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Calcitonin gene-related peptide stimulates stromal cell osteogenic differentiation and inhibits RANKL induced NF- κ B activation, osteoclastogenesis and bone resorption

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Abstract

Previously we observed that capsaicin treatment in rats inhibited sensory neuropeptide signaling, with a concurrent reduction in trabecular bone formation and bone volume, and an increase in osteoclast numbers and bone resorption. Calcitonin gene-related peptide (CGRP) is a neuropeptide richly distributed in sensory neurons innervating the skeleton and we postulated that CGRP signaling regulates bone integrity. In this study we examined CGRP effects on stromal and bone cell differentiation and activity *in vitro*. CGRP receptors were detected by immunocytochemical staining and real time PCR assays in mouse bone marrow stromal cells (BMSCs) and bone marrow macrophages (BMMs). CGRP effects on BMSC proliferation and osteoblastic differentiation were studied using BrdU incorporation, PCR products, alkaline phosphatase (ALP) activity, and mineralization assays. CGRP effects on BMM osteoclastic differentiation and activity were determined by quantifying tartrate-resistant acid phosphatase positive (TRAP⁺) multinucleated cells,

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pit erosion area, mRNA levels of TRAP and cathepsin K, and nuclear factor- κ B (NF- κ B) nuclear localization. BMSCs, osteoblasts, BMMs, and osteoclasts all expressed CGRP receptors. CGRP (10^{-10} - 10^{-8} M) stimulated BMSC proliferation, up-regulated the expression of osteoblastic genes, and increased ALP activity and mineralization in the BMSCs. In BMM cultures CGRP (10^{-8} M) inhibited receptor activator of NF- κ B ligand (RANKL) activation of NF- κ B. CGRP also down-regulated osteoclastic genes like TRAP and cathepsin K, decreased the numbers of TRAP⁺ cells, and inhibited bone resorption activity in RANKL stimulated BMMs. These results suggest that CGRP signaling maintains bone mass both by directly stimulating stromal cell osteoblastic differentiation and by inhibiting RANKL induced NF- κ B activation, osteoclastogenesis, and bone resorption.

Keywords

Neuropeptide; Calcitonin gene-related protein; Bone marrow stromal cell; Osteoblast; Osteoclasts; Mineralization

Introduction

Skeleton is a living and continuously remodeling tissue, which exhibits abundant sensory neuron innervation.(1,2) 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.(3) 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. (4-6) 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, and somatostatin) that are depleted by systemic capsaicin treatment in adult rats. (3,7,8) In the current study we examined the effects of CGRP on bone cells proliferation, differentiation, and function.

There is compelling evidence that CGRP signaling has anabolic effects in bone. Firstly, in bone tissue there are numerous nerve fibers immunostaining for CGRP in the periosteum, bone marrow, and in the epiphyseal trabecular bone.(9) Secondly, the CGRP receptor complex is expressed in osteoblasts. The CGRP receptor is a dimer complex of two molecules, the G-protein coupled calcitonin receptor-like receptor (CRL) and a receptor activity-modifying protein (RAMP1), both of which are required for physiologic activation by CGRP. CRL and RAMP1 receptors are expressed in mature osteoblasts.(10-14) Thirdly, the administration of CGRP has stimulatory effects in osteoblasts. CGRP increases intracellular levels of cAMP and calcium,(1) and insulin-like growth factor expression in osteoblast cultures.(15) Finally, transgenic mice over-expressing CGRP in their osteoblasts have increased bone formation activity and trabecular bone mass,(16) while α CGRP deficient mice display a decreased bone formation rate and accelerated bone loss with aging.(11,17) While numerous investigators have identified the presence of CGRP receptors and observed CGRP stimulatory effects in osteoblasts, there have been no previous studies examining CGRP receptors or CGRP osteogenic effects in BMSCs.

Osteoclasts are derived from mononuclear precursors in the myeloid lineage of bone marrow hematopoietic cells. M-CSF is required for these progenitor cells to differentiate into BMMs, but RANKL is critical for their further differentiation, fusion into multinucleated osteoclasts, activation and survival.(18) 1,25-dihydroxyvitamin D₃ can also stimulate osteoclastogenesis in cultured bone marrow cells, possibly due to an increase in RANKL expression in stromal cells.(19) Previous investigators have detected mRNAs and protein (by Western blot) for the CGRP receptors CRL and RAMP1 in mouse nonadherent bone marrow cell cultures stimulated with M-CSF, but were unable to observe CGRP receptors in bone cell cultures using immunocytochemical techniques.(20) Another group observed that isolated osteoclasts from vitamin D₃ stimulated mouse bone marrow cultures expressed CRL, but not RAMP1 mRNA. (21) In the current study we examined the time course of CGRP receptor expression in M-CSF and RANKL stimulated mouse nonadherent bone marrow cell cultures and in RAW 264.7 cells.

