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The Inhibitory Effects of Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide on Osteoclast Formation Are Associated with Upregulation of Osteoprotegerin and Downregulation of RANKL and RANK

Hitoshi Mukohyama,*,‡,§ Maria Ransjö,*,† Hisashi Taniguchi,§ Takashi Ohyama,¶ and Ulf H. Lerner*,‡,[1]

**Department of Odontology, Oral Cell Biology, Umeå University, Umeå, Sweden; †Department of Odontology, Orthodontics, Umeå University, Umeå, Sweden; ‡Center of Musculoskeletal Research, National Institute for Working Life, Umeå, Sweden; §Department of Maxillofacial Prosthetics Maxillofacial Reconstruction and Function, Division of Maxillofacial/Neck Reconstruction, Graduate School, Faculty of Dentistry, Tokyo Medical and Dental University, Tokyo 113-8549, Japan; and ¶First Department of Prosthodontics, Faculty of Dentistry, Tokyo Medical and Dental University, Tokyo 113-8549, Japan*

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The presence of a network of peptidergic nerve fibers in the skeleton, expressing several neuropeptides including vasoactive intestinal peptide (VIP), has been demonstrated. This observation, together with our findings *in vitro* showing that VIP can regulate the activities of osteoblasts and osteoclasts as well as the recruitment of osteoclasts, has suggested the existence of a neuro-osteogenic interplay in bone metabolism. In the present study, the effects of VIP and pituitary adenylate cyclase-activating polypeptide (PACAP), two members of the VIP/secretin/glucagon superfamily, on osteoclast formation and mRNA expression of three key regulatory proteins involved in osteoclast formation have been investigated. VIP, PACAP-27, and PACAP-38, at concentrations of 10^{-6} M, all significantly inhibited formation of tartrate-resistant acid phosphatase-positive multinuclear cells (TRAP + MNC) in mouse bone marrow cultures stimulated by 1,25(OH)$_2$-vitamin D3 (D3; 10^{-8} M). By using semiquantitative RT-PCR, it was found that D3 upregulated the mRNA expressions of receptor activator of NF-κB ligand (RANKL) and receptor activator of NF-κB (RANK), whereas the expression of osteoprotegerin (OPG) was downregulated in mouse bone marrow cultures stimulated by D3 for 7 days. Both VIP and PACAP-38 decreased the stimulatory effects of D3 on RANKL and RANK expression, whereas the inhibitory effect of D3 on OPG expression was reversed by VIP and PACAP-38. These observations indicate that the inhibitory effects of VIP and PACAP on osteoclast recruitment are due to regulation of the expression of key proteins involved in later stages of osteoclast differentiation.

Bone tissue in the skeleton is continuously remodelled through the concerted activities of bone forming osteoblasts and bone resorbing osteoclast. The activities of osteoblasts and osteoclasts are controlled by several systemic hormones, including parathyroid hormone (PTH) and 1,25(OH)$_2$-vitamin D3 (D3), and a variety of local factors such as osteotropic cytokines and growth factors (1). Based upon the findings that skeletal bone tissues contain a network of peptidergic nerve fibers and that neuropeptides expressed in these skeletal nerves have the capacity to regulate the activities of both osteoblasts and osteoclasts, we have suggested that neuro-osteogenic interactions also are important in bone metabolism (2).

Vasoactive intestinal peptide (VIP) is a neuropeptide which is present in skeletal sympathetic nerve fibers (2). Receptors for VIP of the VIP-2 receptor subtype are expressed on osteoblasts (3–5) and activation of these receptors leads to enhanced activity and mRNA expression of alkaline phosphatase, as well as increased

[1] To whom correspondence should be addressed at Department of Odontology, Oral Cell Biology, Umeå University, Umeå, SE-901 87 Sweden. Fax: +46-90-139289. E-mail: Ulf.Lerner@odont.umu.se.

