Neuropeptides and the puzzle of bone remodeling

State of the art

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Bone metabolism is dependent on cells of the osteoblast and osteoclast lineage. These cells play a major role in the synthesis and degradation of osteoid and in its mineralization and demineralization. Bone cells are under the influence of various systemic and local auto/paracrine factors. One further regulatory element that can play both a sensory/afferent and a regulatory/efferent role, consists of neuropeptide-containing nerves. In particular, the calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) have been implicated; their distribution in bone and their molecular biology are discussed in some detail. Bone neuropeptides can function as direct bone cell regulators, with additional amplifying indirect effects mediated by vascular endothelial cells, monocyte/macrophages and mast cells and their mediators. Recent experimental and clinical work has implicated bone nerves in processes varying from normal remodelling to fracture healing and non-union. Apart from systemic endocrine influences on bone stock and osteoclast/osteoblast coupling (activation–resorption–formation cycle) mediated by local auto/paracrine factors, bone nerves/neuropeptides may explain why various inputs/outputs are transformed in a meaningful way to altered mass and quality of bone.

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Bone is living and continuously remodeling tissue consisting of bone cells in and on an organic osteoid matrix largely impregnated with hydroxyapatite crystals. Osteoid matrix contains fibers of interstitial triple helical type I collagen (85–90% of the total bone protein). Collagen fibers are decorated with numerous noncollagenous proteins (NCP), which on a molar basis are as many as the collagen fibers. NCPs regulate attachement of the hydroxyapatite crystals to bone collagen, cell attachment and binding of various growth factors to bone matrix. The mineral attached to collagen in bone is carbonated, basophilic hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], with many biologically relevant impurities like fluoride. At the cellular level, bone cells of the osteoblast and osteoclast lineage play a major role in the synthesis and degradation of osteoid and in its mineralization and demineralization, respectively. Recently, neuronal cells containing peptides with actions on cells of the osteoblast and osteoclast lineage have been recognized in bone tissue (Figures 1–8). The regulation of these cells/events by neuronal factors, in particular neuropeptides, is discussed in this report in some detail.

Systemic regulatory factors

All bone cells are controlled by various systemic regulatory factors (parathyroid hormone, sex steroids, glucocorticoids, 1,25-dihydroxyvitamin D3, calcitonin, insulin, growth hormone, thyroid hormone etc) and by availability of other important participants in bone turnover, such as calcium, phosphorus, fluoride etc. Abnormalities in these systemic factors usually lead to more or less generalized metabolic bone changes which, however, may call attention to them as local pathologic events, such as osteoporotic fractures.

Local auto/paracrine factors

Bone cells are also to some extent controlled by various local growth factors and cytokines deposited in the bone matrix. Examples include platelet-derived growth factor (A and/or B chain-containing) binding to perlecan HS (heparan sulfate proteoglycan) and to SPARC (osteonectin); basic fibroblast growth factor (bFGF) binding to syndecan HS/CS (CS chondroitin

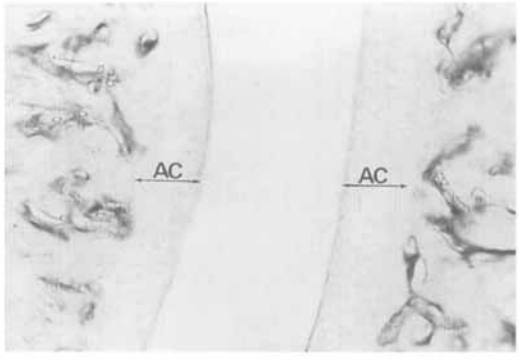

Figure 1. Calcitonin gene-related peptide (CGRP) -immunoreactive fibers in the narrow marrow spaces of rat tarsal bone. Note the very thin, varicosal appearance of the fibers (open arrows). AC Articular cartilage of the talus and the navicular bones. Original magnification ×160.