Calcitonin has been widely studied as a therapy for postmenopausal osteoporosis because of its inhibitory effects on bone resorption.(22,23) Structurally homologous to calcitonin,(24) CGRP has been demonstrated to inhibit vitamin D₃ stimulated osteoclastogenesis(25-28) and bone resorption.(26,29,30) Bone resorbing osteoclasts are formed by the fusion of mononuclear progenitors of the monocyte/macrophage system. Two molecules produced by bone marrow stromal cells are required for osteoclast formation and activity; macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL). (18) NF- κ B activation in osteoclast progenitors induces osteoclastic differentiation, activity, and survival.(31) Furthermore, CGRP inhibits PMA and ionomycin activation of NF- κ B in thymocytes,(32) suggesting that CGRP inhibitory effects on osteoclast formation and activity may be attributable to inhibitory effects on RANKL activation of NF- κ B in osteoclast precursors.

The objectives of the current study were to; 1) determine when the CGRP receptor dimer complex is expressed in BMSC culture, 2) determine whether CGRP stimulates cell proliferation and osteogenic differentiation in BMSCs, 3) identify the stages of BMSC osteoblastic differentiation at which CGRP can effectively stimulate osteoblastic gene activity, and 4) determine whether CGRP can inhibit RANKL induced NF- κ B activation, osteoclastogenesis, and bone resorption in BMMs.

Materials and methods

BMSC and MC3T3-E1 osteoblastic cell culture

Bone marrow stromal cells from male C57 BL/6 mice were harvested using established techniques.(33) Briefly, for each experiment using BMSCs, 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 a growth medium containing Minimum Essential Medium Alpha (α -MEM, Invitrogen) supplemented with 10% (V/V) fetal bovine serum (FBS, HyClone Laboratories Inc.), 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 the 20-gauge needle. Cells were then seeded into 6 well plates, 5.6×10^6 cells /well (Day 0) and grown in culture medium. Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C. On day 5 the culture medium for BMSCs was changed to an osteogenic medium consisting of α -MEM supplemented with 10% FBS, 50 μ g/mL ascorbic acid (Sigma), 5mM β -glycerol phosphate (Sigma), 0.25 μ g/mL Fungizone, and 1% penicillin and streptomycin.

MC3T3-E1 cells were obtained from American Type Culture Collection (ATCC) and maintained in growth medium, containing α MEM, 10% FBS, and 1% penicillin and

streptomycin. The MC3T3-E1 cells were seeded in the 24 well plates and cultured in growth media supplemented with 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate to promote differentiation.

BMM and osteoclastic cell culture

Bone marrow macrophages (BMMs) were cultured as previously described.(34) 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) in growth medium overnight. Starting on day 1, cells were treated in an osteoclastogenic medium consisting of αMEM supplemented with 10% fetal bovine serum, 50 ng/mL RANKL (R&D Systems), and 10 ng/mL M-CSF.

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). 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 CGRP used in these studies was αCGRP (Sigma), which differs from βCGRP by just one amino acid. The αCGRP and βCGRP peptides have equipotent affinity for the CGRP receptor (calcitonin receptor-like receptor, CLR) and equipotent pharmacologic activity. The CGRP was dissolved in distilled water to a final concentration of 10^{-4} M, and then was aliquoted and stored at - 20°C. Immediately before using, the CGRP was diluted to the appropriate concentration in the culture medium. Starting on day 1 the mouse BMSCs or BMMs were continuously stimulated with CGRP at concentrations of 10^{-14} , 10^{-12} , 10^{-10} , and 10^{-8} M, and the medium was changed every two days.

Immunofluorescence confocal microscopy

Mouse BMSCs, MC3T3-E1 cells, and BMMs were cultured on cover slips. The BMSCs were separately stained on days 7, 14, and 21 post-seeding, the MC3T3-E1, BMMs, and RAW 264.7 cells were stained on day 7 post-seeding. The cells were fixed for 20 minutes with 3.7% (V/V) paraformaldehyde at room temperature and then permeabilized with ice-cold ethanol for 5 min.(35) BMSCs and MC3T3-E1 cells 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 CGRP receptor, CRL (Santa Cruz Biotechnology) and mouse alkaline phosphatase (R&D Systems) or mouse RAMP1 (Santa Cruz Biotechnology) overnight at 4°C. Cells were then treated with the PE- or CY3- 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 CRL and mouse RAMP1 (Santa Cruz Biotechnology) or mouse TRAP (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.

Cell proliferation

The effect of CGRP on the proliferation of adherent bone marrow stromal cells was determined by measuring BrdU incorporation using a Roche Cell Proliferation ELISA kit (Roche Diagnostics Corp). Briefly, the adherent bone marrow stromal cells were plated into 96 well plates at a seeding density of 1×10^5 cells / well and cultured on the condition described previously. On day 4, BrdU was added to the culture medium 4 hour prior to the assay. The incorporated BrdU in each culture was quantified by the Molecular Device microplate system according to the manufacturer's instruction.