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mineralization of bone nodules in cultures of mouse calvarial osteoblasts (6). VIP also stimulates release of calcium from mouse calvarial bones *in vitro* (7). This effect may be due to activation of terminally differentiated multinucleated osteoclasts, since we have found that VIP, in the presence of stomal cells/osteoblasts, can stimulate the bone resorbing activity of isolated rat osteoclasts incubated on slices of bovine bone (8). The degree of bone resorption in the skeleton is dependent not only on activation of multinucleated osteoclasts, but also on recruitment of osteoclasts from osteoclast progenitor cells (1). Recently, we have demonstrated that VIP inhibits osteoclastogenesis in mouse bone marrow cultures stimulated by D3 or PTH (9), suggesting that VIP has unique, dual and opposite effects on the two processes involved in bone resorption.

Osteoclastogenesis is a complex process regulated primarily by PTH- and D3-sensitive stromal cells/osteoblasts. The stromal cells control the proliferation and differentiation of preosteoclasts to multinucleated osteoclasts, processes requiring cell-to-cell contact (1, 10). Recently, three novel molecules have been discovered that are crucial for osteoclastogenesis (10, 11). Stromal cells and osteoblasts express on their surface a long sought after protein called osteoclast differentiation factor (ODF; 12). The amino acid sequence of ODF is identical to a newly discovered type II membrane protein in the tumor necrosis factor (TNF) ligand family called TNF-related activation-induced cytokine (TRANCE; 13), or receptor activator of NF-κB ligand (RANKL; 14). As proposed by Suda *et al.* (11), the term RANKL is used in the present paper. The expression of RANKL is upregulated by PTH and D3 and addition of this cytokine (together with macrophage colony-stimulating factor) to preosteoclasts leads to osteoclast formation (12). Mice deficient of RANKL suffer from osteopetrosis due to a lack of osteoclasts (15). RANKL expressed on stromal cells interacts with a receptor expressed on preosteoclasts called RANK (16). RANK is a type I transmembrane receptor of the TNF receptor superfamily. Activation of RANK by RANKL results not only in osteoclast formation but is also important for osteoclast activation (17). The RANKL dependent activation of RANK in terminally differentiated multinucleated osteoclasts is associated with translocation of NF-κB to the nucleus, elevation of intracellular calcium, and activation of c-Jun N-terminal kinase (18). The importance of RANK for osteoclastogenesis is clearly demonstrated by the finding that *rank*$^{-/-}$ mice develop osteopetrosis due to a lack of osteoclasts (19). The interaction between RANK and RANKL is antagonized by another member of the TNF-receptor superfamily, which is called either osteoprotegerin (OPG; 20) or osteoclast inhibitory factor (OCIF; 21). OPG/OCIF lacks transmembrane spanning domain and is a soluble protein that is released from stromal cells/osteoblasts and which has affinity to RANKL. Binding of OPG to RANKL abolishes osteoclast formation via a "decoy receptor mechanism." Mice overexpressing OPG become osteopetrotic because of a lack osteoclasts (20) and *opg*$^{-/-}$ mice develop osteopenia because of enhanced number of osteoclasts (22).

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the VIP/secretin/glucagon family of peptides (23). The N-terminal sequence of PACAP shows 68% homology with VIP. In most cell types, including osteoblasts, PACAP is a more effective stimulator of cyclic AMP formation than VIP (5).

The aims of the present study were to examine whether the expression of RANK, RANKL, and OPG in mouse bone marrow cultures are regulated by VIP and to investigate if PACAP shares the activities of VIP on osteoclast formation and expression of osteoclastogenic cytokines.

