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Neuropeptidergic regulation of bone resorption and bone formation

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Abstract

Immunohistochemical studies have revealed an extensive network of nerve fibers in the vicinity and within the skeleton, not only in the periosteum of bone but also in cortical and trabecular bone as well as in the bone marrow. Phenotyping of the skeletal nerve fibers have demonstrated the expression of a restrictive panel of different signalling molecules including neuropeptides, neurotransmitters and neurotrophins. In this review, the presence of receptors for the neuropeptides vasoactive intestinal peptide, calcitonin gene-related peptide and substance P on osteoblasts and osteoclasts and the capacity of these receptors to regulate bone formation, osteoclast formation and activity are described. These findings, together with data obtained by chemically and surgically targeted nerve deletion and observations made in paraplegic patients, strongly suggest that neuro-osteogenic interactions play an important role in skeletal function.

Keywords: Bone, Neuropeptides, Vasoactive Intestinal Peptide, Calcitonin Gene-Related Peptide, Substance P

Introduction

During the recent decade several groups have reported interesting effects on bone resorptive and bone forming cells by molecules known primarily to be present in the nervous system. The effects by these substances are in general as profound as those caused by well known osteotropic hormones, cytokines and growth factors. These studies have very much been prompted by the immunohistochemical demonstrations of an intensive network of sensory and autonomic nerve fibers within the skeleton, not only in the periosteum but also within the cortical bone, bone marrow and epiphyseal growth plate[1,2]. Although many skeletal nerve fibers are associated with the blood vessels, several blood vessel-unrelated nerves and free nerve endings have also been demonstrated. There are also reports which show a very close proximity between nerve endings and bone cells[3]. Since bone cells express receptors for many of the neuronal messengers present in these skeletal nerve fibers and since activation of such receptors lead to profound effects on the activity of both osteoblasts and osteoclasts, the existence of neuro-osteogenic, or neuro-immuno-osteogenic, interactions has been suggested[2,4], in analogy with the more well known neuro-immune or neuro-immune-endocrine system. Most interestingly, Delgado et al.[5] recently reported that treatment with the neuropeptide vasoactive intestinal peptide (VIP) resulted in not only decreased joint swelling, but also inhibition of cartilage and bone destruction in experimentally induced arthritis, suggesting an important role in inflammation and bone metabolism by the neuronal system.

The importance of the nervous system is also suggested by several clinical observations as well as experimental findings. Thus, increased fracture rate and extensive callus formation has been reported in paraplegic children, in patients with spinal cord lesions and in stroke patients[2,4]. It has been argued that the skeletal pathology in these patients are mainly due to disuse. However, in a recent study we have found that the hemi-osteoporosis in stroke patients is very rapidly developing during the first months and is associated with increased serum levels of bone resorption markers without any change of bone formation markers, suggesting that the loss of bone is due to enhanced resorption rate[6]. Similarly, in patients with spinal cord lesions, the loss of trabecular bone is associated with increased concentrations in the urine of bone resorption markers[7,8]. Since it is generally decreased bone formation rate that has been associated with disuse and since the stroke patients studied by us was a group of patients totally immobilized and still developing