Alkaline phosphatase activity

Increased alkaline phosphatase activity is an early marker of osteoblastic differentiation. Cell layer lysates of BMSCs and MC3T3-E1 cells were collected in 0.1% Triton-X and alkaline phosphatase activity was measured as a function of release of p-nitrophenol from p-nitrophenylphosphate at pH 10.2.(36) Protein content of the cell layer was determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co). The results of this assay were confirmed by repeating the experiment 3 times.

Mineralization

CGRP was added to the BMSC culture medium over a 21-day period. At the end of incubation, the medium was removed and the culture was briefly washed with phosphate-buffered saline (PBS) followed by fixation in 4% paraformaldehyde for 20 min. Calcium deposits in the BMSC cultures were stained with 2% Alizarin red (Sigma) for 10 minutes as previously described. (37) The Alizarin red bound to the calcium salts in the cell matrix was eluted with 10% cetylpyridinium chloride (Sigma), and the staining intensity was quantified by measuring absorbance at 540 nm and calculated according to a standard curve. One molar Alizarin red selectively binds about two moles of calcium.(38) Then the relative cell numbers in the same culture were estimated by crystal violet staining.(37) The values of mineralization were normalized by the absorbance of crystal violet staining at 590 nm in this study.

Quantitative real time PCR

Total RNA from the BMSCs and BMMs grown in 6-well plates was extracted using the RNeasy Mini Kit (Qiagen) 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 Lab). Real time PCR reactions were conducted using the SYBR Green PCR master mix (Applied Biosystems). 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.

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

BMMs were plated in 48-well plates at a seeding density of 5×10^5 cells / well and cultured for 7 or 8 days in media containing 10 ng/mL M-CSF and 50 ng/mL RANKL. At the end of the culture period TRAP staining was performed using a leukocyte acid phosphatase kit (Sigma) to identify TRAP⁺ osteoclasts containing 3 or more nuclei. TRAP⁺ osteoclast numbers were counted using a Bioquant Image Analysis system.

To evaluate the effects of CGRP treatment on osteoclast resorption activity, BMMs were cultured on BD BioCoat Osteologic Discs Multi Test Slides (BD Biosciences) at a seeding density of 1×10^5 cells / well for 10 days. All cells were then removed with bleach and erosion area was visualized and measured by using the Bioquant Image Analysis system (Nashville, TN).

Western blot

To study the effects of SP on NF- κ B activation in BMMs, western blot analysis was used to measure expression of the dominant NF- κ B subunit, the p65 protein, in nuclear extracts prepared from BMMs as previously described.(39) 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

MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 µg/ml leupeptin, and 6.4% Nonidet P-40 and incubated for 15 minutes on ice. After another 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 µg/ml pepstatin A and 5 µg/ml leupeptin. After incubation on ice for 30 minutes, the nuclear extract was collected, boiled with 3 × sodium dodecyl sulfate (SDS) sample buffer, and then subjected to SDS electrophoresis.

The concentration of protein in the samples containing NF-κB p65 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 difluoride 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 mouse anti-NF-κB p65 antibody (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-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). 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. The results of this assay were confirmed by repeating the experiment 3 times.

Statistical analysis

Statistical analysis was performed using Prism 4.02 (GraphPad Software). All data was evaluated using an analysis of variance (ANOVA) followed by Bonferroni post hoc testing. Data are presented as the mean ± standard error of the mean (SEM) and a $p \leq 0.05$ was considered statistically significant. Each experiment was repeated at least two to three times in this study to ensure the validity of the data. The data presented are from a single experiment.

Results

Expression of CGRP receptors in osteoblasts

Confocal microscopy demonstrated co-expression of the CGRP receptor CRL and the osteoblast marker alkaline phosphatase in the cytoplasm and plasma membrane of adherent BMSCs, as shown in Figs. 1A, B, and C. All adherent BMSCs grown in osteogenic medium expressed both alkaline phosphatase and CRL after 14 days of cell culture. Co-labeling for both CRL and its chaperone protein RAMP1 was observed in BMSCs at 7, 14, and 21 days of cell culture (Figs. 1D - F). Osteoblastic MC3T3-E1 cells also co-expressed CRL and RAMP1 receptors (Figs. 1G - I). To further characterize CGRP receptor mRNA expression during osteogenic differentiation, real time PCR was performed to quantify mRNA levels of CRL and RAMP1 in BMSCs at various time points of cell culture (Figs. 1J, K). After normalization to 18S, there was a 57% decline in RAMP1 mRNA expression in BMSCs over time, from day 3 to day 21 post-seeding. In contrast, no temporal changes were observed in CRL expression in BMSCs. CRL and RAMP1 mRNAs were also expressed at high levels (relative to 18S) in the osteoblastic MC3T3-E1 cells and in brain tissue homogenate.

