resorption. The low concentrations of SP required for its osteogenic effects in vitro are close to the SP concentrations observed in human serum (10^{-11} M), and synovial fluid (10^{-10} M), [45–47] and in rat trabecular bone (10^{-9} M), [4] data suggesting that SP could be a physiologic activator of bone formation in vivo. Higher concentrations of SP are observed in the synovial fluid of rheumatoid and osteoarthritis arthritis patients, [46–48] and enhanced SP signaling has the potential to induce osteoclastogenesis and bone resorption in the arthritic joint. Future studies examining the role of SP signaling in animal models of bone acquisition and loss are required to further investigate these hypotheses.

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Fig. 1.

Laser scanning confocal microscopy of mouse bone marrow stromal cells (BMSCs). Alkaline phosphatase and the substance P (SP) NK1 receptors were co-localized in the cytoplasm and on the plasma membrane of BMSCs. **(A)** The punctuated presence of green fluorescence indicates the presence of alkaline phosphatase. **(B)** The homogeneous presence of red fluorescence indicates the presence of NK1 receptors. **(C)** The presence of yellow fluorescence indicates the presence of the two proteins overlapping in the cellular microcompartment. **(D)** RT-PCR analysis of NK1 receptor gene (TACR-1) expression in mouse BMSCs demonstrates stable levels over the cell life cycle. The mRNA gel band of TACR-1 and 18S at respective time points were quantified using densitometry, separately. **(E)** NK1 mRNA is present in BMSCs, murine osteoblast-like MC3T3-E1 cells, and mouse brain.

Fig. 2.

Effects of SP treatment (10^{-8} M, 10^{-10} M, 10^{-12} M) on BMSC cellular proliferation. (A) The highest concentration of SP (10^{-8} M) did increase BrdU incorporation in mouse BMSCs at 72 h post-seeding. Relative changes were calculated by the percentage of cells positive for BrdU incorporation relative to total nuclei positive for propidium iodide. SP treatment for 21 days had no effect in the later stages of BMSC cellular proliferation as measured by (B) crystal violet staining or (C) soluble cellular protein at 21 days post-seeding. Values are means $\pm$ SEM for six wells (* $p < 0.05$ vs. control).

Fig. 3.

This figure illustrates the time course of BMSC differentiation *in vitro*. BMSC osteoblastic gene expression was evaluated on days 3, 7, 14, and 21 post-seeding, as measured by real time PCR for (A) alkaline phosphatase, (B) osteocalcin, (C) collagen type I, and (D) Runx2 mRNA levels. Gene expression increased for all osteoblastic markers over time. Values are means $\pm$ SEM for six independent cultures (** p < 0.01; *** p < 0.001 vs. day 3 mRNA levels).

Fig. 4.

Effects of 3, 7, 14, and 21 days of SP treatment (10^{-8} M, 10^{-10} M, 10^{-12} M) on mouse BMSC gene expression measured by real time PCR for (A) alkaline phosphatase, (B) osteocalcin, (C) Runx2, and (D) collagen type I mRNA levels (* $p < 0.05$ vs. the day 3 mRNA level for the same SP concentration). Effects of SP on osteoblast-specific protein expression in mouse BMSCs were also measured on days 7, 14, and 21 post-seeding. (E) Alkaline phosphatase activity was determined by staining with a solution consisting of equal parts *p*-nitrophenol phosphate (Sigma) and alkaline buffer solution (Sigma) and then was normalized by cell number determined by crystal violet staining. Values are means $\pm$ SEM for six wells (* $p < 0.05$ vs. control). Levels of (F) osteocalcin in culture medium and (G) Runx2 produced by BMSCs were determined by ELIA and western blot, respectively. (H) Effect of 21 days of SP treatment on mineralization in BMSCs was determined by Alizarin red staining and normalized to crystal violet staining. Values are means $\pm$ SEM for six wells (** $p < 0.01$ vs. control).