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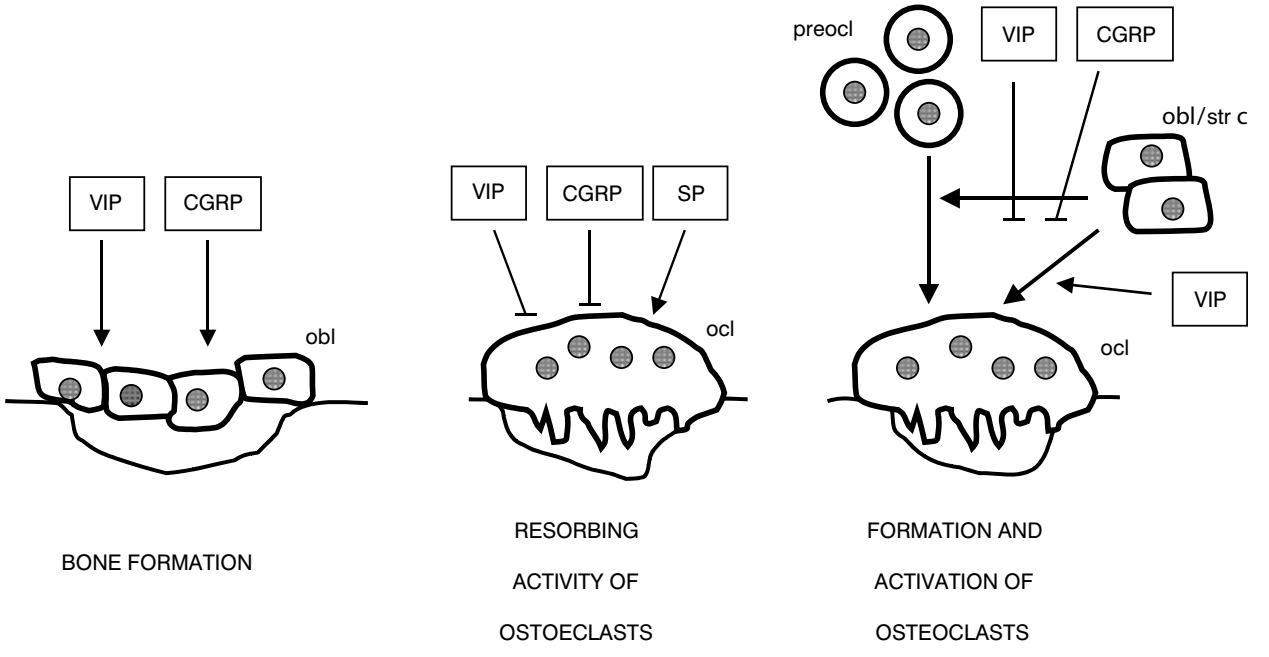

Figure 1. Pleiotropic effects of vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and substance P (SP) on bone formation, bone resorbing activity and formation/activation of osteoclasts. Receptors for VIP and CGRP are present on osteoblasts and activation of these receptors stimulates bone formation both in vitro (VIP/CGRP) and in vivo (CGRP). Terminally differentiated osteoclasts express receptors for VIP and CGRP linked to an acute, transient inhibition of the osteoclasts, whereas the SP receptors are linked to enhancement of the activity. Stromal cells/osteoblasts also are equipped with receptors for VIP and CGRP and activation of these receptors by VIP leads to inhibition of osteoclast formation and stimulation of the activity of already present osteoclasts. CGRP, similar to VIP, decreases the formation of osteoclasts. No studies have been performed showing if CGRP, similar to VIP, also can enhance the activity of osteoclasts mediated by the stromal cell receptors.

only hemi-osteoporosis, it is more likely that it is the loss of functional innervation that is the cause of the loss of bone. Interestingly, Demulder et al.[9] have recently reported that the number of osteoclasts formed *ex vivo* in bone marrow from iliac crest (below lesional level) is significantly increased compared to the number of cells formed in sternal bone marrow cultures (above lesion) established from paraplegic patients with spinal cord lesions.

Surgical and chemical denervation in animals also lead to a skeletal phenotype, not only in loaded parts of the skeleton, but also in unloaded bones, further suggesting that the skeletal pathology can not be solely explained by disuse. Thus, treatment of rats with guanethidine results in a decrease of neuropeptides such as VIP and neuropeptide Y (NPY) and a substantial increase of the number of osteoclasts in the mandibule without any changes in periosteal bone formation[10]. In addition, surgical and pharmacological sympathectomy in Mongolian gerbils result in enhancement of osteoclast numbers and resorptive surface in ear bones, also a bone not being exposed to mechanical loading[11]. Moreover, surgical denervation reducing calcitonin gene-related peptide (CGRP) and substance P (SP) expressing nerve fibers in the rat hind foot results in reduced growth of metatarsal bones, a phenomenon not observed in rats immobilized by tenotomy[12].