Effect of CGRP on BMSC proliferation

CGRP effects on osteoprogenitor cellular proliferation were assessed using the BrdU incorporation assay. Three different concentrations of CGRP (10^{-12} , 10^{-10} , and 10^{-8} M) were tested in BMSCs at day 4 post-seeding, but only the 10^{-10} M concentration significantly

increased BrdU incorporation by 36% ($p < 0.05$), compared to vehicle-treated control cultures (data not shown).

Effect of CGRP on osteoblast differentiation

This study was designed to examine the concentration-dependent effects of CGRP (10^{-12} - 10^{-8} M) on mouse primary BMSC osteogenic differentiation through the three distinct stages of cellular activities; proliferation, extracellular matrix maturation, and matrix mineralization. (40) Osteoblastic gene expression was evaluated by real time PCR and total mRNA was isolated from BMSCs on days 7, 14, and 21 of the culture period. Genes of interest included alkaline phosphatase, osteocalcin, collagen type I, and Runx2 (Fig. 2). Alkaline phosphatase and collagen type I were selected as early markers and osteocalcin was selected as a late marker of osteoblastic differentiation. Runx2, a transcriptional factor necessary for osteoblast differentiation, was also examined. The addition of CGRP (10^{-10} - 10^{-8} M) to the cell media significantly increased BMSC gene expression of alkaline phosphatase, collagen type I, osteocalcin, and Runx2 in BMSCs at 7 and 14 days of cell culture, but not day 21 (Fig. 2). CGRP (10^{-12} , 10^{-8} M) had no effect on osteocalcin, collagen type I, and Runx2 gene expression in osteoblastic MC3T3-E1 cell cultures at 4 or 7 days post-seeding, but CGRP (10^{-12} and 10^{-8} M) did increase alkaline phosphatase expression in MC3T3-E1 cells on day 4, but not day 7 of cell culture (data not shown).

Next, we evaluated the effect of chronic CGRP treatment on BMSC alkaline phosphatase activity. The effect of CGRP treatment on BMSC alkaline phosphatase activity was examined on days 7, 14, and 21 of the culture period (Fig. 3A). CGRP increased the alkaline phosphatase activity in BMSCs in a time and concentration-dependent manner. During the first 7 days, the alkaline phosphatase activity was not altered by CGRP treatment (data not shown). Compared to untreated time-matched controls, CGRP at the 10^{-10} M concentration increased alkaline phosphatase activity by 7.4 fold at day 14 and by a lesser extent at day 21. Similarly, the 10^{-12} M concentration of CGRP significantly stimulated alkaline phosphatase activity in MC3T3-E1 cells at day 4 and to a lesser extent at day 7 post-seeding (data not shown).

Finally, we examined the effect of CGRP on BMSC mineralization and cellular proliferation (Fig. 3). Continuous CGRP treatment (10^{-10} M to 10^{-8} M) over 21 days stimulated mineralization in BMSC cultures at day 21 post-seeding, as measured by Alizarin red staining (Fig. 3B). Continuous CGRP treatment (10^{-12} M to 10^{-8} M) modestly decreased BMSC cell numbers, as estimated by crystal violet staining, over a 21 day cultural period (range 14-21%, Fig. 3C). When mineralization was normalized to relative cell numbers (Alizarin red/crystal violet) the CGRP stimulatory effects on mineralization persisted (Fig. 3D).

Expression of CGRP receptors in osteoclasts and their progenitors

The expression of CGRP receptors was studied in the mouse bone marrow derived osteoclast cultures by confocal microscopy. Immunofluorescence for CRL was mainly detected in the cytoplasm and on the plasma membrane of TRAP⁺ BMMs and osteoclasts (Fig. 4A - C). The CRL immunostaining was more intense in BMMs and preosteoclasts than in mature osteoclasts. Furthermore, CRL and RAMP1 immunofluorescence was co-localized in BMMs and osteoclasts on day 7 of culture period (Fig 4.D - F). CRL and RAMP1 immunostaining was also observed in the macrophage RAW 264.7 cell line (data not shown).

Real time PCR was performed to quantify mRNA levels for RAMP1 and CRL in BMMs and osteoclasts on day 4 and day 7 of the culture period (Fig. 4G, H). After normalization to 18S, there was a 46% decline in RAMP1 mRNA expression by day 7 and a 38% increase in CRL expression.