MATERIAL AND METHODS

Materials. Highly purified porcine VIP was purchased from Professor Viktor Mutt, Karolinska Institute (Stockholm, Sweden), PACAP-38 and PACAP-27 from Penninsula (Belmont, CA), bovine serum albumin, gentamycin sulphate, and the kit for tartrate resistant acid phosphatase (TRAP) staining from Sigma Chemical Co. (St. Louis, MO), α-modification of Minimum Essential Medium (α-MEM), heat inactivated fetal calf serum (FCS), L-glutamic acid, TRIzol LS reagent kit, deoxyribonuclease I (amplification grade) and oligonucleotide primers from Life Technology (Renfrewshire, UK), bensylpenicillin from ASTRA (Södertälje, Sweden), streptomycin from Heyl (Berlin, Germany), multi-well plates and culture dishes from Costar (Corning, NY), the 1st strand cDNA synthesis Kit and PCR core kit from Boehringer-Mannheim GmbH (Mannheim, Germany). HotStarTaq PCR kit from Qiagen (Hilden, Germany). 1,25$(OH)_2$-vitamin D_3 (D_3) was kindly supplied by Roche-Produkter (Helsingborg, Sweden).

Isolation and culture of bone marrow cells. Bone marrow cells were isolated from 5- to 9-week old CsA mice as previously described (9, 24). The bone marrow cells were plated in 24 multi-well plates at a cell density of 1×10^6 cells/cm^2 in 1 mL of α-MEM supplemented with 10% FCS and antibiotics. After a 24 h attachment period, cells were cultured in medium with or without D_3 (10^{-8} M), in the absence or presence of VIP, PACAP-27, or PACAP-38, all at concentrations of 10^{-6} M. The cells were cultured for 7 days with 2 day intervals of medium change. Approval of animal experiments was obtained from animal use committee of Umeå University.

Counting osteoclast number. At the end of culture periods, media were removed and cells were washed twice with phosphate buffered saline (PBS, pH 7.4). Then, the cells were fixed with phosphate buffered formalin (pH 7.4) for 10 min. After fixation, cells were stained to detect TRAP positive multinucleated cells (TRAP + MNC) using a commercially available kit and by following the manufacturer's instruction. Cells positive for TRAP having more than three nuclei were counted as TRAP + MNC.

RNA isolation and first-strand cDNA synthesis. Mouse bone marrow cells were plated in 55 cm^2 culture dishes at a density of 1×10^6 cells/cm^2. After 7 days of culture, with or without test substances, cells were lysed with TRIzol LS reagent and total RNA was extracted following manufacturer's protocol. The RNA was quantified spectrophotometrically and the integrity of the RNA preparations was examined by agarose gel electrophoresis. Only RNA preparations

showing intact species were used for subsequent analysis. Extracted total RNA was treated with Deoxyribonuclease I to eliminate genomic DNA. One microgram of total RNA, following DNAse treatment, was reversed transcribed to cDNA with a 1st strand cDNA synthesis kit using random primers. After incubation at 25°C for 10 min and at 42°C for 60 min, cDNA was kept at −20°C until used for polymerase chain reaction (PCR).

Polymerase chain reaction. Two microliters of the first-strand cDNA mixture was processed by PCR using a PCR core kit. The conditions for PCR of glyceraldehyde-phosphate dehydrogenase (GAPDH) were: denaturing at 94°C, followed by annealing at 57°C, polymerizing at 72°C. For the PCR of RANKL and RANK, the temperature cycling was as follows: denaturing at 94°C for 1 min, annealing at 65°C for 2 min, and polymerizing at 72°C for 3 min for 10 cycles. In subsequent cycles, the primer annealing temperature was decreased stepwise by 5°C every 5 cycles. After the last cycle, the mixtures were incubated at 72°C for 7 min. For the PCR of OPG, hot start method was used. Prior to denaturation, HotStarTaq polymerase was used initially at 95°C for 15 min for activation, followed by denaturation at 94°C for 1 min, annealing at 65°C for 2 min, and polymerizing at 72°C for 3 min for 10 cycles. In subsequent cycles, the primer annealing temperature was decreased stepwise by 5°C every 5 cycles. After the last cycle, the mixtures were incubated at 72°C for 7 min. The sequences of primers used were GAPDH sense, ACTTTGTCAAGCTCATTTCC and antisense TGCAGCGAACTTTATTGATG; RANKL sense GGTCGGGCAATTCTGAATT and antisense GGGAATTACAAAGTGCACCAG; OPG sense TGGAGATCGAATTCTGCTTG and antisense TCAAGTGCTTGAGGGCATAC.; RANK sense CACAGACAAATGCAAACCTTG and antisense GTGTTCTGGAACCATCTTCCTCC. The expression of these factors was compared at the logarithmic phase of the PCR. The PCR products were electrophoretically size fractionated in 2% agarose gel. The size of the PCR products were GAPDH 270 bp, TRANCE 810 bp, RANK 400 bp and OPG 720 bp. The sizes of the bands were confirmed by a 100-bp ladder.