Phenotyping of the skeletal nerve fibers have demonstrated the presence of a wide variety of different transmitter substances including VIP, pituitary adenylate cyclase-activating peptide (PACAP), substance P (SP), CGRP, NPY, neurokinin A, met-enkephalin, serotonin, glutamate, catecholamines, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). Several of these molecules have been shown to be able to control the activities of osteoblasts and osteoclasts[2]. In the present paper the effects of the neuropeptides VIP, CGRP, and SP are summarized.

Vasoactive intestinal peptide

VIP was originally discovered as an amidated 28 amino acid peptide in the gut with vasodilator activity. Since then several peptides with homology to VIP have been described, forming a family denoted the VIP/secretin/glucagon family of neuropeptides, consisting of VIP, secretin, glucagon, gastric inhibitory peptide, growth hormone-releasing hormone, peptide histidine isoleucine amide, pituitary adenylate cyclase-activating peptide (PACAP) 27 and 38, as well as the reptilian peptides helodermin, helospectin and exendine.

The presence of VIP not only in the gut, but also in the brain and in the peripheral nervous system, has led to the introduction of VIP as a neuropeptide.

A broad range of biological actions have been linked to activation of VIP receptors including effects on blood vessels, gastrointestinal tract, lungs and the immune system. Tashjian and co-workers were the first to demonstrate effects on bone, by showing that VIP stimulates calcium release from organ cultured neonatal mouse calvariae[13].

The presence of skeletal nerve fibers expressing VIP has been demonstrated in the periosteum in several different species[14-16] and also in the rat epiphyseal growth plate[15,16]. The periosteal VIP-positive nerves are only rarely associated with blood vessels. The possibility exists that these nerves mainly may have a sensory function, but the increasing evidence that VIP can regulate bone cell activities has prompted the hypothesis that skeletal nerve fibers expressing VIP may also have a neuro-hormonal function[2,4,17].

Receptors

The presence of receptors for VIP linked to enhanced formation of cyclic AMP has been demonstrated in human, rat and mouse osteoblasts[18-21]. During recent years, three different subtypes of VIP receptors have been pharmacologically characterized and recently also cloned. These receptors are called VIP-1, VIP-2 and VIP/PACAP receptors[22]. All three receptors are seven transmembrane, G-protein coupled receptors and members of the VIP/secretin/parathyroid hormone receptor superfamily.

By comparing the rank order of response of peptides in the VIP/secretin/glucagon family on cyclic AMP formation and in radioligand binding studies, we have found that mouse calvarial osteoblasts express PACAP preferring VIP-2 receptors[21]. The use of atomic force microscopy (AFM) to study the physical binding of agonists to binding sites on the surface of cells has confirmed the presence of VIP-2 receptors and the lack of VIP-1 receptors on mouse calvarial osteoblasts, an observation further supported by the fact that these cells express mRNA for VIP-2, but not for VIP-1 or VIP/PACAP receptors[21]. When the osteoblasts were cultured for longer periods of time to induce bone noduli formation and subsequent mineralization it was found that, at the same time as mineralization started, VIP-1 receptors were expressed. Very few studies have been directed to study a possible differentiation-dependent expression of different receptors in bone cells, but recently Deckers et al.[23] described an up regulation of receptors for vascular endothelial growth factor in mouse osteoblasts, Bhangu et al.[24] a down regulation of the expression of the glutamate transporter in rat osteoblasts and Gong et al.[25] an up regulation of the LDL receptor-related protein 5 in differentiated mouse osteoblasts. The functional role of the VIP-1 receptors in osteoblasts is presently unknown.

As discussed below, occupancy of VIP receptors on osteoblasts has been shown to affect bone formation, expression of molecules regulating osteoclast formation, production of cytokines, regulation of cytokine receptor expression and the expression of signal transducing proteins linked to cytokine receptors.