Effect of CGRP on osteoclast formation and function

CGRP effects on osteoclastogenesis from non-adherent bone marrow cells were evaluated by counting the number of multinucleated TRAP⁺ cells at day 7 post-seeding (Fig. 5A). In addition, CGRP effects on osteoclast resorption activity were examined in non-adherent bone marrow cells grown on BD Biocoat osteologic discs for 10 days, after which the erosion area was quantified (Fig. 5B). All cell cultures were treated with 10 ng/ml M-CSF and 50 ng/ml of RANKL. Without the addition of RANKL no osteoclastogenesis or resorption activity was observed. Only the 10⁻⁸M concentration of CGRP decreased osteoclastogenesis, reducing the TRAP⁺ cell number by about 50%. Osteoclasts derived from non-adherent bone marrow cells formed resorption pits after 10 days of culture and CGRP treatment decreased the erosion area in a concentration-dependent manner. Compared to untreated cells, the 10⁻¹⁰M concentration of CGRP reduced erosion area by 64% and the 10⁻⁸M concentration of CGRP reduced erosion by 80%.

Effect of CGRP on osteoclast differentiation

Real time PCR was used to quantify the effects of CGRP on gene expression for TRAP and cathepsin K in non-adherent bone marrow cell cultures at day 7 post-seeding (Figs. 6A and 6B). All cell cultures were treated with 10 ng/ml M-CSF and 50 ng/ml of RANKL. CGRP (10⁻⁸M) treatment decreased TRAP mRNA levels by 33% and reduced cathepsin K mRNA levels by 23%.

CGRP activation of NF-κB in BMMs

CGRP (10⁻⁸M) treatment alone had no effect on nuclear NF-κB-p65 levels in BMMs, but adding the receptor activator of NF-κB (RANKL) to the BMM culture media caused a 3-fold increase in NF-κB activation within 30 minutes. When the BMMs were treated with both RANKL and CGRP there was no increase in NF-κB activation (Fig. 6C). These results indicate that CGRP inhibits RANKL induced NF-κB activation in osteoclast progenitors, at critical step in osteoclastogenesis and resorption activity.

Discussion

CGRP acts at the cellular level by binding to its seven trans-membrane domain G protein-coupled receptor.(41) A functional CGRP receptor requires heterodimerization of CRL with a single-transmembrane domain protein, the receptor activity-modifying protein-1 (RAMP1), which allows the receptor complex to reach the cell surface.(42,43) CRL and RAMP1 mRNAs have been detected in the mouse MC3T3-E1 osteoblastic cell line, the human MG63 osteoblastic cell line, rat primary calvarial osteoblasts, and in human primary osteoblast cell cultures.(10,11,13,14) CRL and RAMP1 proteins have been identified in the MC3T3-E1 and MG63 cell lines using the Western blot assay, and immunocytochemical studies in MG63 cells demonstrate co-labeling of plasma membrane sites with anti-CRL and anti-RAMP1 antibodies. (10,14) The current study now extends these findings to osteoblast precursor cells, demonstrating for the first time the co-localization of CRL and RAMP1 proteins on the plasma membrane of adherent BMSCs and in MC3T3-E1 cells (Fig. 1). In addition, BMSCs and MC3T3-E1 cells expressed mRNA for CRL and RAMP1 receptors.(Fig. 1 J, K) Expression of RAMP1 mRNA in BMSCs peaked at day 3 post-seeding and then gradually declined during osteoblastic differentiation, while CRL mRNA levels remained stable between days 3 to day 21 post-seeding.

Numerous studies have looked at CGRP effects on osteoblastic cell lines and in primary rat or human osteoblast cell cultures.(13-15,44-49) CGRP has been shown to bind to the cell membrane and to increase intracellular cyclic AMP (cAMP) and calcium in osteoblastic cells. Furthermore, the addition of CGRP to osteoblast cell culture media also stimulates cell

proliferation, synthesis of cytokines and growth factors (including insulin-like growth factor I), and collagen synthesis.

The osteogenic effects of CGRP on bone marrow stromal cells, the primary source of osteoprogenitor cells, have not been previously described. Osteoblastic differentiation *in vitro* is marked by three distinct stages of cellular activities: proliferation, extracellular matrix maturation, and matrix mineralization. Being directed by Runx2, the master transcription factor regulating bone formation, BMSCs can differentiate towards the osteoblastic lineage, accompanied by increased alkaline phosphatase activity and production of type I collagen and osteocalcin.(50) The current study examined mouse BMSCs throughout the cell life cycle to determine at which time points CGRP regulated cell proliferation and osteoblast differentiation occurs.