Statistical analysis. Data were analyzed by ANOVA followed by Fisher's protected least significance difference. $P < 0.05$ was considered statistically significant.

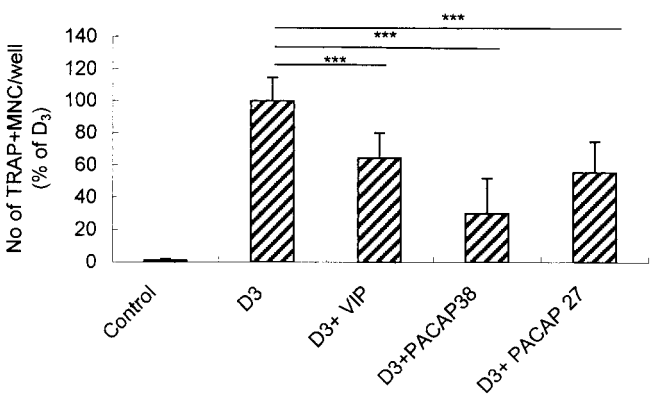

FIG. 1. The effect of VIP (10^{-6} M), PACAP-38 (10^{-6} M), and PACAP-27 (10^{-6} M) on the formation of TRAP + MNC in mouse bone marrow cultures stimulated with D3 (10^{-8} M). Results represent the mean of 3 different experiments in which the number of TRAP + MNC in D3 stimulated cultures was set to 100%. In each experiment, the number of wells per treatment group was 6. The number of TRAP + MNC formed in D3 stimulated wells was for each experiment 125 ± 14, 96 ± 9 and 182 ± 44 (mean value ± SD), respectively. The vertical bars represent SD. The asterisks denote significant differences between D3 alone and D3 + VIP/PACAP-27/PACAP-38 (***$P < 0.001$).

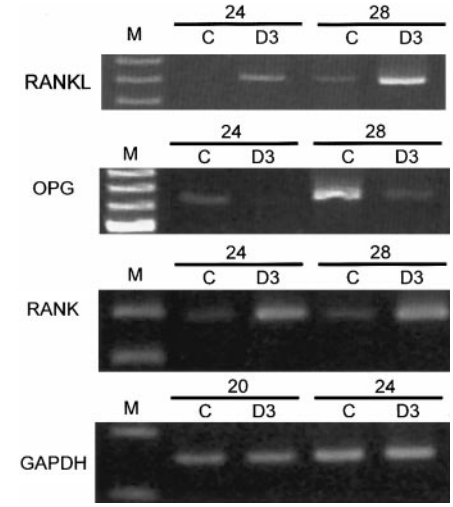

FIG. 2. The expression of mRNA for RANKL, OPG, RANK, and GAPDH in mouse bone marrow cultures cultured in the absence or presence of D3 (10^{-8} M) for 7 days. Total RNA was extracted from the cells and processed by RT-PCR with the cycles in the PCR reactions stopped at the indicated number of cycles; 24 and 28 for RANKL, OPG, and RANK, 20 and 24 for GAPDH. Results shown are representative of 3 different experiments.