Much less is known about VIP receptors in cells of the osteoclastic lineage. However, AFM studies have shown that isolated rat osteoclasts are equipped with binding sites for VIP[26] and reversed transcriptase polymerase chain reaction analysis of mouse bone marrow osteoclasts isolated by micromanipulation have demonstrated the expression of mRNA for VIP-1 and VIP/PACAP receptors, but not for VIP-2 receptors[27]. Whether or not the profile of VIP receptor subtypes are different during osteoclastic differentiation is yet not known.

In mouse bone marrow cultures, mRNA for VIP-1 and VIP-2, but not VIP/PACAP receptors have been described[28]. Presently it is unknown in which cell types these receptors are present. However, we have found that both VIP-1 and VIP-2 receptors are functionally linked to osteoclastogenesis (see further below), indicating the presence of VIP receptors in bone marrow cells involved in osteoclast formation.

Effects on bone formation

Possible effects of VIP on bone formation has been assessed *in vitro* using mouse calvarial osteoblasts. These findings suggest that VIP may have anabolic actions in bone. Thus, VIP stimulates the activity and mRNA expression of alkaline phosphatase[29]. This effect is mediated by the PACAP-preferring VIP-2 receptors since the effect of VIP is shared with PACAP-38 but not with secretin[30]. Stimulation of osteoblasts in long term cultures by VIP results in increased mineralization at early time points but not to increased mineralization totally[29], suggesting that VIP enhances the differentiation of committed osteoblasts rather than stimulating the differentiation of non-committed cells, a view supported by the observation that VIP does not enhance the mRNA expression of type I (α1) procollagen[30]. The findings that VIP can regulate the expressions of non-collagen bone matrix proteins, including osteocalcin, bone sialoprotein, osteonectin and osteopontin[31], further indicate that VIP affects bone formation *in vitro*.

Effects on bone resorption

As mentioned above, Tashjian and co-workers were the first to report that VIP has an effect on bone cells by demonstrating a stimulation of calcium release from neonatal mouse calvariae in organ culture[13]. It was necessary to add the peptide repeatedly to the cultures, an observation suggested to be due to degradation during culture. The finding was in line with previous observations demonstrating that parathyroid hormone and other compounds stimulating cyclic AMP enhance bone resorption *in vitro*. However, calcitonin also raising cyclic AMP in bone causes an acute inhibition of bone resorption by decreasing the activity of termi-

nally differentiated, multinucleated osteoclasts[32]. The divergent effects by PTH and calcitonin are due to the presence of PTH receptors in osteoblasts, linked to a paracrine activation of osteoclasts, and to the presence of calcitonin receptors in osteoclasts causing a contraction of the cells, ceased motility and decreased release of proteolytic enzymes. The observation by Hohmann et al.[13] suggested that the bone resorptive effect of VIP was due to VIP receptors present in osteoblasts and that the effect of VIP was similar to that of PTH. However, when cyclic AMP is raised in mouse calvariae by compounds stimulating adenylate cyclase or by analogues of cyclic AMP an acute, calcitonin-like inhibition of bone resorption is obtained[33]. This effect is transient, similar to that of calcitonin, and followed by a delayed, PTH-like, stimulation of bone resorption. Since there are no calcitonin receptors in osteoblasts, the escape from calcitonin induced inhibition is not followed by a delayed stimulation of bone resorption and since there are no PTH receptors in osteoclasts no initial inhibition of bone resorption is obtained by PTH. Since only the accumulated release of calcium was determined in the experiments by Tashjian and co-workers[13], these observations raise the possibility that the bone resorptive effect of VIP was due to a delayed stimulation of bone resorption caused by osteoblasts which may have been preceded by an initial, transient inhibition of bone resorption. In fact, studies on isolated rat osteoclasts in the absence and presence of osteoblasts have suggested that VIP may cause both a calcitonin-like effect on osteoclasts and a PTH-like stimulation of osteoclasts mediated by a paracrine osteoblastic activation[26].