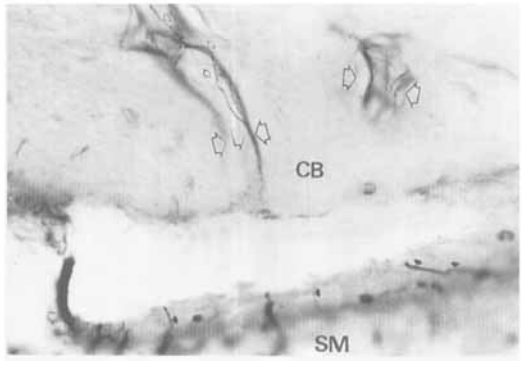

Figure 2. CGRP-immunoreactive fibers in a Volkmann's canal (large open arrows) in association with a blood vessel. Note the very thin, varicosal, intraosseous fibers (small open arrows) in contrast to the thick, synovial fibers (solid arrows). CB Cortical bone, SM Synovial membrane. Original magnification ×160.

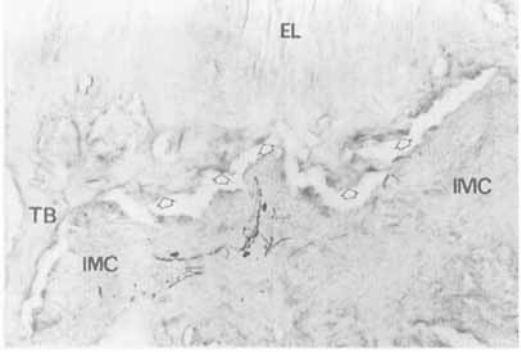

Figure 3. A photomicrograph showing regenerating, intramedullary CGRP-immunoreactive fibers in a rat with adjuvant-induced arthritis. Open arrows indicate the trabecular bone of the talus (TB), which has been resorbed close to the intramedullary cell accumulations (IMC). Note the thick and speckled appearance of the CGRP-immunoreactive fibers (solid arrows). EL Enthesis of a ligament. Original magnification ×160.

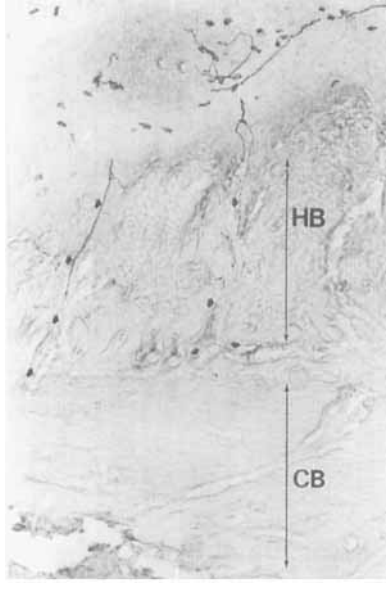

Figure 4. A photomicrograph demonstrating regenerating CGRP-immunoreactive fibers (arrows) in newly formed hypertrophied bone (HB). Hypertrophied bone was formed on the original cortical bone (CB) in an adjuvant-induced arthritis. Original magnification ×160.

sulfate) and to perlecan HS; and probably, in particular, transforming growth factor betas (TGFß), including bone morphogenetic proteins (BMPs), binding to betaglycan CS/DS (DS dermatan sulfate) and decorin CD/DS. These kinds of local growth and/or differentiation factors regulate the so-called coupling of bone resorption and bone formation. They are deposited and bound to solid substrates, a binding that protects them from diffusion and proteolytic and/or oxidative degradation. Following demineralization by dissolution of hydroxyapatite crystals, degradation of the organic bone matrix mediated by acid lysosomal carboxyl/aspartic acid and thiol/cysteine proteinases follows. Upon degradation of the structural backbone of the growth factor depositories, i.e., upon bone resorption, growth factors are released into their immediate surroundings, in which they may attain signficant concentrations. These local growth factors may induce the formation of new bone at the very site of resorption. Such local growth factors may thus form the foundations of the so-called activation-resorption-formation (ARF) cycle, coupling resorption with new bone formation.