RESULTS

The stimulatory effect of D3 (10^{-8} M) on formation of TRAP + MNC in mouse bone marrow cultures was significantly inhibited by VIP (10^{-6} M; Fig. 1), in agreement with our previous study (9). PACAP-38 (10^{-6} M) and PACAP-27 (10^{-6} M) also caused a significant inhibition of D3-stimulated formation of TRAP + MNC, with PACAP-38 being a more effective inhibitor than VIP and PACAP-27 (Fig. 1).

D_3 treatment (10^{-8} M) enhanced the expression of mRNA levels for RANKL and RANK, whereas the expression of OPG mRNA was decreased (Fig. 2), as expected from previous studies (25, 26). Treatment of the bone marrow cells with VIP (10^{-6} M) and D3 (10^{-8} M) resulted in decreased expression of RANKL and RANK mRNAs compared to the levels seen in cells only treated with D3 (Fig. 3). The expression of mRNA level for OPG was increased in cells treated with both D3 and VIP as compared to that seen in cells only treated with D3 (Fig. 3). The effects of VIP on all three cytokines in D3 stimulated mouse bone marrow cells were mimicked by PACAP-38 (10^{-6} M; Fig. 3). No reaction product could be seen when the PCR reactions were performed without the initial RT-step, indicating that we did not amplify genomic DNA.

DISCUSSION

This report shows that the previously reported inhibitory effect of VIP on osteoclast formation in mouse

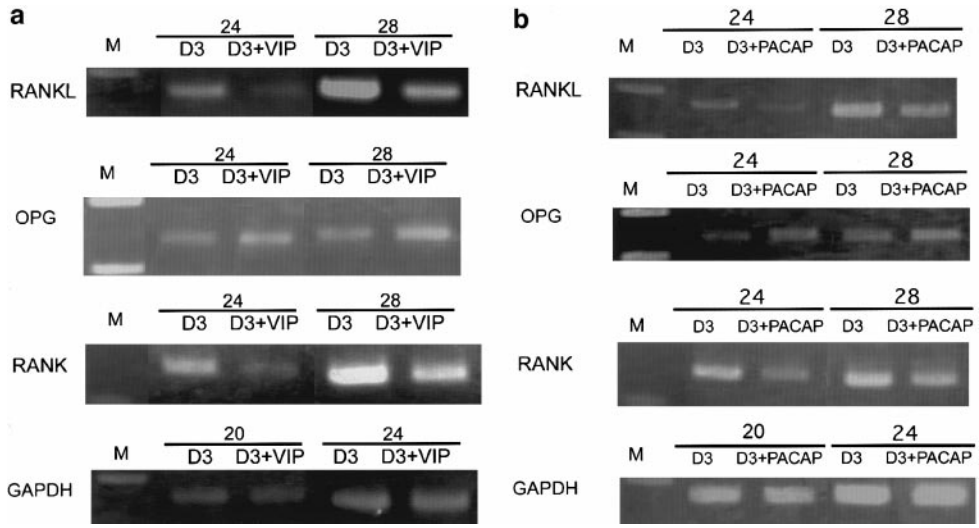

FIG. 3. The expression of mRNA for RANKL, OPG, RANK, and GAPDH in mouse bone marrow cultures cultured in the presence of D3 (10^{-8} M) without and with either VIP (10^{-6} M) or PACAP-38 (10^{-6} M) or 7 days. Total RNA was extracted from the cells and processed by RT-PCR with the cycles in the PCR reactions stopped at the indicated number of cycles; 24 and 28 for RANKL, OPG, and RANK, 20 and 24 for GAPDH. Results shown are representative of three different experiments.