When BMSCs were treated with CGRP (10^{-10} - 10^{-8} M) for 7 or 14 days there was an increase in osteoblastic gene expression, including Runx2, alkaline phosphatase, osteocalcin, and collagen type I (Fig. 2). CGRP stimulatory effects on BMSC osteoblastic differentiation were only observed during the first 2 weeks of cell culture, prior to the onset of mineralization. (51) In addition, CGRP (10^{-10} M) stimulated alkaline phosphatase activity in BMSCs at day 14 and to a lesser extent day 21 post-seeding, and in MC3T3-E1 cell cultures at day 4 and to a lesser extent day 7 of cell culture (Fig. 3). CGRP (10^{-8} M) treatment for 21 days also increased BMSC mineralization, as measured by the Alizarin red assay (Fig. 3). We also observed that CGRP (10^{-10} M) treatment stimulated BMSC proliferation (data not shown). However, compared to the impact on osteoblast gene expression, the effects of CGRP on osteoblast progenitor proliferation are rather moderate. Our result is line with the findings that α CGRP knockout mice have decreased bone formation, but normal osteoblast numbers.(17) Collectively, these novel data are the first to demonstrate CGRP's stimulatory effects on osteoblastic differentiation in bone marrow osteoprogenitors. CGRP's stimulatory effects were restricted to the earlier stages of osteoblast development, suggesting that its osteogenic effects are primarily due to its ability to direct stromal progenitors into the osteoblastic lineage.

Previous studies have presented conflicting data regarding the presence of CGRP receptors in BMMs.(20,21) In the current study both CRL and RAMP1 mRNAs were detectable in nonadherent mouse bone marrow cells at 4 days post-seeding (Fig. 4G, H) and in the RAW 264.7 macrophage cell line (data not shown). In addition, immunocytochemistry was used to identify CGRP receptor protein at the cellular level. TRAP⁺ monocytes and multinucleated osteoclasts derived from mouse bone marrow cells, and macrophages from the RAW 264.7 cell line all expressed both CRL and RAMP1 proteins (Fig 4 A-F, RAW 264.7 data not shown).

CGRP significantly inhibits vitamin D₃ induced osteoclastogenesis in macrophages.(25-27) In addition, Ishizuka et al observed that CGRP (10^{-7} M) inhibited BMM osteoclastogenesis only in the presence of RANKL.(28) Furthermore, the addition of CGRP (10^{-11} to 10^{-7} M) to enriched osteoclast cultures inhibits bone resorption activity.(26,29,30) In the current study we have confirmed and extended these observations, showing that CGRP (10^{-10} to 10^{-8} M) inhibited; 1) the formation of TRAP⁺ multinucleated cells, 2) the erosion area on coated BD BioCoat osteologic discs, and 3) the expression of TRAP and cathepsin K mRNA in M-CSF and RANKL stimulated bone marrow cell cultures (Figs. 5, 6).

Osteoclast precursor differentiation and activation by RANKL requires the sequential activation of NF- κ B, c-Fos, and NFATc1.(18,31) Millet et al observed that CGRP (10^{-8} M) selectively interfered with the nuclear accumulation of NF- κ B in thymocytes by increasing the levels of I κ B proteins, which normally retain NF- κ B in the cytoplasm, thus preventing nuclear translocation.(32) Now we have observed that CGRP (10^{-8} M) blocked RANKL induced activation of NF- κ B in BMMs, but the addition of CGRP without RANKL had no effect on

the basal activation of NF- κ B in BMMs (Fig. 6). Collectively, these data suggest that CGRP inhibition of osteoclastogenesis is due to its inhibitory effects on RANKL induced activation of NF- κ B in BMMs.

We have observed that selective sensory nerve ablation in rats can induce a chronic increase in trabecular bone osteoclast surface per bone surface (Oc.S/BS) and osteoclast number (N.Oc), with a concurrent reduction in bone volume, suggesting that sensory signaling has an inhibitory effect on bone resorption.(3) Furthermore, daily injections of the sensory transmitter CGRP can prevent bone loss in ovariectomized rats primarily by reducing bone resorption activity, additional evidence that sensory neuropeptides can have inhibitory effects on osteoclast function.(52) Collectively these data suggest that sensory neuropeptide signaling can inhibit osteoclast formation and function *in vivo*. Unexpectedly, α CGRP deficient mice have normal bone resorption parameters. There is no change in osteoclast number per bone surface (N.Oc./BS), osteoclast surface per bone surface (Oc.S/BS), or in urinary deoxypyridinoline levels. (11) These data suggest that either CGRP has no effect on bone resorption or perhaps the loss of just the α CGRP signal is not enough to alter osteoclast formation and resorption activity. In sensory nerves there are actually two different calcitonin gene-related peptides, termed α CGRP and β CGRP, belonging to the calcitonin family of peptides. The α CGRP peptide is generated by alternative splicing of the calcitonin gene *Calca*, while β CGRP is derived from a separate gene, termed *Calcb*, that is located in close proximity to *Calca*, both in mice and humans. The sequence homology between α CGRP and β CGRP is over 90% and they both activate the same CGRP receptor complex. Conceivably, in α CGRP deficient mice the release of β CGRP from sensory terminals could compensate for the loss of the α CGRP signal, thus maintaining CGRP inhibitory effects on osteoclastogenesis and bone resorption.