Several local auto/paracrine factors are known at present, which regulate cells of the osteoblast and os-

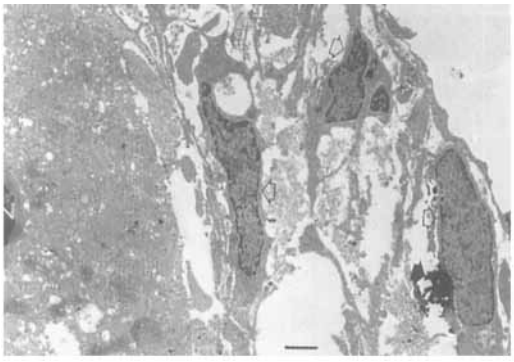

Figure 5. A CGRP containing nerve (arrow) in close association with an osteoblast (open arrows) in a group of such cells. Part of a giant multinucleated osteoclast is seen to the left, with one of the nuclei (N) and cytoplasm-containing rough endoplasmic reticulum, Golgi stacks, numerous transport vesicles and mitochondria are also visible. An immunoelectron micrograph, bar = 1 μm.

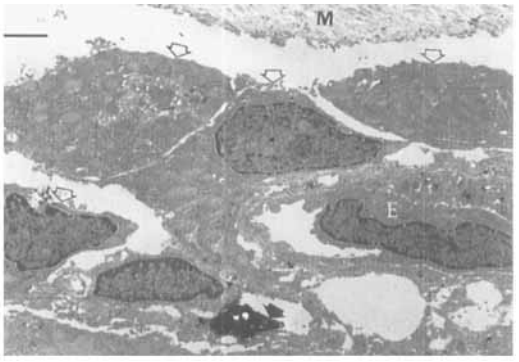

Figure 6. A CGRP-containing nerve (arrow) near an osteoblast (open arrows) in a group of such cells. Note the light staining of the nuclear euchromatin-associated with a relatively well developed rough endoplasmic reticulum, which can be seen in this view. Calcified bone matrix (M) and an endothelial cell (E) of a blood vessel in the bone marrow are visible in the vicinity of the axon. An immunoelectron micrograph, bar = 1 μm.

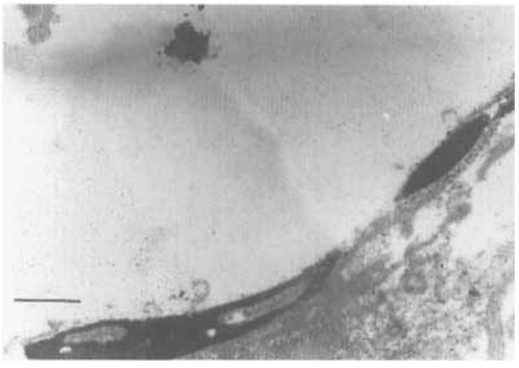

Figure 7. This immunoelectron micrograph demonstrates CGRP-containing nude nerve fibers running in longitudinal direction along and on the endosteal surface of a bone. Bar = 1 μm.

Figure 8. An immunoelectron micrograph demonstrating CGRP-containing terminal varicosities/ nerve terminals in bone tissue. In contrast to nerve fibers mainly containing axoplasm and cytoskeletal components, the terminal varicosities often (and also in this case) contain various cell organelles, mitochondria being particularly visible in this view. Bar = 1 μm.

teoclast lineage; most of these we have systematically quantitated in the periprosthetic tissues of loosening total hip prosthesis (Konttinen et al. an invited review, in preparation). Some of these are mainly classified as osteoblast/ osteogenesis-stimulating and include FGFs, TGFßs and the related BMPs, epidermal growth factor (EGF), transforming growth factor alfa (TGFα) and insulin-like growth factors (IGF-I, IGF-II). Some seem to stimulate osteoclasts/osteolysis and include tumor necrosis factors (TNFα and TNFß), interleukins (IL-1α,IL-ß, IL-6, IL-8, IL-11) and colony-stimulating factors (GM-CSF and M-CSF). Classification into a specific class is naturally not unequivocal (e.g., PDGFs, namely PDGF-AA, -AB and -BB) and, in some cases (e.g., IL-3), in part because of the organization of cytokines into a "network". Finally, lipids of the prostanoid class, in particular PGE_2, produced in excess in many pathological bone diseases by cytokine-induced cyclooxygenase-2 (one of the PGH_2 isoenzymes), constitute an important local auto/paracrine factor.