When rat osteoclasts, isolated in a manner resulting in very few contaminating stromal cells/osteoblasts, are exposed to VIP for a short period of time (<24 h) a rapid contraction of the osteoclasts and ceased motility of the cells are obtained and when VIP treated osteoclasts are cultured on bone slices a decreased number and area of resorption pits are seen[26]. These effects are identical to those caused by calcitonin. Similar to calcitonin, the effects on osteoclast contraction and motility disappear over time. We assume that the effects are caused by VIP receptors present on osteoclasts, a view supported by the facts that binding of VIP to the osteoclasts can be demonstrated using AFM[26], that VIP causes a very rapid increase of intracellular calcium in osteoclasts[26] and that mRNA expression VIP-1 and VIP/PACAP receptors have been shown[26]. In preliminary studies we have seen that both secretin, acting on VIP-1 receptors and PACAP 38 acting on VIP/PACAP receptors, also cause a rapid inhibition of osteoclast activity.

When rat osteoclasts, isolated in a manner giving a relatively high number of contaminating stromal cells/osteoblasts, are treated with VIP for longer periods of time (>48 h), the initial decrease of osteoclast contraction and motility has disappeared and when the cumulative resorption activity is followed by counting the number of resorption pits it appears that VIP, similar to PTH, causes a significant enhancement of the number of resorption pits[26]. These findings suggest that the delayed stimulatory effect of VIP is due to the presence of VIP receptors on stromal cells/osteoblasts in analogy with PTH. The demonstration of binding sites for VIP on osteoblasts, using either AFM or radioligand binding, and the expression of mRNA for VIP-2 receptors in these cells further support that hypothesis[21].

Effects on osteoclast formation

The results obtained in mouse calvariae and in isolated osteoclasts suggest that VIP stimulates bone resorption by enhancing the activity of differentiated osteoclasts. An alternative explanation may be that VIP stimulates bone resorption by an effect on osteoclast formation, similar to that by PTH. We, therefore, have studied effects of VIP and related peptides on osteoclastogenesis in mouse bone marrow cultures. However, in contrast to PTH and 1,25(OH)2-vitamin D3 (D3), VIP does not stimulate osteoclast formation[28,34]. On the contrary, VIP inhibits the stimulatory effects of PTH and D3 on the number of $TRAP^+$ multinucleated cells, as well as on the number of calcitonin binding sites and the number of bone resorption pits formed when the bone marrow cultures were performed on slices of devitalized bone[28]. Osteoclast formation stimulated by D3 is associated with stimulation of mRNA for calcitonin receptor, TRAP and cathepsin K, effects down regulated by VIP. These findings together demonstrate that VIP inhibits osteoclast formation and does so by inhibiting the differentiation of preosteoclasts.

Recent findings have demonstrated that osteoclastogenes is controlled by several members of the tumor necrosis factor (TNF) receptor and ligand superfamilies[35,36]. Thus, activation of receptor activator of NF-kB (RANK), expressed on preosteoclasts, by RANK ligand (RANKL), expressed on stromal cells/osteoblasts, is crucial for osteoclast formation. This interaction can be blocked by osteoprotegerin (OPG) released from stromal cells/osteoblasts, since this TNF receptor related soluble cytokine can bind to the TNF ligand related molecule RANKL and thereby inhibiting the interaction between RANKL and RANK, the latter also displaying homology to the TNF receptor superfamily. The stimulatory effect of PTH and D3 on osteoclast formation is associated with increased expression of RANKL and decreased OPG. VIP and PACAP 38 decrease the stimulatory effect of D3 on RANKL and counteract the decrease of OPG in cultured bone marrow cells[34]. In addition, VIP and PACAP 38 decrease the expression of RANK[34]. All these effects can explain the inhibition osteoclastogenesis by VIP. The observations further indicate that VIP receptors are present both on stromal cells and on preosteoclasts, a possibility that is presently studied using clonal cell lines.