Fig. 5.

Laser scanning confocal microscopy of bone marrow macrophages (BMMs) and osteoclasts derived from M-CSF and RANKL stimulated mouse non-adherent bone marrow cells. **(A)** Green immunofluorescence indicates the presence of tartrate-resistant acid phosphatase (TRAP⁺), a marker of osteoclasts and osteoclast precursors. **(B)** Red indicates the presence of NK1 receptors. **(C)** Yellow indicates the presence of the two proteins in the same cellular microcompartment. **(D)** Green immunofluorescence indicates the presence of CD 14, a marker of monocyte/macrophages. **(E)** Red indicates the presence of NK1 receptors. **(F)** The presence of yellow fluorescence indicates the presence of the two proteins in the same cellular microcompartment. **(G)** RT-PCR measurements of NK1 receptor mRNA levels in mouse

BMMs demonstrate a slight decline in NK1 expression at day 7. The mRNA gel band densities of TACR-1 and 18S at respective time points were quantified using densitometry, separately. **(H)** NK1 receptor mRNA is present in BMMs, RAW 264.7 cells, and mouse brain. Values are means $\pm$ SEM for six wells (***) $p < 0.001$ vs. day 4).

Fig. 6. Effects of SP treatment on BMM (A) BrdU incorporation, (B) pit erosion area, and (C) multinucleated TRAP⁺ cell numbers in BMMs. (D) Effect of SP treatment on multinucleated TRAP⁺ cell numbers in RAW 264.7 cell culture. SP (10^{-8} M) treatment had no effect on BrdU incorporation, but dramatically increased the number of TRAP⁺ cells and the pit erosion area, as compared to controls. (E) Photomicrographs illustrating the effects of SP (10^{-8} M) on TRAP⁺ cell numbers and dentine disc erosion area in BMMs (** $p < 0.01$; *** $p < 0.001$ vs. control).

Fig. 7. Effects of SP on NF-κB nuclear translocation in BMMs and RANKL production in BMSCs. (A) RANKL (100 ng/ml) induced NF-κB nuclear translocation in BMMs in a time dependent manner. (B) SP (10⁻⁸ M) also induced NF-κB nuclear translocation in BMMs in RANKL free media and there was no additive effect with RANKL. (C) SP concentration-dependently stimulated RANKL protein levels in the cell layer of BMSC cultures (* p < 0.05 vs. control).

Table 1

Primer sequences for the neurokinin 1 receptor (TACR-1), osteocalcin (OCN), alkaline phosphatase (ALP), Collagen type 1 (COL1), Runt related transcription factor 2 (Runx2), and 18S rRNA gene (18S). Sources represent the GenBank accession number.

Gene	Source	Sequence	Predicted length (bp)
TACR -1	NM_009313	Forward primer 5'-GAAGGCTATCCTCGGTTTCC-3' Reverse primer 5'-TGTGGCTGAGAATGACTTCG-3'	156
OCN	NM_031368	Forward primer 5'-TTGGTGCACACCTAGCAGAC-3' Reverse primer 5'-ACCTTATTGCCCTCCTGCTT-3'	151
ALP	NM_007431	Forward primer 5'-AACCCAGACACAAGCATTCC-3' Reverse primer 5'-GCCTTTGAGGTTTTTGGTCA-3'	200
COL1	NM_007742	Forward primer 5'-GAGCGGAGAGTACTGGATCG-3' Reverse primer 5'-GCTTCTTTTCCTTGGGGTTC-3'	158
RUNX2	NM_009820	Forward primer 5'-GCCGGAATGATGAGAACTA-3' Reverse primer 5'-GGACCGTCCACTGTCACCTT-3'	200
18S	M35283	Forward primer 5'-AGGAATTGACGGAAGGGCAC-3' Reverse primer 5'-GTGCAGCCCCGGACATCTAAG-3'	323