Innervation

Peptidergic periosteal nerves are arranged into networks of nerves on the surface of the bone and are more numerous at the epiphysis than in the mid-shaft region of diaphyseal bones. Both CGRP- and VIP-containing nerves are seen in all four layers of the periosteum consisting mainly of bone-lining cells (probably osteoblasts), precursor cells, loose connective tissue and a more superficial dense connective tissue. In addition, blood vessels, visualized in all layers, are

innervated by CGRP- and VIP-containing nerves, but also by neuropeptide Y/dopamine-ß-hydroxylase containing nerves. Both free and perivascular nerve fibers are found among these periosteal nerve fibers, which mostly originate from the surrounding muscle layers. These periosteal fibers bifurcate and often terminate in beaded varicosities at the bone-periosteum interface. In addition, small branches or single fibers of the periosteal nerves enter the cortical bone, usually associated with blood vessels located in Volkmann's canals or in Haversian canals (Hill and Elde 1991, Hukkanen et al. 1992).

Relatively thick nerves or branches of nerves enter the medullary canal with the nutrient blood vessels, whereas the epiphyseal nerves entered the bone through the arterial and venous channels. Small branches of the nutrient nerve enter the cortical bone, whereas the rather thick main nerve branched into smaller nerves as it entered the medullary canal. Nerve fibers were found in bone marrow in association with arteries, veins and some medullary sinusoids. Both varicose and non-varicose nerve fibers were observed. Small nerves were also located on the endosteum, from which many fibers entered the cortical bone (Hukkanen et al. 1992, 1994, Buma et al. 1995).

Peptidergic innervation of the epiphyseal growth plate deserves some special attention. The osteochondral junction of the growth plate is richly innervated on the side of the epiphysis, whereas the metaphyseal side of the growth plate contains hardly any peptidergic nerves at all. The epiphysis is also otherwise richly innervated, whereas peptidergic nerves in the diaphysis are not so common and are quite rare in the metaphysis. The osteochondral junction of the articular cartilage (subchondral bone) is sparsely innervated compared to the epiphyseal growth plate (Hukkanen et al. 1992, 1994).

Effects of the nervous system on bone

There are many functional studies indicating a role of the nervous system on embryonal development (Frenkel et al. 1990), during fracture healing (Smith and Dunsford 1955, Hulth and Olerud 1965, Quilis and Gonzáles 1974, Frymoyer and Pope 1977, Aro et al. 1981, 1982, 1985, Aro 1985, Nordsletten et al. 1994, Madsen et al. 1996), on peri-implant bone remodelling (Buma et al. 1995), on denervation (Hill et al. 1991) and on heterotopic bone formation (Spencer 1987, Bjurholm et al. 1990, Wildburger et al. 1994). Recently, it has become apparent that at least some of these neural influences on bone may be mediated by neuropeptides released from the sensory nerve fibers and from the postganglionic, autonomic nerve fibers (vide infra).

Neurogenic regulatory factors

A third category of regulatory factors, which we would like to emphasize, seems to be neuropeptides (and their amplifiers such as mast cells, monocyte/macrophages and endothelial cells) from bone nerves (Lerner 1992). They appear to be involved even on theoretical grounds, because bone remodeling is by no means a totally random process. Although it occurs at many different sites and phases as a delicate mosaic, it still responds to continuously changing demands. According to the much quoted law of Wolff, still unexplained as to mechanisms mediating it, physical activity is transformed into changes in bone mass or quality. Like all regulatory factors, neuropeptides do not by themselves participate in the subsequent reactions as enzymes or building blocks, but we believe, that they can at low concentrations affect the bone remodeling cycle and the net results of it. Alternatively, due to stimulus-coupled release of neuropeptides stored in nerve terminals, relatively high concentrations can perhaps be reached close to the nerve terminals. They do not have to be in action all the time, because the ARF cycle is relatively slow, with the resorption phase taking approximately three weeks and followed by a more slowly proceeding formation, so that the whole cycle takes approximately five months. Therefore, factors regulating the initial activation may have self-perpetuating effects in the long term; the regulatory factors would only be responsible only for the site- and time-specific location of these remodeling areas, making it all into a delicate composite of smaller pieces forming a pattern meaningful from the point of view of the actual strains and stresses. According to this "mosaic hypothesis", neuropeptidergic nerves provide a means both to sense the mechanical demands (sensory function of the neuropeptide nerves) and to respond to them (the effector function of sensory and autonomic nerves) and thus fill the "gap" in-between systemic and solely local regulators.