Effects on osteotropic cytokines

VIP may affect bone cells not only by direct effects caused by VIP receptors on stromal cells/osteoblasts and preosteo-

clasts/osteoclasts, but also by affecting cells in the vicinity of bone and by an indirect, paracrine mechanism stimulate or inhibit bone cells. Such interactions are commonly mediated by cytokine and growth factors. In macrophages which are very closely related to osteoclasts, Ganea and Delgado have reported that VIP inhibits the release of pro-inflammatory cytokines such as IL-6 and TNF-α and stimulates the release of anti-inflammatory cytokines such as IL-10[37]. No studies, besides the observation that VIP decreases the mRNA expression of RANK in bone marrow cells[34], have been directed to study effects of VIP on cytokine expression in osteoclasts. In stromal cells[38] and in an osteosarcoma cell line[39], however, VIP stimulates IL-6 production, findings which have been confirmed in mouse calvarial osteoblasts[40]. In the latter cells, the effect of VIP is mediated by VIP-2 receptors. Whether or not the VIP-1 receptors that are induced during osteoblastic differentiation may also be involved in the control of IL-6 expression is not presently known. The fact that the bone resorptive effect of PTH in isolated rat osteoclasts can be inhibited by an antiserum neutralizing IL-6[41] suggests the intriguing possibility that the stimulatory effect of VIP on osteoclast activity may be due to VIP induced IL-6 release from stromal cells/osteoblasts.

Recently, we have found that VIP also affects the capacity of osteoblasts to respond to cytokines. Thus, VIP decreases the expression of mRNA for the IL-6 receptor and for gp 130 in mouse calvarial osteoblasts[40].

Calcitonin gene-related peptide

CGRP is a 37 amino acid peptide belonging to a superfamily of peptides including calcitonin, CGRP-I, CGRP-II, amylin and adrenomedullin[42]. CGRP is produced both in neuronal and non-neuronal cells by tissue-specific alternative splicing of the initial transcript encoding the precursor of calcitonin. Amylin and adrenomedullin are not expressed in neuronal tissues. Pancreatic β-cells express high levels of amylin, whereas adrenomedullin is expressed in a variety of cell types.

Based upon the homology between calcitonin and CGRP, it was initially demonstrated that CGRP can inhibit bone resorption in organ cultures and in isolated osteoclasts[43-45]. Although, the homology is limited to very few amino acids in the very amino terminal parts of the molecules, it has been postulated that the effect of CGRP on bone resorption is mediated by calcitonin receptors. More recent data indicate that the receptors are different but closely related (see below). Since then, effects of CGRP on osteoblasts have also been described and it seems as if both VIP and CGRP have pleiotropic effects on bone.

Receptors

The receptors for calcitonin, CGRP and the other peptides in this superfamily are all seven transmembrane, G-protein coupled, receptors linked to activation of adenylate cyclase. CGRP, similar to adrenomedullin, binds to a calcitonin receptor-like receptor (CRLR) which needs the interactions with single transmembrane proteins, so called receptor activity-modifying proteins (RAMPs)[46], to be activated. Interaction between CRLR and RAMP 1 leads to receptors sensitive to CGRP, whereas the interaction with RAMP 2 or 3 creates a receptor sensitive to adrenomedullin[47]. Interestingly, interaction between the calcitonin receptor and RAMP 1 or 3 gives rise to an amylin sensitive receptor. The expression, regulation and importance of CRLR and RAMPs in bone cells have not yet been elucidated.

Effects on osteoblasts

The presence of functional receptors for CGRP on osteoblasts have been demonstrated by showing that CGRP stimulates cyclic AMP in a variety of different osteoblasts, including the rat osteosarcoma cell line UMR 106-01, the human osteosarcoma cell line Saos-2, the mouse calvarial osteoblastic cell line MC3T3-E1 and primary cultures of mouse calvarial osteoblasts[18,48].