This study is unique in that it comprehensively investigated the concentration-dependent effects of CGRP 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. CGRP receptor expression was also observed at various time points during cell differentiation in both primary osteoblast and osteoclast cell cultures and in cell lines. CGRP directed the differentiation of BMSCs towards mature osteoblasts and inhibited RANKL induced activation of NF- κ B, osteoclastogenesis, and bone resorption in BMMs. These *in vitro* effects suggest that the relative concentration of CGRP in bone could be an important determinant of bone mass and strength, a hypothesis supported by the osteopenic skeletal phenotype observed in α CGRP deficient adult mice. (11,17) Although it is impossible to generate and characterize the skeletal phenotypes of the mice lacking the CRL component of the CGRP receptor, due to embryonic lethality,(53) this lethality is due to the loss of adrenomedullin signaling that is mediated by the CRL and RAMP 2 or RAMP 3 receptor dimer complex. Future characterization of the skeletal phenotypes of mice lacking expression of both α CGRP and β CGRP, or in mice lacking the RAMP1 component of the CGRP receptor dimer complex, will undoubtedly further our understanding of neuro-osseal regulation of bone metabolism and integrity.

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Fig. 1.

Laser scanning confocal microscopy demonstrated the presence of the calcitonin gene-related protein (CGRP) receptors in adherent mouse bone marrow stromal cells (BMSCs). The CGRP receptor is a dimer complex of two molecules, the calcitonin receptor-like receptor (CRL) and a receptor activity-modifying protein (RAMP1), both of which are required for physiologic activation by CGRP. Alkaline phosphatase and the CRL receptor co-localized in the cytoplasm and cell membrane of BMSCs on day 14 of cell culture. The presence of green fluorescence indicates the presence of alkaline phosphatase (A), the presence of red fluorescence indicates the presence of CRL (B), and the presence of yellow fluorescence indicates co-labeling for the two proteins in the same cellular microcompartment (C). RAMP1 and CRL co-localized in the

BMSC derived osteoblasts on day 14 (**D**, **E**, and **F**) and in MC3T3-E1 osteoblast-like cells (**G**, **H**, and **I**) on day 7 of cell culture. RAMP 1 immunofluorescence (green) was observed in the cytoplasm, cell membrane, and nucleus (**D**, **G**) and CRL immunostaining (red) was localized in the cytoplasm and on the plasma membrane (**E**, **H**). The presence of yellow fluorescence indicates co-labeling for the two proteins in the same cellular microcompartment (**F**, **I**). Real time PCR analysis of RAMP1 (**J**) and CLR (**K**) mRNA levels are shown for mouse BMSCs, brain and MC3T3-E1 cells. RAMP1 mRNA levels in BMSC cultures gradually dropped from day 3 to day 21 post-seeding, while CRL expression remained stable over time. Values are means $\pm$ SEM for six wells and CRL and RAMP1 mRNA levels were normalized to 18S mRNA levels.

Fig. 2.

Effects of 7, 14, and 21 days of CGRP treatment (10^{-10} M and 10^{-8} M) on mouse BMSC gene expression measured by real time PCR for (A) alkaline phosphatase, (B) collagen type I, (C) osteocalcin, and (D) Runx2 mRNA levels. CGRP stimulated osteoblastic gene expression in the earlier stages of osteoblastic cell differentiation. Values are means $\pm$ SEM for six wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Fig. 3.

Panel A illustrates the effects of 14 and 21 days of CGRP (10^{-14} M, 10^{-12} M, 10^{-10} M, 10^{-8} M) treatment on alkaline phosphatase activity in mouse adherent BMSCs. Cell lysates were used for analyzing alkaline phosphatase activity and activity was normalized to the amount of cell protein. The 10^{-10} M concentration of CGRP significantly stimulated alkaline phosphatase activity in BMSCs at both time points. CGRP (10^{-12} - 10^{-8} M) added to the BMSC culture medium for 21 days increased mineralization in BMSCs as determined by Alizarin red staining (B) and reduced cell proliferation, as estimated by crystal violet staining (C). When mineralization was normalized to cell number (D) there was a 923% increase after treatment with CGRP (10^{-10} M) and a 614% increase after treatment with CGRP (10^{-8} M). Values are means $\pm$ SEM for six wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Fig. 4.

Confocal microscopy demonstrating CGRP receptors in M-CSF and RANKL stimulated mouse nonadherent bone marrow stromal cell cultures at day 7 post-seeding. Under these conditions the majority of stromal cells in the culture medium are transformed into bone marrow macrophages (BMMs), preosteoclasts, and osteoclasts. Green fluorescence indicates the presence of tartrate-resistant acid phosphatase (TRAP), a marker for osteoclasts and osteoclast precursors (**A**), red fluorescence indicates CRL receptors (**B**), and yellow fluorescence indicates the co-labeling of the two proteins in the same cellular microcompartment (**C**). RAMP 1 and CLR immunostaining was observed in monocytes and multinucleated osteoclasts in BMM cultures at 7 days post-seeding. RAMP1

immunofluorescence (green) was observed in the cytoplasm, cell membrane, and nucleus (**D**) and CRL immunostaining (red) was localized in the cytoplasm and plasma membrane (**E**). Yellow fluorescence indicates co-staining for the two proteins in the same cellular microcompartment (**F**). Real time PCR measurements of RAMP1 (**G**) and CRL (**H**) mRNA levels are shown in BMM cultures at days 4 and 7 post-seeding. Values are means $\pm$ SEM for six wells and CRL and RAMP1 mRNA levels were normalized to 18S mRNA levels. *** $p < 0.001$ vs. day 4.