Neuropeptide-containing nerves in bone

In contrast to systemic and local factors, neuropeptides in the sensory and autonomic nervous systems are synthesized in dorsal root or local sympathetic/parasympathetic ganglia, respectively, from where they are transported in a protected environment in

dense core vesicles in an intraaxonal, extravascular space to their site of storage. Non-stimulated nerves do not seem to release their peptides to any great extent, but release from the nerve terminals may lead to significant local concentrations upon stimulation or trauma of the nerves. The presence of such nerves in bone has recently been established (Hohmann et al. 1983, Bjurholm et al. 1988a,b, Hukkanen, 1994). In addition, "neuropeptides" can be produced and/or released also by many non-neuronal cells, e.g., VIP - like peptides by mast cells and NPY by megakaryocytes/platelets.

Not only are neuropeptides present in normal bone, but changes in neuropeptide-containing nerves in various pathobiological conditions suggest that they are actively involved in local disease processes, such as arthritis, heterotopic bone formation in demineralized allogeneic bone matrix and fracture healing (Bjurholm et al. 1990, Hukkanen et al. 1992, 1993, 1995). Accordingly, denervation affects fracture healing and local neuropeptide expression (Hukkanen et al. 1995), whereas non-union is characterized by diminished local expression of neuropeptides (Santavirta et al. 1992, Nordström et al. 1994). In amphibians, an intact nerve supply is essential for the development and regrowth of amputated limbs. In particular, calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) have been implicated.

Calcitonin gene-related peptide (CGRP)

CGRP is a 37 amino acid peptide produced by tissue-specific alternative processing of the primary RNA transcripts of the calcitonin gene. Two isoforms of CGRP, CGRP-I and CGRP-II, derived from related CALC-I and CALC-II genes, respectively, have been identified in man; they differ in only three amino acids. CGRP is often localized in sensory neurons together with substance P and, accordingly, CGRP-immunoreactivity decreases following capsaicin treatment (Hill and Elde 1991). It has been postulated that substance P and CGRP containing fibers are primary sensory neurons involved, not only with nociception, but also with thermo- and mechanoreception. However, CGRP is also found in other neurons, such as spinal motoneurons and motor end-plates of striated muscles. In bone, the distribution pattern of substance P and CGRP-containing peptidergic nerves is similar, although CGRP-containing fibers are more numerous, and found, in particular, near the epiphyseal plate, in the bone marrow, and in the periosteum, also around blood vessels such as in the Volkmann's canals and less frequently in Haversian canals. Effects of CGRP, in general, are mediated via $CGRP_1$ and $CGRP_2$ receptors distinct from calcitonin C receptor, to which CGRP may also bind, but with low affinity. CGRP receptors are coupled to G_s linked to adenylate cyclase and thus to increased cytoplasmic cAMP and protein kinase A activation. CGRP lowers blood calcium and inhibits bone resorption (Roos et al. 1986), in part by inhibiting recruitment of macrophages into osteoclast-like cells (Owan and Ibaraki 1994) and by inhibiting osteoclastic bone resorption (D'Souza et al. 1986, Zaidi et al. 1987a,b). The last-mentioned influence seems to be a G protein-dependent effect on osteoclast motility, which is inhibited (quiescence or Q effect). In addition, osteoblastic cell lines are equipped with receptors for CGRP (Bjurholm et al. 1992) and CGRP stimulates osteogenesis (Bernard and Shih 1990).