There is strong evidence that CGRP, amylin and adrenomedullin have anabolic activities in the skeleton. Thus, all three peptides stimulate the proliferation of osteoblasts *in vitro*[49-51]. Targeted expression of CGRP to osteoblasts under the control of the osteocalcin promotor, results in enhanced trabecular bone density, trabecular bone volume and increased bone formation rate in mice[52]. Injection of CGRP into ovariectomized rats partly prevents the osteoporotic phenotype[53]. Similarly, treatment of rats or mice with amylin or adrenomedullin results in enhanced trabecular bone volume and increased mechanical bone strength[51,54]. Although the effects of CGRP and the related peptides on the skeleton are mainly attributed to their effects on osteoblasts, the possibility exists that decreased bone resorption has also contributed to the anabolic response *in vivo* (see further below).

Effects on osteoclasts

CGRP is a strong inhibitor of bone resorption, as assessed by findings both *in vitro* and *in vivo*. Thus, injection of CGRP into rabbits and rats causes hypocalcemia, similar to calcitonin[55]. This effect is most likely caused by decreased rate of bone resorption since CGRP inhibits bone resorption in organ cultures[43,44] and the pit forming activity of isolated rat osteoclasts on bone slices[45]. The effects of CGRP on serum calcium and bone resorption are shared by amylin, suggesting that CRLR and RAMP 1 are expressed in osteoclasts. In contrast, adrenomedullin does not inhibit bone resorption in mouse calvariae[51], indicating that RAMP 2 and 3 may not be expressed in osteoclasts. The inhibitory effects by CGRP and amylin on bone resorption are transient, similar to those of calcitonin and VIP. Whether or not CGRP and the related peptides may cause a delayed stimulation of bone resorption due to osteoblastic paracrine activation of osteoclasts,

similar to VIP, is presently studied in our laboratory. CGRP 8-37 does not block the inhibitory effect of CGRP on bone resorption in organ culture but counteracts the stimulatory effect of CGRP on osteoblast proliferation, indicating that different receptor subtypes may be present in osteoblasts and osteoclasts, respectively.

Effects on osteotropic cytokines

Very few studies have been performed exploring the possibility that CGRP may affect cytokine expression in bone cells. It has, however, been reported that CGRP increases IGF-I and IL-6 and decreases TNF-α in rat calvarial osteoblasts[56,57].

Substance P

The information regarding effects of SP on bone is very limited. There is one report claiming that osteoblasts do not express mRNA for SP receptors[58] and another, using immunocytochemistry, stating that SP receptors can be found, although only weakly, in osteoblasts[59]. No information is available whether or not SP can stimulate or inhibit anabolic activities in osteoblasts.

In contrast, osteoclasts have been found to express SP receptors[59] and the fact that SP causes an acute rise of intracellular calcium in osteoclasts and stimulates the pit forming activity of rabbit osteoclasts[60], suggest that the SP receptors in osteoclasts are functional.

Conclusions

Phenotyping of skeletal nerve fibers has demonstrated the presence of a panel of signalling molecules including the neuropeptides VIP, CGRP and SP. Using several experimental approaches, it has been shown that osteoblasts and osteoclasts express mRNA for the different receptor subtypes recognizing these neuropeptides and that the receptors are functional. Activation of the different receptors leads to regulation of proliferation and/or differentiation of osteoblasts, to regulation of the mechanisms involved in differentiation and fusion of preosteoclasts to multinucleated osteoclasts, as well as to activation or inhibition of osteoclastic bone resorption (Figure 1). The responses to the skeletal neuropeptides in bone cells are similar to those induced by osteotropic hormones and cytokines and suggest that the nervous system may be part of the endo- and paracrine control of bone metabolism. This suggestion is further supported by data obtained by chemically and surgically targeted nerve deletion and observations made in paraplegic patients.

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