Fig. 5.

Effects of CGRP on osteoclast formation and resorption in M-CSF and RANKL stimulated mouse nonadherent bone marrow stromal cell cultures. Osteoclast formation was measured by counting multinucleated TRAP⁺ cells per well (**A**) and osteoclast resorption was evaluated by measuring total erosion area on BD BioCoat osteologic disc (**B**). CGRP (10^{-8} M) treatment reduced osteoclastogenesis and bone resorption in the BMM cell cultures. Values for TRAP⁺ cells are means $\pm$ SEM for six wells, and erosive areas are means $\pm$ SEM for six to eight discs. Panel **C** shows representative photomicrographs of TRAP⁺ osteoclasts in controls (**a**) and after CGRP (10^{-8} M) treatment (**b**) and the erosion area in controls (**c**) and after CGRP (10^{-8} M) treatment (**d**). * $p < 0.05$, ** $p < 0.01$ vs. control.

Fig. 6.

Effects of 7 days of CGRP treatment on TRAP (**A**) and cathepsin K (**B**) gene expression in M-CSF and RANKL stimulated nonadherent bone marrow stromal cell cultures. CGRP (10^{-8} M) reduced TRAP mRNA levels by 33% and cathepsin K levels by 23% compared to controls not treated with CGRP. Panel **C** shows the effects of RANKL (100ng/mL), CGRP (10^{-8} M), and the combination of RANKL (100ng/mL) and CGRP (10^{-8} M) on nuclear NF-κB-p65 levels in BMMs after 30 minutes of treatment. RANKL activated NF-κB in BMMs and CGRP completely blocked this effect. CGRP alone had no effect on NF-κB activation. The densitometry reading of each band was normalized by its β actin value. The readings shown at the bottom of the panel are the relative fold change over the values of control BMM samples treated only with M-CSF. * $p < 0.05$, ** $p < 0.01$ vs. control.

Table 1

Primer sequences for the Calcitonin-Gene Related Peptide (CGRP) receptor gene (CALCRL), CGRP receptor activity-modifying protein 1 (RAMP1), osteocalcin (OCN), alkaline phosphatase (ALP), Collagen type 1 (COL1), Runt related transcription factor 2 (RUNX2), tartrate-resistant acid phosphatase (TRAP), Cathepsin K (CTSK) and 18S rRNA gene (18S). Sources represent the GenBank accession number.

Gene	Source	Sequence	Predicted length (bp)
CALCRL	NM_018782	Forward primer 5'-ATTGGATAGCCAGCAAATGG-3'	224
		Reverse primer 5'-CCTGGGAGTTGTTTGTGCTT-3'	
RAMP 1	NM_016894	Forward primer 5'-ACGTGAAGAGGGTGCTGTCT-3'	235
		Reverse primer 5'-CACCCCAAAGTGCTTTGATT-3'	
OCN	NM_031368	Forward primer 5'-TTGGTGCACACCTAGCAGAC-3'	151
		Reverse primer 5'-ACCTTATTGCCCTCCTGCTT-3'	
ALP	NM_007431	Forward primer 5'-AACCCAGACACAAGCATTCC-3'	200
		Reverse primer 5'-GCCTTTGAGGTTTTTGGTCA-3'	
COL1	NM_007742	Forward primer 5'-GAGCGGAGAGTACTGGATCG-3'	158
		Reverse primer 5'-GCTTCTTTTCCTTGGGGTTC-3'	
RUNX2	NM_009820	Forward primer 5'-GCCGGAATGATGAGAACTA-3'	200
		Reverse primer 5'-GGACCGTCCACTGTCACTTT-3'	
TRAP	NM_007388	Forward primer 5'-CAGCAGCCAAGGAGGACTAC-3'	190
		Reverse primer 5'-ACATAGCCCACACCGTTCTC-3'	
CTSK	NM_007802	Forward primer 5'-CAGCTTCCCCAAGATGTGAT-3'	165
		Reverse primer 5'-AGCACCAACGAGAGGAGAAA-3'	
18S	M35283	Forward primer 5'-AGGAATTGACGGAAGGGCAC-3'	323
		Reverse primer 5'-GTGCAGCCCCGACATCTAAG-3'	