Vasoactive intestinal peptide (VIP)

VIP is a 28 amino acid cleavage product of preproVIP, which is the parent molecule also for peptide histidine methionine (PHM), pituitary adenylate cyclase-activating polypeptide (PACAP) and peptide histidine valine (PHV-42). VIP can be found in postganglionic sympathetic nerves in bone (Hill and Elde 1991), but has usually been found mainly in parasympathetic nerves (Lundberg 1981) and to a minor extent in primary sensory neurones (Lundberg et al. 1979). Interestingly, tyrosine hydroxylase and neuropeptide Y (markers of postganglionic sympathetic fibers)—containing nerves were found perivascularly, whereas VIP-ergic nerves had a different localization, being found in the epiphysis and periosteum and not so often around blood vessels (Bjurholm et al. 1988b). Effects of VIP are mediated by high-affinity, low-capacity and low-affinity, high-capacity receptors. VIP receptors have been demonstrated on human osteoblastic osteosarcoma cell line SaOs-2 (Hohmann and Tashjian 1984). VIP receptors can be coupled to a stimulatory G protein (G_s protein), which stimulates formation of cAMP, or to G_{plc} protein, which stimulates formation of inositol phosphates and diacylglycerol (Brenneman and Eiden 1986, Lutz et al. 1993, Fatatis et al. 1994); VIP may also stimulate PGE_2 production in human osteoblast-like cells (Rahman et al. 1992). Effects in bone seem to be mediated via the traditional and well-established cAMP-mediated second messenger pathway, which stimulates osteolysis, suggesting an indirect mode of action on osteoclasts (Hohmann et al. 1983). VIP-producing tumors (VIPomas) are a rare cause of hypercalcemia.

However, some preliminary results suggest an inhibitory effect on bone resorption as well (Bax et al. 1993, Winding and Hou 1994). The VIP effects are terminated by degradation mediated by enzymes like neutral endopeptidase (also known as enkephalinase) rather than by uptake. Other enzymes which come into question include mast cell tryptase and chymase. Neuropeptide effects might be affected by the local microenvironment, such as bone extracellular fluid at the site of release.

Neuropeptide nerves vs endothelial cells and mast cells

In addition to systemic and auto/paracrine local factors, bone remodeling and development may in part be controlled by neural factors/neuropeptides. Neuropeptide-containing nerves are most frequent in metabolically active bone areas. Neuropeptides released from nerve terminals may utilize various amplifying systems—in bone as well as in other tissues—like endothelial cells, monocyte/macrophages, bone cells and mast cells (Konttinen et al. 1994). For example, CGRP and VIP are both efficient vasodilators and both also modulate the adhesive properties of endothelial cells. CGRP is mitogenic to endothelial cells (Konttinen et al. 1994). Interestingly, the non-adrenergic, non-cholinergic (NANC) effects of CGRP may to some extent be dependent on nitric oxide (NO; Lambrecht et al. 1993, Wiencke et al. 1994). NO as well as other endothelial cell products, such as endothelin, hydrogen peroxide and prostaglandins, may play a role in bone metabolism (for a recent review, see Zaidi et al. 1993, Bergmann and Schoutens 1995). VIP and substance P modulate mast cell function (Johnson and Erdös 1973, Tomioka et al. 1989, Inoue et al. 1995). Recently, mast cell activation has been reported to comprise not only release of preformed and stored histamine and heparin but also production *de novo* of various lipid (e.g., LTB_4 and PGD_2) and cytokine (e.g., IL-6 and TNFα) mediators, some of which are also affected by stimulation with substance P (Ansel et al. 1993, Cocchiara et al. 1995). It is therefore possible, although not proven, that some neuropeptide effects on bone might, in part, be mediated via mast cells.

Although nerve fibers in bone are few, they represent sophisticated and specialized regulatory elements able to deliver time- and site-specific stimuli according to demand. Bone remodeling is to some extent self-perpetuating and bone nerves may provide a mean of fine adjustment, such as in remodeling, according to the local needs, so that the inflow of mechanical and chemical influences will be adequately perceived and responded to. Bone nerves may represent the sensor/effector part of this tissue, so that various inputs/outputs make sense, since they are transformed to alterations in the structure and function of specialized bone tissue (Hukkanen 1994), as has been already accepted generally for most other tissues.

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