bone marrow cultures (9) is mimicked by PACAP-38 and PACAP-27, two other members of the VIP/secretin/glucagon family of peptides. The effects of VIP and related peptides are mediated by three distinct receptor subtypes called VIP-1, VIP-2, and PACAP receptors (27). We have recently reported that mouse bone marrow cultures express mRNAs for both VIP-1 and VIP-2 receptors (9). Osteoblasts isolated from 5- to 6- day-old mouse calvariae express mRNA for VIP-2 receptors, but not for VIP-1 or PACAP receptors (5). VIP-1 receptors are likely to be more important than VIP-2 receptors as inhibitor of osteoclast formation, since secretin (but not glucagon) inhibits osteoclast formation in D3 stimulated mouse bone marrow cultures (28). The fact that the present study demonstrates that not only VIP (9), but also PACAP-38 and PACAP-27 inhibit D3 stimulated osteoclast formation indicate the possibility that mouse bone marrow cells also express PACAP receptors and studies are underway to examine this possibility, using both pharmacological and molecular approaches.

In mouse bone marrow culture, the expression of RANKL is reported to be up-regulated and OPG to be downregulated by the treatment with osteotropic factors (25, 26). We here confirm the reciprocal expression of RANKL and OPG in mouse bone marrow cultures stimulated by D_3 and further demonstrates that RANK expression in mouse bone marrow cells is up-regulated by D3 treatment. The information regarding the control of RANK expression is very limited. In an isolation of mouse calvarial osteoblasts, most likely containing some mononuclear preosteoclasts, D3 was found to enhance RANK expression (29), similar to our results obtained in mouse bone marrow cultures.

The stimulatory effects of D3 on RANKL and RANK expression were decreased by both VIP and PACAP-38. Since RANKL and RANK are crucial for osteoclast formation (10, 11), our findings provide a reasonable molecular explanation for the inhibitory effects of VIP and PACAP on osteoclastogenesis. The fact that VIP and PACAP-38 enhance the expression of OPG further demonstrates the anti-osteoclastogenic effects of VIP and PACAP. Thus, several mechanisms may explain the molecular pathways involved in VIP-induced inhibition of osteoclast formation, and the relative importance of the three mechanisms indicated by our findings in the present study is not know. In addition, we can not exclude the possibility that the decrease of osteoclast number after VIP treatment is due to enhanced apoptosis, since Lacey *et al.* (30), in a preliminary report, recently demonstrated that RANKL is necessary for osteoclast survival in mouse bone marrow cultures as well as *in vivo.*

The finding that VIP inhibits D3 and PTH stimulated osteoclast formation does not reveal whether or not stromal cells or osteoclast progenitor cells are the main target cells. RANK is expressed by osteoclast progenitor cells and RANKL by stromal cells and since VIP/PACAP can regulate the mRNA expression of both molecules our data indicate that stromal cells as well as osteoclast progenitor cells possess functional VIP receptors. The fact that OPG expression is affected by VIP and PACAP-38 further indicates the presence of VIP receptors in stromal cells. The observations, show-

ing that osteoblasts are equipped with VIP receptors linked to cyclic AMP formation (3–5), are also in line with the view that stromal cells are one of the cell types expressing VIP receptors in bone marrow cultures. Previously, we reported that addition of VIP during the last two days of bone marrow culture is sufficient to decrease osteoclast formation (9), indicating that VIP has a role in the differentiation of late osteoclast precursor cells and/or fusion of these cells to TRAP + MNC. The observations in the present study further indicate that VIP inhibits osteoclastogenesis by interfering with the differentiation of osteoclast progenitor cells.

In summary, we here present the first evidence that a signaling molecule from the nervous system can regulate molecular mechanisms known to be crucially involved in osteoclast formation. This observation is likely to explain the recent finding that the neuropeptide VIP, abundantly expressed in peptidergic skeletal nerve fibers, inhibits osteoclast formation in mouse bone marrow cultures (9). These observations further the evidence for the suggestion that skeletal nerve fibers may play a role in bone metabolism via neuro-osteogenic interactions (2). Since VIP is predominantly expressed in sympathetic nerve fibers (31–33), the observations that surgical and pharmacological sympathectomy leads to enhanced number of osteoclasts (34–38) may also be explained by the observations in the present study.

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